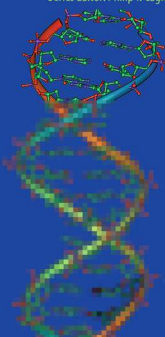


Cherie H. Dunphy
Editor

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Molecular Pathology of Hematolymphoid Diseases



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Molecular Pathology of Hematolymphoid Diseases

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Series Preface

The past two decades have seen an ever-accelerating growth in knowledge about molecular pathology of human diseases, which received a large boost with the sequencing of the human genome in 2003. Molecular diagnostics, molecular targeted therapy and genetic therapy, are now routine in many medical centers. The molecular field now impacts every field in medicine, whether clinical research or routine patient care. There is a great need for basic researchers to understand the potential clinical implications of their research whereas private practice clinicians of all types (general internal medicine and internal medicine specialists, medical oncologists, radiation oncologists, surgeons, pediatricians, family practitioners), clinical investigators, pathologists and medical laboratory directors and radiologists require a basic understanding of the fundamentals of molecular pathogenesis, diagnosis, and treatment for their patients.

Traditional textbooks in molecular biology deal with basic science and are not readily applicable to the medical setting. Most medical textbooks that include a mention of molecular pathology in the clinical setting are limited in scope and assume that the reader already has a working knowledge of the basic science of molecular biology. Other texts emphasize technology and testing procedures without integrating the clinical perspective. There is an urgent need for a text that fills the gap between basic science books and clinical practice.

In the *Molecular Pathology Library* series, the basic science and the technology is integrated with the medical perspective and clinical application. Each book in the series is divided according to neoplastic and non-neoplastic diseases for each of the organ systems traditionally associated with medical subspecialties.

Each book in the series is organized to provide specific application of molecular pathology to the pathogenesis, diagnosis, and treatment of neoplastic and non-neoplastic diseases specific to each organ system. These broad section topics are broken down into succinct chapters to cover a very specific disease entity. The chapters are written by established authorities on the specific topic from academic centers around the world. In one book, diverse subjects are included that the reader would have to pursue from multiple sources in order to have a clear understanding of the molecular pathogenesis, diagnosis, and treatment of specific diseases. Attempting to hunt for the full information from basic concept to specific applications for a disease from varied sources is time-consuming and frustrating. By providing this quick and user-friendly reference, understanding and application of this rapidly growing field is made more accessible to both expert and generalist alike.

As books that bridge the gap between basic science and clinical understanding and practice, the *Molecular Pathology Series* serves the basic scientist, the clinical researcher and the practicing physician or other health care provider who require more understanding of the application of basic research to patient care, from “bench to bedside.” This series is unique and an invaluable resource to those who need to know about molecular pathology from a clinical, disease-oriented perspective. These books will be indispensable to physicians and health care providers in multiple disciplines as noted above, to residents and fellows in these multiple disciplines as well as their teaching institutions and to researchers who increasingly must justify the clinical implications of their research.

New York, NY

Philip T. Cagle, MD

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Section I

Molecular Pathology of Hematolymphoid Neoplasms: General Principles

1 Molecular Oncogenesis

Aniruddha J. Deshpande, Christian Buske,
Leticia Quintanilla-Martinez, and Falko Fend

Introduction

The history of molecular pathology is inseparable from the advances in neoplastic hematopathology, since many advances, both in understanding mechanisms of disease development and progression, as well as of technical aspects of molecular pathology, are intimately linked with landmark findings in hematologic disorders. The detection of the Philadelphia chromosome in chronic myelogenous leukemia, which was subsequently shown to represent a translocation involving chromosomes 9 and 22 t(9;22)(q34;q11.2) resulting in the BCR–ABL fusion gene (see Chap. 30), marks the beginning of an exciting journey, which in turn has led to the development of targeted therapies against this defining molecular aberration.

The first clinical areas where molecular testing was incorporated into routine diagnosis and clinical management of patients were hematology and hematopathology. Molecular studies are nowadays an integral part of state-of-the-art diagnostics of hematologic neoplasms. Correct performance and interpretation of molecular studies in these disorders require an understanding of the underlying principles of oncogenesis. Therefore, this chapter tries to summarize the molecular changes that are important for the development and progression of hematolymphoid malignancies.

The Initiation and Maintenance of Oncogenic Programs: Genetic and Epigenetic Changes

Human tumors are often a result of the abnormal and limitless clonal expansion of one renegade cell. Like normal cells, tumor cells propagate by the transmission of their genetic and epigenetic information to daughter cells. The difference is that in tumor cells, this information is changed, usually in many ways, and the faithful propagation of this abnormal change is the key to the expansion of the tumor. These changes can occur at many levels, one of the most important being

the change in genetic information due to changes in DNA sequence that is characteristic of most cancers. Recently, epigenetic changes or changes in genetic information without alterations in the sequence of DNA have been in the limelight because they have profound effects on gene expression and the maintenance of genome integrity. Genetic and epigenetic lesions are acquired by somatic cells, often progressively, and can work in tandem to induce tumor formation.

Types of Genetic Changes in Hematolymphoid Neoplasms

Recent studies involving genetic and molecular techniques have provided tremendous insights into the biology of hematopoietic neoplasms. Genetic changes in hematopoietic and lymphoid malignancies are the result of either chromosomal alterations or epigenetic changes that induce deregulation of gene expression.

Since the discovery of the Philadelphia chromosome, recurrent chromosomal abnormalities such as translocations, deletions, inversions and duplications associated with several types of leukemia, lymphoma, and certain types of epithelial tumors have been identified.^{1–3} These chromosomal abnormalities are often somatic mutations acquired by a clonally expanded malignant population. As is the case with CML, certain chromosomal abnormalities can be associated with specific types of disease, and the characterization of these abnormalities can be used for diagnosis, as well as for the determination of disease prognosis. Moreover, treatment regimens can be optimized to suit discrete subgroups divided according to these abnormalities. Chromosomal aberrations can be numerical (changes in chromosome numbers) or structural (changes in chromosome structure such as those arising from translocations, inversions, deletions, etc.). Even though several hundred different types of chromosomal alterations have been reported⁴, most of them occur at a very low frequency, with some recurrent translocations accounting for most of the cases. These translocations can, however, be

broadly classified into those that lead to the juxtaposition of oncogenes to strong regulatory elements, such as those of the immunoglobulins or chromosomal translocations that lead to oncogenic fusion gene formation. The former leads to the aberrant overexpression of structurally normal oncogenic gene products and are mostly observed in lymphoid malignancies. The latter types of gene rearrangements lead to the formation of aberrant fusion genes, many of which have been shown to be oncogenic in models of tumor formation.

In contrast to the chromosomal translocations, other acquired somatic mutations such as point mutations, deletions, and insertions have been more difficult to detect. However, mutations in protein-coding genes constitute a significant proportion of genetic changes and may impact tumor progression. These mutations occur in a diverse set of genes, some of the most common being in genes governing signal transduction pathways or in lineage-specific transcription factors. While mutations in signaling pathway genes confer proliferative advantage to cells, abnormal changes in lineage-specific transcription factors impair differentiation of cells. These two types of mutations, as described below in the two-hit model of leukemogenesis, are often seen to be complementary and sequentially acquired steps. Although the assumption that signaling pathway alterations mostly affect proliferation, and transcription factor deregulation that mostly affects differentiation is simplistic and not entirely correct, for didactic purposes, this division is helpful and will be used to describe the two classes of mutations in more detail in the next subsections. Since the molecular mechanisms responsible for triggering leukemia and lymphoma are so different, the chapter is divided into two sections; one section deals with molecular mechanisms of leukemias and myeloid disorders and the second section deals with molecular mechanisms of lymphoid neoplasms.

Genetic Changes in Leukemia and Myeloid Disorders

Multistep Pathogenesis and the Cooperativity of Genetic Alterations

Cancer is now widely recognized as a multistep process involving progressive accumulation of multiple mutations involving the activation of oncogenes and the inactivation of tumor suppressor genes. Often, the deregulation of distinct pathways and processes by these accumulating mutations is a necessary prerequisite for tumor formation. Several observations suggest that single mutations are insufficient for tumor development. Cells carrying certain leukemia- or lymphoma-specific lesions may be detected in normal individuals, albeit at low frequencies.⁵⁻⁷ A simplistic model for cooperative mutations in acute myeloid leukemias (AML) proposed by Gilliland and Griffin⁸ postulates that these

can be broadly classified into two major complementary subgroups: (1) mutations that confer proliferation or survival signals (usually involving aberrantly activated tyrosine kinases) and (2) mutations that impair differentiation (usually involving transcription factors) (Figure 1.1).⁹ It is hypothesized that the combined action of these two classes of mutations is necessary for a full-blown AML to develop. This is supported by the fact that mutations in two genes belonging to the same sub-group are rarely seen in the same patient. In line with the finding that abnormal gene fusions can be found in normal individuals, the fusion genes *AML1/ETO* (*RUNX1-RUNX1T1*) as a result of a t(8;21)(q22;q22) and *TEL/AML1* (*ETV6-RUNX1*) occurring as a result of t(12;21)(p13;q22) have been reported to occur at low frequencies without inducing disease. Accordingly, it was also shown that these fusion genes can rarely initiate complete leukemogenesis in murine models in the absence of cooperating mutations.^{10,11} However, the introduction of appropriate “second hits,” which support the hypothesis of collaborative action, can induce a leukemic phenotype, resembling the corresponding human malignancy. For example, aggressive leukemias could be induced by the combined, but not separate, expression of the *AML1/ETO* (*RUNX1-RUNX1T1*) fusion protein and a mutated version of *FLT3* (*FLT3* internal tandem duplication).¹² Similar evidence for a multistep pathogenesis exists for malignant lymphomas, both derived from experimental data, as well as clinical observations. For example, in monoclonal gammopathy of unknown significance (MGUS), clonal plasma cells carrying the pathognomonic immunoglobulin translocations characteristic for multiple myeloma may be detected in a significant percentage of normal elderly individuals. Transformation to overt multiple myeloma or lymphoma occurs at a rate of approximately 1% per year, again demonstrating the necessity to acquire additional genetic alterations for a fully malignant phenotype. In view of these findings, it is clear that full blown hematologic malignancies result from the deregulation of multiple different pathways and that understanding them is the key to the establishment of treatment strategies.

The most frequent recurrent translocations and mutations in acute myeloid leukemia are listed in Tables 1.1 and 1.2. These abnormalities are also discussed in Chaps. 34 and 35, respectively.

Proliferation and/or Survival Signals

The most frequently observed molecular abnormality in AML, are mutations in nucleophosmin (NPM), which usually involve exon 12 of the *NPM1* gene (Table 1.2). NPM is a ubiquitously expressed nucleolar phosphoprotein, which shuttles continuously between the nucleus and the cytoplasm. The prevalence of *NPM1* in all de novo AML is roughly 35%. Furthermore, more than half of the AML patients with no cytogenetic abnormality bear this mutation

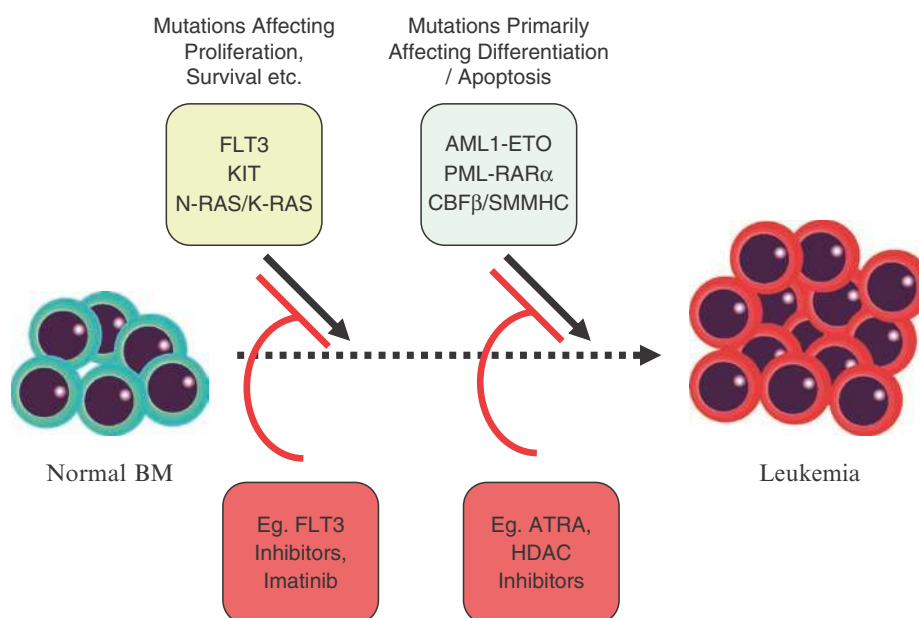


FIG. 1.1. The two-hit model of leukemogenesis. This figure shows collaborating mutations between genetic alterations in factors that affect differentiation and activating mutations in genes causing

proliferative/survival advantages. Potential therapeutic interventions are depicted below. Adapted and permission granted from Kuchenbauer et al.⁹

(normal karyotype). This mutation appears to show a female predominance.¹³ In AML, mutations in the *NPM1* gene lead to increased nuclear export and aberrant accumulation of the NPM protein in the cytoplasm, which is thought to contribute to tumorigenesis by increasing proliferation and/or inhibiting the programmed cell death.¹⁴ A number of recent studies have increased our understanding of the role of *NPM1* in leukemia, which are becoming very important for developing new therapeutic strategies to target this pathway. AML with mutated *NPM1* and a normal karyotype, has in general a favorable prognosis and a good response to induction therapy.

Malignant changes in signal transduction pathways confer survival and proliferative properties to leukemic cells. The alteration of these signal transduction pathways is often mediated by genetic changes in key signaling molecules such as the receptor tyrosine kinases (RTKs) or the RAS family of guanine nucleotide-binding proteins. An impressive body of evidence in the last decades has highlighted the role of aberrantly activated RTKs in leukemia. While some RTKs are involved in the formation of leukemia-specific fusion genes such as ABL, JAK2, PDGFRs, SYK, and FGFRs, others such as JAK2, FLT3, and the KIT have been shown to be activated by gain of function mutations in myeloproliferative disease and myeloid leukemia.

One of the most common examples of a kinase activated due to chromosomal translocation in leukemia is the

BCR-ABL kinase, which is generated by the t(9;22) (q34;q11.2) translocation, which is present in all cases of CML and in a proportion of cases with ALL. The inhibition of this kinase is seen to be crucial to the therapy of t(9;22) positive leukemias.¹⁵ In AML, overexpression or aberrant constitutive activation of class III RTKs like FLT3 or KIT through point mutations, duplications etc., has been reported.^{16–19} A class of tyrosine kinases termed Janus kinases (JAKs), which mediate cytokine/growth factor signaling are frequent targets of mutation in myeloproliferative disorders. The JAK2 V617F mutation in the pseudokinase domain of JAK2 is found in >95% polycythemia vera patients, essential thrombocythemia (EM, 50% of patients) and primary myelofibrosis (PMF, 50% of patients).²⁰ In these disorders, hypersensitivity to growth factor signaling leads to uncontrolled increase in mature hematopoietic elements with normal or near-to-normal function. At the molecular level, mutations in RTKs could affect dimerization, kinase function, receptor conformation, or phosphorylation, leading to their constitutive activation.²¹ The common pathological consequence of this constitutively active kinase signaling is uncontrolled proliferation, which is an important component in the pathogenesis of leukemia.

Finally, mutations in p53 gene, which is probably the most frequently mutated gene in cancer, is observed at a much lower frequency in leukemia than in solid tumors; whereas RAS mutations, most of which involve the N-Ras gene, may be found in as much as 30% of the AML cases.^{22,23}

TABLE 1.1. Examples of chromosomal translocations in patients with AML.

Translocation	Involved genes	Protein function
Translocations involving the “core binding factor” (CBF) family		
t(8;21)(q22;q22)	AML1 ETO	Transcription factor and CBF complex subunit Putative transcription factor
t(3;21)(q26;q22)	AML1 EV11	Transcription factor and CBF complex subunit Transcription factor
t(3;21)(q26;q22)	AML1 EAP	Transcription factor and CBF complex subunit Ribosomal Protein
t(3;21)(q26;q22)	AML1 MDS1	Transcription factor and CBF complex subunit Unclear
inv(16)(p13;q22)	CBFβ MYH11	Heterodimeric Partner of AML1 Smooth muscle myosin heavy chain
t(12;21)(p13;q22)	TEL AML1	ETS related transcription factor Transcription factor and CBF complex subunit
Translocations involving the retinoic acid receptor α (17q11)		
t(15;17)(q21;q11)	PML1	Zinc finger protein
t(11;17)(q23;q11)	PLZF	Transcriptional repressor
t(5;17)(q31;q11)	NPM	Nuclear phosphoprotein
t(11;17)(q13;q11)	NUMA	Mitotic spindle component
Translocations involving the “mixed lineage leukemia” (MLL) gene (11q23)		
t(11;16)(q23;p13.3)	CBP	Histone acetylase
t(11;22)(q23;q13)	P300	Histone acetylase
t(9;11)(p22;q23)	AF9	Transcription factor?
t(11;19)(q23;p13)	ENL	Transcription factor
t(6;11)(q27;q23)	AF6	Signal transduction protein?
Translocations involving the nucleoporin family		
t(2;11)(q31;p15)	NUP98 HOXD13	Component of the nuclear pore complex Homeobox gene
t(7;11)(p15;p15)	NUP98 HOXA9	Component of the nuclear pore complex Homeobox gene
t(6;9)(q23;q34)	DEK CAN (NUP214)	Putative transcription factor Component of the nuclear pore complex
Normal Karyotype	SET CAN	Histone binding protein Component of the nuclear pore complex

TABLE 1.2. Examples of some common mutations in protein coding genes described in AML.

Name	Description	Mutation type
NPM1	Nucleophosmin	Point mutations leading to altered protein localization
FLT3	Tyrosine kinase	Internal tandem duplications in the Juxtamembrane domain, Point mutations in the “activation loop”
KIT	Tyrosine kinase	Point mutations in the “activation loop”
N-RAS and K-RAS	RAS viral oncogene homologs	Activating mutations in codons 12, 13 or 61
CEBPA	Transcription factor	Loss of function point mutations
AML1	Transcription factor	Loss of function point mutations

Block of Differentiation

Another important subset of genes that are frequently mutated in acute leukemias of both lymphoid and myeloid origin are transcription factors with essential functions in hematopoiesis. Mutations in lineage-specific transcription factors are thought to lead to a block in differentiation and, therefore, contributing both to cellular transformation and the characteristic immature phenotype of acute leukemia. Deletions of the IKAROS gene occur in over 80% of patients with BCR-ABL positive B-ALL, but not in CML. These deletions result either in loss of expression or the expression of a dominant

negative form of IKAROS in the tumor cells suggesting that the loss of function of this transcription factor is an important step in the development of Ph+ B-ALL. Moreover, the loss of IKAROS might explain the difference in maturation between Ph+ B-ALL and CML despite the common presence of the BCR-ABL.

Point mutations in the granulocytic differentiation factor CEBPα have been reported in over 10% of all AML patients,^{24–26} whereas 7% of patients harbor mutations in the transcription factor PU.1.²⁷ The myeloid transcription factor RUNX1 (also known as AML1), which is recurrently involved in chromosomal translocations, is also mutated in

a subset of patients with AML, predominantly in the M0 subtype.^{28–32} Mutations in these genes lead to loss of function of these transcription factors, which plays a major role in malignancy. The role of transcription factor mutations in acute lymphoblastic leukemia (ALL) is also coming into focus in recent years, and with the advent of high throughput sequencing technologies, several such mutations have been documented. In patients with pediatric B-ALL, deletions, amplifications, and point mutations in several B-lineage associated transcription factors, such as PAX5 and EBF, have been reported.³³ In T-ALL, activating mutations in the NOTCH1 gene may be observed in over 50% of patients,^{34,35} suggesting that also in ALL, the deregulation of transcription factors plays a major role in oncogenic transformation.

Epigenetic Changes and Their Impact on Leukemogenesis

Epigenetic mechanisms such as DNA methylation, post-translational histone modifications, and nucleosome remodeling are now recognized as major players in the control of gene expression and the maintenance of normal processes of cell growth and differentiation. In addition to genetic alterations, aberrant changes in these epigenetic mechanisms may lead to the initiation and progression of disease. Profound epigenetic alterations such as aberrant DNA methylation or histone modifications have been found to be associated with human tumors. The most well-studied DNA modification is the methylation of cytosine at CpG dinucleotides. Regions near the promoters of genes are seen to be enriched for these potentially “methylable” CpG dinucleotides. These regions, termed CpG islands, are usually unmethylated in normal cells, thereby rendering these regions accessible to transcriptional activation by transcription factors. In contrast, tumor cells often show hypermethylation of CpG islands near tumor suppressor genes, thereby leading to their epigenetic inactivation. Such a hypermethylation at specific tumor suppressor gene promoters may be observed in DNA from CLL patient samples,³⁶ although there is an overall decrease in the global DNA methylation as compared to normal.^{37–39} In AML, a classic example of epigenetic dysregulation is the retinoic acid receptor α (*PML-RAR α*) fusion gene, a product of the t(15;17)(q22;q12) translocation seen in patients with acute promyelocytic leukemia (APL). The expression of this fusion gene has been shown to induce hypermethylation of *RAR α* target genes, including the tumor suppressor *RAR α 2*, which results in its epigenetic silencing.⁴⁰ Similarly, the *AML1-ETO* (*RUNX1-RUNX1T1*) fusion gene, a product of the relatively common t(8;21)(q22;q22) translocation in AML has also been shown to recruit HDACs and DNA methyltransferase, resulting in the potent transcriptional repression of *AML1* target genes,⁴¹ including the p14(*ARF*)

tumor suppressor.⁴² Recently, epigenetic suppression of the myeloid transcription factor, CCAAT/enhancer binding protein α (*C/EBP α*), has been reported in 51% of AML patients studied.⁴³ The silencing of this gene is associated with a block in terminal differentiation, which could contribute to leukemogenesis. It is possible that such epigenetic silencing of tumor suppressors or transcription factors could prime cells for malignant transformation. In T-ALL, methylation of the *PAX5* promoter region has been observed in the majority of cases.⁴⁴

In addition to the DNA modifications, the modification of chromatin structure, which is believed to constitute a heritable “cellular memory,” could lead to major changes in gene expression in tumor cells. Two of the most important gene families involved in the modification of chromatin structure are the Trithorax (*Trx*) and the Polycomb group (*PcG*) families. These families have opposing effects on the expression of a large number of developmental target genes by altering the accessibility of DNA to their transcription factors. One such family of developmental regulators, which is now known to be deregulated in a large number of hematological malignancies, is the *Hox* gene family.^{45,46} Members of the *Trx* and *PcG* family control the gene expression of these developmental regulators. Translocations of the trithorax group mixed lineage leukemia (*MLL*) gene can be seen in approximately 15% of human leukemias.⁴⁷ Studies on *MLL* fusion partners in leukemia strongly point to the role of aberrant histone modification leading to the dysregulation of gene expression.^{48,49} Moreover, the polycomb group gene *AF10*, which partners with *MLL*, as well as the endocytosis related *CALM* gene in two distinct and recurrent t(10;11) translocations, interacts with the H3K79 methyltransferase *hDOT1L*. This interaction results in the activation of *HOX* genes due to aberrant H3K79 histone methylation and this has been shown a critical step in the leukemogenesis of both *MLL-AF10*,⁴⁸ as well as *CALM-AF10* fusion genes.⁵⁰ More recently, *DOT1L* mediated epigenetic activation of the *Hox* gene cluster has also been demonstrated in myeloid and lymphoid leukemias initiated by the *MLL-AF4* oncogene⁵¹ making this an important target in leukemias with aberrant *HOX* gene activation.

More recent data points to the heterochromatic silencing of microRNAs by leukemia specific fusion genes such as the silencing of miR-223 by *AML1-ETO* (*RUNX1-RUNX1T1*) by the recruitment of histone deacetylases and DNA methyltransferases.⁵² Moreover, mir-124a, a regulator of *CEBP α* , is epigenetically silenced in leukemia cell lines and can be upregulated by epigenetic treatment.⁴³ These results suggest that epigenetic alterations in cancer are better “druggable” candidates due to the relative ease of reversing these changes, as opposed to changes in the DNA sequence. Therefore, a clearer understanding of these mechanisms and their contribution to normal and malignant processes will be one of the prime focuses in cancer research in coming years.

The Involvement of Stem Cells and Stem Cell Characteristics in Leukemia

Most cancers are now viewed to be driven by a population of cells with stem cell characteristics. These cells, termed “cancer stem cells” (CSCs), have been shown to be a distinct isolatable sub-component of the tumor and are thought to be responsible for tumor propagation and maintenance. Currently, this term is used as a “working definition” for defining cells within a tumor that can reconstitute an identical tumor in suitable recipient animals. CSCs in leukemia, termed leukemia stem cells (LSCs) have been shown to be responsible for leukemia propagation, and preliminary studies⁵³ support the notion that the refractoriness of these LSCs to currently used therapies could account for the frequent tumor relapse seen in patients with leukemia. Evidence from AML elegantly showed that an identifiable sub-component of cells with stem cell characteristics is exclusively responsible for tumor propagation,⁵⁴ kick starting efforts for CSC identification in other tumors. Since most tumor propagating cells were shown to possess stem cell characteristics, it was interesting to speculate that most tumors arise from tissue stem cells. However, in a series of elegant studies using highly purified hematopoietic subfractions, it was demonstrated that the expression of appropriate oncogenes in more downstream progenitor cells could also lead to leukemia formation.^{55–57} This datum is in line with the observation that some LSC candidates resemble differentiating progenitor cells.^{58–60} In some myeloid leukemias, it was demonstrated that the acquisition of stem cell characteristics by myeloid progenitors and the activation of a stem-cell associated or “stemness” transcriptional signature resulted in LSC formation.^{58,61,62}

The subversion of the molecular circuitry of “stemness” is now seen as a critical milestone, leading to oncogenesis. An understanding of the molecular changes responsible for this process are therefore of paramount importance in the design of therapies. Stem cell programs may either be retained in tissue stem cells which acquire mutations, or may be aberrantly reactivated by mutated downstream progenitors. These stemness characteristics, especially the property of self-renewal, are thought to be indispensable for the limitless propagation of tumor cells. The property of hematopoietic self-renewal is mediated by several pathways, such as the CDX-HOX pathway, the WNT signaling cascade, Hedgehog and NOTCH signaling, and the Polycomb/Trithorax network. The subversion of these pathways for the aberrant acquisition of leukemic self-renewal, specifically the CDX-HOX and the WNT signaling pathways, has been demonstrated in AML and CML, respectively, offering new therapeutic targets. Aberrant transcriptional activation of the clustered homeobox (*HOX*) genes, especially genes of the *HOX A* cluster, have been shown to be a feature common to many leukemias.^{45,46} There are several routes to this dysregulation, some of the most prominent being the involvement of these genes in chromosomal translocations, notably those involving the

NUP98 gene, or their upregulation by leukemia specific fusion proteins, such as *MLL* fusions,^{49,63,64} *CALM-AF10*,^{50,65} *SET/NUP214*,⁶⁶ or the proto-oncogene *CDX2*, an upstream regulator of *HOX* genes, which is aberrantly overexpressed in the bone marrow of a vast majority of AML patients.^{67,68} A few years ago, our group demonstrated that the ectopic expression of this gene in murine bone marrow progenitors may lead to the induction of an aggressive AML.⁶⁹ The expression of this gene leads to aberrant activation of *HOX A* genes, which have been shown to be key regulators of normal, as well as leukemic, self-renewal.⁶⁸ Aberrant acquisition of self-renewal by myeloid progenitors, which activate WNT signaling, has been shown to be a crucial step in the initiation of CML, and for its progression to the more aggressive acute form (or blast crisis) of CML.⁵⁸ Apart from self-renewal, the acquisition of stem cell programs is thought to confer other stem cell properties, such as quiescence, niche dependence, and multidrug and radiation resistance; although a detailed dissection of the molecular events underlying these changes awaits elaboration.

Therapies Targeting Leukemia-Specific Molecular Alterations

Although contemporary therapies for leukemia induce remission in a majority of patients, a significant number of patients still relapse and succumb to the disease. Since the understanding of the molecular oncogenesis of leukemic transformation is growing, the treatment of leukemia has progressed from common strategies to more specific approaches. These strategies are devised from studies on morphological and molecular characterization, response to specific therapeutic regimens, and the rate of disease recurrence in each disease sub-type. The characterization of specific, acquired molecular lesions in leukemia has led to the understanding of the biological processes that are subverted in the development of the malignancy. For example, the use of all-trans retinoic acid (ATRA) for APL associated with the *PML-RARα* translocation reverses the repression of retinoic-acid-responsive genes by *PML-RARα*.⁷⁰ The use of ATRA has dramatically improved the prognosis of APL, and was the first model of a drug targeting the specifically altered molecular event in leukemia. Another example of targeted molecular therapy is the tyrosine kinase inhibitor imatinib mesylate (or GleevecTM). This inhibitor was specifically designed to target the constitutive tyrosine kinase activation mediated by the BCR-ABL fusion protein and may cause decreased proliferation and enhanced apoptosis of BCR-ABL positive cells. This drug is effective for the treatment of t(9;22) positive CML and ALL, and the inhibition of this abnormal kinase activity has greatly improved treatment outcome in Ph positive patients.¹⁵ The success of these drugs has raised hopes of such targeted therapies in other leukemias. Therefore, the understanding of the molecular pathways that are affected in each of these leukemias is of paramount importance.

It is important to note that although particular types of leukemia may respond well to treatment regimen, targeted or otherwise, there is frequently the emergence of drug-resistant clones following some years of therapy, which may lead to an aggressive relapse of the disease. The involvement of mutant long-term self-renewing stem cells in leukemia, as discussed earlier, is a likely cause of this frustrating clinical scenario. In CML, one study has shown that quiescent cells are more resistant to treatment with imatinib mesylate.⁷¹ Recently, Costello et al demonstrated that normal and leukemic CD34+/CD38− cells exhibited a decreased sensitivity to the chemotherapeutic drug, daunorubicin, as compared to CD34+/CD38+ cells. Another recent study showed that following treatment with the standard chemotherapeutic agent, cytosine arabinoside (Ara-C), the relatively quiescent AML LSCs represented the chemoresistant fraction of the tumor.⁷² Therefore, targeting of LSCs is now considered critical in the complete eradication of the disease. Recent studies have begun to address this in some detail. Work from John Dick's laboratory has shown that the inhibition of the CD44 antigen, which is expressed in high levels on AML LSCs, using anti-CD44 antibodies may inhibit engraftment of leukemic cells into humanized mouse recipients.⁷³ Moreover, treatment of mice engrafted with leukemia with this antibody may also lead to a significant reduction in disease burden, suggesting its clinical relevance. In AML, the sesquiterpene lactone parthenolide has been found to inhibit primitive AML cells in vitro and inhibit LSCs in NOD/SCID mice.⁷⁴

The inhibition of the aberrantly activated self-renewal pathways seems to be crucial to the elimination of LSCs. Emerging data in CML suggest that the targeted inactivation of the WNT signaling pathway in CML LSCs may be critical to anti-LSC therapies in that disease. In AML, the PTEN pathway has been recently implicated in the survival of LSCs. The treatment of AML leukemic blasts with rapamycin, an inhibitor of the PI3K/PTEN pathway, before or after engraftment has been shown to reduce the leukemic burden in secondary mice.⁷⁵ Several studies in AML have shown that the ablation of key components of the *HOXA* genes and their cofactors may inhibit leukemia propagation.^{76,77} The identification of downstream targets of this pathway and their inhibition may thus prove to impair leukemic self-renewal in AML.

Oncogenesis of Malignant Lymphoma

Programmed Genetic Changes of Antigen Receptor Genes during Normal Lymphocyte Development

The development of a functional immune response depends on the development of a highly diversified repertoire of antigen receptors expressed by B- and T-cells. This is achieved by means of programmed rearrangements of genes encoding for T- and B-cell receptors in early lymphoid progenitors.

These targeted rearrangements, which depend on the sequential expression of sets of genes committing lymphoid precursors to the B-, T- or NK-cell lineage, allow for the generation of a virtually unlimited variety of antigen-specific receptors by means of stochastic recombinations of a limited number of variable (V), diversity (D, present only in a part of receptor gene families) and joining (J) genes of the four T-cell receptor loci and the immunoglobulin heavy and light chain genes, respectively.^{78,79} In B-cells, two additional rounds of programmed genetic alterations, namely somatic hypermutation (SHM) and heavy chain switch recombination (CSR), happen at a later time during B-cell maturation in the germinal centers of peripheral lymphoid organs, resulting in the generation of high affinity antibodies of different immunoglobulin isotypes.

Generation of Antibody Diversity and B-Cell Lymphoma Development

Primary Immunoglobulin Gene Rearrangement

Following expression of genes leading to commitment to the B-cell lineage, the RAG complex is activated, initiating a strictly hierarchical sequence of genetic recombinations.⁸⁰ The first target is the immunoglobulin heavy chain locus (IGH) on 14q32, which consists of approximately 40–50 functional variable (V) genes in 7 families, 23 functional diversity (D) genes, 6 joining (J) genes, and 9 genes encoding for the constant regions of the B-cell receptor and secreted antibody molecules.⁷⁹ Rearrangements of antigen receptor genes are precisely targeted by recognition signal sequences (RSS), consisting of a palindromic heptamer and a nonamer separated by nonconserved 12 or 23 base pair spacer flanking the coding regions.⁸¹ In pro-B cells, in one of the two alleles of the immunoglobulin heavy chain locus, a D gene and a J gene are recombined by excision of the intervening DNA sequences, followed by a V-DJ joining. If this results in an in-frame sequence without stop codons, thus encoding for a potentially functional receptor protein, the process is followed by recombination of one of the kappa light chain alleles located on 2p11–12. On the other hand, if the resulting IGH rearrangement is nonfunctional, the second allele is activated. This principle is called allelic exclusion, explaining the fact that mature B-cells usually express only a single light chain molecule. Similarly, a non-functional rearrangement of the first IGK allele will result in activation of the second allele. If both kappa rearrangements are nonfunctional, the lambda light chain genes on 22p11 are rearranged. Rearrangement of the four T-cell receptor loci TCR δ (14q11), TCR γ (7q15), TCR β (7q34), and TCR α (14q11) takes place in a similar fashion, in this sequential order.⁸² Since malignant lymphomas are derived from a single transformed progenitor, the detection of clonal IG or TCR rearrangements is an important diagnostic tool in the molecular diagnosis of lymphoma. The techniques to determine clonality are discussed in more detail in Chap. 8.

Oncogene Activation Caused by Illegitimate Recombination during Immunoglobulin Gene Rearrangement

These programmed genetic changes sequentially taking place during lymphocyte maturation, however, present a risk factor for the development of oncogenic alterations, since they involve the generation of DNA double-strand breaks. The rejoining of double-strand breaks is not a fail-safe mechanism. It may lead to mis-joining with parts of other chromosomes, or to insertion of fragments of the IG genes into other genetic regions, resulting in transcriptional activation of oncogenes. Spatial proximity within the interphase nucleus, as well as DNA sequences with similarities to RSS sequences, seem to play a role for the frequency at which certain oncogenes are involved.^{83,84} Due to this specific susceptibility, non-Hodgkin malignant lymphomas, especially of the B-cell line (B-NHL), are characterized by a unique spectrum of genetic alterations, mainly translocations involving antigen receptor genes, which sets them apart from other types of neoplasms.^{1,85}

Translocations in lymphoma are usually recurrent, reciprocal, balanced translocations that involve exchange of chromosomal parts without apparent loss of genetic material. In B-NHL, the IGH locus at 14q32 is most commonly involved. Sometimes, cryptic deletions, inversions, or insertions may cause an identical disease phenotype without cytogenetically detectable involvement of the gene in question, which then requires FISH to identify the lesion.⁸⁶ In addition, some translocations may be cytogenetically silent due to their location close to the telomeric part of the involved chromosomes, such as the t(4;14)(p16;q32) in multiple myeloma.^{87,88} The involved oncogene usually is structurally normal, and the pathogenetic effect is due to inappropriate overexpression independent of regulatory signals, caused by the strong influence of juxtaposed immunoglobulin enhancer regions. The involved oncogenes may be at a large distance of 100 Mb or more from the breakpoint, sometimes making it difficult to identify the gene responsible for oncogenic transformation. Potentially, more than one oncogene may be deregulated by a single translocation. This is exemplified by the t(4;14) translocation in myeloma mentioned above, in which the translocation separates the strong 3' alpha and mu enhancers of the IGH locus onto two different chromosomes, resulting in overexpression of the fibroblast growth factor receptor 3 (*FGFR3*) and the *MMSET/WHSC10NSD2* gene.⁸⁹ Of interest, *FGFR3* is overexpressed in only 70–75% of t(4;14)+ cases, indicating that *FGFR3* is perhaps not the relevant target gene.⁹⁰ The *IG* light chain loci may also be involved in translocations, albeit at a much lower frequency, and account for some cases which are considered translocation-negative with standard detection assays. The best known examples of translocations involving IG light chains are the t(2;8)(p12;q24) and the t(8;22)

(q24;q11) in Burkitt lymphoma, accounting for 20–25% of *C-MYC* translocations.⁸⁵

Although the common recurrent translocation partners, such as *C-MYC*, *BCL-2*, and *CCND1* (*BCL-1*) have been recognized for a long time and make up for a significant proportion of translocations involving the immunoglobulin gene loci in B-NHL cases, there are a wide variety of less commonly found partner genes more recently identified by a variety of techniques. This is especially true for extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT)-type and multiple myeloma (MM), highlighting the fact that errors in IG gene receptor rearrangement are a dominant oncogenic mechanism in B-NHL.^{91–94} The more common translocation partners observed in lymphoma and the resulting diseases are listed in Table 1.3.

Although by virtue of their oncogenic genetic alterations, B-NHLs seem to be independent from the survival and proliferation signals mediated by appropriate B-cell receptor activation through antigen binding, combined with co-stimulatory signals provided by T-cells, many B-NHLs still show evidence for the importance of antigen for lymphoma development. Most mature B-NHLs carrying IGH translocations exhibit a functional rearrangement on the other allele, resulting in the expression of a B-cell receptor and immunoglobulin production. This indicates that functional B-cell receptor signaling is still required for the survival of many lymphoma cells, with the notable exception of classical Hodgkin lymphoma, which lacks detectable IG at the mRNA and protein level.⁹⁵

IGH Translocations may Often be Detected in the Absence of Clinical Disease

IG translocations are early events and represent necessary, but not sufficient, steps for the development of malignant lymphomas. Of note, the *BCL-2* translocation has been found in 25–60% of healthy elderly individuals using sensitive nested PCR techniques, whereas the *CCND1* (cyclin D1) translocation is much less common, occurring in only around 1% of probands.^{6,96} In addition, so-called follicular or mantle cell lymphoma “in situ,” consisting of cells with overexpression of *BCL-2* or cyclin D1 and the presence of the t(14;18) or t(11;14), respectively, as incidental finding limited to one or few B-cell follicles in lymph nodes removed for other reasons have recently been described.^{97,98} Some of these patients do not show evidence of clinical disease during a long follow-up period. Similarly, in MGUS, a common precursor lesion for MM detectable in approximately 3% of healthy individuals aged over 50, the recurrent translocations characteristic of MM may be observed by FISH in isolated plasma cells of MGUS patients at about the same frequency as in MM.⁹⁹ Since the risk of transformation is only about 1% per year, this again highlights the necessity of secondary alterations for the development of a malignant phenotype.

TABLE 1.3. Common nonrandom translocations in malignant lymphoma and myeloma.

Genetic aberration	Involved genes	Disease	Frequency	Function
t(14;18)(q32;q21)	IGH-BCL2	Follicular lymphoma Diffuse large cell lymphoma	70–85% 20–30%	Inhibition of apoptosis
t(11;14)(q13;q32)	IGH-BCL1 (CCND1)	Mantle cell lymphoma Multiple myeloma	>95% 20%	Deregulation of cell cycle, Rb phosphorylation
t(8;14)(q24;q32)	IGH-CMYC	Burkitt lymphoma	80% remaining cases	Transcription factor
t(8;22)(q24;q11)	IGK-CMYC			Cell proliferation
t(2;8)(p12;q24)	IGL-CMYC			
t(4;14)(p16;q32)	IGH-FGFR3/WHSC1	Multiple myeloma	10–15%	cell cycle deregulation, adhesion
t(6;14)(p21;q32)	IGH-Cyclin D3	Multiple myeloma	5%	Cell cycle deregulation
t(14;16)(q32;q23)	IGH-MAF	Multiple myeloma	<5%	Transcription factor
t(11;18)(q21;q21)	API2-MALT1 ^a	Extranodal marginal zone lymphoma	30–50% ^b	NFκB activation, inhibition of
t(14;18)(q32;q21)	IGH-MALT1	(MALT-lymphoma)	10% ^c	apoptosis
t(1;14)(p22;q32)	IGH-BCL10	MALT-lymphoma	Rare	Transcription factor
t(3;14)(p14.1;q32)	IGH-FOXP1	MALT-lymphoma, DLBCL	<10%	
Rearrangements of 3q27	BCL6	DLBCL FL grade 3B	30% 20–50%	Regulation of transcription
t(2;5)(p23;q35)	NPM-ALK ^a	Anaplastic large cell lymphoma	70–80%	Constitutively active ALK kinase,
t(1;2)(q21;p23)	TPM3-ALK ^a	ALK+ (ALCL)	10–20%	Induction of proliferation,
others	TFG, ATIC, CTLC ^a		Rare	anti-apoptosis

^aGeneration of chimeric fusion protein^bIn MALT lymphomas of lung and stomach^cIn MALT lymphomas of liver, lung, ocular adnexa

The Germinal Center Reaction and Lymphomagenesis

B-cells, which have undergone successful *IG* gene rearrangement and express a functional B-cell receptor on their surface are stimulated by the encounter with an appropriate antigen, resulting in production of IgM antibodies of low affinity, representing the primary immune response. Once the B-cell enters the germinal center, somatic hypermutation (SHM) is activated, targeting a region within about 1.5 kb of the promoter of the *IGH* and *IGL* genes.^{79,82} SHM represents introduction of replacement mutations in the variable, antigen-binding region of the immunoglobulin heavy and light chain genes. If this stochastic process leads to a higher antigen affinity of the hypervariable region, the B-cell is positively selected; otherwise, it undergoes programmed cell death. SHM is not entirely specific and affects non-*IG* genes, primarily *BCL6*.¹⁰⁰ SHM is thought to play a role both for the development of oncogenic point mutations, as well as for the induction of immunoglobulin translocations.^{101,102} Translocated oncogenes such as *C-MYC*, *BCL-6*, and *BCL-2* commonly show mutations, which are also thought to result from SHM and may further enhance the oncogenic property of the protein.¹⁰³ Furthermore, mutations caused by aberrant SHM are found in a variety of oncogenes in B-NHL, especially diffuse large B-cell lymphomas (DLBCLs).¹⁰⁴

Class switch recombination (CSR) replaces the constant region of the rearranged *IG* gene with another constant region segment, leading to a new *IG* isotype with different biological functions. Whereas the majority of translocations in B-NHL

seem to arise during primary *IG* gene rearrangement, the majority of *IGH* translocations in MM and other plasma cell neoplasms, which show promiscuous translocation partners, are the result of errors in switch recombination, mirroring their terminal state of differentiation.⁹³ A schematic representation of B-cell maturation and corresponding B-NHL is depicted in Figure 1.2.

A key molecule for induction of SHM, as well as CSR is activation induced deaminase (AID), an enzyme selectively expressed in germinal center B-cells.¹⁰⁵ AID induces U:G mismatches by deaminating cytidine nucleotides in the variable and switch regions, which are then processed and either generate a mutation in case of SHM, or a double strand break rejoined by nonhomologous end joining, in case of CSR. AID^{-/-} knock-out mice fail to develop translocations involving the Ig heavy chain locus, demonstrating the relevance of the SHM/CSR machinery for the development of B-NHL specific translocations.¹⁰⁶

Mutational Analysis of IGH Genes Captures the History of Malignant B-Cell Clones

SHM and CSR leave undeletable traces in the sequence of the immunoglobulin heavy chain genes, giving evidence of the molecular maturation state of the neoplastic B-cell. Lymphomas without SHM-induced mutations, such as the majority of mantle cell lymphoma and a subset of B-cell chronic lymphocytic leukemia (CLL), are regarded as pregerminal center lymphomas; neoplasms with evidence of subclones with different *IGH* sequences carrying additional point mutations indicative of ongoing SHM are called germinal center type lymphomas, exemplified by follicular

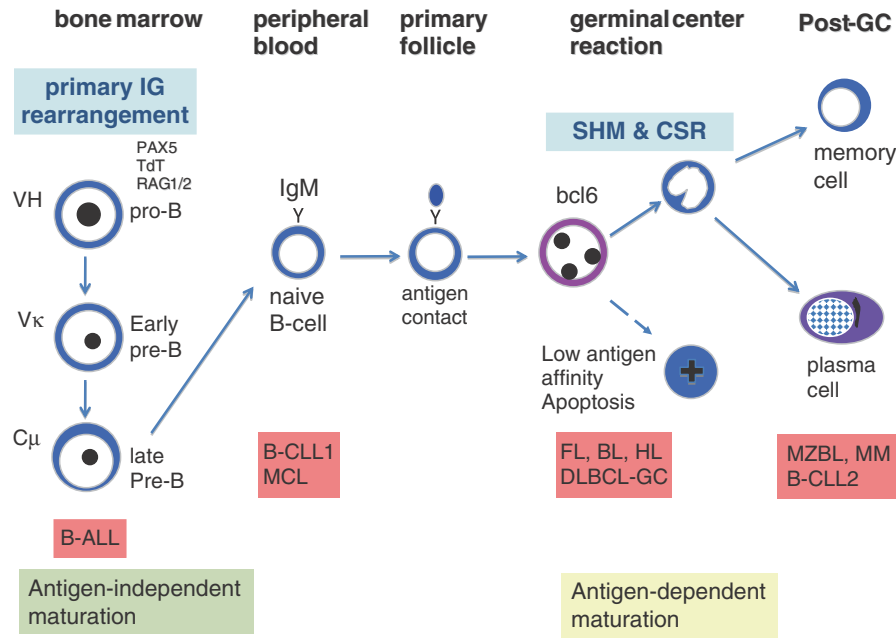


FIG. 1.2. Schematic representation of B-cell development and lymphomagenesis. *B-CLL* B-cell chronic lymphocytic leukemia, *MCL* mantle cell lymphoma, *FL* follicular lymphoma, *HL* Hodgkin lymphoma, *DLBCL-GC* diffuse large B-cell lymphoma germinal

center type, *BL* Burkitt lymphoma, *B-ALL* B-lymphoblastic leukemia, *MM* multiple myeloma, *MZBL* marginal zone B-cell lymphoma, *SHM* somatic hypermutation, *CSR* class switch recombination.

lymphoma; and tumors with somatic mutations, but lack of sequence variation, are designated postgerminal center lymphomas, such as MM or most DLBCLs. Of interest, some entities, which appear morphologically and phenotypically homogeneous, contain subsets of cases with mutated and unmutated *IGH* genes.^{101,102,107} In CLL, cases with evidence of SHM make up for about 50% and show a significantly better prognosis than CLL with germline *IGH* sequence.¹⁰⁸ Also see Chap. 16. Of note, analysis of the distribution of somatic mutations in the *IGH* genes shows possible evidence of antigen selection pressure or conservation of antibody structure in many mature B-NHLs (i.e., in MALT-type lymphomas), further emphasizing the role of B-cell receptor signaling in the development of B-NHL mentioned above.^{102,107}

Genetic Alterations in Peripheral T-Cell Lymphoma

Mature lymphomas derived from B-cells are much more common than those originating from T-cells. Although the exact reasons for this are not known, the three steps of programmed genetic alterations required for complete B-cell maturation – immunoglobulin heavy and light chain rearrangement, somatic hypermutation, and heavy chain rearrangement – in contrast to the single step of T-cell receptor rearrangement – present more chances for

B-cells to acquire oncogenic genetic alterations than T-cells. Consequently, translocations involving the *TCR* gene loci are much less common, compared to *IGH* rearrangements, but do occur in certain mature T-cell neoplasms. The *TCL-1* gene on 14q32.1 (or occasionally the related *MTCP1* gene) is activated through juxtaposition to *TCR* gene loci, most commonly in form of an inversion 14(q11;q32) or a t(14;14)(q11;q32) in T-cell prolymphocytic leukemia.^{109,110}

Overall, very little is known about specific genetic alterations in T-cell NHLs. Although mutations in commonly altered cancer genes, as well as activation of a variety of signaling pathways, have been described, only a few disease-causing or disease-specific recurrent alterations have been identified to date, with the notable exception of translocations involving anaplastic lymphoma kinase (*ALK*) in *ALK*-positive anaplastic large cell lymphoma (Table 1.3). This is one of the few examples from the group of malignant lymphomas, where formation of a chimeric fusion protein is considered the seminal oncogenic mutation. The nucleophosmin (*NPM*)-*ALK* translocation or one of its variants – a variety of partners have been identified at lower frequencies – leads to constitutive activation of the *ALK* tyrosine kinase through oligomerization mediated by the N-terminal oligomerization motif of *NPM*, with subsequent activation of downstream signaling pathways, such as the signal transducer and activator of transcription 3 (*STAT-3*) pathway and induction of the transcription factor *C/EBPβ*.^{111–113}

Oncogenes and Oncogenic Pathways in Malignant Lymphoma

Proto-oncogenes are normal cellular genes with the potential to contribute to neoplastic transformation, if they are overexpressed or mutated, resulting in aberrant function. They are involved in a variety of cellular processes, including proliferation and growth, differentiation, antiapoptosis, and induction of angiogenesis. Deregulation in hematological tumors occurs through the common mechanisms known from other neoplasms, including mutations, amplifications, and translocations, but the frequency distribution of molecular alterations is significantly different for malignant lymphomas. In general, genomic instability and, thus genetic complexity, is less in hematopoietic neoplasms, as compared to solid tumors. Many lymphomas and leukemias show a relatively simple karyotype with hallmark molecular alterations, which often are disease-specific, and may be used as molecular markers for specific diagnosis and follow-up. In lymphoma, this is mainly due to the impact of errors in antigen receptor gene recombination, which may lead to inappropriate expression of structurally normal genes. Formation of chimeric fusion genes with novel functions is relatively rare in lymphoma, in contrast to acute leukemias, with the notable exceptions of the t(2;5) and variant translocations in ALCL mentioned above, and the t(11;18), occurring in a subset of extranodal marginal B-cell lymphomas, which activates the *MALT1/MLT* gene.¹¹⁴ Gene amplifications resulting in an increased gene dosage are another common mechanism of oncogene activation, and examples include *BCL2*, *REL*, *CDK4*, and others.^{115,116} Activation of oncogenes or inactivation of tumor suppressor genes by point mutations or small insertions/deletions may be observed in two classes of genes in hematopoietic tumors: (1) genes which are found to be commonly altered in a broad variety of tumors, such as the RAS family genes or the TP53 tumor suppressor gene, indicating an important cell type-independent role in the maintenance of basic cellular growth-related functions and (2) genes which are mutated either in specific tumor entities or certain groups of hematological neoplasms, indicating a crucial dependence on cell type. Examples for this group are the *BCL6* gene in B-NHL and the *JAK2* V617F and *JAK2* exon 12 mutations in chronic myeloproliferative neoplasms.^{117–119}

The Properties of the Activated Oncogene Determine the Biologic and Clinical Behavior of Lymphomas

The function of protein deregulated by a translocation is an important determinant of the biological behavior of the resulting lymphoma, although secondary alterations modulate this behavior and may override the features of the primary oncogene, mostly toward a more aggressive phenotype.

In a simplistic way, two main groups of oncogenic alterations may be discerned in lymphoma: (1) inactivation of apoptosis and activation of survival pathways, which allows the

malignant cell to escape programmed cell death, which is the invariable fate of lymphocytes lacking survival signals through antigen receptor engagement and costimulatory signaling and (2) activation of cell proliferation and cell cycle deregulation. Lymphomas, in which the dominant alteration belongs to the first group, usually behave in an indolent way, with slow disease progression through constant accumulation of long-living tumor cells with low proliferation rate. In contrast, lymphomas in which alterations favoring rapid and uninhibited progression through the cell cycle are predominant, show an aggressive clinical behavior, but commonly respond well to therapeutic intervention. The following oncogenes and oncogenic pathways are commonly deregulated in malignant lymphoma and serve to explain the various mechanisms of tumor development and progression in these neoplasms.

BCL-2

The *BCL-2* (B-cell lymphoma 2) gene was originally detected through its involvement in the translocation t(14;18)(q32;q21) and encodes an antiapoptotic member of the bcl-2 family of proteins. It exerts its antiapoptotic influence through stabilization of the mitochondrial membrane by sequestering the pro-apoptotic family members. The t(14;18) is the hallmark lesion of follicular lymphoma, but is also found in about 30% of DLBCLs which show a germinal center-type expression profile.^{120,121} The techniques to detect this translocation are discussed in Chap. 9. It allows lymphoma cells to evade negative selection and to persist in the germinal center microenvironment. Follicular lymphoma usually is an indolent disease with long evolution, but a relatively high risk of secondary transformation due to the acquisition of secondary genetic alterations, mainly of tumor suppressor genes.¹²² Furthermore, bcl-2 protein is expressed in a variety of other lymphomas through different mechanisms, including gene amplification in DLBCL.¹¹⁵

CCND1 (Cyclin D1)

Cyclin D1, the product of the *CCND1* gene at 11q13 is a cell cycle protein dysregulated in most cases of mantle cell lymphoma and a subset of multiple myeloma through the t(11;14)(q13;q32) involving the *BCL-1* locus.^{123–125} The techniques to detect this translocation are discussed in Chap. 9. Although shortening of the cell cycle, phosphorylation of the retinoblastoma protein, and sequestration of the cyclin dependent kinase inhibitor (CDKI) p27/Kip-1 are thought to be the main oncogenic properties of cyclin D1, other non-katalytic effects of cyclin D1 are still suspected.^{126,127} Cyclin D1 overexpression alone is not lymphomagenic in mouse models,¹²⁸ and sh-RNA mediated suppression of cyclin D1 has only minor influence of cell proliferation in MCL cell lines.¹²⁹ Therefore, cyclin D1, similar to other deregulated

proteins, needs collaboration of other oncogenes. Of interest, translocations involving cyclin D2 in MCL and cyclin D3 in MM have revealed that other D-type cyclins may induce similar tumorigenic mechanisms.^{130,131}

C-MYC

C-MYC at 8q24 is a classic oncogene initially identified in Burkitt lymphoma. Of interest, the breakpoints on chromosome 8 vary between endemic and sporadically occurring cases, indicating different mechanisms of translocation.^{132,133} The techniques to detect a *C-MYC* translocation are discussed in Chap. 9. The effects of *C-MYC* overexpression are complex and only in part understood. *C-MYC* forms heterodimers with *MAX* and perturbs a variety of cellular processes leading to maximally stimulated proliferation. Of interest, *C-MYC* overexpression may induce apoptosis, and disruption of tumor suppressor genes, such as p53 mutations, p16 deletions, or promoter methylations are common secondary alterations, supposedly helping to circumvent induction of apoptosis. Burkitt lymphoma is characterized by the highest proliferation rate of all lymphoma subtypes, prominent apoptosis, and high sensitivity to chemotherapeutic agents. *C-MYC* is a common target of secondary translocations, involving both *IG* as well as non-*IG* loci. Whereas secondary *C-MYC* translocations involving the *IG* locus in B-NHL carrying other primary translocations result in very aggressive disease with poor prognosis, the impact of translocations into other chromosomal regions as commonly observed in MM is less clear.^{134–137}

BCL-6

BCL-6 is a sequence-specific repressor of transcription expressed only in germinal center B- and T-cells. *BCL-6* is necessary for the formation of germinal centers and allows B-cells to undergo the genetic changes of the germinal center reaction, namely SHM and CSR.^{117,119} Translocations involving *BCL-6* at 3q27, which are found in 20–40% of DLBCLs and a minority of follicular lymphomas lead to promoter substitution of the gene, thus aborting normal downregulation and permanent activation of the germinal center reaction, resulting in the arrest of neoplastic cell at this developmental stage.^{119,138} As mentioned earlier, *BCL-6* is commonly targeted by SHM, and mutations in the 5′-noncoding region of the gene are encountered in approximately 30% of GC and post-GC B-cells.¹⁰⁰ However, some of these mutations, which are also very common in DLBCL, may also lead to deregulated expression of bcl-6 protein and thus contribute to lymphomagenesis.^{104,119}

Deregulation of the NF-κB Pathway

Nuclear factor kappa-B (NF-κB) is a small family of inducible transcription factors, playing a pivotal role in the activation and survival of immune cells. Constitutive NF-κB activation is a hallmark lesion of classical Hodgkin lymphoma, which lacks functional B-cell receptor signaling.^{95,139} NF-κB activation occurs through a variety of mechanisms,

including aberrant activation of tyrosine kinases, oncogenic proteins of EBV, mutation of inhibitory protein IκBα, and c-REL amplification.¹³⁹ Mediastinal large B-cell lymphoma, and the so-called activated B-cell type of DLBCL identified by large-scale gene expression profiling, also show constitutive NF-κB activation and are characterized by a distinct set of genetic alterations.^{140,141} Constitutive NF-κB signaling is required for survival of the neoplastic cells.¹⁴² Another mechanism of NF-κB activation is found in extranodal B-cell lymphomas of MALT-type. Translocations t(11;18)(q21;q21), t(1;14)(p22;q32), and t(14;18)(q32;q21) involve the *MALT1* and *BCL-10* genes and are exclusively found in extranodal marginal zone B-cell lymphomas, and share as common functional motif the activation of NF-κB through self-oligomerization of MALT1 and BCL10, resulting in cell proliferation and survival.^{114,139,143–145}

Tumor Suppressor Genes in Lymphoma

Mutational and epigenetic inactivation of tumor suppressor genes is common in malignant lymphoma, in contrast to acute leukemia. The classical tumor suppressor genes and related pathways, which are found altered in a wide range of human tumors, such as *p53*, retinoblastoma protein (*RB*), and *p16/INK4a* show a high frequency of aberrations and are frequently associated with aggressive disease and high-grade transformation (i.e., in CLL, transformed follicular lymphoma, blastoid mantle cell lymphoma, MM, and peripheral T-cell lymphoma).^{146–148} Most aggressive lymphomas show mutational or deletional inactivation of either the p53 or the p16-RB pathways, and these mutations are usually mutually exclusive.¹⁴⁹ However, concurrent inactivation (i.e., by promoter methylation) results in a very aggressive course.^{148,150,151} Of special interest is the *ATM* (ataxia-telangiectasia mutated) tumor suppressor. Germ line truncating mutations in *ATM* lead to immunodeficiency chromosomal instability and an increased incidence of lymphoma with translocations involving antigen receptor loci. In addition to activating the DNA damage signaling pathways in response to double-strand breaks, activating cell cycle checkpoints, and apoptosis, *ATM* is directly involved in maintaining DNA ends in repair complexes, generated during antigen receptor rearrangements, and leads to the deletion of lymphocytes with free DNA ends, generated by failed end joining during VDJ recombination.^{152,153} This explains the unique association of *ATM* mutations with lymphoid malignancy. Of interest, in contrast to the inherited syndrome, most mutations in sporadic lymphomas are missense mutations. T-cell prolymphocytic leukemia, mantle cell lymphoma, and CLL are the neoplasms with the highest proportion of *ATM* mutations.¹⁵⁴

Inhibition of Death Receptor Signaling

FAS (CD95, Apo-1) is a death receptor of the TNF-receptor family and induces externally triggered cell death upon ligand binding, relevant for negative selection in the germinal center. Up to 20% of germinal center and postgerminal center

B-NHLs show *FAS* mutations, probably as result of aberrant SHM; however, other mechanisms, including downregulation, also play a role in deficient *FAS* signaling.¹⁵⁵

MicroRNA Deregulation

Another class of genetic alterations relevant for lymphomagenesis is deregulation of microRNA expression (miRNA). miRNAs are a class of short, noncoding mRNAs derived from larger precursor mRNAs that regulate the expression of target mRNAs by binding to partially matching sequences mainly, but not exclusively, in the 3' untranslated region (3'-UTR). Important examples for miRNA deregulation are the loss of miRNAs miR-15 and miR-16 in CLL showing deletion of the 13q14 region¹⁵⁶ and upregulation of miR-155 and its precursor mRNA BIC (B-cell integration cluster) in DLBCL and classical Hodgkin lymphoma.^{157,158} However, this field is rapidly evolving, and many of the published data so far on associations of miRNA deregulation and associations with clinical outcome need to be confirmed by other groups.

Infectious Agents Contributing to Lymphomagenesis

Infectious agents have been implicated as contributing factors for the development of malignant lymphoma based on several different mechanisms. Firstly, oncogenic viruses are present in the neoplastic cells of certain lymphoma subtypes. They can contribute to malignant transformation by insertional mutagenesis resulting in the activation of oncogenes. In most instances, however, the virus is present in episomic form and acts through expression of viral oncogenes. For the Epstein Barr virus (EBV) and human Herpes virus 8 (HHV8), two lymphotropic gamma Herpes viruses, and HTLV-1, a T-lymphotropic retrovirus, their oncogenic role in lymphomagenesis has been amply documented by epidemiological, molecular and functional evidence. Viral oncogenesis is described in detail in Chap. 7. Secondly, certain chronic bacterial and viral infections result in chronic immune stimulation with increased proliferative activity of lymphoid cells, thus indirectly increasing the risk for malignant transformation. Bacterial pathogens are involved in extranodal marginal-zone B-cell lymphomas of MALT-type, such as *Helicobacter pylori* in gastric MALT-lymphoma, and eradication of the infection can lead to tumor regression in a subset of cases. This highlights the fact that at least a fraction of these tumors are still dependent on chronic immune stimulation and pro-survival signaling provided by external sources, such as T-helper cells.^{159,160}

Conclusion

The use of systematic cytogenetic and molecular approaches to hematolymphoid malignancies has shown that there are a number of distinct, molecularly classifiable diseases with distinct

morphological, cytogenetic, and molecular characteristics. Very often, these characteristics may be useful in diagnosis as well as in determining prognosis, treatments, and their outcomes. Several deregulated molecular pathways have been identified in leukemia and lymphoma, and the dissections of these genetic and epigenetic changes that lead to this deregulation are underway. With the advent of new technologies that enable investigation of molecular pathways underlying leukemia initiation and progression, the stage has been set for the formulation of intelligent therapies targeting individual subsets of the disease. While some agents, such as imatinib mesylate and ATRA, have shown great promise, several key molecular targets and their clinically effective inhibitors remain to be discovered. In the coming years, the ever-increasing amount of information on the molecular oncogenesis of leukemia holds great promise for the invention of novel targeted drugs for the treatment of this disease.

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2

Genetic Predispositions for Hematologic and Lymphoid Disorders

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Introduction

A wide spectrum of inherited and sporadic genetic abnormalities predisposes individuals to increased risks of developing hematopoietic and lymphoid disorders. Each of these genetic abnormalities is associated with a syndrome with characteristic clinical and laboratory features. These predispositions to hematolymphoid disorders may be placed into one of two broad categories: (1) bone marrow (BM) failure syndromes; and (2) primary immune deficiency syndromes. The majority of individuals in either of these two groups will have an inherited genetic abnormality. The disorders predisposing to hematolymphoid neoplasias addressed in this chapter are presented in Table 2.1. An attempt was made to include in this table the processes where there is sufficient documentation of increased risks of developing hematologic or lymphoid neoplasms. This table also attempts to group the different hematolymphoid predisposition entities by major function of their mutated gene's normal counterpart. This is not an entirely satisfying approach. For example, patients with Dyskeratosis congenita (DC) and a mutation of *DKC1* will have an abnormal dyskerin, the normal counterpart of which is involved in RNA biogenesis and telomerase activity. Similarly, some disorders like Ataxia-telangiectasia (AT) or Wiskott-Aldrich syndrome are included in classifications of primary BM failure syndromes as well as in those of primary immune deficiency diseases. Space does not allow for providing an extensive discussion of the clinical and laboratory features of each of entities to be presented, but this information is available in many current reviews and texts.¹⁻⁴

Neurofibromatosis

Neurofibromatosis type 1 (NF1) is a disorder with clinical manifestations involving neural crest-derived tissues. Persons with NF1 neurofibromatosis, also known as von Recklinghausen disease, have multiple peripheral neurofibromas, café-au-lait spots, axillary and inguinal freckling, and iris

Lisch nodules.⁵⁻⁷ Individuals with NF1 may also develop scoliosis, pseudoarthrosis, and vertebral dysplasia. They are at a substantially increased risk for developing malignant neural crest-derived tumors including malignant peripheral nerve sheath tumor, gliomas, pheochromocytoma, gastrointestinal stromal tumor, and myeloid malignancies. Neurofibromatosis type 2 (NF2) is another disorder of nerve tissue but should not be confused with NF1. NF2 is characterized by bilateral vestibular schwannomas, with resulting hearing loss, tinnitus, and balance dysfunction. NF2 is also referred to as "central neurofibromatosis" to distinguish it from NF1 or "peripheral neurofibromatosis." Individuals with NF2 may also develop schwannomas of cranial and peripheral nerves, meningiomas, and rarely ependymomas, and astrocytomas but not hematolymphoid neoplasms. The diagnoses of NF1 and NF2 are based on clinical findings and family histories, but confirmatory molecular testing for both entities are available.

The prevalence of NF1 is placed at 1 in 3,000 individuals, making it one of the most common dominantly inherited disorders.⁸ Heterozygous mutations of the *NF1* gene, located on chromosome 17q11.2, are responsible for the majority of patients with NF1 neurofibromatosis. NF1 is inherited in an autosomal-dominant manner but half of affected persons have NF1 as the result a sporadic *NF1* gene mutation. The disease manifestations of *NF1* mutations are extremely variable, but café-au-lait spots are almost always present at birth and over 90% of individuals develop intertriginous freckling. Café-au-lait spots may also be observed in other processes not associated with NF1, such as Noonan syndrome (NS), DNA repair syndromes, McCune-Albright syndrome (large café-au-lait spots and polyostotic fibrous dysplasia), or an autosomal dominant process of multiple café-au-lait spots without other features of NF1. Manifestations of NF1 neurofibromatosis may be so slight in very young children as to escape clinical detection unless specifically looked for and, as discussed below, may be of clinical significance in some children who present with juvenile myelomonocytic leukemia (JMML). The clinical variability of NF1 results from a combination of genetic, nongenetic, and stochastic factors.

TABLE 2.1. Inherited and sporadic genetic conditions predisposing to the development of hematologic and lymphoid neoplasia.

Syndrome	Mechanism	Inheritance	Altered gene	Associated hematopoietic and/or lymphoid neoplasia
<i>Signal transduction</i>				
Neurofibromatosis		AR, S	<i>NF1</i>	JMML, MDS, AML, (ALL, NHL) ^a
Noonan's syndrome		AD, AR, S	<i>PTPN11</i> , <i>KRAS</i> , <i>SOS1</i> , <i>RAF1</i>	JMML, ALL
<i>DNA repair</i>				
Fanconi anemia		AR, XL ^b	<i>FANC</i> genes (see Table 2.3)	MDS, AML, (ALL) ^a
Bloom's syndrome		AR	<i>BLM</i>	DLBCL, FL, HL, ALL, AML, MDS ^c
Nijmegen breakage syndrome		AR	<i>NBS1</i> (~50%) ^d	DLBCL, BL, HL, T-ALL/LL, B-ALL
Ataxia telangiectasia		AR	<i>ATM</i>	TCL, T-PLL, T-ALL, NHL, HL, (AML) ^a
Seckel syndrome ^e		AR	<i>ATR</i>	(AML) ^a
Dubowitz ^e		AR	? gene(s)	(AL, NHL) ^a
<i>Defective telomerase activity</i>				
Dyskeratosis congenita		XL, AR, AD	<i>DKC1</i> (others see Table 2.4)	MDS, ET, PNH
Cartilage-hair hypoplasia ^e		AR	<i>RMRP</i>	NHL, (?)AML
<i>RNA biogenesis</i>				
Shwachman–Diamond syndrome		AR, S	<i>SDBS</i>	MDS, JMML, AML, (ALL) ^a
Blackfan–Diamond anemia		AD, AR, S	Multiple, see Table 2.5	MDS, AML, ALL, NHL, HL
<i>Primary immuno-deficiency states</i>				
X-linked lymphoproliferative disorder		XL	<i>SH2D1A</i>	EBV-related BL and DLBCL, (HL, T-ALL) ^a
Hyper-IgM syndromes		XL	<i>XIAP</i>	Not known
		XL	<i>CD40L</i>	HL, (MALT, LGLL, TCL) ^a
Hyper-IgE		AR	<i>CD40</i> , <i>AID</i> , <i>UGO</i> , <i>NEMO</i>	
		S, AD, AR	<i>STAT3</i>	DLBCL, BL, (MCL, HL, TCL, ALCL) ^a
Common variable immune deficiency		AR	<i>TYK2</i>	Not known
Wiskott–Aldrich syndrome		S, AR, AD, XL	<i>TNFR</i> , <i>ICOS</i> , <i>CD19</i>	MALT, DLBCL, BL, (HL, PEL, TCL) ^a
Severe combined immune deficiency		XL	<i>WAS</i>	DLBCL, (BL, HL, TCL, FL) ^a
		R	<i>ADA</i>	EBV-associated lymphomas ^f
<i>Apoptotic defect</i>				
Autoimmune lymphoproliferative syndrome		AD	<i>FAS</i> , <i>FASLG</i> , <i>CASP10</i>	HL, DLBCL, BL, FL, MZL, TCRBCL ^g
<i>Granulopoiesis</i>				
Congenital neutropenic syndromes				
Severe congenital neutropenia		AD, S	<i>ELA2</i> , others see Table 2.9	MDS, AML
Kostmann syndrome		AR	<i>HAX1</i>	MDS, AML
Cyclic neutropenia		AD	<i>ELA2</i>	No associations
<i>Megakaryopoiesis</i>				
Congenital amegakaryocytic thrombocytopenia ^e		AR	<i>MPL</i>	AML
Amegakaryocytic thrombocytopenia with radioulnar synostosis ^e		AD	<i>HOX11</i>	AML
Familial platelet disorder with associated AML ^e ? gene		AD	<i>AML1</i>	MDS, AML
Thrombocytopenia with absent radii ^e		?AR		AML

AD autosomal dominant, AR autosomal recessive, XL X-linked, S sporadic, JMML juvenile myelomonocytic leukemia, MDS myelodysplastic syndrome, AML acute myeloid leukemia, ALL acute lymphoblastic leukemia, NHL non-Hodgkin lymphoma, DLBCL diffuse large B-cell lymphoma, FL follicular lymphoma, HL Hodgkin lymphoma, BL Burkitt lymphoma, TCL T-cell lymphoma, T-PLL T-cell prolymphocytic leukemia, ET essential thrombocythemia, PNH paroxysmal nocturnal hemoglobinuria, EBV Epstein–Barr virus, MALT mucosal-associated lymphoid tissue lymphoma, LGLL large granular lymphocytic leukemia, MCL mantle cell lymphoma, ALCL anaplastic large cell lymphoma, PEL primary effusion lymphoma, MZL mantle zone lymphoma, TCRBCL T-cell rich B-cell lymphoma.

^a Uncommon associated neoplastic conditions are in parenthesis.

^b Only BRCA2, also known as FANCD1, is X-linked inherited.

^c MDS and some AML cases in Blooms' syndrome are thought to be secondary to another cancer treated with chemotherapy and/or radiation.

^d The genetic abnormality is not known in approximately 50% of Nijmegen syndrome patients.

^e Rare disorder, not discussed in text.

^f Not discussed in text. Reported instances of lymphoma have largely been in patients following bone marrow transplantation.

^g Only patients with mutations of *FAS* are associated with lymphoma.

For example, whole deletion of *NFI* is associated with early appearance of cutaneous neurofibromas, and more cognitive abnormalities and 3-base pair in-frame mutations of exon 17 are associated with typical café-au-lait spots without subcutaneous neurofibromas.

Molecular Pathogenesis of NF1

The *NF1* gene is very large consisting of ~350 kb and 60 exons.⁹ Four introns separate the gene into four major regions. Intron 27b contains the coding sequences for three other genes, *OMGP*, *EVI2B*, and *EVI2A*, which are all transcribed in the opposite orientation to *NF1*. The *NF1* gene encodes for cytoplasmic neurofibromin, which is a 327 kD peptide of 2818 amino acids. Neurofibromin is a member of the GTPase-activating protein family and inhibits RAS signaling by hydrolysis of active RAS-GTP to inactive RAS-GDP (Figure 2.1).^{10,11} The peptide domain encoded by exons 21 to 27a activate the GTPase of RAS proteins. Neurofibromin also appears to regulate adenyl cyclase activity, and intracellular cyclic AMP generation.

More than 800 different mutations of *NFI* have been identified. Mutations occur over the entire gene without any single group of mutations accounting for the majority of patients. Mutations may be due to amino acid substitutions, stop codon mutations, insertions, deletions, intronic alteration affecting splicing, and chromosomal rearrangements. More than 80% of germline mutations result in marked truncation of the gene product. Mutations of *NFI* result in loss-of-function and, hence, an increase in RAS activity. Thus, mutations of *NFI* are the functional equivalents of *RAS* activating mutations.

Hematolymphoid Disorders Associated with NF1

Individuals with NF1 are at increased risk of developing myeloid neoplasms, principally JMML, and also myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML).^{12,13} Increased risk of acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma was reported in one study.¹⁴ The predisposition to myeloid malignancies is restricted to childhood, and boys are more affected than girls. Although JMML is an uncommon complication of children with NF1, approximately 14% JMML patients have NF1.¹⁵

JMML is a clonal hematopoietic disorder of children, characterized by myelocytic and monocytic proliferations.¹⁶ Typically, these children present with marked hepatosplenomegaly, increased white blood cell counts with an absolute monocytosis ($>1 \times 10^9/\text{L}$), anemia, and thrombocytopenia.¹⁵ Leukemic skin infiltrates are not uncommon. Mutations of *NRAS/KRAS*, or *PTPN11*, or inactivation of *NF1* are present in ~25, ~35, and ~30% of patients with JMML, respectively.^{17,18} A key diagnostic finding in JMML is hypersensitivity of the leukemic progenitor cells to low concentrations of granulocyte-macrophage colony-stimulating factor (GM-CSF), as a result of deregulated signaling through the RAS pathway.¹⁹ RAS proteins are signal switch molecules that regulate cell proliferation in response to extracellular stimuli by cycling between an active guanosine triphosphate (GTP)-bound state and an inactive guanosine diphosphate (GDP) state (Figure 2.1). RAS mutations in myeloid malignancies introduce amino acid substitutions, that result in accumulation of GTP-bound RAS and, thus, increased RAS activity. As discussed above, mutations of *NF1* result in increased RAS activity. Mutations of *PTPN11* associated with NS also increase RAS activity.

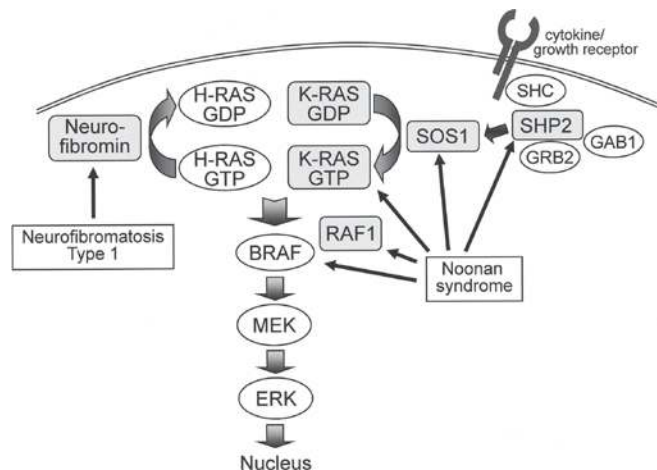


Fig. 2.1. The RAS/ERK/MAPK pathway with proteins involved by genetic defects in Neurofibromatosis 1 and Noonan syndrome (NS). The RAS/ERK/MAPK signaling pathway plays a crucial role in cell proliferation, differentiation, and survival in response to various growth factors and cytokines. The *NF1* gene encodes for cytoplasmic neurofibromin, a member of the GTPase-activating protein family that inhibits RAS signaling by hydrolysis of active

RAS-GTP to inactive RAS-GDP. RAS activity is increased in individuals with germline mutations of *NF1*. The majority of patients with NS have a germline mutation of *PTPN11* that encodes for SHP-2 which also plays a key role in the RAS-MAPK pathway. Other genes implicated in NS include *KRAS*, *SOS1*, *RAF1* and *BRAF* that encode for proteins known to be regulatory components or the RAS pathway.

In children with NF1 and JMML, RAS hyperactivity is due to inactivation of both alleles of the *NF1*.^{17,20,21} Similarly, JMML in non-NF1 patients may also be associated with biallelic defects of *NF1*.¹ Heterozygous *NF1* mutant mice are susceptible to fibrosarcoma and pheochromocytoma; with spontaneous somatic loss of the normal *NF1* allele, they also develop a JMML-like myeloproliferative disorder.²² Thus, *NF1* appears to function as a tumor-suppressor gene, which regulates myeloid growth through its effect on RAS. Monosomy 7, 7q deletions, and other chromosomal abnormalities are present in ~40% of patients but occur independently of *PTPN11*, *NRAS/KRAS*, and *NF1* mutations.^{18,23} It is not presently clear whether loss of *NF1* is sufficient to produce JMML or if additional genetic abnormalities are required.

Tests for NF1

DNA testing for *NF1* mutations is not necessary in patients fulfilling the diagnostic clinical criteria for NF1.²³ Presently, detection of specific *NF1* mutations is not predictive for development of JMML in NF1 patients. As mentioned earlier, some children that develop JMML display very few of the signs and no family history of NF1 while having a germline mutation of *NF1*.¹⁸ Patients with NF1 neurofibromatosis may present with various cutaneous manifestations, including juvenile xanthogranuloma (JXG).^{24–26} Children with NF1 and JXG have an increased incidence of JMML. Thus, studies of *NF1* may be warranted in young children to distinguish de novo JMML from NF1-related JMML.

Existing testing procedures for *NF1* are time-consuming and expensive. Identification of *NF1* mutations is challenging, due to the gene very large size, absence of localized mutation clustering, wide array of mutation types, and the presence of highly homologous partial *NF1* pseudogene-like sequences in the human genome.²⁷ Tests include protein truncation test that detects ~80% mutations, single-strand conformational polymorphism, denaturing gradient gel electrophoresis, denaturing high-performance liquid chromatography, long range RT-PCR, and fluorescence in situ hybridization.^{23,28–31} However, DNA sequence analysis is required to confirm the nature of the specific mutation. LOH and array CGH are also valid methods for detecting large genomic deletions.^{20,21}

Gene microarray expression profiling may identify gene expressions or pathway alterations that are associated with the development of myeloid neoplasms in NF1.

Noonan Syndrome

Individuals with NS have short stature, characteristic facies with ptosis, low-set ears, hypertelorism, and webbed, short necks, resembling Turner syndrome.^{32–34} Mild mental retardation is relatively common, and congenital heart defects affect 50–80% of these individuals. Up to 20% of NS individuals have generalized or peripheral lymphedema, or pulmonary or intestinal lymphangiectasia. Coagulation problems are described in over one-third of those with NS. The diagnosis may be difficult, due to the varied clinical presentations and the regression of some clinical features with increasing age.³⁴ Thus, several scoring systems have been proposed to aid in the clinical diagnosis.^{35,36} With the fairly recent identification of the genetic defects underlying NS, molecular testing should allow for better diagnosis and possibly prognostic assessment.

Molecular Pathogenesis of NS

NS is predominantly an inherited autosomal dominant disorder, but autosomal recessive and sporadic forms are also described.^{34,37–39} Up to 60% of familial and 37% of sporadic cases have a mutation of *PTPN11* located on chromosome 12q24.^{40–42} The majority of NS individuals with no mutation of *PTPN11* will have mutations of *KRAS*, *SOS1*, *RAF1*, or *BRAF* (Table 2.2).^{32,43–46} The *PTPN11* gene encodes for SHP-2, a protein tyrosine phosphatase (PTP) with a cytoplasmic domain that functions as a signal transducer (Figure 2.1). SHP-2 contains a single PTP domain and two tandem Src homology 2 domains, N-SH2 and C-SH2. The SH2 domains function as phosphor-tyrosine-binding domains and mediate the interaction of SHP-2 with its substrates. SHP-2 is widely expressed in fetal and adult tissues and plays a key role in the RAS-MAPK (mitogen-activated protein kinase) signaling cascade, that controls cell proliferation, differentiation, and survival in response to various growth factors and cytokines.

TABLE 2.2. Genes associated with Noonan syndrome.

Gene	Gene name	Chromosome	Protein	% of patients with mutation ^{39–46}
<i>PTPN11</i>	Protein tyrosine phosphatase, nonreceptor type 11	12q24.1	SHP-2	~50
<i>KRAS</i>	v-Ki-ras2/Kirsten rat sarcoma viral oncogene homolog	12p12.1	K-RAS	<5
<i>SOS1</i>	Sons of sevenless homolog 1	2p22.1-p21	SOS1	10–15
<i>RAF1</i>	RAF proto-oncogene serine-threonine-protein kinase	3p25	RAF1	10–15
<i>BRAF</i>	V-raf murine sarcoma viral oncogenes homolog B1	7q34	B-RAF	<2
Unknown	?	?	?	5–10

The close interaction of between the N-SH2 and PTP domains keep this phosphatase in an autoinhibited, closed conformation (Figure 2.2). When SHP domains bind to phosphotyrosine motifs, the closed conformation is opened with release of catalytic domain and subsequent facilitation of RAS-MAPK signaling. The majority of *PTPN11* mutations in NS result in amino acid substitutions at the interface between the N-terminal Src homology 2 (N-SH2) and catalytic PTP domains (Figure 2.2).⁴⁷ These mutations are predicted to promote SHP-2 gain-of-function by interfering with the switch between the active and inactive conformation of the protein, resulting in a shift to the active form.^{41,48,49} One-half of the cases of NS are caused by gain-of-function mutations of *PTPN11*.^{34,48} Approximately, 75% of mutations reside in exons 3 or 8; the remainder, largely in exons 4, 7, and 13.^{34,48} One mutation, N308D, is responsible for up to one-third of cases.

Similarly, *KRAS*, *SOS1*, *RAF1*, and *BRAF* are also key components of the RAS/ERK/MAPK signaling pathway (Figure 2.1).⁴³⁻⁴⁶ Mutations of *KRAS* result in gain-of-function. RAS proteins regulate cell activity by cycling between active GTP-bound and inactive GDP-bound conformations. *SOS1* (son of seven less homology 1) has two RAS binding domains. *SOS1* missense mutations cluster at codons, encoding residues

implicated in maintenance of *SOS1* in its autoinhibited form. Thus, *SOS1* mutations release the autoinhibition, and thereby increase and prolong RAS activation.

Hematolymphoid Disorders Associated with NS

Individuals with NS may have a variety of hematologic abnormalities, including amegakaryocytic thrombocytopoenia, platelet functional defects, bleeding problems related to factor (i.e., VIII, XI, X11, and protein C) deficiencies, von Willebrand disease, pancytopenia with a hypercellular BM, and hematolymphoid malignancies.^{38,50-55} Children with NS are at increased risk for JMML and ALL.^{53,56} The 218C>T mutation of *PTPN11* is associated with a predisposition to a myeloproliferative disorder that may resolve spontaneously.^{34,39,53} Mutations of *PTPN11* at codons 61, 71, 72, and 76 are associated with an increased risk of developing JMML. In a study of *PTPN11* mutations in seven children with NS and JMML, germline missense mutations were found in all cases.⁵⁶ Five of the seven cases JMML in NS patients had a Thr73Ile substitution. Similar findings were observed in another study, in which 8 of 19 NS patients with JMML carried the Thr73Ile substitution.⁵³ Interestingly, non-NS patients with JMML may have a poorer prognosis

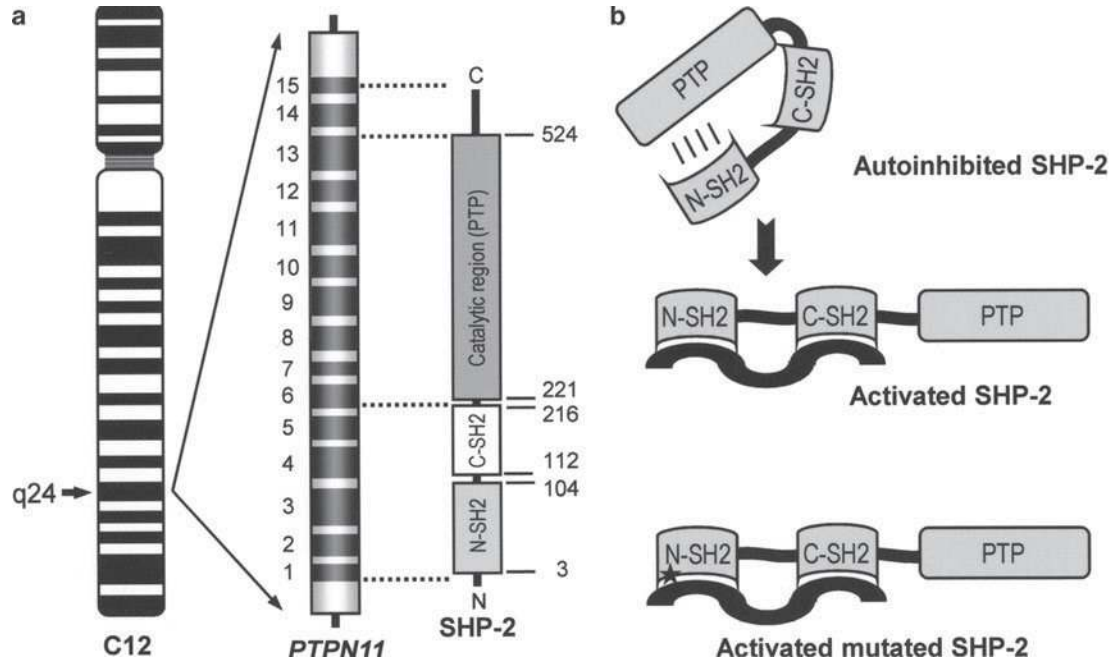


FIG. 2.2. The formation and function of SHP-2. (a) The *PTPN11* gene is located on the q arm of chromosome 12 (C12) and encodes for SHP-2. The SHP-2 protein is a tyrosine phosphatase, which contains a single protein tyrosine phosphatase (PTP) and two tandem Src-homology-2 (N-SH2 and C-SH2) domains. (b) The close interaction between the N-SH2 and PTP domains keep this

phosphatase in an autoinhibited, closed conformation. Binding to a phosphotyrosine results in an open, activated PTP domain. (c) The majority of *PTPN11* mutations in Noonan syndrome individuals produce amino acid substitutions in N-SH2 or PTP that are predicted to result in an “unfolding” of SHP-2 and hence a gain-of-function.

than NS patients with JMML.^{53,57} ALL is markedly less common than JMML in NS.^{54,55} Little has been reported on the mutations of *PTPN11* in these patients. In one study of six NS patients with ALL, a germline mutation affecting exon 3 was found in one patient.⁵⁵

Abnormalities of *PTPN11* have been extensively studied in hematolymphoid disorders of non-NS patients. Investigations of nonsyndromic children with JMML suggest that *PTPN11* mutations play a significant role in the genesis of this neoplasm. In a study of 62 samples of JMML, 34% had missense *PTPN11* somatic mutations in exons 3 and 13.⁵⁶ In other studies of pediatric myeloid neoplasms, including JMML and AML, mutations of *PTPN11* were identified in 35 and 4%, respectively.^{58–60} The frequency of *PTPN11* mutations in adult MDS, AML, and chronic myelomonocytic leukemia (CMML) is lower than that observed in pediatric cases.^{61–63} Mutations of *PTPN11* are present in less than 2% of adult AMLs and in very few CMMLs. Studies of ALL in non-NS patients found *PTPN11* mutation in 7% of cases, all of precursor B-cell type.^{58,59,64} The types of mutations in ALL are like those of JMML, consisting of missense changes affecting exons 3 and 13 of *PTPN11*. Chromosome hyperdiploidy may be observed in ALL in NS patients, but the common nonrandom chromosomal translocations associated with pediatric ALL are conspicuously absent.

The *PTPN11* mutation (resulting in a Thr73Leu substitution common to NS individuals) is not found in non-NS JMML patients. Furthermore, the clinical features of JMML in patients with NS differ from non-NS JMML patients. Patient with NS and JMML are younger and may even present at birth; their clinical course is commonly less aggressive.^{52,53,65,66} The overall survival rate for non-NS JMML patients is less than 20%⁶⁶; whereas, NS patients with JMML commonly show improvement or spontaneous remissions.^{34,50,67,68} Although the clinical course is usually relatively benign for JMML in NS patients, some may have a very aggressive course, and transformations to AML have been reported.^{50,60} Several investigators hypothesize that the genotype/phenotype relationship observed in patients with somatic and germline *PTPN11* mutations may be due to different gain-of-function effects of the altered SHP-2. Consistent with this hypothesis, somatic JMML-associated *PTPN11* mutations are predicted to have strong gain-of-function effect, while germline mutations in NS-associated JMML would have weaker hematologic effects, possibly resulting in milder myeloproliferative processes.^{53,57}

Tests for NS

The confirmation of a suspected diagnosis of NS may be established in over 90% of individuals by molecular testing for germline mutations of *PTPN11*, *KRAS*, *SOS1*, *RAF1*, and *BRAF* (Table 2.2). The tissue (or cells) used for testing should not be involved by a neoplastic process. A stepwise testing

approach for diagnosis is recommended.³² This consists of an initial sequence analysis of exons 3, 8, 9, and 13 of *PTPN11*, and if not informative, then perform sequence analysis of exons 1–23 of *SOS1*. Approximately 60–75% of individuals with NS will have a detectable mutation with these initial analyses. If no mutation is identified in *PTPN11* or *SOS1*, sequence analysis of the remaining 11 exons of *PTPN11* and exons 7, 14, and 17 of *RAF1* should be performed. If the preceding investigations are negative, sequence analysis of the remaining exons of *RAF1* and exons 1–6 of *KRAS* should be carried out. Rare individuals with NS may have partial or whole gene deletions of *PTPN11* that require a different testing approach, such as array CGH. Upwards of 5–10% of individuals with clinical features of NS will have no detectable abnormalities of the four genes presently associated with NS, either because they harbor another genetic abnormality yet to be associated with NS, or possibly they have a different syndrome that closely resembles NS.

Some individuals may have very subtle features of NS and their diagnosis will not be suspected until they present with JMML or another neoplastic process. The diagnosis of NS in these persons requires testing of tissues or cells not involved by the proliferative process. One-third of nonsyndromic patients with JMML and lesser numbers of patients with de novo MDS or AML have a mutation of *PTPN11*.⁵⁶ However, the molecular lesions of *PTPN11* in de novo and NS-associated JMML, MDS, and AML are mutually exclusive.⁵⁶

Fanconi Anemia

Fanconi anemia (FA) is an inherited disorder characterized by physical abnormalities and progressive BM failure. The classic anomalies of FA include a short stature, abnormal thumbs, microcephaly, café-au-lait, and hypopigmented spots, and characteristic facies consisting of epicanthal folds, broad nasal base, and micrognathia.^{69–71} The list of other external dysmorphic features associated with FA is extensive and one is referred to reviews of FA.^{69,70} Additionally, most patients have one or more structural or functional abnormalities of kidneys, genitalia, gastrointestinal tract, heart, internal ear, lung, and bones.^{69,70} However, as many as 25–40% of patients (usually older children and adults) appear entirely normal.^{69,70} Persons with FA also display cellular hypersensitivity to interstrand DNA cross-linking agents, such as cisplatin and melphan.^{69,72} Evidence of hematopoietic failure manifests in 90% of patients by the fifth decade of life.⁷³ The hematologic presentations are highly variable and include hematolymphoid malignancies.

Patients with FA are also at increased risk of developing solid tumors.^{73–78} About 28% of patients develop a nonhematologic malignancy by 40 years of age.⁷³ The most frequent solid tumors are squamous cell carcinomas of the upper respiratory and gastrointestinal tracts, followed by

carcinomas of the vulva and uterine cervix. In one study of patients with FA, a high rate of papillomavirus infections preceded the development of cervical or vulvar squamous cell carcinoma, but this was not confirmed in another study.^{73,79} Liver tumors, including adenomas, hepatoma, and hepatocellular carcinoma, are also relatively common. Most patients with liver tumors are in their third decade of life, and most have received androgen therapy for aplastic anemia.^{80,81} Heterozygous carriers of mutations in FA genes are not at increased risk for cancer except for *BRCA2*, whose heterozygous carriers are susceptible to breast and ovarian cancers.⁸²

Molecular Pathogenesis of FA

FA is inherited in an autosomal-recessive manner except for mutations of *FANCB*, which is inherited in an X-linked manner. The prevalence is thought to be 1 to 5 per million and the heterozygous carrier frequency is estimated at 1 in 300 but may be higher.^{72,73,83} Mutations of FANC genes are particularly high in Ashkenazi Jews, South African Afrikaans, Spanish gypsies, and sub-Saharan Africans.^{84–87} Thirteen FA complementation groups, based on somatic cell fusion studies, have been identified (Table 2.3). These cooperate in a pathway called the “FA-BRCA pathway/network.”^{83,88–91} The protein products of *FANCA* and *FANCC*, the products of genes *FANCB*, *FANCE*, *FANCF*, *FANCG*, *FANCL*, and *FANCM*, and FAAP24 and FAAP100 form the “FA core complex,” a nuclear multisubunit ubiquitin ligase complex (Figure 2.3).^{70,83} This complex, through the E3 ubiquitin ligase activity of FANCL, mediates monoubiquitination of FANCD2 and FANCI, during normal S phase or in response to DNA cross-link damage.^{90,92} Ubiquitination is a post translational modification in which ubiquitin, a 76-residue protein, is covalently attached to a target protein. Monoubiquitination confers signal regulating of protein targeting, membrane trafficking, histone function, transcriptional regulation, or DNA

repair. Monoubiquitination of FANCD2 results in a complex with other FA proteins, including BRCA2, BRCA1, PALB2, RAD1, FANCI, and other proteins, which in turn leads to the formation of DNA damage repair foci.^{90,92} This nuclear complex protects the cell from DNA cross-linking and participates in DNA repair.^{90–93} The vast majority of patients with FA have mutations of FANCA, C, or G (Table 2.3).^{83,94} Mutations of FANCD2 affect cells by a different mechanism downstream of the FANC complex (see Figure 2.3). Mutations of *FANCI* and *FANCI* also result in a defective monoubiquitination of FANCD2.

Hematologic Disorders Associated with FA

The hematologic presentations in FA patients are highly variable.^{69–71} Most patients have mild to moderate thrombocytopenia and/or leucopenia, which slowly progresses to pancytopenia. Erythrocytes are frequently macrocytic with or without mild anisopoikilocytosis, even in the absence of significant anemia. The BM may initially be normocellular, but with time progresses to a mix of focal hypocellular and hypercellular areas. Increasing pancytopenia is reflected by marked BM hypocellularity or aplasia with relative increases of stromal cells, mast cells, mature lymphocytes, and plasma cells. The BM findings may be identical to patients with aplastic anemia of different etiologies. Erythropoiesis may be left-shifted and display megaloblastoid features. Fetal hemoglobin and serum erythropoietic levels may be increased. Some individuals with FA, usually a family member of another FA patient, have neither physical nor hematologic abnormalities. Several retrospective studies have shown that the type and number of congenital abnormalities are predictive of BM failure.^{76–78,95} Patients with abnormal radii have an increased risk of developing BM failure. However, independent of abnormal radii, the risk of developing BM failure is increased with the number of heart, kidney, head, hearing, and developmental defects.

TABLE 2.3. Genes involved in Fanconi anemia and percent of patients with an associated mutation.^{70,78,83,90,91}

Gene	Chromosome	Protein name	% of patients with a mutation
<i>FANCA</i>	16q24.3	FA group A protein	55–70
<i>FANCB</i>	Xp22.2	FA group B protein	<1
<i>FANCC</i>	9q22.3	FA group C protein	8–15
<i>BRCA2/FANCD1</i>	13q12.3	Breast cancer type 2 susceptibility	3–4
<i>FANCD2</i>	3p25.3	FA group D2 protein	3
<i>FANCE</i>	6p21.3	FA group E protein	1–3
<i>FANCF</i>	11p15	FA group F protein	2
<i>FANCG</i>	9p13	FA group G protein	8–10
<i>FANCI</i>	15q26.1	FA group I protein	Rare
<i>FANCI/BRIP1</i>	17q23.1	FA group J protein (partner of BCRA1)	~2
<i>FANCL</i>	2p16.1	E3 ubiquitin-protein ligase	<1
<i>FANCM</i>	14q21.3	FA group M protein	Rare
<i>PALB2/FANCN</i>	16p12.1	Partner and localizer for BRCA2	<1

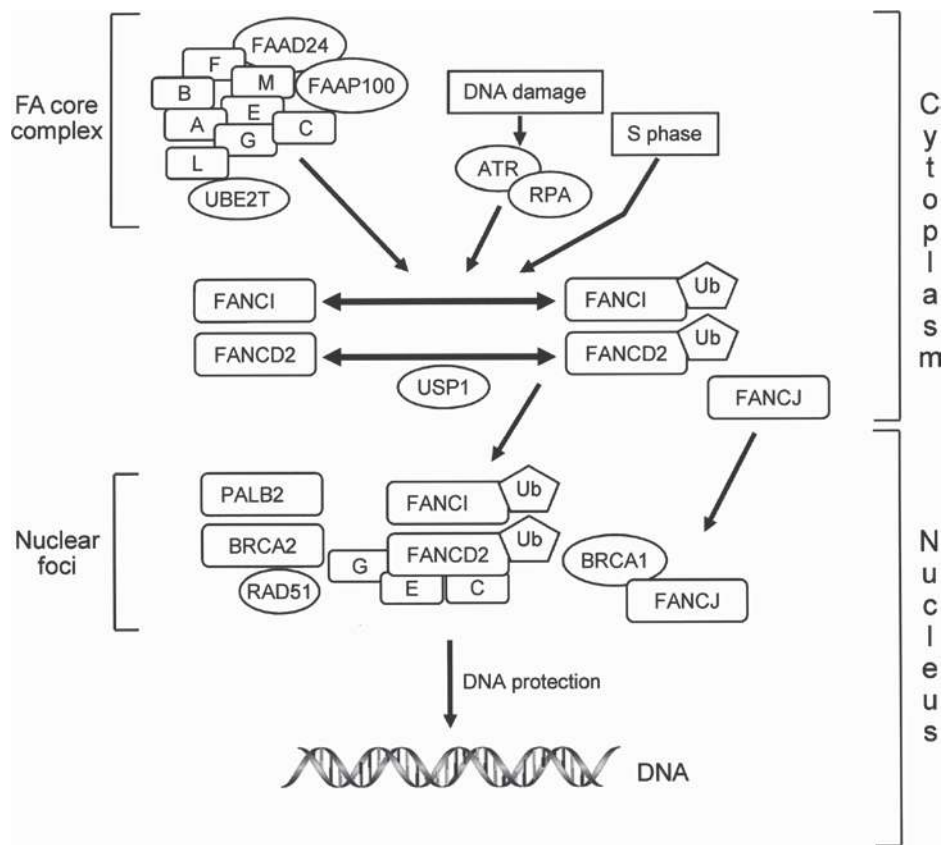


FIG. 2.3. Schematic of the activation cascade of the Fanconi anemia (FA) pathway (adapted from refs.^{70,83,90,91}). A large nuclear complex of 8 FA proteins (FANCA, -B, -C, -E, -F, -G, -L, and -M), a FANCM-interacting protein (FAAP24), and another unidentified factor (FAAP100) comprise the FA core complex. FANCL associates with UBE2T, an ubiquitin conjugating enzyme, to impart E3 ubiquitin ligase activity to the core complex. In response to DNA damage or during S phase progression of the cell cycle, the FANCD2 protein

is monoubiquitinated in a FA core complex-dependent manner. The monoubiquitinated FANCD2 (FANCD2-Ub in the diagram) is translated to the site of DNA damage or synthesis, the so called Nuclear Foci. Also included in the Nuclear Foci and required for ubiquitinylation of FANCD2 are ATR, DNA damage-activated signaling kinase, and RPA, and a single-strand DNA binding protein. In the nuclear foci, FANCD2-Ub co-localizes with BRCA1, BRCA2, RAD51, and FANCI/BRIPI to stabilize or confer resistance to DNA damage.

The risk for MDS and AML are markedly increased in patients with FA. The cumulative risk for developing hematologic neoplasms by age 50 is 33%.^{73,74,77} The relative risk for AML is 868-fold over that of the general population.⁷⁷ The median age for developing AML is 14 years, as compared to 68 years for the general population.⁷⁴ In a retrospective study of 1,301 FA patients, the frequency of AML, MDS, and ALL was 8.3, 6.8, and 0.5%, respectively.⁷⁴ Another independent study of 754 patients confirmed these findings.⁷³ In the former study, all subtypes of AML (except AML, M3) were represented with an excess of AML, M4 and AML, M5 subtypes.

The MDS in FA patients may be different from de novo MDS in non-Fanconi patients. Only 13 of 89 FA patients with MDS progressed to AML.⁷⁴ Additionally, patients with FA may have MDS for long periods of time with clonal fluctuation.⁹⁶ The presence of a clonal cytogenetic abnormality is not sufficient proof of MDS or AML. FA patients with no evidence of a myeloid neoplastic process frequently have

clonal cytogenetic abnormalities that may be transient, show evidence of clonal evolution, or develop new clones.^{96,97} Morphologic features of MDS may be more strongly associated with poor prognosis than the presence of a cytogenetic clone without MDS morphology.⁹⁶ The cytogenetic abnormalities of FA-associated AML are more like secondary AML of non-FA patients. The cytogenetic abnormalities commonly encountered in FA patients with MDS or AML include monosomy 7 or 7q rearrangements, abnormalities of chromosomes 1p, 1q, and 3q, rearrangements of 11q22–25, and chromosome 5 abnormalities (including monosomy 5).^{81,96–99} FA patients with AML do not have chromosomal translocations t(8;21), inv(16), t(15;17), or t(11q23;V), which are common to AML of non-FA patients.

Studies have demonstrated a genotype–phenotype relationship for some patients with FA. The most common *FANCC* mutation, IVS4+4A>T, and also the p.Arg548X and p.Leu554Pro mutations, which are prevalent in Ashkenazi

Jews, are associated with lower risk for congenital anomalies and late development of BM failure.^{100,101} Individuals with homozygous mutations of *FANCA* (and no *FANCA*) may have an earlier onset of anemia and a higher incidence of leukemia than persons with mutations that result in production of an abnormal *FANCA* protein.¹⁰² Mutations of *BRCA2* are associated with early onset of leukemia and solid tumors.^{82,103} Mutations of *FANCG* may be associated with severe cytopenia and a higher incidence of leukemia.¹⁰²

Pathogenesis of Neoplasms Associated with FA

Recent investigations have added significantly to the understanding of the functions of Fanconi proteins and pathway, but myeloid neogenesis in FA remains poorly understood. Based on what is known about the FA pathway, homozygous loss of a Fanconi protein may be expected to disrupt normal DNA repair, resulting in gene mutations or chromosomal breakage and rearrangements. Although defective DNA repair is expected to lead to cellular apoptosis in most cells, some cells may gain a proliferative advantage predisposing to MDS or AML.¹⁰⁴ The cytokine hypersensitivity of some FA cells may lead to a selective inducement (or an environment conducive) for mutations, resulting in an outgrowth of cytokine-resistant clones.⁹⁴ More recently, Briot and coworkers have proposed that FANCA gene mutations activate MAPK signaling, inducing MMP-7 overexpression and leading to TNF- α oversecretion.¹⁰⁵ TNF- α may in turn sustain or amplify MAPK and NF- κ B activations, which are implicated in myeloid leukemogenesis.

Tests for FA

The current diagnostic test for FA is the quantitation of chromosome breakage, using metaphase preparations of phytohemagglutinin-stimulated cultures of peripheral blood lymphocytes. Patients with FA have increased spontaneous chromosome breaks, gaps, exchanges, rearrangements, and endoreduplications. Breakage is markedly increased by cross-linking agents [i.e., mitomycin C (MMC) and diepoxybutane (DEB)]. The DEB-induced chromosome breakage test (DEB test) is the standard diagnostic test for FA.^{106,107} Some patients with FA will not demonstrate increased numbers of spontaneous chromosomal breakages, but testing with MMC or DEB results in increased breakage. The breakage in homozygotes is three to ten times that of normal individuals.¹⁰⁸ The DEB test may be difficult to interpret in those patients whose lymphocytes develop mosaicism. With clonal evolution to MDS or AML, FA cells might also acquire resistance to DEB and MMC.¹⁰⁹ Patients with other rare chromosome breakage syndromes, such as the Nijmegen breakage syndrome (NBS), may also have a positive DEB test.¹¹⁰ Spontaneous chromosome breaks are common in patients with Bloom's syndrome (BS) and AT, but these are not increased with MMC or DEB testing.¹¹¹ FA heterozygotes may also demonstrate increased

chromosome breakage with clastogenic agents, but this breakage overlaps with that of normal individuals.

Flow cytometry may also have a role in the initial screening of patients suspected of having FA. Cells of FA patients have an abnormal cell cycle with increased numbers of cells arrested at G2, likely since FA cells fail to carry out the DNA repair and thus remain in the G2 phase of cell cycle. The increased number of G2 cells is the basis for a flow cytometric assay of the response of lymphocytes to DEB in nonleukemic FA patients.^{112,113} One advantage of the flow cytometry assay is that it is faster than the cytogenetic DAB test and may detect FA patients with somatic mosaicism.¹¹⁴

Although a positive DEB test is indicative of FA, molecular analysis is required to demonstrate the pathogenic mutations in a FA gene. Identification of the type of mutation is important for clinical management, genetic counseling, and assessing prognosis.^{70,115} Molecular genetic testing is complicated by the presence of multiple mutations in 13 different genes. Presently, testing for FA mutations include the following: retrovirus-mediated complementation, multiple ligation-dependent probe amplification, cell fusion, FANCD2 immunoblotting, sequence analysis for *FANCA*, *FANCB*, *FANCC*, *FANCE*, *FANCF*, *FANCG*, and *FANCI*, PCR for common Ashkenazi Jewish *FANCC* mutations, and SNP analysis for deletions of one or more exons of *FANCA*.^{116–119} However, these test methods are not applicable for all mutations, and most are only available in research laboratories. All testing for FA should start with the DEB chromosome breakage test with subsequent molecular testing for FANCA gene mutations (Figure 2.4). Recently, Ameiziane and coworkers described an effective and relatively rapid mutation screening approach for the majority of FA patients.¹¹⁹

Bloom's Syndrome

Patients with BS present with marked prenatal and postnatal growth retardation. Within the first 2 years of life, they develop photosensitive malar telangiectatic erythema, resembling lupus erythema.¹²⁰ Patches of hyper- and hypopigmentation of the skin are also common. Early female menopause and male infertility are characteristic. Major anatomic abnormalities are not a feature of this disorder. Recurrent infections, chronic pulmonary disease, and noninsulin-dependent diabetes mellitus are also common manifestations. Low immunoglobulin levels and defective cell-mediated immunity account for recurrent infections. Cancer is a frequent complication and is the most common cause of death.¹²¹

Molecular Pathogenesis of BS

The clinical findings in BS patients are due solely to mutations of the BS gene (*BLM*) located on chromosome 15q26.1.¹²² *BLM* is comprised of 22 exons and encodes for a RecQ helicase homolog protein, which functions to resolve

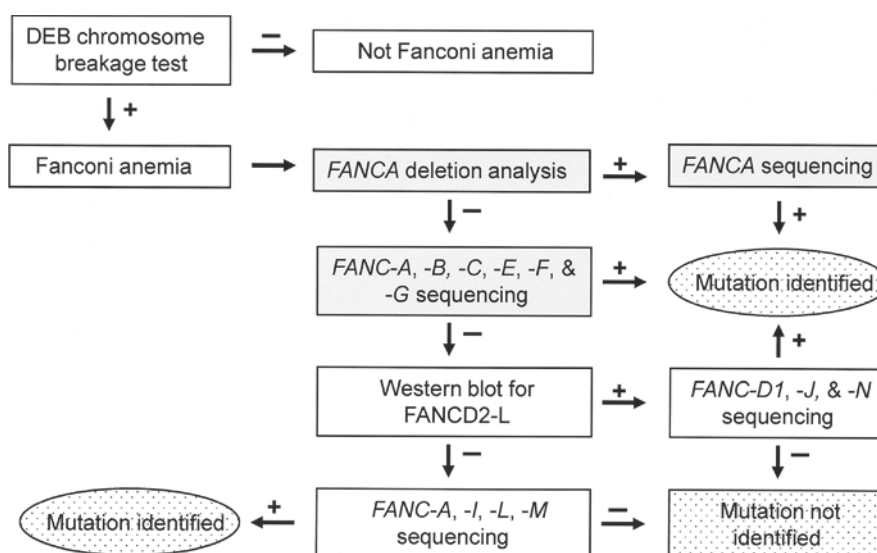


FIG. 2.4. Testing for Fanconi anemia (FA). Testing for FA should start with the diepoxybutane (DEB) chromosome breakage test. Although a positive DEB test is highly indicative of FA, a molecular analysis of the *FANC* genes is required to confirm the diagnosis and for genetic counseling. Molecular testing should begin

with testing for mutations of *FANCA* since this gene is mutated in 55–70% of FA patients. In the absence of a *FANCA* mutation, studies of *FANCC*, *-E*, *-F*, and *-G* will detect an additional 10–30% of patients. Less than 5% of FA patients will have mutations of *FANCI*, *-J*, *-L*, or *-M*.

Holiday junctions, suppress recombination, and repair double-stranded DNA breaks.^{123–126} BLM is a cell cycle-regulated protein found in the nucleus and concentrated in bodies, referred to as PML (promyelocytic leukemia) bodies.¹²⁷ Mutations of *BLM* result in loss of helicase activity and genomic instability. Thus, the BLM protein is involved in maintaining the cell's genomic integrity.

In a recent report from the Bloom's Syndrome Registry, 64 different *BLM* mutations were found in 125 persons with BS, but only 19 were recurrent mutations.¹²⁸ Mutations consist of nucleotide insertions and deletions (that interfere with localization of BLM in the nucleus), intron mutations (causing splicing defects), nonsense mutations (resulting in truncated BLM), and missense mutations (resulting in non-functional BLM protein).¹²⁰ Although rare in Ashkenazi Jews, approximately a quarter of persons in the Bloom's Syndrome Registry are of Ashkenazi Jewish ancestry.¹²⁰ Most persons of Ashkenazi Jewish heritage with BS have the so-called *BLM*^{Ash} mutation.¹²⁹ Another mutation less commonly observed in this population is insT2407.¹²⁰ While over 95% of BS individuals of Ashkenazi Jewish ancestry will have at least one *BLM*^{Ash} mutation, this mutation is detected in only about 5% of BS persons of non-Jewish heritage.

Hematolymphoid Disorders Associated with BS

Patients with BS have one of the highest rates of cancer among the inherited disorders of DNA repair. Cancer types include hematologic, lymphoid, epithelial, connective tissue, germ

cell, nervous system, and renal.^{120,121} An individual with BS may develop multiple different cancers in their lifetime.¹²¹ Murine studies show that BML is critical for development and function of $\alpha\beta$ T-cells,¹³⁰ and its absence compromises B-cell development and function.¹³¹ Mice deficient in BLM are prone to developing B-cell lymphoma.¹³¹ According to a report of neoplasms in the Bloom's Syndrome Registry, 38% of BS individuals develop lymphoid or hematologic neoplasms.¹²¹ Lymphoid malignancies include diffuse and follicular B-lineage non-Hodgkin lymphomas and (less commonly) ALL and Hodgkin lymphoma. Myeloid neoplasms include AML and MDS. The development of MDS usually is preceded by another cancer treated with chemotherapy and/or radiotherapy.¹²⁰ Case reports of BS patients with myeloid neoplasms point to a higher incidence of chromosome 7q deletions and monosomy 7 than in non-BS patients with AML.¹³²

The mechanism for developing hematolymphoid malignancies in BS is unknown. The BLM helicase functions as part of a protein complex of the same name that, through its combined DNA nicking and unwinding, catalyses the resolution of double-cross-shaped DNA structures, known as double Holiday junctions. Double Holiday junctions arise from reciprocal sister chromatid exchanges (SCEs) of homologous single strands of DNA during the process of homologous recombination. The BLM complex prevents the formation of crossovers that would lead to loss of heterozygosity,^{133,134} the latter of which is thought to contribute to the development of malignancies. The risk of cancer in carriers of a *BLM* mutation is not known.

Tests for BS

Testing of individuals with suspected BS should start with cytogenetic testing, which can exclude non-BS cases. Cytogenetic studies of blood lymphocytes (or cultured fibroblasts) commonly show increased numbers of chromatid gaps, breaks, and rearrangements and particularly quadriradial configurations.¹²⁰ The hallmark of BS is a very high rate of SCEs. BS lymphocytes exposed to bromodeoxyuridine will have ten times the number of SCEs than normal lymphocytes.¹³⁵ Immunoblot and immunohistochemical analysis for the detection of BML protein and nuclear dots, respectively, using polyclonal anti-BML have been described, but they may be relatively labor-intensive and are not readily available.¹³⁶ Molecular testing for *BML* mutations may be indicated to confirm a suspected diagnosis of BS (based on the preceding cytogenetic studies) and for carrier testing. Targeted mutation analysis for *BML*^{Ash} and insT2407 should be initially performed in individuals of Jewish ancestry. Screening for the presence of a *BML*^{Ash} mutation may also be performed in those with no known Jewish ancestry; however, the yield will be low. If one of these mutations is not detected, more laborious sequence analysis or mutation scanning studies may be undertaken; however, these methods are primarily only available in research laboratories.

Nijmegen Breakage Syndrome

Individuals with NBS have short stature with progressive microcephaly and loss of cognitive skills, recurrent sinopulmonary, and urinary infections due to immunodeficiency, gonadal failure, radiation hypersensitivity, and increased risk of cancer (particularly lymphoma).^{137,138} Microcephaly is present at birth in the majority of cases and is evident in all infants during the first month life. By 3 years of age, characteristic facial features are present, consisting of a sloping forehead, prominent nasal bridge with a long nose, upslanting palpebral fissures, large ears, and small jaw. Less common are hypo- and hyperpigmentation of the skin. Congenital abnormalities, such as clinodactyly, syndactyly, cleft lip and palate, tracheal hypoplasia, horseshoe kidney, hypospadias, and hip dysplasia are present in one-half of cases. Intellectual ability declines with age, and mental retardation is present in most children with NBS by tens years of age. Pulmonary infections may cause early death. Individuals with NBS have a combined B- and T-cell immune deficiency. Abnormal serum immunoglobulin levels are a common finding and include agammaglobulinemia, selective IgA deficiency, or increased IgM with decreased IgG and IgA.^{137,138} T-cell deficiency includes progressive T-cell lymphopenia and decreased T-cell function.¹³⁹ Malignancy develops in about half of individuals by their adolescent years.¹³⁸

Molecular Pathogenesis of NBS

NBS is an autosomal recessive DNA repair disorder, due to a mutation of *NBS1*, a gene consisting of 16 exons on chromosome 8q21.^{137,138} The *NSB1* gene encodes for nibrin (or p95-nibrin) and p70-nibrin, which are expressed in all tissues. Protein product nibrin/p95-nibrin is involved in repair of DNA double-strand breaks and in cell cycle checkpoints.^{140,141} Nibrin is part of a trimeric complex (Mre11/Rad50/nibrin), which is phosphorylated at several sites, in response to DNA double-strand breaks and damage, and thereby contributes to ATM activation.¹⁴² The p70-nibrin form also may bind to Mre11 and Rad50 and stimulate ATM.¹⁴² The role of nibrin in cell cycle control is not fully understood, but in some way interacts with ATM to coordinate cell cycle arrest with DNA repair.¹⁴³ Thus, it is not surprising that the clinical and cellular features of NBS and AT overlap. Two recent gene expression studies show that both of these disorders regulate several genes in common, but still have distinct patterns of gene expression.^{144,145}

A mutation of *NBS1* is the only genetic abnormality associated with NBS.^{137,138} However, half of children with a clinical history and physical findings of NBS lack a mutation of *NBS1*. This suggests that another abnormal gene (or genes) may be responsible for a very similar disorder. Over 90% of all patients are homozygous for a five base-pair truncating mutation, 657de15, and the majority of individuals are of Central-East Europe origin.^{137,138} All mutations described to date are predicted to be truncating mutations. Chromosomal instability is thought to be the direct result of these mutations. Rearrangements of chromosome 7 and 14 with break-points at the loci of T- and B-cell receptor genes are common in NBS.^{146,147} These arrangements may be the consequence of incorrect rejoining during V(D)J recombination, resulting from defective Nibrin function.¹⁴⁸

Hematolymphoid Disorders Associated with NBS

Approximately 40–50% of individuals with NBS develop a malignancy before 21 years of age, a higher rate than for other DNA repair disorders.^{147,149} Non-Hodgkin B-cell lymphoma comprise 50–75% the lymphoid malignancies with Hodgkin lymphoma, T-cell lymphoma, and T- and B-cell ALL accounting for the rest.^{138–140,147–153} In a very informative investigation of hematolymphoid malignancies of patients entered in the Polish NBS Registry, diffuse large B-cell lymphomas and T-cell ALL/lymphoblastic lymphoma developed in 8 and 3 of 14 patients, respectively.¹⁴⁷ The other three patients developed Burkitt-like lymphoma, classical Hodgkin lymphoma, and angioimmunoblastic-like T-cell lymphoma. All patients had common homozygous 657de15 *NBS* mutations. Evidence of breaks of Ig loci was found in six of eight diffuse large B-cell lymphomas by FISH studies of paraffin sections. Two of the three T-cell lymphoblastic malignancies had T-cell receptor breaks. Only two cases of

diffuse large B-cell lymphoma had evidence of EBER by immunohistochemical studies. By contrast, EBV is commonly associated with B-cell lymphomas of patients with a secondary immunodeficiency. This suggests that EBV does not play a significant role in lymphoma genesis in NBS.¹⁴⁷ Other reported malignancies in NBS include medulloblastoma, glioma, and rhabdomyosarcoma.^{137,138} Heterozygotes without manifestations of NBS are cancer prone.^{154,155} A genotype–phenotype relationship exists between the severity of NBS and mutation status of patients.^{156,157} Patients who are compound heterozygotes for the 657de15 mutation and the missense mutation R215W have a more severe phenotype than classical NBS individuals homozygous for 657de15.¹⁵⁶ The expression level of nibrin appears critical in relation to the age at which individuals with NBS present with lymphoma or other malignancies. NBS individuals with lower levels of the p70 form of nibrin develop lymphoma earlier in life.¹⁵⁷

Tests for NBS

Preliminary confirmation of a suspected diagnosis of NBS may be accomplished with tests for spontaneous or radiation-induced chromosomal instability. Spontaneous chromosome instability analysis is performed with mitogen-stimulated cultures of peripheral blood lymphocytes. One-hundred metaphases are examined for chromosome and chromatid breaks, and the frequency of abnormal metaphases are compared with age-matched controls. Translocations and inversions of chromosome 7 and 14 (T- and B-cell receptor loci) may be expected in 10–50% of metaphases of individuals with NBS. Induced chromosomal instability requires that lymphoblastoid cell lines

(or fibroblast cultures) be established from patients and scored for the number of induced chromatid breaks after G2-phase low-dose radiation. The number of chromatid breaks in the test specimen is compared to that of cells from a healthy non-NBS person. An immunoblot assay of a patient's lymphoblastoid cell line using polyclonal antibodies to nibrin may also be performed to confirm a suspected diagnosis of NBS.

Definitive confirmation of the diagnosis of NBS requires molecular testing for mutations of the *NBS1* gene. To date, all disease-related mutations are within exons 6–10.¹³⁷ Individuals from Poland, Czech Republic, and Ukraine are homozygous for 657de15. In the United States, 70% of NBS patients are homozygous for 657de15, and the other 30% are either heterozygous for 657de15 plus a second mutation, or homozygous for a unique mutation.¹³⁷ Thus, molecular testing should begin with targeted mutation analysis for the common 657de15 mutation (Figure 2.5). In the absence of the 657de15 mutation, nibrin should be looked for by an immunoblot assay. If nibrin is not detected, sequence analysis of *NBS1* may be pursued to identify the mutation. Patients may have a different DNA repair defect disorder if no mutation of *NBS1* is identified by sequence analysis.

Ataxia-Telangiectasia

AT is an inherited autosomal recessive disorder, characterized by progressive cerebellar ataxia, oculomotor apraxia, telangiectasias of conjunctivae, recurrent infections, combined immunodeficiency, increased incidence of malignancies, and premature aging.^{158–160} The most common initial presentation is progressive cerebellar ataxia, which usually

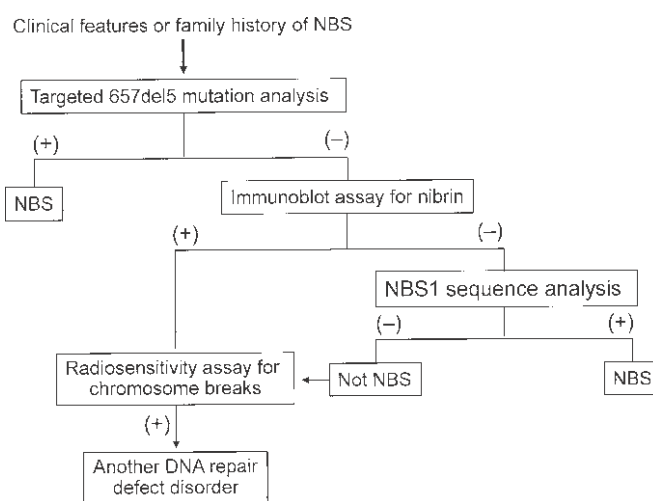


FIG. 2.5. Testing for Nijmegen Breakage Syndrome (NBS). Initial testing for NBS should target the common 657de15 mutation that is present in over 90% of patients in the United States. If this mutation is

not detected, either an immunoblot assay for nibrin or full sequence analysis of *NBS1* can be initiated. A DNA repair defect other than NBS may be present in the absence of a mutation of *NBS1*.

manifests in the first 4 years of life. Other neurologic signs and symptoms are discussed in the references cited above. Telangiectasias of the face, ear, eyelids, bulbar conjunctivae, flexor folds of the neck extremities, and dorsal surfaces of the hands and feet occur later in life. Sinopulmonary infections afflict most patients on a recurring basis and may lead to chronic bronchiectasis and respiratory insufficiency. Defects in organ development and tissue differentiation commonly result in endocrine abnormalities, including delayed growth, insulin-resistant diabetes mellitus, hirsutism in females, hypoplastic or absent ovaries, and hypogonadism in males. Premature aging and graying of hair are also characteristic. The absence of a thymus (or a poorly developed thymus with absent Hassell's corpuscles and lymphoid hypoplasia) contributes to the immunodeficiency of these individuals. Individuals with AT are hypersensitive to agents that cause DNA strand breaks, including ionizing radiation and chemicals, such as bleomycin. Approximately one-third of patients are at risk for developing a malignancy.^{161–163} Considerable heterogeneity exists among patients with AT, ranging from the classic form with onset in infancy to milder forms with late appearance or slow progression.

Molecular Pathogenesis of AT

The genetic defect underlying AT are mutations of the *ataxia telangiectasia mutation (ATM)* gene.¹⁶⁴ *ATM* was identified by positional cloning on chromosome 11q22–23. The *ATM* gene is large at 150 kb and consists of 66 exons, which encode for a 350 kD protein kinase with a phosphatidylinositol-3-kinase (PI3-kinase) domain, a leucine zipper motif, and a p53 binding site.¹⁶⁵ The ATM protein is involved in multiple signaling pathways of cellular response to DNA double-strand breaks, and control of the cell cycle check points, DNA replication, recombination, and repair.¹⁶⁶ ATM is involved in activating the G1-S, S, and G2-M cell cycle check points after DNA damage by phosphorylation of different targets.¹⁶⁶ These check points prevent damaged DNA from being replicated and inhibit cell division until DNA strand breaks have been repaired. Decrease or absence of ATM leads to loss of these cell cycle check points and inappropriate progression of cells through the cell cycle.

Over 500 unique mutations of *ATM* are described with no known hot spots. Mutations are found throughout the gene, and most mutations inactivate the ATM protein by truncation, large deletions, or prevention of initiation or termination. Less common are missense mutations in the PI3 kinase domain and the leucine zipper motif. The more severe forms of AT with the classic presentation of early development of cerebellar ataxia and ocular telangiectasia are associated with total absence of ATM as a result of compound heterozygosity or homozygosity for truncating mutations.¹⁶⁷ Milder clinical AT phenotypes may be the result of missense or splice site mutations, which allow for some expression of mutant (or decreased but normal) ATM protein.^{168,169}

Hematolymphoid Disorders Associated with AT

Approximately 10–15% of patients with AT develop a malignancy in childhood and up to another 10–20% later in life.^{158,161} Lymphoid malignancies predominate throughout life, but older individuals are also at risk for other cancers, including breast and ovarian cancer, melanoma, gastric cancer, brain tumors, and sarcomas. The lymphoid malignancies consists of T-cell lymphoma, T-cell prolymphocytic leukemia (T-PLL), precursor T-ALL, B-cell non-Hodgkin lymphoma, and Hodgkin lymphoma, the latter of which is mostly of the lymphocyte-depleted subtype.¹⁶¹ T-ALL may precede the diagnosis of AT. As a result of the high spontaneous rate of chromosomal abnormalities (due to loss or deficient ATM function), chromosomal breaks and inversions as well as translocations at the T-cell receptor loci (7p13-p14, 7q32-q35, and 14q11) and the B-cell receptor loci (2p12, 14q32, and 22q12) are frequent in AT individuals.^{170,171} Approximately 10% of AT patients have clonal expansions of T cells with translocations involving the chromosomes 7 and 14.^{170,171} It is postulated that these translocations lay the ground work for the development of lymphoid neoplasms; however, additional genetic abnormalities, or unregulated signaling pathway, are required for the development of leukemia and lymphoma.^{172,173} Heterozygous carriers appear to be at increased risk for cancer, primarily breast cancer.^{174–176}

Mutations of ATM are not infrequent in sporadic cases of T-PLL, B-cell chronic lymphocytic leukemia, and mantle cell lymphoma.^{177–180} A low frequency of ATM mutations have been reported in adult precursor B-ALL, diffuse large B cell lymphoma, and Hodgkin lymphoma.^{177,181–183} ATM mutations are rare in sporadic case of T-ALL.¹⁸⁴ In contrast to the predominance of truncating mutations in AT patients, most ATM alterations in sporadic leukemia and lymphoma are missense mutations.^{185,186}

Tests for AT

The clinical diagnosis of AT is supported by neuroimaging for cerebellar atrophy, serum alpha fetal protein testing, immunoglobulin assays and immunophenotyping, cytogenetic studies for chromosome 7 and 14 rearrangements, assessment of in vitro lymphoblastoid cell radiosensitivity, ATM kinase activity, and flow cytometry studies (Table 2.1). Serum AFP is elevated in 95% of patients with AT. Disorders of the B- and T-cell immune system are present in 60–80% of patients and may consist of: decreased serum levels of IgA, IgE, and/or IgG2; increased serum levels of IgM or (less commonly) IgG or IgA; decreased numbers of T-cells with marked increases of gamma-delta T-cell receptor forms; and delayed cutaneous hypersensitivity responses. Stimulated lymphocytes will frequently display chromosomal rearrangements involving T-cell receptor or B-cell immunoglobulin genes, reflecting defective repair of double-strand DNA breaks. Recently, rapid flow cytometry assays for ATM

have been described that assess ATM- or H2AX histone-phosphorylation, following induced DNA damage.^{187–191} All of the preceding tests lack specificity or sensitivity for AT. About 90% of individuals with AT have no detectable ATM protein; 10% have only trace amounts; and 1% have normal ATM that lack kinase activity. Thus, immunoblotting for ATM protein has been a good test for confirming the diagnosis in 90% of patients but requires significant amounts of protein lysate, is complicated, and costly.¹⁹¹

Due to the large size of *ATM* and the lack of mutation “hot spots,” screening of genomic DNA for all possible mutations is greatly complicated. Mutation analyses include a protein truncation assay,^{192,193} single-strand conformation polymorphism (SSCP),^{194,195} and restriction endonuclease fingerprinting.^{196,197} These begin with separate amplification of individual *ATM* exons from genomic DNA or RNA transcripts by PCR or RT-PCR, respectively. Unfortunately, these assays do not detect intronic mutations and heterozygous deletions. Mutational analysis by hybridization-based methods, using high-density oligonucleotide arrays (DNA chips), offers another testing approach.^{177,198} Gene expression profile studies may also prove useful for identifying heterozygous carriers of AT.¹⁹⁹

Dyskeratosis Congenita

DC is an inherited disorder, characterized by the clinical triad including: (1) reticulated hyperpigmentation of the face, neck, and shoulder; (2) dystrophic nails of hands and feet consisting of longitudinal ridges; and (3) mucous membrane leukoplakia.^{200–205} Abnormalities of the eyes and teeth are present in ~40 and ~20% of patients, respectively. Other physical abnormalities include developmental delay, skeletal anomalies and short stature, premature hair graying and loss, genitourinary and gastrointestinal tract abnormalities, and gonadal anomalies.^{201–204} Skin pigmentation and dystrophic nails usually appear by 10 years of age but may disappear later. Hematologic abnormalities commonly develop by the second decade of life with evidence of BM failure

in up to 90% of patient by age 30.^{203,204} Blood cytopenias may sometimes precede skin and nail abnormalities. Early mortality is primarily due to infections and hemorrhage secondary to BM failure and immune-deficiency, pulmonary complications, and malignancy.^{202–204} Studies of skin fibroblasts demonstrate abnormal growth rates and chromosomal abnormalities, including dicentrics, tracentrics, and unbalanced translocations.^{202,205} Thus, similar to FA, DC may be considered a chromosomal instability disorder but with a tendency towards chromosomal rearrangements, rather than the breaks, duplications, and deletions as observed in FA.^{202,206}

Molecular Pathogenesis of DC

Three patterns of inheritance have been described for DC: (1) X-linked recessive; (2) autosomal recessive; and (3) autosomal dominant (Table 2.4).^{200–203} Some of the patients with presumed autosomal inheritance may be X-linked with X-inactivation. The X-linked form is associated with younger age of onset and a more severe phenotype and includes patients with the clinical diagnosis of Hoyeraal–Hreidarsson syndrome. The autosomal recessive forms show considerable clinical heterogeneity. The X-linked form is associated with mutations of *DKC1*, which encodes for dyskerin, a nucleolar protein.^{207,208} Dyskerin binds small nucleolar RNAs and is involved in ribosome biogenesis, but it also is a subunit of the telomerase complex (Figures 2.6 and 2.7).^{209,210} Studies of dyskerin have not detected defective rRNA processing but have shown reductions of telomerase activity.²¹¹ The dominant form of DC has been linked to mutations of *TERC*, a 451 nucleotide RNA component of telomerase, and less commonly *TERT*, the reverse transcriptase of the telomerase complex.^{212,213} Mutations of *NOP10*, encoding for a small core component of the snoRNA and the telomerase complex, and *TERT* have been identified in several families with autosomal recessive DC.^{214,215} Recently, mutations of *NHP2* and *TINF2*, which encode for NHP2 and TIN2 subunits of the telomerase and shelterin complex, respectively, have been described in families with DC.^{216,217}

TABLE 2.4. Genetic abnormalities associated with Dyskeratosis congenita and associated hematologic disorders.

Gene	Chromosome	Product	Function	Inheritance	% of cases	Associated hematologic disorders
<i>DKC1</i> ^a	Xq28	Dyskerin	Telomere maintenance and ribosomal biogenesis	XLR	~40	AA
Not known	—	—	—	AR, S	~50	ND
<i>NOP10</i>	15q14	NOP10	Telomere maintenance	AR	<1	ND
<i>TINF2</i> ^a	14q12	TIN2	Telomere maintenance	AD, S	<1	ND
<i>NHP2</i>	22q13.2	NHP2	Ribosomal biogenesis	AR	<1	ND
<i>TERC</i>	3q21-3q28	<i>TERC</i> ^b	Telomere maintenance	AD	~5	AA, PF, PNH, MDS, ET
<i>TERT</i>	5p15.33	TERT	Telomere maintenance	AD, AR	<5	AA, PF

XLR X-linked recessive, AR autosomal recessive, S sporadic, AD autosomal dominant, AA aplastic anemia, ND none described, PF pulmonary fibrosis, PNH paroxysmal nocturnal hemoglobinuria, MDS myelodysplastic syndrome, ET essential thrombocythemia.

^a Associated with Hoyeraal–Hreidarsson syndrome, a severe form of recessive Dyskeratosis congenita.

^b *TERC* is a 451 nucleotide RNA component of telomerase.

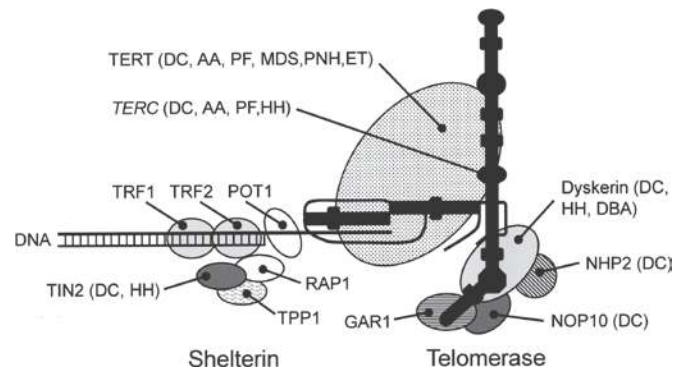


FIG. 2.6. The telomerase complex and constituents involved in Dyskeratosis congenita (DC). Telomerase is a polymerase that adds telomeric repeats (TTAGGG) to the end of the 3'-lagging strand of DNA after replication. The telomerase complex is a ribonucleoprotein composed of a catalytic structure, TERT, which adds the repeats and plus TERC that act as the template. Shelterin complex I is comprised of at least six proteins (TIN2, TPP1, RAP1, POT1, TRF1, and TRF2) and has at least three functions: (1) determines the structure of the telomeric terminus; (2) controls

synthesis of telomeric DNA by telomerase; and (3) may be involved in the generation of t-loops. Dyskeratosis congenita (DC) has been associated with mutations affecting the production of dyskerin, TERT, TERC, NOP10, NHP2, and TIN2. Aplastic anemia (AA), essential thrombocythemia (ET), pulmonary fibrosis (PF) myelodysplastic syndrome (MDS), and paroxysmal nocturnal hemoglobinuria (PNH) have also been associated with defects of the telomerase complex. Hoyerall-Hreidarsson (HH) is now considered a severe form of DC.

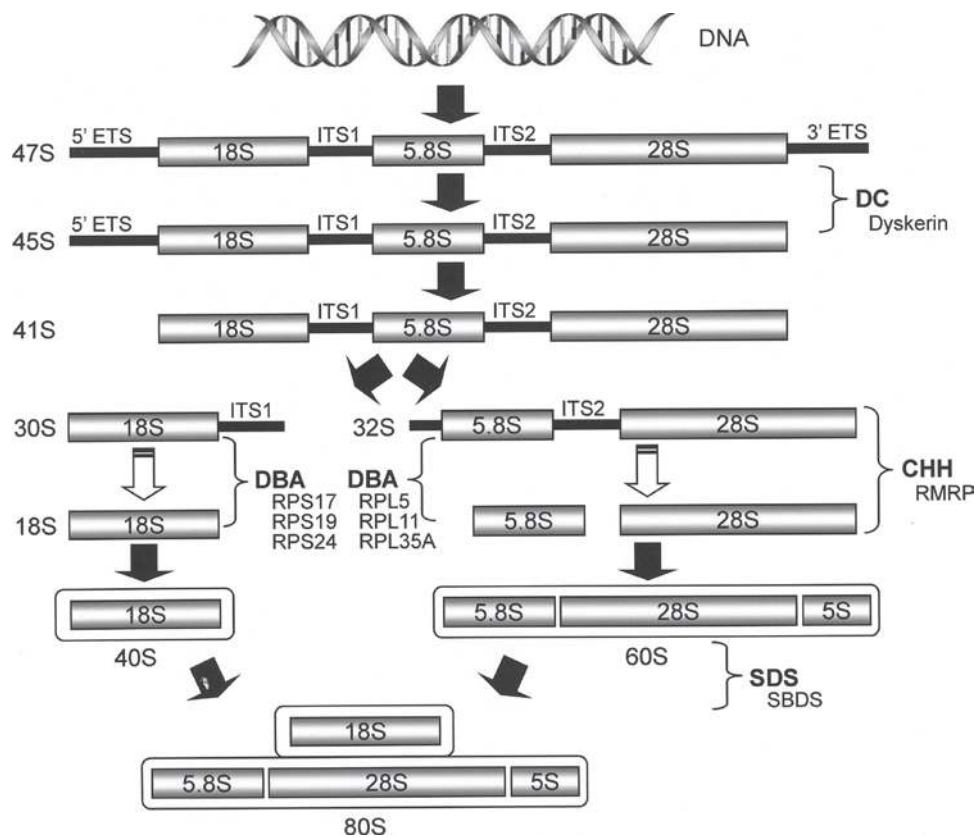


FIG. 2.7. Schematic overview of ribosomal biogenesis. Ribosomal biogenesis is a complex process involving almost 200 nonribosomal proteins and small RNAs. Biogenesis begins in the nucleolus where 28S, 18S, and 5.8S ribosomal RNAs (rRNA) are transcribed by RNA polymerase into a rRNA precursor. A series of regulated cleavages and posttranscriptional modifications form the 40S and 60S ribosomal subunits. The final assembly of the 40S and 60S subunits to form the 80S ribosome takes place in the cytoplasm. This highly regulated

and balanced process is disrupted by deficiencies or excesses of any of the pathway proteins or RNA components. Dyskeratosis congenita (DC), Diamond-Blackfan anemia (DBA), cartilage-hair hypoplasia (CHH), and Shwachman-Diamond syndrome (SDS) are due to mutations of genes encoding proteins involved in ribosomal biogenesis. The putative interactive sites of dyskerin, RPS17/RPS19/RPS24, and RPL5/RPL11/RPL35A, RMRP, and SBDS that are deficient in DC, DBA, CHH, and SDS are indicated in the diagram.

Telomerase is a ribonucleoprotein, which is comprised of a catalytic component, TERT (that adds repeats), and an RNA component, *TERC* (which provides the nucleotide template). Other constituents of telomerase include dyskerin and NOP10, NHP2, TIN2, and GAR1. Since only mutations of *DKC1* (dyskerin), *TERC*, *TERT*, *NOP10*, *TINF2*, and *NHP2* have been detected thus far in DC patients and because these patients have shortened telomeres, DC is thought to be principally due to defective telomerase activity.^{201,205,218}

Hematolymphoid Disorders Associated with DC

Aplastic anemia occurs in ~50% of patients with a median age of presentation at 11 years.^{200–202} More specifically, aplastic anemia develops in 30 and 60% of patients with the X-linked and autosomal recessive forms, respectively. In some patients, the recognition of DC follows the development of hematologic abnormalities. Hematologic abnormalities often begin with thrombocytopenia, followed later by pancytopenia. The anemia is macrocytic. Fetal hemoglobin levels are increased. Initially, BMs may be hypercellular, but eventually become hypocellular due to a trilineage hypoplasia. Chromosomal rearrangements may be present in the absence of MDS or leukemia. The etiology of BM failure is postulated to be a consequence of abnormalities of hematopoietic stem cells and stromal cells, as a result of decreased telomerase activity.^{202,205,219,220} It is postulated that telomerase deficiency results in increased telomere shortening in stem cells of DC and consequently, loss of cells of tissues that need constant renewal, such as hematopoietic stem cells and gastrointestinal epithelium.²⁰⁵ Abnormalities of genes associated with DC may be the etiology of some non-DC patients with idiopathic aplastic anemia (IAA). Like individuals with DC, patients with IAA may have shortened telomeres.^{202,221} Mutations of *TERC* and *TERT* have been identified in some patients with IAA and may represent occult forms of DC.^{221–225}

Approximately 15% of DC patients develop cancer, most commonly squamous cell carcinoma of the oropharyngeal region and gastrointestinal tract cancer. Other cancers include bronchial adenocarcinoma and nonmelanotic skin cancer. MDS and leukemia are uncommon neoplasms in patients with DC. Paroxysmal nocturnal hemoglobinuria, MDS, essential thrombocytosis, and idiopathic pulmonary fibrosis have been reported in patients with DC and mutations of *TERC*.²⁰¹

Tests for DC

Distinguishing DC from FA by clinical features alone may be difficult. Earlier studies by some investigators reported increased spontaneous and induced chromosomal breakage;

however, in a careful study by Coulthard and coworkers, no difference in breakage was demonstrable between lymphocytes from normal and DC individuals.²²⁶ Thus, DC may be differentiated from FA by induced chromosomal breakage studies and mutational analyses of genes associated with FA and DC. Several reference laboratories perform mutational studies of *DKC1* and *TERC* to confirm suspected cases of DC, identify carriers and provide antenatal diagnosis in X-linked families. Testing is performed by bidirectional sequencing of the coding regions of exon 1–15 of *DKC1*, or the nontranslated single exon of *TERC*.

Shwachman–Diamond Syndrome

Shwachman–Diamond syndrome (SDS) is a clinical disorder that includes pancreatic exocrine insufficiency, hematologic dysfunction, and skeletal abnormalities.^{227–231} SDS is the second most common cause of inherited pancreatic insufficiency after cystic fibrosis. The exocrine pancreatic insufficiency usually presents in infancy but may improve with age. Histologically, the pancreas shows fatty replacement of acini with preservation of islets and ducts. As a result of intestinal malabsorption, patients may display symptoms of deficiencies of the fat soluble vitamins A, D, E, and K. Pancreatic exocrine dysfunction may be documented by an abnormally high 72-h fecal fat study, low serum levels of isoamylase and cationic trypsinogen, or decreased pancreatic enzyme secretion following pancreatic stimulation with intravenous cholecystokinin or secretin. However, abnormal pancreatic function may be difficult to detect in early childhood.^{232,233} Neutropenia, which may be persistent or intermittent, is the other hallmark of SDS. Anemia and thrombocytopenia may also be present or develop later in life. Characteristic skeletal changes, which are not uncommonly subclinical, appear to be present in almost all individuals with SDS.²³⁴

Molecular Pathogenesis of SDS

SDS is an autosomal recessive disorder with an estimated incidence of 1 in 76,000.²³⁵ In most patients, the disorder is due to mutations of the Shwachman–Bodian–Diamond syndrome (*SBDS*) gene.^{236,237} Although the parents of most patients are carriers, de novo mutations have also been described. The *SBDS* gene is located on chromosome 7q11 and is composed of 5 exons, encoding for the SBDS protein of 250 amino acids.^{238,239} The *SBDS* gene resides in a 305 kb segment, which is duplicated and located 5.8 Mb distally.²³⁷ The duplication contains a nonfunctional copy of *SBDS* [i.e., a pseudo-*SBDS* (*SBDSP*)], which is 97% identical to *SBDS* but contains deletions and nucleotide changes, interfering with coding for a functional SBDS. The SBDS is expressed in all human tissues

and is found in the cytoplasm, nucleus, and prominently in the nucleolus. *SBDS* moves in and out of the nucleolus in a cell-cycle fashion. Evidence is mounting that *SBDS* contributes to the regulation of ribosomal RNA synthesis and possibly results in mitotic spindle stabilization (Figure 2.7).^{240–243}

Mutations of *SBDS* are detectable in 90% of patients.^{227,237,244–246} In a study of 158 SDS families, 74% of the disease-associated mutations appeared to be the result of gene conversion, while 89% of patients harbored at least one such mutation.²³⁷ In the process referred to as gene conversion, a small segment of *SBDS* is replaced by a segment copied from the *SBDSP* pseudogene.²⁴⁷ Sequences of the inserted segment of *SBDSP* may then inactivate normal *SBDS* gene expression or result in decreased translation of *SBDS* protein. Three converted pathologic alleles account for over 75% of individuals with SDS.^{227,237} Mutations of *SBDS* disrupt normal ribosomal RNA synthesis or processing; however, it is not known how these mutations cause the variable tissue and organ manifestations of SDS (Figure 2.7). Based on murine studies, loss of both *SBDS* alleles is thought to be incompatible with life.²⁴⁸ Thus, at least one of the mutant alleles in SDS patients is expected to result in decreased (or partially functioning) protein.²³⁷ Approximately 10% of individuals with clinical features of SDS do not have a mutation of *SBDS*, suggesting that abnormalities of another gene or genes may be responsible for their disorder. No genotype–phenotype correlations have been described for *SBDS* mutations.^{234,249,250}

Hematopoietic Disorders Associated with SDS

Neutropenia is the presenting finding in 98% of children and is usually moderate with an absolute neutrophil count between 1 and $1.5 \times 10^9/\text{L}$.^{249,251} However, neutropenia may be intermittent, and, therefore, its documentation may require multiple CBC studies over a 3 month period.²⁵² In addition to neutropenia, neutrophils may have impaired chemotaxis.^{230,250,253} Anemia is present in ~40% of patients and may be normocytic or macrocytic. Anemia may be due to erythropoietic failure or iron deficiency, the latter due to intestinal malabsorption. Fetal hemoglobin is elevated in 80% of patients.²²⁸ Initially, 30% of patients present with thrombocytopenia ($<150 \times 10^9/\text{L}$), but this may develop in another 40% with increasing age. The BM is commonly hypocellular with maturation arrest but may be normal or even increased relative to age. Similar to other BM failure syndromes, myelocytic, erythroid, and megakaryocytic lineages may display mild dyspoietic or dysplastic changes. The risk for progression to panhypoplasia and aplastic anemia is estimated at 20–25%. BM failure has been attributed to defects in stem cells and the stromal compartment, increased apoptosis, and shortened telomeres.^{234–258}

From 5 to 33% of patients with SDS may develop myeloid neoplasms, including MDS, JMML, AML, and uncommonly ALL.^{252,259–261} AML is frequently preceded by a myelodysplastic phase. Reported cases of AML are primarily myelocytic and/or monocytic, but all FAB subtypes (except AML M3 and AML M7) have been reported.^{252,262,263} Evidence is lacking for a genotype–phenotype relationship for hematologic abnormalities.²⁵⁰ The risk for malignancies other than hematolymphoid does not appear to be increased.

Aplastic anemia may be difficult to differentiate from MDS on morphologic BM studies as both conditions may have dysplastic morphologic features. Therefore, it is important to perform cytogenetic studies in parallel with BM examinations to document clonal evolution. Clonal cytogenetic abnormalities described in MDS and AML patients with SDS include monosomy 7, 7q deletions and chromosome 7 translocations, chromosome 5q deletions, and complex karyotypes, similar to those observed in non-SDS patients with secondary MDS and AML.^{252,259,263–267} Chromosomal abnormalities iso(7q) and del(20q) have been described in SDS patients without morphologic evidence and development of MDS.^{250,252,261,268} Similar to other inherited BM failure syndromes, the mechanism for developing MDS or AML is not known. Since less than 35% of individuals with SDS develop MDS or AML, it is most likely that an additional genetic abnormality(s) is/are required for myeloid neogenesis. A recent leukemia-related gene expression study of BM cells from SDS patients without MDS or leukemia found upregulation of oncogenes *LARG*, *TAL1*, or *MLL* and downregulation of tumor suppressor genes *DLEU1*, *RUNX1*, *FANCD2*, and *DKC1*.²⁶⁹ Whether over- or underexpression of these genes contributes to development of MDS or AML is not known, since this report provided no information on the clinical course of the studied patients. Others have speculated that genomic instability resulting from abnormal or decreased *SBDS* co-localization with the mitotic spindle leads to myeloid neogenesis.²⁴¹

Tests for SDS

SBDS is the only gene at present known to be associated with SDS. Thus, targeted mutation analysis and sequencing of *SBDS* are the only indicated and available tests for SDS.²⁷⁰ Over 90% of SDS patients will have at least one abnormal allele, due to gene conversion; 76% of these mutations involve one of three gene conversions.²²⁷ A stepwise approach to clinical testing, which moves from initial targeted mutation analysis for the three most common mutations to sequencing of the whole *SBDS* coding region, is shown in Figure 2.8. There is no evidence that patients with MDS or AML and no clinical features of SDS have mutations of *SBDS*.²³⁰

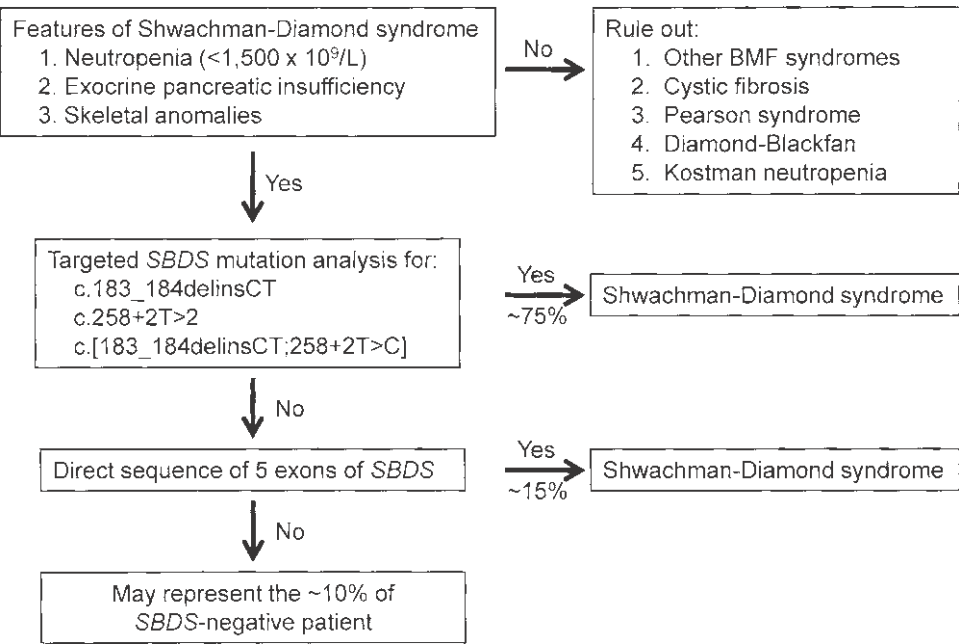


FIG. 2.8. Molecular testing approach for Shwachman–Diamond syndrome (SDS). At least one allele in >90% of patients with SDS have a mutation that occurred as a result of gene conversion. As shown in the figure, approximately 75% of patients with diagnostic

clinical and laboratory features of SDS will have one of three conversion mutations of *SBDS*. Over 35 other less common and rare mutations account for an additional ~15% of *SBDS* mutations that may be detected by direct sequencing the *SBDS* gene.

TABLE 2.5. Diamond–Blackfan anemia associated genetic abnormalities.

Gene	Chromosome	Role in RNA biosynthesis	Frequency ^{280,281,284,285}
<i>RPS19</i>	19q13.2	RP component of the 40S subunit	~25%
<i>RPS17</i>	15q25.2	RP component of the 40S subunit	1–4%
<i>RPS24</i>	10q22-23	RP component of the 40S subunit	1–2%
<i>RPL35A</i>	3q29	RP component of the 60S subunit	2–3%
<i>RPL11</i>	1p36.12	RP component of the 60S subunit	~7%
<i>RPL5</i>	1p22.1	RP component of the 60S subunit	~10%
<i>RPS7</i>	2p25	RP component of the 40S subunit	<2%
Unknown	?	?	~50%

RP ribosomal protein.

Diamond–Blackfan Anemia

Diamond–Blackfan anemia (DBA) is a congenital BM failure syndrome, characterized by anemia and occasionally thrombocytopenia and neutropenia, usually presenting in the first year of life.^{271–275} Congenital abnormalities, such as craniofacial, cardiac, genitourinary, short stature, and hand and upper limb malformations, are also present in half or more of patients. The first cases of DBA were described in 1938 by Diamond and Blackfan, but it was not until 1999 that first of several genes responsible for this syndrome was described.^{276,277} Updated criteria for diagnosing DBA were described in a recent international conference.²⁷⁵ The presenting features may vary greatly and may sometimes initially be difficult to separate from other BM failure syndromes and a variety of acquired anemias, particularly transient erythroblastopenia of childhood. It should be noted that not infrequently some individuals with DBA

will be diagnosed in adulthood, and others will present with congenital abnormalities without anemia.^{271,275}

Molecular Pathogenesis of DBA

The inheritance of DBA is autosomal dominant in some families and recessive in others, but most cases appear to be sporadic, due to extremely variable penetrance of the underlying genetic defect.²⁷¹ The etiology of DBA is due to defects of proteins involved in ribosomal biogenesis (Figure 2.7). A family history of DBA is present in 10–15% of cases, but DBA-related abnormalities, such as elevated erythrocyte adenosine deaminase (ADA) activity, have been detect in another 30% of cases, lending support to the impression of frequent incomplete penetrance.^{273,278,279} Approximately 25, 4, and 2% of DBA patients have mutations in the *RPS19*, *RPS17*, and *RPS24* genes, respectively (Table 2.5).^{273,280–283} Patients with mutations of *RPL35A*, *RPL11*, *RPL5*, and *RPS7* genes encoding for

ribosomal-associated proteins are also described (Table 2.5).^{284,285} The finding of only mutations in genes coding for proteins involved in ribosomal biogenesis points to problems with ribosomal synthesis as the underlying cause of the abnormalities in DBA. The highest concentration of RPS19 is in the nucleolus, the location of initial ribosome construction. RPS19 is one of 33 ribosomal proteins that make up the 40S ribosomal subunit and appears to be required for maturation of 18S rRNA and 40S ribosomal subunit assembly.²⁷³ Thus far, studies of RPS19 indicate that haploinsufficiency (with the inability of the normal *RPS19* allele to compensate) is the underlying disease mechanism in most patients.^{286,287} Mutations of *RPL35A* result in disruption of ribosomal 60S subunit biogenesis, but whether all patients with DBA have defects in ribosomal synthesis is unknown. The molecular defect is unknown in about 50% of DBA patients.

Hematopoietic Disorders Associated with DBA

By definition, all patients with DBA have anemia, which is usually macrocytic with variable anisopoikilocytosis (tear drop cells).²⁷¹ White blood cell counts are usually normal but often decrease with age. Up to 25% of patients will have thrombocytopenia at least on one occasion during their life time. Almost all patients have reticulocytopenia. Hemoglobin F is usually increased as is often observed in other primary and secondary causes of BM failure. Serum levels of iron, B12, and folate are commonly elevated. Red cell ADA is frequently elevated. Erythropoietin is higher in patients with DBA than expected for their level of anemia. BM examinations show normal myelopoiesis and megakaryopoiesis and increased numbers of lymphocytes. About 90% of patients present with erythroid aplasia or hypoplasia that is left-shifted with increased numbers of proerythroblasts. The remaining 10% of patients may show increased, left-shifted erythropoiesis or normal, orderly erythropoiesis. Dyspoietic or dysplastic changes may be observed in the BMs of some patients, and rare instances of ringed sideroblasts have been described. Colony forming unit (CFU) and other studies indicate that the defect in DBA is intrinsic to the erythroid progenitor cells and not due to extrinsic factors. Recent studies of *RPS19* mutations point to disrupted ribosome biogenesis or imbalance in the erythroid lineage as the cause of anemia, but just how this happens is not known.^{272,273,288} Reports of patients with *RPS19* mutations have a wide variety of phenotypic expression, but as compared to DBA patients with no *RPS19* mutations, these patients have a more severe malformations, a lower age of presentation, a lower degree of anemia, and a poorer response to steroid treatment for anemia.²⁸⁹

Information with regard to cancer predispositions is largely confined to case reports and may reflect an overreporting bias.^{271,275} The frequency of cancer in DBA ranges from 1.9 to 6.6%, compared to an expected 0.5% after adjustment for age, sex, and birth cohort.^{275,290–294} Reports of hematolymphoid malignancies include MDS, AML, ALL, and Hodgkin and

non-Hodgkin lymphoma.^{275,290–292,295} No genotype–phenotype correlation for these malignancies has been reported.

Tests for DBA

The identification of *RPS19* on chromosome 19q13 has been based on a study of a patient with DBA whose cells had a t(X;19)(p21;13) translocation.^{277,296} However, this translocation and others involving *RPS19* have not been reported in other patients with DBA. Thus, classical cytogenetic studies are not helpful in confirming the diagnosis of DBA. Commercial testing for mutations of *RPS19* is available but not for other genes implicated in causing DBA. Using genomic DNA obtained from buccal swabs or blood, each of the 5 exons coding for *RPS19* are screened by directional sequence analysis. The *RPS19* gene consists of 6 exons. Exon 1 corresponds to an untranslated region of mRNA. Nucleotides 172 and 173 on exons 2 and 3, respectively, are the hot spots for mutations of *RPS19*.^{282,283,289} Mutations of *RPS19* have not been described in malignant cells of non-DBA patients with leukemia or lymphoma.

Primary Immune Deficiency Disorders

Primary immunodeficiency diseases (PID) are a heterogeneous group of disorders that effect cellular and humoral immunity. Also included are nonspecific host defense systems mediated by natural killer cells, phagocytes, and the complement system. Approximately one in 500 individuals in the United States carries a defect in some component of the immune system.²⁹⁷ For most affected individuals, PID manifests in the first year of life, but there are many exceptions. The most recent catalogue of PID by the International Union of Immunological Societies in 2007 lists 150 disorders, many of which are exceedingly rare.²⁹⁸ Over 20 additional primary immune deficiency disorders have since been recognized.^{299–302} Classifications have traditionally been based on the phenotypic expression of the disease; however, the discovery over the past 10 years of the underlying genetic abnormalities, responsible for most of the recognized PIDs, strongly suggests that future classifications will be based in genotype–phenotype if not genotype alone. Individuals with PIDs are susceptible to infections, autoimmune diseases, and malignancy. The risk of developing hematologic and lymphoid neoplasms is related to the type of PID. Fortunately, these neoplasms develop in only a minority of the recognized PIDs. An overview of the PIDs to be discussed in the following sections is provided in Table 2.1. Ataxia–telangiectasia, in addition to being recognized as a disorder of DNA repair, is also included in most classifications of PID. As this disorder has been discussed earlier, it will not be revisited here. Patients with severe combined immunodeficiency syndromes (SCID) frequently die early of infections, if not treated with BM transplant. Thus, lymphoma and other neoplastic processes are not well-documented in this entity.

However, patients with SCID receiving a BM transplant are at risk for developing lymphoproliferative processes associated with BM transplants in non-SCID patients. SCID is also discussed in Chap. 38.

The hematopoietic and lymphoid neoplasias associated with PIDs have several features in common. Most are diagnosed in infancy or early childhood and involve males more often than females. They more often are lymphoid, as opposed to myeloid neoplasms. Many are associated with EBV infections or other infectious processes that chronically stimulate the immune system. The majority of lymphomas has a mature B-cell phenotype and not infrequently display aggressive morphologic features. Hodgkin lymphoma appears to be more common than expected. Chronic benign or polyclonal lymphoproliferations not infrequently precede the development of lymphoma. And lastly, the lymphomas commonly present in extranodal sites, such as the gastrointestinal tract and central nervous system.

X-Linked Lymphoproliferative Disorder (Also See Chap. 38)

X-linked lymphoproliferative disease (XLP) is a rare inherited immunodeficiency disorder, usually presenting in early childhood with fatal or near-fatal Epstein–Barr virus-induced infectious mononucleosis. Rare sporadic cases of XLP have been described that appear to be relatively milder and develop in older children and young adults. The three clinical manifestations common to this disease include: (1) fulminating infectious mononucleosis; (2) dysgammaglobulinemia; and (3) lymphoproliferative process.^{303–306} Less commonly, patients (initially or later in their course) present with an autoimmune process, such as vasculitis, psoriasis, blood cell cytopenias including aplastic anemia and pure red cell aplasia, nephritis, and colitis.

Fulminating infectious mononucleosis is the most common presentation.^{305,307} As such, the clinical findings are that of overwhelming infectious mononucleosis with fever, fatigue, sore throat, lymphadenopathy, hepatosplenomegaly, atypical lymphocytosis, and frequently hypo- or hypergammaglobulinemia. Lymph nodes contain a polyclonal lymphoid infiltrate with EBV-bearing B-cells and increased numbers of CD4 and CD8 lymphocytes.^{308–310} Histiocytosis and hemophagocytosis are common. The patient's cytotoxic immune system is unable to counter the EBV infection. The release of cytokines, by persistently stimulated cytotoxic T-cells, is thought to contribute to tissue destruction.³¹¹ Mortality associated with EBV infection is about 90%, and is usually due to hepatic or BM failure.^{305,312} Interestingly, patients do not appear exceptionally susceptible to other herpes viruses, such as herpes simplex and cytomegalovirus.

Approximately 25–30% of patients present with dysgammaglobulinemia, manifested by hypogammaglobulinemia of one or more immunoglobulin subclasses or, less

commonly, increased levels of IgM or IgA.^{297,307,312,313} The dysgammaglobulinemia may precede, or follow, EBV infection. Another 25–30% of patients present with lymphoproliferative processes, most often consisting of high grade B-cell Burkitt and large B-cell lymphomas.³⁰⁵ The lymphomas commonly present in extranodal sites, such as the ileocecum, tonsils, liver, kidney, and brain.^{314,315} Small numbers of patients develop Hodgkin lymphoma, precursor T-ALL/lymphoblastic lymphoma, lymphomatoid granulomatosis, or angiocentric lymphoproliferative processes.^{305,310,312,316,317} Rarely, patients present with a lymphoproliferative process, including lymphoma without an antecedent EBV infection.^{312,318–320} Thus, the role EBV in the genesis of the lymphoma is unclear.

Molecular Pathogenesis of XLP

XLP is inherited in an X-linked recessive manner. The International Union of Immunological Societies Primary Immunodeficiency Disease Classification of 2007 recognizes mutations of *SH2D1A* [*Src homology 2 (SH2) domain-containing gene 1A*] as the cause of XLP; however, others more recently describe rare patients presenting with clinical features almost identical to XLP but with mutations of *XIAP* (*X-linked inhibitor of apoptosis protein*).^{298,321} The *SH2D1A* gene, located on chromosome Xq12, was identified by three laboratories in 1998.^{322–324} This gene consists of four exons, and its transcript encodes for SLAM associated protein (SAP), which contains a SH2 domain and a 26 amino acid carboxy-terminal extension. SAP is present in NK-cells, T-thymocytes, mature CD4 and CD8 T-lymphocytes, and some B-cell subpopulations.^{303,305,322,324–328}

Defects in the cytotoxic armamentarium of the immune system appear largely responsible for the susceptibility of XLP patients with mutations of *SH2D1A* to infections with EBV. The SAP protein interacts with CD150 (SLAM), a member of the CD2 subfamily of Ig receptors consisting of CD2, CD58, CD48, CD84, CD229, CD244 (2B4), CRACC, and NTB-A.^{324,329–334} CD150/SLAM is expressed on the surface of T-thymocytes, T-cells, B-cells, dendritic cells, and macrophages.^{331,335,336} Following lymphocyte activation, CD150 is upregulated. The SAP protein binds to the cytoplasmic domain of CD150 and possibly other members of the CD2 subfamily of Ig receptors and is postulated to participate in regulation of signaling pathways involved in the activation NK-cells.^{337,338} NK-cell numbers are normal in XLP patients, but unlike normal NK-cells, the cytotoxic activity of XLP-derived NK-cells is deficient when activated through receptors known to be associated with SAP.^{339,340} Furthermore, NK-cells from XLP individuals are unable to kill EBV-transformed B-cell lines.³⁴¹ Cytotoxic T-cells are decreased in patients with XLP and probably also contribute to the inability of patients to effectively fend off an EBV infection.³⁴²

The number of B-lymphocytes in XLP individuals with mutations of *SH2D1A* may be decreased, normal, or slightly increased without active disease; however, maturation of

naïve B-cells is dysfunctional, and there is a marked decrease of memory B-cell.^{343–345} The B-cell deficiencies are in part due to the inability of CD4 T-cells to differentiate into effective IL-10 producing helper cells.³⁴³ As IL-10 is a growth differentiation cytokine for B-cells, a decreased level or absence of IL-10 may contribute to decreased numbers of B-cells and decreased levels of serum immunoglobulin.^{343,344,346,347}

In the classical form of XLP, mutations of *SH2D1A* span the length of the gene, but most are concentrated in exon 2.³³⁸ Only rare patients with no family history of XLP will have a sporadic mutation of *SH2D1A*. Mutations of *SH2D1A* consist of large and small deletions or insertions, single nucleotide substitutions, and splice-site abnormalities. These mutations alter the SH2 domain of SAP directly, or indirectly destabilize it. The loss or altered SAP function underlies the immunologic defects in XLP, as described above. A genotype–phenotype correlation has not been identified, and clinical manifestations with a family may vary considerably. This suggests that other host or external factors influence the clinical presentation and course of XLP.³⁴⁸

The few studied patients with defects of the *XIAP* gene located on chromosome Xq25 have a disorder closely resembling XLP.^{321,349,350} Similar to patients with *SH2D1A* mutations, individuals with *XIAP* mutations have low numbers of NK-T cells, develop lymphohistiocytosis in response to EBV infections, and may have hypogammaglobulinemia. But unlike patients with *SH2D1A* mutations, these patients do not have impairments of 2B4 mediated NK cell cytotoxicity, and it is unknown if they are susceptible to developing lymphoma.³⁴⁹

Testing for XLP

The confirmation of XLP is by molecular testing for mutations of *SH2D1A*, and in rare families, for mutations of *XIAP*. Over 60 disease-related mutations involving the

four exons of *SH2D1A* have been described.^{338,351} PCR-gene amplification with sequencing analysis of the entire coding region of *SH2D1A* and exon/intron boundaries identifies ~97% of *SH2D1A* mutations in affected males who have two or more maternally related family members with an XLP phenotype and about 75% of *SH2D1A* mutations in carrier females. By this method, large deletions of *SH2D1A*, which account for up to 25% of mutations in families with XLP, will result in the absence of amplification of the *SH2D1A*. Screening for SAP expression by flow cytometry may be used for screening of individuals suspected of having XLP, but it does not replace definitive confirmation by molecular testing.³⁵²

Hyper-IgM Syndromes (Also See Chap. 38)

Hyper-IgM (HIGM) encompasses a group of disorders, which have in common normal to increased levels of serum IgM, decreased IgG and IgA, and recurrent bacterial and often opportunistic infections.^{353,354} Over the past several years, the cellular and molecular mechanisms causing these diseases have been characterized and are now known to interfere with immunoglobulin class switching recombination (Ig CSR) and somatic hypermutation (SHM)^{353,355–358} As such, it has been suggested that these diseases be referred to as “defective Ig class switch recombination” disorders.

Five underlying genetic abnormalities account for over 85% of the cases of HIGM.^{355–359} The HIGM subtypes, based on their associated genetic anomalies are listed in Table 2.6, for comparison of their salient features. An X-linked form, caused by deficiency of CD40L, is the most common cause of HIGM and accounts for 65–70% of reported cases.^{355,359,360} These individuals present in infancy with features suggestive of combined immune deficiency,

TABLE 2.6. Features of hyper-IgM syndromes.

Type	Inheritance	Defect	Frequency	Defect in CSR	Defect in SHM	Function	Associated with LH	Associated with ML
1	XL, AR ^a , AD ^a	CD40 ^b	65–70%	Yes	Yes	CD4+ T cell surface molecule that regulates B cell function by engaging CD40 on B-cells	Rare	?
2	AR, AD	AID	10–15%	Yes	Yes	A ssDNA deaminase required for conversion of deoxycytidine to deoxyuridine	Yes	No
3	AR	CD40	<2%	Yes	Yes	Cross-linking to CD40L results in germinal center cell proliferation with protection from apoptosis	No	No
4	AR, S	?	<1%	Yes	No	Defect either in CSR-specific factor of DNA repair or in survival signals delivered to switched B cells	Yes	No
5	AR	UNG	~5%	Yes	No	mediates deglycosylation & removal of deoxyuridine residues on ssDNA	Yes	Yes
6	XL	NEMO	<2%	Yes	Yes	regulatory subunit, IKK γ , of the kappaB kinase (IKK) complex which regulates NF- κ B signaling. NF- κ B is required for AID and UNG expression.	No	?

CSR class switch recombination, SHM somatic hypermutation, LH lymphoid hyperplasia, ML malignant lymphoma, XL X-linked, AR autosomal recessive, AD autosomal dominant, S sporadic, CD40L CD40 ligand, NEMO nuclear factor κ B essential modulator, UNG uracil DNA glycosylase.

^aRare/uncommon modes of inheritance.

^bAlso called CD154.

including respiratory infections with *Pneumocystis carinii* and protracted diarrhea due to *Cryptosporidia*. Infections with *Cryptosporidia* may contribute to the high frequency of sclerosing cholangitis in these patients and possibly for the increased incidence of tumors of the liver and biliary tract. Patients have normal numbers and functional B-cells in the blood, but IgD-, CD27+ memory B-cells are markedly decreased. Serum IgG and IgA are markedly reduced, while levels of IgM are normal or increased. A defect in T-cell antigen presenting is suggested by susceptibility of these patients to *Pneumocystis* and *Cryptosporidia* infections. Lymph nodes of these patients have primary follicles but lack germinal centers.³⁶¹

A very rare autosomal recessive form of HIGM is due to a complete lack of CD40 on lymphocytes, monocytes, and dendritic cells.^{362,363} Like deficiency of CD40L, patients with lack of CD40 present at an early age with opportunistic infections and failure to thrive. They have normal numbers of B cells but decreased numbers of memory B-cells. In contrast to B-cells from patients with CD40L deficiency, B-cells from these patients are unable to produce IgG or IgA in vitro in response to anti-CD40 ligation or IL-10.³⁶² These individuals cannot mount recall responses after antigenic challenges. Their serum IgG and IgA levels are markedly reduced, but IgM concentrations are normal to increased. Similar to CD40L-deficient patients, the lymph nodes of these patients contain primary follicles but lack germinal centers. The lymphoid hyperplasia often decreases in patients treated with IVIG therapy, suggesting that the hyperplasia is due to continuous B-cell stimulation by infectious processes.

A rare autosomal recessive form of HIGM is the result of deficiency of activation-induced cytidine deaminase (AID).^{364,365} These patients often present in their teens or twenties with histories of repeated bacterial sino respiratory and gastrointestinal infections but not opportunistic infections with *Pneumocystis* and *Cryptosporidium*. Autoimmune processes, especially hemolytic anemia and thrombocytopenia, are common. These individuals have normal numbers of B-cells and memory B-cells, as well as intact T-cell functions, but their serum IgG and IgA levels are decreased and IgM levels are normal to increased. Over half of these patients have lymphadenopathy, due to follicular hyperplasia with giant germinal centers.^{356,364,366} The germinal centers are comprised of proliferating B-cells that coexpress IgM, IgD, CD38, and nuclear Ki-67 – a phenotype characteristic of germinal center founder cells.³⁶⁷

Very rare individuals with a deficiency of uracil N-glycosylase (UNG) have been discovered in an Ig CSR study of patients with high levels of serum IgM levels, profound decreases of IgG and IgA, recurrent bacterial infections, and no deficits of CD40L, CD40, or AID.^{368,369} These patients have normal numbers of B- and T-cells; however, in contrast to cases of CD40 and CD40L deficiency, CD27+ memory B-cells are detectable. Two of three studied patients had lymphadenopathy, but no histologic information was recorded.

A subset of individuals with the rare syndrome of hypohidrotic ectodermal dysplasia-A (EDA), characterized by abnormal development of hair, teeth, and sweat glands, are immunodeficient. Patients with the immunodeficient type of hypohidrotic EDA are susceptible to bacterial infections and particularly to infections with atypical mycobacterium. They have low serum levels of IgG and IgA with normal to increased levels of IgM and impaired response to polysaccharide antigens. Whereas mutations of *EDAI* (*ectodysplasin-A*) or *EDAR* (*ectodysplasin-A receptor*) are responsible for hypohidrotic EDA, mutations of *NEMO* (*NFκB essential modulator*) result in the rare syndrome of hypohidrotic EDA with immunodeficiency.^{370–372}

Pathogenesis of HIGM

All patients with HIGM have a defect in Ig CSR and/or SHM.^{355–358} Mature post-BM naïve B-cells have surface membrane IgM or IgM plus IgD but no “switched” IgG, IgA, or IgE. The surface IgM of naïve B-cells have low affinity for foreign antigens or extracellular pathogens. Normally, after engagement of IgM B-cells with foreign antigen, effective humoral response requires further maturation of B-cells. This is accomplished by the production of antibodies of various isotypes with high affinity for foreign antigens. Antibody “maturation” takes place in the germinal centers of secondary lymphoid tissues and requires two independent processes: (1) CSR, which results in production of antibodies of different isotypes (IgG, IgA, and IgE); and (2) SHM, which introduces mutations, mostly in the V region of immunoglobulins, resulting in the production of high affinity immunoglobulins (Figure 2.9).^{373,374} These two processes require close T- and B-cell cooperation, involving CD40 ligand (CD40L/CD154), a molecule transiently expressed on activated T-helper cells, and CD40, which is constitutively expressed on B cells (Figure 2.10). The Ig CSR is a DNA recombinatory process between two constant regions of the immunoglobulin heavy gene. It results in the replacement of the μ heavy chain region with a downstream $C\gamma$, $C\alpha$, or $C\epsilon$ heavy chain region, leading to the generation of IgG, IgA, or IgE, respectively, with the same V regions as the parent IgM molecule. With SHM, point mutations occur at a high rate in the V regions of immunoglobulin genes, resulting in an expansion of and selection of high-affinity antigen-specific antibodies. Both CSR and SHM are interdependent and nonredundant and require AID and UNG.^{355,375} The role of *NEMO* in CSR and SHM is more complex. The *NEMO* gene encodes $IKK\gamma$, which is a component of the $I\kappa B$ kinase, a complex of $IKK\alpha$, $IKK\beta$, and $IKK\gamma$. This complex is a regulator of NF- κB activation and its translocation to the cell nucleus. If NF- κB translocation to the nucleus is defective, multiple NF- κB -dependent proteins, including AID and UNG, cannot be expressed.^{357,376} Thus, mutations of the genes encoding for CD40L/CD154, CD40, AID, UNG, and *NEMO* may all potentially interfere with Ig class switching and production of IgG, IgA, and IgE.

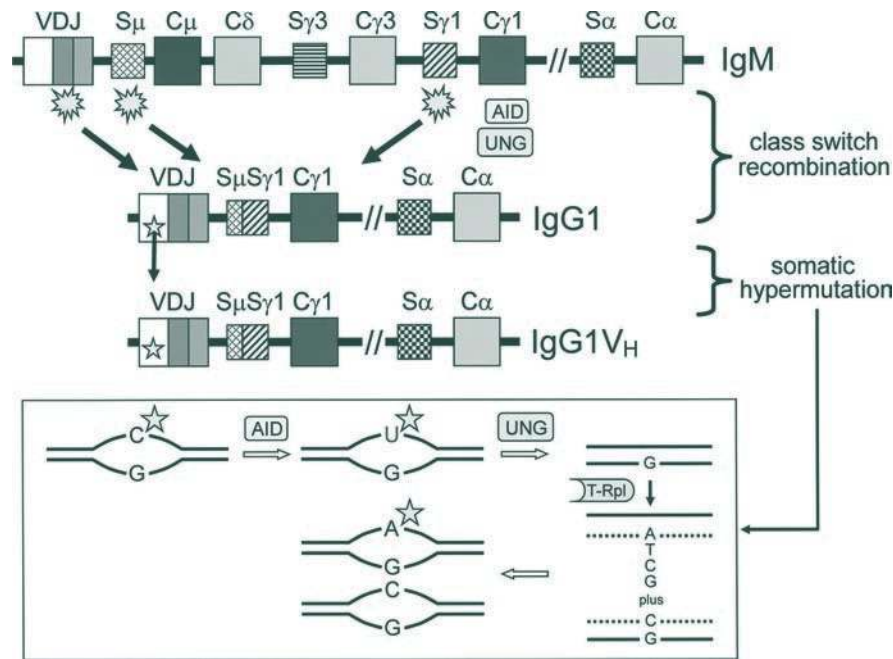


FIG. 2.9. Schematic of class switch recombination (CSR) and somatic hypermutation (SHM) resulting hypermutated IgG1 (IgG1V_H). Sites of involvement of activation-induced cytidine deaminase and uracil N-glycosylase are indicated by AID and UNG, respectively.

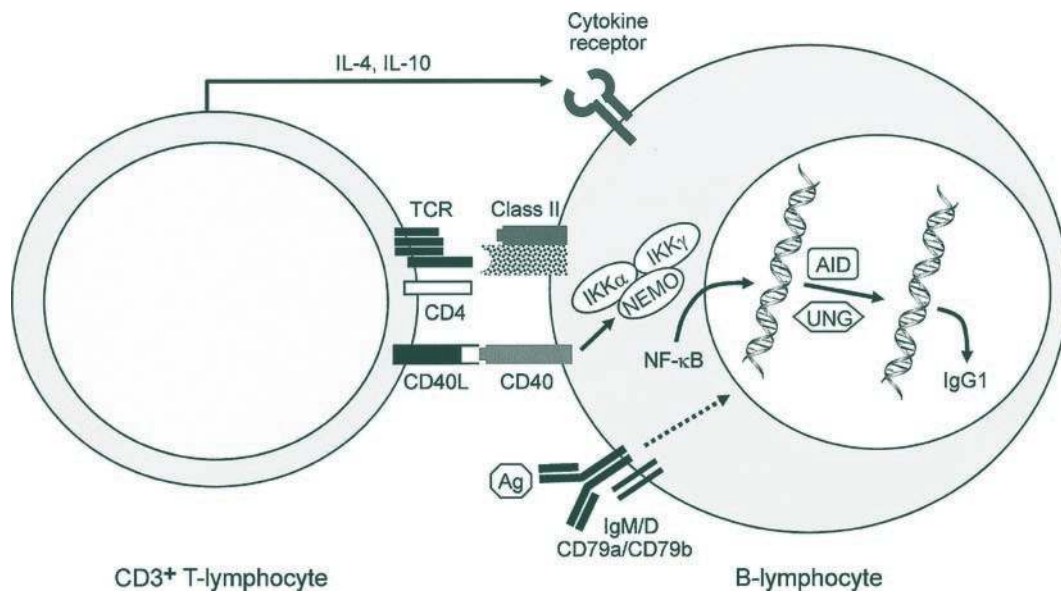


FIG. 2.10. Graphic representation of known molecular defects leading to Hyper-IgM syndromes. Ineffective interaction of CD40 and CD40L due to defects of these molecules result in impaired class switch recombination (CSR) and somatic hypermutation

(SHM). Downstream defects of NF-κB essential modulator (NEMO), activation-induced cytidine deaminase (AID), or uracil DNA glycosylase (UNG) similarly result in blocks of CSR and/or SHM.

Hematolymphoid Disorders Associated with HIGM

Very few hematologic and lymphoid neoplasms have been reported in association with HIGM. This is in-part due to the rarity of the condition and relatively short life span of

patients with HIGM. However, patients with HIGM appear to have an increased risk of lymphoma, particularly Hodgkin lymphoma in association with EBV infection.³⁷⁷ Case reports of lymphoma and leukemia in HIGM include MALT lymphoma, large granular lymphocytic leukemia, and suppressor T cell lymphoma.^{378–381}

Tests for HIGM

Prior to molecular testing, the diagnosis of HIGM should be ascertained by low levels of IgG and IgA with normal to increased IgM levels, normal numbers and distribution of CD4+ and CD8+ T-cell subsets, normal T-cell proliferation in response to mitogens, and normal numbers of B-cells.³⁵³ Since over 70% of HIGM patients have a mutation of *CD40L*, the next step is to test patients satisfying the preceding criteria for the ability of their stimulated T-cells to express CD40L/CD154.^{382,383} If CD40L/CD154 is not expressed, molecular genetic testing for mutations of *CD40LG* may be performed by sequence analysis. If CD40L/CD154 is not detectable, the patient may have a defect of *CD40*, *UNG*, or *AID*. The rare patient with a mutation of *NEMO* should be suspected if the patient has cutaneous and hair anomalies characteristic of hypohidrotic EDA. Approximately 20% of patients with clinical and laboratory features of HIGM may have another genetic abnormality that has not yet described. Patients with a CD40 mutation may be screened by analysis of their monocytes for expression of CD40. For patients with a family history and known inherited gene abnormality, molecular testing for the abnormal gene should be performed for confirmation and genetic counseling.

Hyper-IgE Syndromes

The hyper-IgE syndrome (HIES), first described as “Job’s syndrome,” is characterized by high serum IgE, sinopulmonary bacterial infections, chronic dermatitis with eczematous eruptions prone to severe superinfections with *Staphylococcus aureus*, and susceptibility to *Aspergillus fumigatus* pneuminitis.^{384–388} Additionally, patients may also have skeletal, connective tissue, cardiac, and brain abnormalities. The major immunologic findings are elevated serum levels of IgE, eosinophilia, and occasionally reduced T-cell response to IL-12 in vitro. Serum IgG, IgM, and IgA are usually normal, but one or more of these may also be low in some patients. The diagnosis is established by clinical and laboratory features and aided by a scoring system.³⁸⁹

Pathogenesis of HIES

The vast majority of cases of HIES are sporadic, but autosomal dominant and rare recessive forms are well documented. Mutations of *STAT3* (*signal transducer and activator of transcription 3*) are responsible for the majority of sporadic and autosomal dominant forms of HIES.^{390,391} *STAT3* is located on chromosome 17q21.31 and encodes for STAT3, a member of the STAT family of proteins. STAT3 is activated through phosphorylation in response to various cytokines and growth factors, such as IFNs, EGF, IL2, IL6, IL10, HGF, LIF, and G-CSF.³⁹² Activated STAT3 forms homo- or heterodimers, which move to the cell nucleus and function as transcription activators. STAT3 is a major signal transduction protein involved in immune pathways, apoptosis, angiogenesis,

wound healing, and cancer. In patients with HIES, mutation of *STAT3* have been described in the SH2 domain and the DNA-binding domain. All mutations so far have been missense mutations or in-frame deletions, which result in full-length mutant STAT3, which is able to exert a dominant negative effect. Although STAT3 is a key regulatory of many immunologic pathways, the immune defects resulting from it deficiency remain unexplained. The high IgE level may be due to defects in STAT3-mediated IL-12 signaling.³⁹³

An autosomal recessive form of HIES is also associated with mutations of *STAT3*.³⁹⁴ Patient with this form of HIES have elevated IgE, severe eczema, and recurrent skin and lung infections but not the connective tissue and skeletal findings of autosomal dominant HIES. A single patient with a null mutation of tyrosine kinase 2 (*TYK2*) and autosomal recessive inherited form of a HIES-like disorder has also been reported.^{395,396} An association of neoplasia with the autosomal recessive form of HIES has not been described.

Hematolymphoid Disorders Associated with HIES

Deficiencies of STAT3 appear to be associated with an increased risk of non-Hodgkin lymphoma and cancers of vulva, liver, and lung.^{397–403} The majority of lymphomas are of B-cell origin with aggressive histology, including diffuse large B-cell lymphoma and Burkitt lymphoma. Rare cases of mantle cell lymphoma, Hodgkin lymphoma, peripheral T-cell lymphoma, and anaplastic large cell lymphoma have also been described.^{398–403} These lymphomas were reported before the association of mutations of *STAT3* with HIES was discovered. Thus, the role of these mutations in the development of lymphoma in HIES is not known.

Common Variable Immune Deficiency (Also See Chap. 38)

Common variable immune deficiency (CVID) encompasses a group of genetically and etiologically different disorders, which share a “late onset” of humoral immunodeficiency, impaired response to immunization, autoimmune processes, recurrent infections, lymphoid and granulomatous proliferations, and a predisposition to malignancy.^{404–411} Late onset is defined as occurring after 24 months of age.⁴¹² Indeed, many individuals are diagnosed in their second and third decades of life. The concentration of serum IgG is markedly below normal and is commonly accompanied by low or absent concentrations of IgA and/or IgM. Additionally, these individuals have an impaired response to immunization. The cellular immune defects are complex and include reduced to normal numbers of B-lymphocytes, low percentages of class switch memory B-cells, low CD4 T-cell numbers, and normal or increased numbers of NK-cells. Functional defects include dysfunctional cytokine production, abnormal T-cell

TABLE 2.7. Genes known to be involved in the heritable causes of common variable immune deficiency.

Gene	Chromosome	Function and associated disease manifestations	Inheritance	Frequency	References
<i>TNFRSF13b^a</i>	17p11.2	Encodes a B-cell surface immunoregulatory member of the TNF; preferentially expressed by transitional B-cells and marginal zone B-cells; involved in B-cell development; regulates class switching of Ig; interacts with BAFF; associated with IgA deficiency, lymphoproliferations, autoimmune processes	AD, AD	10–20%	413–417
<i>ICOS</i>	2q33	Encodes a T-cell surface immunoregulatory molecule expressed by activated T-lymphocytes; enhances NK-cell function; deficiency causes impaired T-cell help resulting in inability of B-cells to undergo Ig class switching; associated with lymphoid hyperplasia, autoimmunity, and susceptibility to malignancy	AR	<2%	404,413,418,419
<i>CD19</i>	16p11.2	Encodes for B-cell CD19 co-receptor of the Ig receptor superfamily; involved in B-cell development; mutations result in absence of CD19 on CD20+ B-cells, low CD5+ and CD27+ B-cells	AR	<1%	404,413,420
<i>TNFRSF13c^b</i>	22q13.1-3	Encodes a B-cell immunoregulatory member of the TNF (BAFF) family; involved in maintaining B-cell homeostasis/survival and production of IgG and IgA	AR	<1%	404,413
Unknown	?	?		>75%	

AR autosomal recessive, AD autosomal dominant, *TNF* tissue necrosis factor, *Ig* immunoglobulin.

^aAlso termed *TAC1*.

^bAlso termed *BAFFR*.

signaling, and activated suppressor T-cells. As a result of the humoral deficiency, most individuals with CVID have recurrent infections, the most common of which are sinopulmonary bacterial infections. Gastrointestinal infections are also common. Many of these individuals develop autoimmune disorders, including autoimmune anemia or thrombocytopenia, rheumatoid-like arthritis, systemic lupus erythematosus-like dermatopathy, hepatitis, thyroiditis, and alopecia areata.

Molecular Pathogenesis of CVID

Most instances of CVID appear to be sporadic, but more recent studies of families with several affected members points to autosomal recessive, dominant, and X-linked modes of inheritance. Recent molecular genetic studies have identified a variety of established or strongly suspected gene abnormalities associated with inherited forms of CVID. These include mutations of *TNFRSF13b*, *ICOS*, *CD19*, and *TNFRSF13c*.^{404,413–420} Together, defects of these genes are found in less than 25% of cases. The genes, their function, mode of inheritance, and genotype–phenotype associations are summarized in Table 2.7.

Hematolymphoid Disorders Associated with CVID

Up to 20% of individuals with CVID will have a systemic, noncaseating granulomatous process and many develop a granulomatous or lymphomatous interstitial lung disease (GLILD).^{404,421} Human herpes virus 8 (HHV8) has been

linked to some, but not all, cases of GLILD.⁴²² Up to 10% of individuals with CVID develop a benign atypical lymphoproliferative process, resulting in significant lymphadenopathy and splenomegaly.⁴²³ The hyperplasia may be so florid, as to mimic lymphoma. A 12- to 47-fold increase of lymphoma has been reported.^{407,424–426} Most lymphomas are B-cell in origin, although rare cases of T-cell lymphoma have also been described.^{423,427–439} The B-cell lymphomas consist of MALT lymphoma, diffuse large B-cell lymphoma, Burkitt lymphoma, and uncommon instances of Hodgkin and HHV8-negative pulmonary effusion lymphomas. The MALT lymphomas involve the lung significantly more often than the stomach, which is just the reverse presentation in non-CVID patients. This is unexpected, since *Helicobacter pylori* (which is implicated in the genesis of stomach MALT) is also a common infection in CVID. EBV is not present in most B-cell lymphomas of these patients.⁴²⁹ In a study of 334 subjects with CVID, The European Common Variable Immunodeficiency Disorders registry defined five distinct clinical phenotypes: (1) no complications; (2) autoimmunity; (3) polyclonal lymphocytic infiltration; (4) enteropathy; and (5) lymphoid malignancy.⁴⁰⁵ Of the five phenotypes, only polyclonal lymphocytic infiltration was associated with an increased risk of lymphoma. Small studies have demonstrated several genotype–phenotype associations, but very little is known about genetic abnormalities and their association with the development of lymphoid malignancy. The development of lymphoma in CVID is postulated to be due a complex interaction between genetic defects, chronic immune stimulation from recurrent infections, and an altered immune system.⁴²⁷

Tests for CVID

The establishment of the diagnosis of CVID rests on family history, clinical examination, and the laboratory tests of immune status and function. Other PIDs, including X-linked agammaglobulinemia (*BTK* gene), X-linked lymphoproliferative disease (*SH2DA1* gene), and Wiskott–Aldrich (*WAS* gene), may have clinical and laboratory features resembling CVID. Thus molecular genetic testing may be indicated for excluding these other disorders, as well as for additional prognostic information and genetic counseling of family members. Unfortunately, the cause of CVID is unknown in over 75% of cases. Molecular testing rests on sequence analysis of genes known to be associated with CVID and of genes associated with PIDs with clinical and laboratory features shared with CVID.

Wiskott–Aldrich Syndrome (Also See Chap. 39)

Wiskott–Aldrich Syndrome (WAS) is an X-linked recessive disorder. Affected males have eczema, recurrent bacterial infections, and a marked microthrombocytopenia.⁴⁴⁰ Viral infections with cytomegalovirus, herpes simplex virus, Epstein–Barr virus, and adenovirus are common. Serum IgM concentrations are decreased, but serum IgG levels are usually normal and IgA and IgE levels are increased. Patients have a poor response to protein antigens and have defective antibody production against the polysaccharide capsule of bacteria. Over time, B cell numbers decrease while T cells increase.⁴⁴¹ Up to 40% of patient develop one or more autoimmune disorders, including hemolytic anemia, immune-mediated neutropenia, immune thrombocytopenic purpura (ITP), vasculitis, inflammatory bowel disease, and immune damage to kidneys or liver.^{440,442}

All individuals with WAS have an abnormal *WAS* gene, located on chromosome Xp11.22.⁴⁴³ *WAS* encodes for a 53 kD proline rich, multidomain WAS-protein (WASP) that regulates cytoskeletal rearrangements mainly in hematopoietic cells.⁴⁴³ WASP is involved in transduction of signals from the cell membrane to the actin cytoskeleton. External cell stimuli may result in actin cytoskeleton reorganization and thereby play a significant role in chemotaxis, cell polarization, adhesion by podosome formation, migration, and phagocytosis.^{444–450}

Over 240 *WAS* mutations have been described. Nonsense and missense mutations, deletions, and insertions of *WAS* may result in partial, defective, or complete absence of WASP. Missense mutations of exons 1 or 2 may result in decreased but detectable WASP.⁴⁵¹ These missense mutations are frequently found in patients with *WAS*-related X-linked thrombocytopenia who have no to little manifestations of immunologic disturbances.^{440,451} Another *WAS*-related entity, X-linked congenital neutropenia is associated with missense mutations within the Cdc42-binding site domain that result in normal levels of (but

constitutively activated) WASP.⁴⁵² Individuals with X-linked congenital neutropenia are not prone to developing autoimmune or a lymphoproliferative process. Other recent studies show that most individuals who produced normal size mutated WASP have thrombocytopenia but are less likely to develop autoimmune disorders or lymphoma; whereas, those with no or truncated WASP have the classic WA phenotype.^{453–456} Thus, the level of functional WASP appears to be a better predictor of the disease severity than the type of mutation.⁴⁵⁷ Spontaneous reversion of *WAS* resulting in somatic mosaicism has been observed in *WAS* patients. Reported reversions are the result of restoration of the reading frame and expression of normal WASP. This is usually confined to T-, B-, and NK-cells and not other tissues.^{457,458}

The immunologic abnormalities observed in *WAS* patients are due to a complex interplay between T- and B-cells, NK-cells, macrophages, and dendritic cells. WASP expression confers a selective growth advantage to T-cells.⁴⁵⁹ The absence of functional WASP directly or indirectly contributes to a reduction of circulating T-cells. CD4 and CD8 T-cells of WA patients have defective Th1 cytokine gene transcription of IL-1, IFN- γ , and TNF- α .⁴⁶⁰ The ability to form synapses and secrete cytotoxic granules is hindered in NK cells deficient in WASP. Expression of WASP in dendritic cells also regulates synapse formation and activation of naïve CD8 T-cells.⁴⁶¹ Recent murine studies suggest that in the absence of WASP, there is inefficient localization of mature B-cells to specific compartments in lymph nodes.^{462,463} Possibly as a result of this, B-cells try to compensate by increasing their proliferative rate.⁴⁶⁴ How the interplay of these defective cells types contribute to autoimmunity and possibly lymphoid neoplasms is not known.

Hematolymphoid Disorders Associated with WAS

Up to 13% of patients with *WAS* develop non-Hodgkin lymphoma at an average age of 9.5 years.^{440,442,465,466} Diffuse large B-cell lymphomas predominate, but patients with EBV-positive T-cell and Hodgkin lymphoma have been reported. Patients with *WAS* may develop lymphomatoid granulomatosis, rarely Burkitt lymphoma, and rarely follicular lymphoma.^{467–469} Lymphomas commonly present in extranodal locations, such as the brain, lung, or gastrointestinal tract. Those patients with autoimmune problems are more susceptible to developing lymphoma. Infection with Epstein–Barr virus also places patients at higher risk of developing lymphoma. One investigation suggests that discordant expression of WASP in T and B cells (i.e., WASP detected in B- but not T-cells) is an indication for increased risk of lymphoma.⁴⁷⁰ Individuals with *WAS* do not appear to be at increased risk for acute leukemia and those with X-linked thrombocytopenia and X-linked congenital neutropenia WASP-related disorders are at low risk for developing lymphoma. Female carriers are not at increased risk for malignancy.

Tests for WAS

The diagnosis of WAS should be suspected in males with marked thrombocytopenia and small platelets plus a history of recurrent bacterial infections, autoimmune disorders, and eczema. The diagnosis is confirmed by tests for the WASP protein or molecular genetic testing. The absence or decreased levels of WASP may be readily detected in peripheral blood lymphocytes by flow cytometry or western blotting,^{471,472} although it should be noted that genetic reversion may confound these assays.⁴⁵⁸ Molecular confirmation of a WAS mutation is performed by SSCP and heteroduplex assays, followed by DNA sequencing. Sequencing of the entire coding region and intron/exon boundaries of WAS will detect over 95% of mutations in males.^{443,473}

Autoimmune Lymphoproliferative Syndrome (Also See Chap. 38)

Autoimmune lymphoproliferative syndrome (ALPS) is a rare lymphoproliferative disorder, that usually presents in childhood and is characterized by lymphadenopathy, splenomegaly, hypergammaglobulinemia, autoimmune processes, increased numbers of double-negative (i.e., CD4-/CD8-) T-cells (DNTs) in the blood, and an increased risk for developing lymphoma.⁴⁷⁴⁻⁴⁷⁸ The lymphadenopathy often fluctuates and may decrease in later life. Lymphadenopathy and splenomegaly are due to benign follicular and interfollicular (T-zone) hyperplasias.⁴⁷⁹ Autoimmune manifestations include hemolytic anemia, thrombocytopenia, and less commonly neutropenia, the latter of which may be immune-mediated or secondary to hypersplenism. Other, rarer autoimmune processes include glomerulonephritis, Guillain-Barre syndrome, autoimmune hepatitis, and IgA dermatopathy. Some patients have eosinophilia, which may be predictive for increased mortality.^{474,480} Circulating autoantibodies are found in up to 80% of ALPS patients.^{474,475} Autoantibodies to platelets or neutrophils are present in up to 46 and 35% of patients, respectively, but their presence is not necessarily accompanied by thrombocytopenia or neutropenia.

The peripheral blood contains an increased number of TCRαβ CD3+/CD4-/CD8- T-cells (DNT) that also lack CD45RO but are CD45RA positive.^{480,481} DNT cells in persons with ALPS may be as high as 40%. This contrasts with non-ALPS individuals that have <1% DNT cells consisting of several distinctly different subpopulations. The DNT cells in ALPS are thought to be derived from chronically activated CD8 T-cells. Other changes in lymphocyte subsets of individuals with ALPS include increases of CD5+ B-cells, CD57+ CD8+ T-cells, TCRγδ-positive T-cells, and a decrease of CD4+/CD25+ T-cells.^{480,481} NK cell numbers are usually normal. Relatives of those with *FAS* mutations, but without the clinical criteria for ALPS, may also have increase numbers of TCRαβ DNT cells, and TCRγδ T-cells. DNT cells secrete

relatively high amounts of IL-10, which might account in part for hypergammaglobulinemia and autoantibody productions.^{481,482} The role DNT cells play in the pathogenesis of ALPS is not known.

Molecular Pathogenesis of ALPS

A little over a decade ago, studies showed that a defect in the Fas-Fas ligand apoptotic pathway was responsible for ALPS.⁴⁸³⁻⁴⁸⁵ Apoptosis of activated T- and B-cells is initiated by the binding of Fas ligand, which in turn transduces a death signal through its cytoplasmic death domain. The cytoplasmic death domain contains a binding site for other proteins that activate the caspase apoptotic cascade.^{486,487} Lymphocytes of individuals with ALPS fail to efficiently undergo apoptosis, leading to lymphadenopathy and splenomegaly. The mechanism for autoimmunity is not known, but impaired apoptosis may lead to an accumulation of lymphocytes, which fail to delete self-reactive cells.⁴⁸⁸

A classification of ALPS, based on mutations of apoptotic pathway associated genes, is presented in Table 2.8. Infants with Type 0 ALPS are homozygous or compound heterozygous for *FAS* mutations and present with marked lymphoproliferations at or shortly after birth. The majority of ALPS individuals have ALPS Type Ia, caused by an autosomal dominant inherited mutation of *FAS* (*TNFRSF6*), which encodes for the CD95 (Fas) death receptor.⁴⁸⁸ The *FAS* gene is located on chromosome 10q24.1 and consists of nine exons. Over 70 mutations of *FAS* have been described and consist largely of heterozygous missense single nucleotide substitutions, affecting the intracellular death domain encoded by exon 9. However, mutations of any domain of *FAS* results in the same clinical phenotype.⁴⁷⁴ These mutations are dominant negative, which may be explained by the requirement a functional trimeric Fas receptor structure.⁴⁸⁸ There is a complex genotype-phenotype association in Type Ia ALPS. Within a given family with the same mutation, the disease manifestation may vary from no to severe symptoms.⁴⁸⁹ This heterogeneous phenotype implies the possibility of the contribution of one or more additional factors to the pathogenesis of ALPS.

TABLE 2.8. Classification of ALPS based on genetic features.^{474,488}

Group	Gene defect	Chromosome	% of ALPS
Type 0 ^a	<i>FAS</i>	10q24.1	Rare
Type Ia	<i>FAS</i>	10q24.1	~75%
Type Ib	<i>FASLG</i>	1q32	Rare
Type Im	Somatic (mosaic <i>FAS</i>)	10q24.1	Rare
Type II	<i>CASP8</i> or <i>CASP10</i>	2q23	Rare
Type III ^b	Unknown	?	20-25%
Type IV ^c	<i>NRAS</i>		

^aThe consequence of homozygous or compound heterozygous *FAS* mutations.

^bPatients with ALPS clinical history and laboratory findings but no detectable genetic defect.

^cOne patient with clinical history and laboratory findings of ALPS and an activating mutation of *NRAS*.

A minority of ALPS patients have mutations in other components of the CD95 pathway and presently include *FASLG* (CD95L) and *CASP10* (caspase 10). A mutation in caspase 8 is associated with an ALPS-like disorder.⁴⁹⁰

Hematolymphoid Disorders Associated with ALPS

Lymph nodes of individuals with ALPS exhibit a marked interfollicular and parafollicular hyperplasia, comprised of small and large immunoblast-like TCR $\alpha\beta$ DNT cells, which are CD57 positive and contain TIA-1. This proliferation is thought to be the result of decreased apoptosis and increased proliferation.⁴⁷⁹ Most lymph nodes also show a florid follicular hyperplasia, sometimes with focal progressive transformation of germinal centers or follicular involution. Plasmacytosis may also be present. In a review of lymph nodes from 44 patients with type Ia ALPS, 41% had a histiocytic component, resembling sinus histiocytosis with massive lymphadenopathy.⁴⁹¹ Like ALPS, sinus histiocytosis with massive lymphadenopathy is associated with autoimmune phenomena and hypergammaglobulinemia. The spleens of ALPS patients display expansions of the marginal zones and periarteriolar lymphoid sheaths.⁴⁹²

Individuals with ALPS have a life-long increased risk for Hodgkin and non-Hodgkin lymphoma. Only ALPS patients with mutations of *FAS* have been reported to develop lymphoma. Lymphoma has been described in 3% in one large series of ALPS patients with mutations affecting the death domain of *FAS*.⁴⁹³ In another study of 130 members of 39 kindreds with inherited *FAS* mutations, the risk of developing Hodgkin or non-Hodgkin lymphoma was 51 and 14 times greater than expected, respectively.⁴⁹⁴ Relatives of affected individuals with death domain mutations of *FAS* also have an increased risk of developing B cell lymphomas.^{494–496} The lymphomas associated with ALPS include: classical Hodgkin lymphoma, nodular lymphocyte predominant Hodgkin lymphoma, Burkitt lymphoma, diffuse large B-cell lymphoma, T-cell-rich large B-cell lymphoma, follicular lymphoma, T-cell lymphoma, and marginal zone lymphoma.^{494,497,498} Thus, the predominance of DNT cells in the prelymphomatous state of ALPS patients does not appear to predispose to a particular type of lymphoma. No significant association of EBV with development of lymphoma has been noted.

Tests for ALPS

In the absence of a family history, the diagnostic approach to ALPS starts with confirmation of a chronic nonmalignant lymphoproliferation plus increased TCR $\alpha\beta$ double-negative T-cells in the blood. Testing for defective apoptotic function is the hallmark of ALPS. Functional apoptotic assays for *FAS* and *CASP10* defects may be performed by flow cytometry.⁴⁹⁹ T-cells from a suspected case of ALPS are

activated and cycled in IL-2 to render them susceptible to apoptosis; or alternatively, induction of cell death is measured after overnight stimulation with anti-CD95/AP01.3 antibodies. The number of living cells after incubation is compared to an unstimulated sample by flow cytometry. Apoptotic assays for *FASLG* mutations are more complicated and not readily available. Because of the limited availability of functional apoptotic assays, gene sequencing has been increasingly used for diagnosing ALPS.

Congenital Neutropenia Syndromes

Congenital neutropenia syndromes consist of a heterogeneous group of disorders, associated with persistent low neutrophil counts that are not secondary to drugs, metabolic disorders, nutritional deficiencies, alloimmune or autoimmune processes, or neoplastic conditions.^{500–502} Neutropenia is defined as an absolute peripheral blood absolute neutrophil count (ANC), which is lower than 1,500/ μ L on repeated testing over 3–4 week intervals. This threshold is based on studies of Caucasians; whereas, other ethnic groups may have a lower normal neutrophil counts.⁵⁰³ Patients with congenital neutropenia frequently have elevated levels of eosinophils and monocytes. Elevated serum levels of immunoglobulins IgG, IgM, or IgA are also common. Clinically, patients with congenital neutropenia have recurrent bacterial infections, including pneumonia, soft tissue abscesses, and frequently septicemia. Congenital neutropenia syndromes may be classified into three types based on clinical and laboratory features: (1) severe congenital neutropenia (SCN); (2) cyclic neutropenia; and (3) congenital neutropenia with hypopigmentation (Table 2.9). Other inherited disorders may also present with neutropenia, such as Wiskott–Aldrich syndrome, SDS, and X-linked agammaglobulinemia. The diagnosis of SCN requires ANC counts of less than 500/ μ L over 3–4 week intervals at least 3 months after birth. Cyclic neutropenia is a condition in which the neutrophil counts oscillate from normal to very low levels, typically in 18- to 21-day cycles. The diagnoses of the entities included in congenital neutropenia with hypopigmentation are based on clinical and laboratory features plus unique molecular genetic defects. SCN and cyclic neutropenia have in common similar clinical manifestations and mutations of *ELA2*. However, only patients with SCN have a substantial increase risk of developing a neoplastic hematopoietic process and will be the focus of further discussions.

Molecular Pathogenesis of Congenital Neutropenia Syndromes

The cellularity of the BM in patients with SCN is normal or decreased, but with a maturation arrest in myeloid

TABLE 2.9. Congenital neutropenia syndromes associated gene abnormalities.

Syndrome	Involved gene	Inheritance	Mutation types/comment
Severe congenital neutropenia	<i>ELA2</i>	AD, S	80% missense, 20% mutations leading to splicing defects or premature stop codons; associated with increased risk of MDS and AML
	<i>HAX1</i>	AR	Associated with Kostmann syndrome SCN patients; risk for MDS and AML not known
	<i>GFI1-1</i>	AD, S	Missense mutations in zinc finger domain; risk for MDS and AML not known
	<i>WAS</i>	XL	Missense mutations leading to gain-of-function
	<i>G6PC3</i>	AR	Missense mutation leading to increase neutrophil apoptosis; risk for MDS and AML not known
Cyclic neutropenia	<i>ELA2</i>	AD, S	Mutations largely different from those of SCN; not associated with increased risk of MDS and AML
Congenital neutropenias associated with hypopigmentation			
Chediak–Higashi syndrome	<i>LYST/CHSI</i>	AR	Neutrophils defective in bacterial killing
Hermansky–Pudlak syndrome, type 2	<i>AP3B1</i>	AR	Other types of Hermansky–Pudlak syndrome not associated with neutropenia
Griscelli syndrome type 2	<i>RAB27A</i>	AR	Other types of Griscelli syndrome not associated with neutropenia
p14 deficiency	<i>P14/MAPBPIP</i>	AR	Neutrophils defective in bacterial killing

AD autosomal dominant, S sporadic, AR autosomal recessive, MDS myelodysplastic syndrome, AML acute myeloid leukemia, SCN severe congenital neutropenia.

differentiation at the promyelocyte–myelocyte stage of differentiation.^{500,502} Normal to increased numbers of promyelocytes and no or very few more differentiated granulocytic forms are present. The promyelocytes may be morphologically normal or display dysplastic features. Eosinophilic and monocytic elements are usually normal in number and morphology. Erythropoiesis and megakaryopoiesis are normal. The blood contains normal or increased levels of G-CSF. The G-CSF receptors on granulocytes are functionally normal. The in vitro granulocytic CFUs of BM samples are decreased with limited granulocytic maturation. G-CSF supplementation partially restores colony formation with evidence of full granulocytic maturation. These features are consistent with either a block in granulocytic maturation or apoptosis at the myeloblast–promyelocyte stage of maturation. Recent studies suggest that the cause of poor colony formation and blood neutropenia is due to an increased susceptibility of myeloid precursors to apoptosis.^{504–508}

Up to 60% of patients with sporadic or autosomal dominant SCN have a mutation of *ELA2*.^{500,509,510} The *ELA2* gene, located on chromosome 19p13.3, encodes for neutrophil elastase (NE). The NE enzyme is processed for inclusion in the primary granules of the early promyelocyte.⁵¹¹ Myeloid cells are thought to be protected from this enzyme's activity during synthesis by terminal peptides that are cleaved as the enzyme is packaged in the primary granules. It is hypothesized that mutations of *ELA2* produce abnormal NE that is not inhibited or is not correctly packaged, resulting in apoptosis of myeloid cells before they mature to neutrophils.^{504,506,512} Over 50 mutations of *ELA2* have been described in SCN, of which about 80% are missense mutations and

20% are mutations leading to splicing defects or premature stop codons. Analysis of the three dimension structure NE predict that these mutations affect the binding site of NE for its substrates.⁵¹³

Homozygous mutations in the mitochondrial protein *HCLS1-associated XI (HAX1)* are present in up to 30% of individuals with Kostmann syndrome, an autosomal recessive form of SCN.⁵¹⁴ The *HAX1* gene encodes for a mitochondrial protein with Bcl-2 homology. The HAX1 protein is involved in signal transduction and cytoskeletal control, and monitors inner mitochondrial membrane potential, to protect cells from premature apoptosis.⁵¹⁴ Autosomal dominant or sporadic mutations of *growth factor independent-1 (GFI1)* are also associated with SCN.⁵¹⁵ The GFI1 protein is a transcription factor involved in T-cell differentiation. Studies show that GFI1 binds to *ELA2* DNA and is postulated to repress *ELA2* resulting in neutropenia. Mutations of *WAS*, encoding for WASP of the Wiskott–Aldrich syndrome, is associated with X-linked inherited SCN. However, unlike Wiskott–Aldrich syndrome which results from loss-of-function mutations, the *WAS* mutations in SCN appear to be gain-of-function mutations that disrupt an autoinhibitory domain of WASP.⁵¹⁶ Rare individuals with an autosomal recessive form of SCN have missense mutations of *G6PC3*, that encodes for glucose-6-phosphatase catalytic subunit-3. Patients with mutations of *G6PC3* have neutrophils and fibroblasts with increased susceptibility to apoptosis.⁵¹⁷ Other genes implicated in a very small number of patients with congenital neutropenia include *AP3B1*, *TAZI*, and *MAPBPIP*.⁵⁰¹

Hematologic Neoplasias Associated with Congenital Neutropenia Syndromes

Individuals with SCN are at increased risk for developing MDS or AML.^{518–521} For many years, a debate ensued over whether or not recombinant G-CSF (rh-G-CSF), the preferred treatment for SCN, was the cause of myeloid neoplasia in SCN. Arguments for an intrinsic defect in SCN predisposing to MDS and AML include: (1) patients with SCN not treated with rhG-CSF may develop AML; (2) many patients with SCN on long-term G-CSF treatment do not develop MDS or AML; and (3) patients with cyclic neutropenia with mutations of *ELA2* and treated with rhG-CSF do not develop myeloid neoplasms. By contrast, a report from one registry of SCN patients showed an increase risk of MDS and AML with increase dosage or length of rhG-CSF therapy.⁵¹⁸ However, the data of that report was interpreted as defining a subgroup of patients requiring higher dosages of rhG-CSF as being an “at risk” group possibly due to a more severe molecular defect. Another registry study showed similar associations of rhG-CSF therapy with risks of developing myeloid neoplasia.⁵²² The risk for developing MDS or AML did not differ between patients with *ELA2* versus non-*ELA2* mutations; however, the data suggested that the risk of myeloid neoplasia may be higher for specific mutations of *ELA2*. Thus, patients with SCN have an intrinsic increased risk for myeloid neoplasia, but G-CSF treatment appears to further increase that risk.

The mechanism responsible for MDS and AML in SCN is not known. Genetic abnormalities found in MDS and AML in patients with SCN are different from those observed in non-SCN patients. Patients with SCN and AML do not have mutations of *FLT3*, *KIT*, *CSF1R*, or *JAK2*, that are common in patients without SCN.⁵²³ The transformation of MDS to AML in SCN patients is associated with the appearance of monosomy 7, trisomy 21, activating *RAS* mutations, or mutations of *CSF3R*.^{268,524,525} The development of mutations of *CSF3R* may be a precursor genetic event in the genesis of MDS or AML. Point mutations of *CSF3R* are present in 78% of SCN patients with MDS or AML associated with either *ELA2* or *HAX1* mutations; whereas, only 34% of patients with SCN and no myeloid malignancy have this mutation.⁵²⁶ Conversely, some patients with a long-standing history of *CSF3R* mutations do not develop leukemia, and not all patients who develop MDS or AML have mutations of *CSF3R*.^{527,528} Thus, mutations of *CSF3R* may be a bystander phenomenon or may reflect an inherent genetic instability in patients with SCN and *ELA2* mutations.⁵²⁹

Tests for Congenital Neutropenia Syndromes

The diagnosis of SCN is based on serial absolute neutrophil counts and clinical findings. Cyclic neutropenia must be distinguished from SCN, since only patients with SCN are at increased risk for developing MDS and AML. Patients with

cyclic neutropenia and up to 60% of those with SCN have mutations of *ELA2*. Testing for *ELA2* mutations is performed by sequence analysis. A patient with no detectable *ELA2* mutation may have a defect in one of the other genes associated with SCN. Patients with SCN are at high risk for developing myeloid neoplasias and should be monitored closely by cytogenetic and molecular genetic studies.

Summary

A wide variety of BM failure and primary immunodeficiency syndromes predispose individuals to hematolymphoid neoplasms. With some exceptions, hematolymphoid neoplasms arise in a only a minority of these patients and, in many instances, malignancies of other types occur as often, if not more frequently. In some individuals, the development of a hematologic or lymphoid neoplastic process may be the first indication of some of these syndromes.

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3

Prognostic Markers

David Bahler

Introduction

The prognosis of hematolymphoid neoplasms varies greatly, not only between different entities but also among tumors that have the same diagnoses. Clinical variation among similar tumors is due largely to genetic and molecular diversity as well as hosts related issues, e.g., age of patient.^{1,2} Molecular and/or genetic variation among similar tumors may reflect differences in a particular tumor pathogenesis relative to others and/or acquisition of additional molecular abnormalities leading to a further loss of normal cell growth control mechanisms.^{1,3-5} Interpreting studies that correlate clinical outcome and other data with molecularly defined differences among similar neoplasms may be complicated by different therapeutic regimens among different studies, as well differences in which specific clinical parameters are evaluated. For example, overall survival, time to first treatment, and risk of relapse may all yield different results for a given prognostic marker. In spite of these complications, molecular prognostic markers have the potential to tie tumor biology to clinical behavior in individual patients and therefore, may be more informative than those based on stage or other clinical or laboratory data.⁶⁻⁸ In addition, molecular techniques may be used to follow levels of minimal residual disease and to better monitor responses to therapy, which is one of the most important general prognostic markers identified to date.^{7,9-13} The molecular and genomic prognostic markers discussed in this chapter for several specific diseases represent those that have recently been shown to have clinical utility or have the most clinical potential (Table 3.1). The discussion of other molecular/genomic markers that may also have prognostic significance may be found in later chapters devoted to specific hematolymphoid neoplasms.

Chronic Lymphocytic Leukemia (Also See Chap. 16)

Immunoglobulin Variable Gene Mutation Analysis

Rearranged immunoglobulin variable region genes expressed by B-cells may undergo a somatic hypermutation process that usually occurs in germinal centers of lymph nodes and is designed to generate antibody molecules with higher antigen binding affinities.¹⁴ The hypermutation process is specific to B-cells and typically generates point mutations in regions that encode the antigen contact areas and occasionally may also produce small deletions or insertions.^{15,16} Approximately, half of chronic lymphocytic leukemia (CLL) cases express mutated immunoglobulin variable genes defined by the expressed heavy chain variable (VH) gene segment showing less than 98% homology to the closest germline VH gene segment present in large databases, e.g., GenBank or IMGT.^{17,18} The 98% cutoff was initially selected to allow for small numbers of polymorphisms that may be present in the germline VH genes of some patients and also because it could separate cases with different prognoses. Patients with CLL, who express mutated VH genes as defined above, typically have more indolent disease with median survivals close to 25 years, while those with unmutated VH genes often have more aggressive disease with median survivals around 8 years.^{17,18} Recent studies have also suggested that separating out CLL cases expressing “lightly” mutated VH genes (i.e., those with germline homologies between 97 and 97.9%) may be warranted, since these patients appear to have a clinical course intermediate between the more heavily mutated (<97%) and unmutated (98% or less) groups.¹⁹ Although VH gene status appears to be an important independent predictor

TABLE 3.1. Prognostic markers.

Neoplasm	Prognostic significance	
	Favorable	Unfavorable
Chronic lymphocytic leukemia	Mutated VH genes	Unmutated VH genes Expression of VH3-21 11q deletion, 17p deletion
Multiple myeloma		t(4;14), t(14;16), t(14;20) 17p deletion
Non-Hodgkin lymphoma		Certain p53 mutations
Diffuse large B-cell lymphoma	Germinal center profile B-cell receptor profile	Nongerminal center profile
Acute myeloid leukemia	NPM1 mutations	FLT3 insertions (ITDs)
Gastric MALT lymphomas		t(11;18)
Splenic marginal zone lymphomas	Mutated VH genes	Unmutated VH genes (with loss of 7q31–32)

of overall survival, and time to first treatment after a diagnosis of CLL is made, it is uncertain whether its prognostic value also holds once treatment is initiated.²⁰

The biological basis for VH gene mutational status being a predictor of CLL prognosis is unclear. Initially, it was proposed that the differences in survival were related to a different cell of origin with mutated CLL, originating from memory B-cells, and the unmutated type of CLL, originating from naïve B-cells.¹⁸ However, studies using gene expression array analysis and other methods have suggested that both the mutated and unmutated types of CLL likely originate from memory or antigen experienced B-cells.^{21,22} Although the two types of CLL share a common expression profile, several genes have been identified, which aid in discriminating between CLL cases that express either unmutated or mutated VH genes, with the most robust discriminator being zeta-associated protein of 70 kDa (ZAP-70).^{23,24} ZAP-70 is a tyrosine kinase, initially thought to be restricted to T-cells, that appears to enhance antigen receptor signaling in CLL cells, and is expressed in most cases with unmutated VH genes.²⁵ Studies comparing the ZAP-70 status of CLL cases, typically determined by flow cytometry, to VH gene mutational status have usually found a good correlation.^{26,27} However, small numbers of discordant cases are always present (i.e., ZAP-70 positive with mutated VH genes), and there are significant technical difficulties measuring ZAP-70 by flow cytometry related to very weak cytoplasmic staining with all of the commercially available antibodies and different methods used to set the negative cut-off value.²⁸ As such, the VH gene mutational status is generally considered to be a more reliable prognostic marker for CLL relative to ZAP-70, and is now an available clinical test in several reference laboratories. Moreover, there is evidence that VH mutational status may also have prognostic value in other small B-cell neoplasms such as splenic marginal zone lymphoma, although additional studies of this possibility are needed.²⁹

The use of particular VH gene segments by CLL has also been shown to have prognostic value. Specifically, cases of CLL that employ the VH3-21 gene segment have a poor prognosis, regardless if this VH gene segment is mutated or unmutated.^{30,31} It is also of interest that many cases of CLL that use VH3-21 also express similar lambda light chain

gene segments and have other conserved areas in the highly variable CDR3, which is encoded by the diversity and joining segments and randomly templated nucleotides.^{30,31} The reason why CLL cases that express VH3-21 do poorly is not clear, but points to the possibility of immunoglobulin receptor signaling mediated by a specific antigen playing a role in CLL development and possible progression.³²

Genomics

Approximately 80% of CLL cases harbor recurrent cytogenetic lesions that may be detected using FISH, with the most common being 13q14 deletion, 11q22–23 deletion, 12q trisomy, and 17p13 deletion, occurring in approximately 55, 18, 16, and 7% of cases, respectively.³³ Retrospective studies have demonstrated that patients with 17p13 or 11q22–23 deletions have worse overall survival and faster disease progression relative to other CLL patients without FISH-detected abnormalities, or those with 12q trisomy or isolated deletions of 13q.^{33,34} Moreover, 17p deletion or 11q deletion appear to be robust independent predictors for decreased survival, which has also been confirmed by recent prospective studies.^{20,35} Using multivariate analysis taking into account VH mutational status with deletion of 11q and 17p, Korber et al³⁴ developed a model that places patients into one of four groups, (1) deletion of 17p regardless of mutation status, (2) deletion of 11q regardless of mutational status, (3) unmutated VH genes without deletion of 11p or 17p, and (4) mutated VH genes without deletion of 11q or 17p. Median survival times for these groups were 30 months for (1), 70 months for (2), 89 months for (3), and not reached for (4) (i.e., 55% survival at 152 months). These risk categories also held when restricted to only the earliest Binet stage A patients. Although several coding and noncoding genes are found in the minimally deleted regions, p53 present on 17p and ATM present on 11q have generated the most interest owing to their well known roles in DNA repair and cell cycle control. Recent studies have also demonstrated that CLL patients with 11q deletions and a mutant ATM gene have significantly reduced survival compared to those with 11q deletions and wild type ATM alleles, suggesting that the loss of ATM alleles may have an additive adverse effect.³⁶

Multiple Myeloma (Also See Chap. 19)

The clinical course of multiple myeloma (MM) patients is highly variable, with survival ranging from months to decades.^{7,13,37} Genetic abnormalities may be easily identified in almost all cases and also provide important prognostic information that may impact clinical management.^{7,38–40} Hyperdiploidy is present in approximately 50% of patients and is typically associated with trisomies of chromosomes 3, 5, 9, 11, 15, 19, and 21.⁴⁰ Deletion of chromosome 13 (85% monosomy and 15% interstitial) also occur in approximately 50% of cases.⁷ Recurrent translocations involving the immunoglobulin heavy chain locus at 14q32 are also common with t(11;14)(q13;q32) occurring in 15–20% of cases, t(4;14)(p16.3;q32) in approximately 15% of cases, t(14;16)(q32;q23) in 5–10% of patients, and t(14;20)(q32;q11) in 2–5%.^{39,41} Usually, these 14q32 translocations are mutually exclusive, and are not present in hyperdiploid cases. In addition, deletion of 17p13, the location of the p53 gene, occurs in approximately 10% of MM cases.^{7,38} Interphase FISH is the preferred method to identify the above mentioned translocations and deletions owing to its much higher sensitivity in low proliferation neoplasm such as MM, compared to conventional cytogenetics. The Mayo Clinic group has recently developed a practical genetic based model that can be easily used to stratify MM patients into high risk and standard risk groups, where the high risk group has a poor prognosis and does not respond well to current therapies.⁴² Patients in the high risk group are those with t(4;14), t(14;16), t(14;20), or deletion of 17p. Other studies by different groups have also established that the high risk features cited by the Mayo group are associated with poor overall survival.^{38,40,41} Although deletion of 13q has been reported to confer a poor prognosis, recent studies have clearly shown that this is due to its frequent associations with t(4;14) and deletion 17p, and that for patients lacking these abnormalities, deletion of 13 does not significantly affect survival.³⁸ Although hyperdiploidy and t(11;14) have been reported to confer a favorable prognosis, these findings are typically not statistically significant and that is why cases showing these abnormalities are considered standard risk.^{7,41} Several recent studies have also used gene expression arrays to identify additional molecular markers of prognosis,^{4,43} but these are not yet ready for clinical use.

Acute Myeloid Leukemia (Also See Chaps. 34 and 35)

The development of acute myeloid leukemia (AML) is associated with the acquisition of genetic abnormalities in hematopoietic progenitor cells. Recurrent cytogenetic abnormalities such as t(15;17)(q22;q11), t(8;21)(q22;q22), and inv(16)(p13) occur in approximately 30% of cases and constitute diagnostic markers that define specific AML subtypes, and also represent prognostic markers for the risk of relapse and overall survival.⁶ However, approximately

40–50% of AML cases do not show abnormalities by conventional cytogenetic analysis and represent a clinically heterogeneous group.^{44,45} Recent studies have identified several molecular markers in karyotypically normal AML that are used to help stratify these patients into different prognostic groups and are further discussed in the following lines.

FMS-Related Tyrosine Kinase 3 (Also See Chap. 35)

The FMS-related tyrosine kinase 3 (FLT3) protein is a receptor tyrosine kinase related to PDGF and KIT that is normally expressed in hematopoietic progenitor cells and plays an important role in proliferation and differentiation.⁴⁶ Approximately 30% of karyotypically normal AML cases harbor internal tandem duplications (ITDs) in exons 14 and 15 of the FLT3 gene that are of variable lengths, always in frame, and result in ligand independent activation of FLT3 and associated downstream effectors.^{46–48} Multiple retrospective studies have demonstrated that patients with FLT3 ITDs have significantly shortened disease free survival and poorer overall survival, compared to karyotypically normal AML patients without FLT3 ITDs.^{44,45} Recent studies have also demonstrated that the level of FLT3 ITD relative to wild type FLT3 is a major determinant of the prognostic effect, and that only those AML patients with high levels of ITD relative to wild type have poor prognoses.^{48,49} Similar to the JAK2 story in myeloproliferative disorders, the ratio of IDT relative to wild type may be greater than 1.0, due to duplication of the ITD on the other allele secondary to uniparental disomy.^{50,51} Although the size of the ITD has also been reported to affect survival (i.e., large ITDs confirming poorer prognosis), the mechanism whereby this may occur is unclear.⁵² In addition to ITDs, approximately 7% of karyotypically normal AML patients have point mutations in the second tyrosine kinase domain of FLT3, usually including the D835 or I836 residues.^{44,53} Although these tyrosine kinase domain mutations also result in constitutive activation, the degree of activation is less than that conferred by ITDs, and the effect of FLT3 kinase domain mutations on overall survival is unclear, with the majority of studies showing no significant effect.^{44,54} However, a recent study of young karyotypically-normal AML patients without FLT3 ITDs demonstrated that FLT3 kinase mutations significantly reduced remission durations relative to FLT3 wild type patients.⁵⁵

Nucleophosmin (Also See Chap. 35)

Mutations in exon 12 of the Nucleophosmin (NPM1) gene occur in approximately half of karyotypically normal adult AML cases and represent the most frequent genetic lesion identified to date in de novo AML.^{56,57} Almost all mutations represent tetranucleotide insertions at position 863 that cause a frameshift in translation, eliminating a nuclear localization signal and resulting in mislocalization of NPM1 protein to the cytoplasm and disruption of normal

function.^{56,57} Wild type NPM1 acts as a chaperone to various proteins as they move between the cytoplasm and nucleus and is known to physically interact with several tumor suppressor proteins, such as p53, ARF, and IRF-1.^{58–60} NPM1 mutations often occur simultaneously with FLT3 ITDs and are more common in FAB M4 and M5 subtypes of AML and in those where the leukemic blasts lack CD34 expression.⁵⁷ It is also important to note that NPM1 mutations are relatively uncommon in childhood AML⁶¹ and rare in chronic myeloproliferative neoplasms.⁶² Mutations in NPM1 confer a more favorable prognosis in terms of relapse risk and overall survival, but need to be interpreted in conjunction with FLT3 status. Retrospective studies analyzing NPM1 mutations together with FLT3 ITDs in young AML patients (age less than 60 years) have identified three major prognostic groups: good (NPM1+, FLT3–), intermediate (NPM1–, FLT3– or NPM1+, FLT3+), and poor (NPM1–, FLT3+).^{49,63,64} In the recent study by Gale et al,⁴⁹ that analyzed 1,217 patients, overall 5-year survival between these groups was 59% (good), 34–35% (intermediate), and 15% (poor). Moreover, survival of karyotypically-normal AML patients in the good prognosis group, defined using NPM1 and FLT3, is similar to that observed in AML patients with karyotypes associated with favorable prognoses, such as t(15;17), and may not benefit from bone marrow transplant.⁴⁵ Detection of mutant NPM1 may theoretically be done by immunohistochemical staining showing abnormal cytoplasmic expression.⁶⁵ However, interpretation of NPM1 staining results may be difficult, due to close packing of AML blasts that have minimal cytoplasm. Moreover, proper interpretation of NPM1 results requires correlation with FLT3 IT status as highlighted above, which requires nucleic acid based testing. Several clinical molecular tests, starting from DNA or RNA, that will detect all described NPM1 mutations, provided the leukemic cells represent 5% or more of cells in the specimen, have been described in the literature.^{66,67} In addition, allelic-specific PCR-based tests have also been described that may be used for the detection of minimal residual disease with higher sensitivities, approaching 1 cell in 10,000, but these only detect the most common mutations.^{68,69}

Non-Hodgkin Lymphoma

Non-Hodgkin lymphomas comprise a diverse group of neoplasms that include follicular lymphomas (FLs), mantle cell lymphomas (MCLs), marginal zone lymphomas, and diffuse large B-cell lymphomas (DLBCLs), among others. In general, lymphomas with complex karyotypic abnormalities have a worse clinical prognosis than those that have single or a limited number of identified abnormalities. In addition, patients with FL with an isolated t(14;18) translocation or MCL with an isolated t(11;14) translocation, may have unusually indolent clinical courses, relative to most patients with these neoplasms.⁷⁰ Recent studies have

also demonstrated that p53 gene mutations shorten the overall survival of patients with DLBCLs and FLs, independent of International Prognostic Index (IPI) score.^{71,72} In the study by Young et al,⁷² 21% of DLBCLs harbored p53 mutations, but only those in the L1 and L3 DNA binding domain loops affected survival, while overall survival was not affected by mutations in the L2 DNA binding domain loop.

Diffuse Large B-Cell Lymphoma (Also See Chap. 22)

The clinical course of DLBCL patients with the same IPI score may be highly variable, presumably reflecting considerable molecular heterogeneity in this disease. Studies using cDNA array-based gene expression profiling initially identified two different types of DLBCL based on similarities in expression of 375 genes to normal germinal center B-cells and activated B-cells.⁷³ Subsequent refinements of this “cell of origin” approach identified a third type of DLBCL, termed type-3 or “other,” representing approximately 40% of cases, and used fewer genes (17) to delineate the germinal center and activated B-cell expression signatures.⁷⁴ Patients with germinal center type DLBCL demonstrate better overall survivals than those with activated B-cell or type-3 DLBCL when treated with anthracycline based (CHOP) chemotherapy.^{74,75} Immunohistochemical staining lymphoma biopsy specimens for proteins, such as CD10, BCL6, and MUM1, which may be easily performed in many pathology laboratories, has also been used to identify germinal center and nongerminal center type DLBCL.^{76,77} Expression array studies by Shipp et al⁷⁸ using different clustering algorithms also identified three subsets of DLBCL termed “oxidative phosphorylation,” “B-cell receptor/proliferation”(BCR), and “host response.” Interestingly, the genes used to define these expression signatures are different than those used for the cell of origin classification, suggesting that the two methods identify different aspects of DLBCL heterogeneity. The BCR subset of DLBCL shows increased expression of genes associated with B-cell antigen receptor signaling and B-cell specific transcription factors, such as BCL6 relative to the other groups. Recent studies using DLBCL-derived cell lines have demonstrated that only those with BCR-type expression profiles are sensitive to the BCL6 peptide inhibitor, BPI, and ceased growing, and that BCR expression profile was better at predicting BPI sensitivity than initial BCL6 expression levels.⁷⁹ Lossos et al⁸⁰ developed a 6-gene model that predicts clinical outcome in DLBCL, that is independent of IPI score, through analysis of 36 genes previously reported to predict survival in DLBCL. Expression of the six genes (LM02, BCL6, FN1, CCND2, SCYA3, and BCL2) is measured by quantitative PCR that may be performed on paraffin-embedded material, making it potentially suitable for routine clinical use.⁸¹ Moreover, the model may predict clinical outcome in DLBCL patients treated with rituximab-augmented anthracycline-based chemotherapy (R-CHOP),

the current standard treatment. It is unclear whether the immunohistochemical identification of germinal center and nongerminal center subtypes of DLBCL has prognostic significance with the addition of rituximab to CHOP-based treatment regimens, as recent studies have shown conflicting results.^{82,83} In addition, interpreting immunohistochemical based tissue staining results may be problematic and may have a misclassification rate of around 20%.⁸² Cases of DLBCL that have coexistent t(14;18) and translocations involving cMYC at 8q24 (i.e., “double hit lymphomas”) generally have a very poor prognosis.^{84,85}

Marginal Zone Lymphoma (Also See Chap. 17)

Extranodal marginal zone lymphomas of mucosa lymphoid tissue (MALT lymphomas) show a variety of chromosomal abnormalities, the most common being translocation t(11;18) (q21;q21).⁸⁶ However, the frequency of t(11;18) in MALT lymphomas varies markedly, being present in approximately 25% of gastric MALT lymphomas and 50% of pulmonary MALT lymphomas, but is only occasionally seen in MALT lymphomas of the salivary gland, skin, or ocular adnexa.^{87,88} Gastric MALT lymphomas with t(11;18) are often disseminated to lymph node and other distal sites at diagnosis and do not respond to *Helicobacter pylori*-eradicating therapies.^{89,90} Interestingly, gastric MALT lymphomas with t(11;18) typically do not display other chromosomal abnormalities, such as trisomy 3 or trisomy 12, which are frequently present in gastric MALT lymphomas, or transform to large cell lymphomas, suggesting that there are two distinct pathways of MALT lymphomagenesis in the stomach.^{91,92}

Up to 40% of splenic B-cell marginal zone lymphomas (SMZL) show allelic loss of 7q31–32, which has been associated with more aggressive disease.⁹³ However, SMZL that express unmutated VH genes have a more aggressive clinical course relative to SMZL that express mutated VH genes, similar to CLL, and 7q31–32 loss is mostly restricted to SMZL cases that have unmutated VH genes.²⁹ In addition, cDNA microarray gene expression profiling studies have demonstrated a shortened survival of SMZL patients is associated with CD38 expression and expression of a set of NK-κB pathway genes, such as TRAF5 and REL.⁹⁴

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Cancer Stem Cells: Potential Targets for Molecular Medicine

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Introduction

The concept of stem cells has become so pervasive in our society that most people have some idea of what stem cells are and some opinion regarding the debates surrounding their use in science and medicine. Stem cells, best studied in the hematopoietic system, are defined by their singular capacity to renew themselves and give rise to more specialized cells that comprise the organs and tissues of the body.^{1–4} Nevertheless, the idea that cancers, too, derive from stem cells or their progenitors is not as widely recognized. Even less well understood are the diagnostic and therapeutic implications of such a hypothesis.

Over the past several decades, research demonstrating that tumors contain rare populations of stem cells, each uniquely endowed with the potential to regenerate the entire tumor, has dramatically changed the way we think about cancer.^{5–10} No longer can it be assumed that all tumor cells are equal and any random cancer cell left behind after surgery or treatment has the potential to incite a relapse. These studies provide compelling data to suggest that only a special subset of cancer cells is responsible for the growth and progression of the disease.^{8,9,11–25} If so, these cells, the *cancer stem cells* (CSC), are a critical target if a cure for malignancies propagated by CSC can ever be achieved.

The Cancer Stem Cell Hypothesis

Traditionally, cancer has been conceived of as arising from a cell that accumulates critical errors in cell cycle regulation. These errors cause the cancer cell to proliferate clonally and produce nascent tumor cells, each with all of the tumorigenicity of the original cancer cell. Accordingly, therapies have been aimed at debulking the tumor and eliminating the rapidly-dividing cancer cells, which constitute most of the tumor. Despite these efforts, relapse and tumor progression are common, resulting in high mortality rates. Even today, cancer remains the leading cause of death among individuals under the age of 85.^{26,27}

Recent evidence suggests, however, that only a rare population of CSC has the capacity for self-renewal, survival, and differentiation required to regenerate the tumor in transplantation models.^{8,9,11–25} Although the majority of current cancer therapies target dividing cells within a tumor, this rare CSC population may be relatively resistant to standard chemoradiotherapy due to their quiescence and/or the acquisition of other protective properties.^{12–14,25} As such, these CSC could be responsible for cancer progression and therapeutic resistance and may represent an important therapeutic target.^{8,9,11–25}

Cancer Stem Cells, Defined

According to a consensus by members of the American Association of Cancer Research Cancer Stem Cells Workshop, CSC are defined as relatively rare, self-renewing cells within a tumor that are capable of generating all of the various cells that comprise the tumor.¹³ Whereas this current definition relies on a phenotypic description of CSC, more research is needed to define these cells on a molecular level and to characterize the mechanisms driving CSC propagation. Recent studies have begun to uncover some of the alterations in the pathways for self-renewal, differentiation, and survival that underlie the oncogenic and regenerative potentials of these CSC.

Historical Perspective

Whereas the concept of a specialized subpopulation of CSC within tumors is relatively new, it has long been recognized that malignancies are comprised of heterogeneous cell types. In 1847, Rudolph Virchow gave leukemia its current name, literally meaning “white blood.” The first description of CSC was made by Lapidot and colleagues in 1994 in acute myeloid leukemia (AML),⁸ which was further refined by Bonnet and Dick⁹ and Blair et al¹⁰ in 1997. It was not until almost a decade later, however, that CSC for breast cancer were described,¹⁸ followed shortly thereafter by descriptions of CSC in brain tumors,^{19,20} and candidate CSC in chronic myeloid leukemia (CML),^{12,15} colon cancer,^{21,22,28} pancreatic

TABLE 4.1. Phenotypic identification of cancer stem cells in human cancers.

Year	Cancer type	Markers	Authors
1994 1997	Acute myeloid leukemia	CD34+, CD38–, CD90–	Lapidot et al ¹⁸ Bonnet and Dick ⁹ Blair et al ¹⁰
2003	Breast cancer	CD44+, CD24 low/–, lineage–	Al-Hajj et al ¹⁸
2003, 2004	Brain tumor	CD133+	Singh et al ^{19,20}
1999	Chronic myeloid leukemia (CML)	CD34+	Holyoake et al ¹⁵
2004	Candidate blast crisis CML	BCR-ABL+, nuclear β -catenin	Jamieson et al ¹²
2007	Colorectal cancer	CD133+, EpCAM high, CD44+	O'Brien et al, ²¹ Ricci-Vitiani et al, ²⁸ and Dalerba et al ²²
2007	Pancreatic cancer	CD44+, CD24+, ESA+	Li et al ²³
2007	T-cell acute lymphoblastic leukemia	CD34+, CD4– or CD34+, CD7–	Cox et al ²⁹
2007	Head and neck squamous cell carcinoma	CD44+	Prince et al ²⁴
2008	Melanoma	ABCBS+	Schatton et al ³⁰
2008	Lung cancer (small cell and non small cell)	CD133+	Eramo et al ³¹
2008	Multiple myeloma	CD138–, CD20+ or CD 27+	Matsui et al ³²

cancer,²³ T-cell acute lymphoblastic leukemia (T-ALL),²⁹ head and neck cancers,²⁴ melanoma,³⁰ lung cancer,³¹ and multiple myeloma³² (Table 4.1). Research continues to focus on uncovering CSC for other human cancers in an effort to identify potentially critical targets in cancer diagnosis and treatment.

The Biology of Cancer Stem Cells

Cancer Stem Cell Identification

Functional Identification

In Vivo CSC Isolation Methods

Because CSC are defined by the property of self-renewal and their capacity to recapitulate the entire tumor in vivo, the gold standard for the identification of CSC is in vivo serial transplantation. Tumor cells from a patient are isolated and transplanted through injection into an immunocompromised neonatal or adult mouse. Cells are injected either subcutaneously, intravenously, intrafemorally, or intrahepatically (due to the favorable milieu of cytokines in the neonatal liver). Those cells with self-renewal potential engraft and expand over a period of weeks to months and are later harvested from tissues. The cells of interest (putative CSC) are then

isolated via fluorescence activated cell sorting (FACS) and transplanted into a second animal, then repeated serially.

Various animal models of xenogenic transplantation have been developed for investigative purposes, the most common utilizing immunodeficient mice such as the NOD/SCID mouse model. The paucity of B and T cells in these mice makes them a favorable host for a cancerous xenograft. More severely immunocompromised mice that also lack natural killer cells, such as NOD/SCID IL-2 receptor γ knockouts, termed NOG, and RAG2 γ c knockouts, permit even higher levels of human engraftment and are thus being adopted more widely by researchers.³³ These animal models allow for the study of tumor growth, survival, metastasis, homing, and response to potential therapies.

In Vitro CSC Assays

Studies in vivo are time-consuming and costly, so acceptable in vitro assays have been developed for identifying CSC based on their molecular characteristics. Most widely accepted is the replating assay, also known as the serial colony forming unit assay, which takes advantage of the CSC's ability to regenerate or, in other words, to self-renew and give rise to other cell types. Cells are plated at low density in methylcellulose or agarose media supplemented with cytokines and incubated in culture for 7–14 days. With time, single cells proliferate to form colonies. These colonies are characterized morphologically and counted. Single colonies are then selected and transferred to new wells, with one colony per well, and later assessed for colony reformation. Only self-renewing cells are capable of recapitulating a colony over several replatings.

Other assays have also been developed but cannot be used alone to identify CSC. The sphere formation assay detects cells capable of forming balls of cells in culture, which are presumed to represent the proliferation of a single CSC at the core. The Aldefluor assay measures expression of aldehyde dehydrogenase, which may be upregulated by a wide range of CSC but is not usually sufficient for CSC identification and isolation. This enzyme converts retinol to retinoic acid, which is important in development and self-renewal and for the detoxification of substances such as alcohol and cyclophosphamide. The Hoechst stain exclusion assay depends on the tendency of CSC to upregulate ABC transporters, active efflux transporters that have been implicated in resistance to chemotherapy and other drugs. The label retention assay identifies CSC as those with low levels of nucleotide labels such as tritiated thymidine, since CSC are quiescent and would thus incorporate less of the label. This assay was originally used by Bayard Clarkson and colleagues to identify putative CSC over 40 years ago.³⁴

Molecular Methods

Studies uncovering the mechanisms driving the stem cell-like property of self-renewal will permit a more precise molecular definition of CSC. Notch, Wnt pathway components

including activated β - and γ -catenin, BMI-1, and hedgehog are involved in self-renewal and differentiation pathways; these pathways are deregulated in cancer and may play a role in CSC propagation.^{12,23,24,35–41} This topic is covered more extensively in the section on molecular changes contributing to CSC propagation in blast crisis of CML.

Phenotypic Identification

Cell Surface Markers

To date, CSC identified through serial transplantation or replating assays have been characterized immunophenotypically in AML,^{8,9} breast cancer,¹⁸ brain tumors such as medulloblastomas and gliomas,^{19,20} chronic myelogenous leukemia,^{12,15} colon cancer,^{21,22,28} pancreatic cancer,²³ T-ALL,²⁹ melanoma,³⁰ small cell and non-small cell lung cancers,³¹ and multiple myeloma³² (Table 4.1).

Universal markers common to all CSC have not been found. The CSC for AML, CML, and T-ALL are all CD34+,^{8,9,12,15,29} whereas breast cancer, pancreatic cancer, colorectal cancer, and head and neck cancers have all been shown to express CD44.^{18,22–24} CSC expressing CD133 have been found in tumors of the brain, colon, and lung.^{19–21,28,31} Although these cellular markers are useful in identifying and sorting these cells, they are by no means unique to CSC, as they are also present on other cell types, including non-cancerous and non-stem cell populations. This fact underscores the need for a more precise molecular characterization of CSC.

In Vivo Monitoring of CSC

Imaging is playing an increasingly important role in pre-clinical research as a noninvasive method of monitoring experimental disease progression and response to potential therapies. Imaging also allows the *in vivo* characterization of CSC homing properties. Once cells are identified and isolated using functional and phenotypic criteria, they can be labeled then transplanted into animal models of disease.

A cell labeling strategy that is commonly used in cancer and imaging research is the isolation and nonspecific labeling of cells *ex vivo* prior to injection or transplantation. This approach allows for the tracking of cells introduced into an animal model of disease. Although PET and MRI have been used for this purpose, optical imaging is the fastest and simplest modality used to follow labeled cells in experimental studies.

A straightforward way to label cells is to incubate them with a lipophilic fluorescent dye, such as DiI, DiO, or DiR, prior to injection and then image them optically with an *in vivo* imaging instrument such as the IVIS 200®. Overlying tissue may attenuate the fluorescent signal, so DiR and other dyes with longer wavelengths are often preferred because of their superior tissue penetration. For this reason, this approach is only feasible in white, nude, or shaven mice. Another limitation of this approach is the dilution of the fluorescent signal with cell division, due to the fact that the

dyes are bound to the cell membrane. Some fluorescent dyes remain detectable through four or five cell divisions, so cell turnover rate may be a critical factor in determining the duration of the signal. Conversely, this property may be exploited to measure cell cycling *in vivo*. Stem cells, for instance, are relatively quiescent, so fluorescent dyes may remain visible for longer, especially when using dyes resistant to photobleaching. Nevertheless, reporter genes are often employed to circumvent this problem.

Viral vectors are used to introduce the reporter genes into cells of interest. For instance, the firefly luciferase gene is transduced into cells using a lentiviral vector. When the substrate luciferin is injected into the animal intraperitoneally or intravenously, the expressed luciferase cleaves the substrate, creating a bioluminescent product that is visible optically. Once transduced into a cell, this gene will be stably expressed and passed on to all nascent cells, thereby allowing the monitoring of tumor growth and metastases. This approach has also been used with herpes simplex viral vectors to introduce PET reporter genes, including herpes simplex virus type I thymidine kinase, which is capable of cleaving various substrates into PET-visible products.⁴²

A limitation of these genetic approaches for studying CSC is that these cells are relatively quiescent, making a lentiviral vector preferred over herpes simplex virus or retroviral vectors, which infect actively-dividing cells. Even with lentiviral vectors, CSC are quite resistant to viral transduction. CSC require the use of high ratios of virions to cells (*multiplicity of infection*, or MOI) in order to achieve efficient transduction. Despite high MOIs, inefficient transductions may result in a mixed population of labeled and unlabeled cells, such that signal pattern and intensity may not correlate with tumor extent and spread. It is thus important to confirm human malignant engraftment by FACS analysis of mouse tissues, immunohistochemistry, and, whenever possible, molecular analysis.

Molecular Pathogenesis of Cancer Stem Cells

The molecular processes that give rise to CSC and the identity of the cells that undergo these original oncogenic changes are the subject of intensive investigation. Although, theoretically, any cell may be conceived to undergo the changes necessary to become CSC, scientists have proposed two general mechanisms based on the data, each involving multiple steps. In the first mechanism, CSC arise from normal stem cells that undergo oncogenic changes; whereas, in the second mechanism, the CSC arise from more committed progenitor cells.

In the first proposed mechanism, normal stem cells acquire genetic modifications that make them cancerous. These modifications generally involve changes in cell cycle regulatory genes or gene products, such as the tumor suppressor p53. In *de novo* AML, the CSC have been shown to be CD34⁺CD38[−] hematopoietic cells,^{8,9} suggesting that

the original cell is similar to a normal stem cell. Additional research has since demonstrated, however, that these AML CSC lack CD90, a hematopoietic stem cell (HSC) phenotypic marker.^{10,43} Therefore, the CSC of AML may resemble human multipotent progenitors more than HSC. The pathogenesis of chronic myelogenous leukemia (CML), on the other hand, represents at least two distinct molecular events corresponding to the different phases of the disease. Whereas the chronic phase of CML arises from an HSC expressing BCR-ABL,^{12,44–49} evolution to acute leukemia does not occur unless additional genetic mutations are acquired and self-renewal capacity is gained by a committed progenitor, as proposed in the second mechanism.¹² The molecular pathogenesis of CML will be explored in greater detail in the next section. Other examples where this second mechanism appears to account for CSC pathogenesis are in several mouse models of AML^{50,51} and T-ALL.²⁹ Clearly, much more research is necessary to uncover the mechanisms and cellular targets of the molecular changes that give rise to CSC.

There is no reason to assume that one mechanism alone accounts for the creation of CSC in different cancers. On the contrary, it is conceivable that CSC may arise through several different mechanisms and, even within a single cancer, more than one CSC may be generated simultaneously or over time and by different mechanisms. Such an occurrence may help to explain the heterogeneity observed in some malignancies and the dramatic shifts in disease course or spread that is sometimes seen in cancer. For instance, CML progresses from a slowly-advancing *chronic phase* to accelerated phase and finally a quickly-progressing period called *blast crisis*. The molecular evolution of CML indicates that, while some mutations are important in disease initiation, other events, such as self-renewal acquisition by a progenitor, promote CSC formation and disease progression. This step-wise progression of CML from chronic to accelerated phase and blast crisis, along with nearly 50 years of research into its molecular pathogenesis, make CML an important paradigm for understanding the genetic and epigenetic events involved in CSC production.^{8,9,11–25}

Pathogenesis of CSC in Blast Crisis of CML (Also See Chap. 30)

Evidence shows that CSC of the hematopoietic system, termed as leukemia stem cells (LSC), subvert the properties normally ascribed to HSC, such as the ability to renew themselves.^{1–3,8,9,11,12,15–17,36,50,52–64} In CML, there appear to be events such as expression of the constitutively active tyrosine kinase BCR-ABL⁶⁵ that initiate chronic phase and promote LSC production,^{12,44–49,65} eventually leading to blast crisis and therapeutic recalcitrance. The hematopoietic cells responsible for the generation of chronic phase CML bear the immunophenotype of normal HSC, but harbor the BCR-ABL translocation, which produces an oncogenic tyrosine kinase fusion protein.^{12,44–49,65} In blast crisis CML, the LSC

resemble granulocyte myeloid progenitor cells (GMP) immunophenotypically, except that they contain aberrant activation of β -catenin via the Wnt signal transduction pathway, a pathway critical for self-renewal in normal stem cells.¹² Although they share a common immunophenotype with normal GMP, only blast crisis CML GMP possess the stem cell-like capacity to renew themselves in vitro, a property that may be inhibited with a specific Wnt pathway antagonist, axin.^{12,66–72} Blast crisis CML myeloid progenitors, which include GMP (and *not* HSC or blast cells), may serially transplant cancer in immunocompromised mice, demonstrating that LSC are enriched within the CML GMP population.⁷³

Molecular Changes Contributing to CSC Propagation in Blast Crisis of CML

CSC have been shown to exhibit properties common to other CSC, including altered self-renewal, survival, differentiation, and inherent resistance to innate and adaptive immune responses. A proportion of patients with blast crisis CML have been shown to contain a novel missplicing of GSK3 β , an inhibitor of the Wnt pathway mediator, β -catenin, which promotes self-renewal.⁷³ Alteration of this pathway could contribute to the LSC self-renewal. Other genes, which have been implicated in self-renewal activation have been notch,^{35,39} hedgehog,^{23,38} and BMI-1.^{24,36} In addition to the property of self-renewal, these LSC also demonstrate enhanced survival associated with changes in apoptotic pathway components such as NF κ B.⁷⁴ Molecular events that ultimately result in CSC generation drive altered differentiation and skewed transcription factor profiles, including Jak2V617,^{75,76} and PU.1,⁵⁷ and aberrant expression of Ikaros in B cell progenitors.⁷⁷ Finally, LSC ensure their own survival by subverting innate and adaptive immune responses and resisting chemoradiotherapy, a topic which will be covered in greater depth in the section on cancer treatment.

Clinical Implications of Cancer Stem Cells

If tumor genesis, progression, and therapeutic resistance depend on CSC, as studies suggest,^{8,9,11–25} then they represent the most clinically-relevant target population for the diagnosis and management of cancer patients. Targeting the CSC could effectively shut down the cancer at its source and limit unnecessary treatments or surgeries for residual masses found not to contain CSC. There are challenges, however, facing the development of such a targeted strategy. CSC represent a relatively rare population, making them difficult to detect. Moreover, the specific identification of these cells is complicated by the facts that they differ phenotypically among various cancers, among patients including those bearing the same diagnosis, and even within the same patient over time. Another challenge of targeting CSC for cancer

TABLE 4.2. Self-renewal and differentiation pathway components altered in human cancers.

Self-renewal pathway component	Cancer	Reference
Notch	T-cell acute lymphoblastic leukemia	Pear et al ³⁹ and Ellisen et al ³⁵
Wnt/ β -catenin/catenin	Chronic lymphocytic leukemia	Lu et al ³⁷
	Chronic myeloid leukemia acute myeloid leukemia (AML)	Jamieson et al ¹² and Zheng et al ⁴¹
	Head and neck squamous cell carcinomas	Yang et al ⁴⁰
Hedgehog	Pancreatic cancer	Li et al ²³
	Multiple myeloma	Peacock et al ³⁸
BMI-1	Head and neck squamous cell carcinomas	Prince et al ²⁴
	AML	Lessard and Sauvageau ³⁶

therapy is that many of the mechanisms subverted by CSC to enhance their propagation are also used by normal cells throughout the body. For example, many CSC have been shown to activate self-renewal pathways that are also used in normal stem cells (Table 4.2). Therefore, pharmacologic targeting of these pathways may also affect normal stem cells, resulting in potentially severe and even permanent side effects, including alopecia, aplastic anemia, and the inability to regenerate the epithelium of the gastrointestinal tract. For these reasons, identification of a unique CSC molecular signature would offer the ideal target for the diagnosis and treatment of cancers.

CSC in Cancer Diagnosis, Prognosis, and Monitoring

The development of CSC-based assays for diagnosing and following cancers will depend on the discovery of better methods for identifying these cells, phenotypically or molecularly, in tumor biopsies, or through CSC-targeted imaging modalities.

Cellular and Molecular Assays

A variety of cellular and molecular assays are being applied for the phenotypic and molecular identification of CSC, including FACS of peripheral blood and bone marrow, PCR, DNA sequencing, and RNA assays. Nevertheless, these techniques are limited by their dependence on a comprehensive characterization of CSC phenotypes and genotypes. Even if these applications are optimized for the identification of CSC in humans, their requirement of a tissue sample for diagnosis makes them less attractive than a noninvasive approach for use in frequent monitoring of disease progression.

Cellular and Molecular Imaging

Newly targeted imaging technologies represent potentially noninvasive ways to visualize CSC for the purposes of determining the diagnosis, prognosis, and progression of disease, as well as the response to treatment. Aside from the obvious advantages of avoiding biopsy and facilitating close monitoring over time, CSC-directed imaging modalities may be used to reveal any nidi of active CSC. Such an application could have implications for prognosis and treatment, since not every mass may necessarily contain CSC and thereby require subsequent treatment. CSC-specific imaging would also be useful in research and clinical trials of new cancer therapies, since selective elimination of the more clinically-salient CSC population may not result in an immediate reduction in tumor size by MRI or CT, which is a common measure of therapeutic response.

The development of such targeted imaging modalities is the subject of ongoing research. A variety of modalities are being adapted for this purpose, including nuclear imaging, PET, MRI, CT, and ultrasound. These modalities all share the essential challenge of concentrating labeling agents within or on the cells of interest at a level that will be visible by imaging and minimize background signal.

The simplest method to label cells is to encourage cellular uptake of a contrast agent, such as indium-111 for nuclear imaging, iron oxide crystals for MRI, or microbubbles for ultrasound. However, CSC may not be phagocytic cells; thus, other methods, such as electroporation, may be required to introduce contrast agents into the cells. A key limitation of this approach is that the cells of interest must be collected, isolated, and treated *ex vivo* before reintroducing them to the patient. Reinjecting patients with their own CSC may result in the seeding of new tumors and the unintentional spread of disease. For that reason, other cell types capable of homing to the tumors, such as immune cells or non-CSC tumor cells, may be better targets for these cell-labeling strategies. The natural propensity for cells of both adaptive and innate arms of the immune system to home to tumor sites makes them potentially useful vehicles for unmasking occult tumors or metastases, or to deliver molecularly-targeted therapies directly to the cancer.

To circumvent these issues and improve specificity, antibody-based approaches have been developed for use with various modalities. Tumor-specific antibodies have been conjugated to radionuclides, such as carbon-11, fluorine-18, or technetium-99m, for use in PET, or conjugated to large dextran backbones bearing multiple gadolinium molecules for use in MRI. While these approaches do have the advantage of specifically labeling cells of interest *in vivo*, they depend on the identification of at least one tumor-specific marker that is present at adequate concentrations on each cell. Furthermore, the labeling efficiency depends highly on antibody-receptor binding kinetics and the time to achieve equilibrium. For PET, if equilibrium is established later than is optimal for the radionuclide being used, a primary

and secondary antibody may be used. For example, an avidin-conjugated primary antibody is injected and allowed to reach equilibrium, then followed by injection of a second biotinylated antibody conjugated to a radionuclide. Despite efforts like these to circumvent the issues associated with antibody-based molecular imaging approaches, they are all limited by circulation and equilibrium time, nonspecific antibody sequestration in the liver and spleen, and excessive background signal. Some of these issues may be minimized through the proper optimization of antibody concentration and circulation times and through the use of multivalent backbones linking two or more specific antibodies. Nevertheless, these problems remain significant challenges in the development of antibody-based contrast agents for imaging.

As more is understood about the molecular features of cancers and their interactions with their niche, more of these properties may be exploited for imaging purposes. Thus, it is critical that cancer researchers and imaging researchers develop effective collaborations with the ultimate goal of developing molecularly-targeted CSC imaging tools, that may be both diagnostic and therapeutic.

CSC-Directed Cancer Treatment

CSC may represent a critical therapeutic target in the eradication of cancer. Their resistance to chemoradiation may explain treatment failures with current therapies, including some cases of CML treated with BCR-ABL inhibitors such as imatinib,⁷⁸ resistant breast cancers,¹⁵ and multiple myeloma.³² For instance, in CML, multiple strategies for CSC evasion of single agent BCR-ABL inhibitor therapy have been proposed based on extensive gene expression analysis of chronic and advanced phase CML progenitors.^{79,80} Similar mechanisms have been implicated in the observed radiation resistance of these cells.¹⁴ Most notably, the relative quiescence of CSC may protect them against the mutagenic effects of chemotherapy and radiation, which damage exposed DNA in rapidly-dividing cells.⁸¹ Moreover, CSC have been shown to activate multiple, redundant pathways for survival, self-renewal and/or differentiation.⁸¹ Another strategy they employ for evading single agent molecularly targeted therapy is the enrichment of ABC transporters, membrane efflux pumps which confer active multi-drug resistance by permitting the extrusion of these drugs and which is defined by the ability to pump out Hoechst.⁸¹

CSC-directed therapies present a potential advantage of targeting therapy to the relevant tumor cell population, thereby increasing the chance for their eradication and a cure. Also, by narrowing the therapeutic target to CSC, especially with therapies designed to target unique CSC molecules and not molecules or mechanisms common to normal cells, these treatments may be less toxic to normal tissues than systemic chemotherapy and radiation therapy. When conjugated with contrast agents for use in molecular and cellular imaging, CSC-specific pharmaceuticals and therapies may one day be exploited both for the diagnosis and treatment of cancer.

As useful as CSC-directed treatments are likely to be, the ideal cancer treatment course is still likely to encompass a dual approach that also focuses on eliminating the bulk of the rapidly-dividing cancer cells, which are often responsible for the characteristic symptoms and paraneoplastic syndromes that afflict so many cancer patients. By reducing the tumor burden and eliminating its regenerative potential, such a combined therapy may finally lead to a cure for one of humanity's most devastating diseases.

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Gene Therapy for Leukemia and Lymphoma

Xiaopei Huang and Yiping Yang

Introduction

With the advancement of knowledge in immunology and virology, technological improvement in the transduction efficiency of existing viral vectors, and the development of new viral vectors in the past two decades, gene therapy targeting diseases at a molecular level has emerged as an exciting and promising strategy for treating hematolymphoid malignancies. Gene therapy is aimed to correct disease processes by introducing a functional gene into target cells to restore or modify cellular functions. Unlike conventional therapies for treating cancers, gene therapy, in theory, could deliver precise, malignancy-specific treatment with minimal side effects on normal cells and biological processes. For many patients with hematolymphoid malignancies who cannot benefit from the therapies available today, gene therapy may hold the promise of future treatment.

This review will mainly focus on three approaches that have been extensively explored in cancer gene therapy with an emphasis on hematolymphoid malignancies:

1. Tumor cells are targeted directly by expression of tumor suppressor genes, or tumor suicide genes, that are introduced into target cells by gene transfer, or by siRNA specific for oncogenic fusion gene transcripts to silence gene expression.
2. Host immune system is manipulated to enhance its responses to tumor cells via gene transfer of immunostimulating cytokines and/or costimulatory molecules.
3. Host T lymphocytes are genetically modified to enhance antitumor effector functions. The current status of development with each approach will be summarized along with evaluations of the outcome of their application in preclinical and/or clinical studies.

Viral Vectors Used for Gene Therapy

A number of viral vectors for gene transfer have been extensively explored, including the retrovirus, adenovirus, adeno-associated virus (AAV), lentivirus, foamy virus, and

herpes simplex virus (HSV). The ideal vector should have high transduction efficiency, should be replication-deficient, and have a very low likelihood of causing an active infection. When transduced tumor cells are designated for use as a vaccine, the vector should not express immune-dominant antigens that might compete with the development of anti-leukemia immune response.¹

Retrovirus is an enveloped RNA virus. The most commonly used oncogenic retroviral vectors are based on Moloney murine leukemia virus (MLV) and have a simple genome.² Retroviral vectors offer the advantage of stable transgene expression; they are engineered to harness their ability to infect and integrate into the host chromosome, while replacing the wild-type viral genes responsible for replication with the therapeutic gene of interest. Recombinant retroviral vectors are generated by introducing this highly modified vector into packaging cells expressing the structural proteins through separate packing (*gag* and *pol*) and envelope (*env*) plasmids. However, the efficiency and efficacy of retrovirus-mediated gene transfer has been disappointing mainly because of the requirement of active cell division for retrovirus to integrate into host genomes.

Adenoviruses are nonenveloped, double-stranded DNA viruses. They are relatively stable, not associated with serious infections, generally cannot transform infected cells, and can be produced to high titer. They can infect postmitotic, nondividing cells.² These features offer advantages over many other viral vectors. However, the use of adenovirus for clinical gene therapy is compromised by their inherent immunogenicity. Therefore, much effort has been devoted to the development in the field of gene therapy for new generation of safer and more effective adenoviral vectors.

Adeno-associated virus is a nonenveloped human parvovirus.² It presents very low immunogenicity, may infect both dividing and nondividing cells, and has the ability to stably integrate into the host cell genome at a specific site, making it more predictable than retroviruses that present threat of a random insertion and of mutagenesis that is sometimes followed by development of a cancer.

Human immunodeficiency virus (HIV)-based lentiviral vectors have gained much interest as a gene transfer vehicle because of their large packaging capacity and their ability to infect a range of different cell types including postmitotic cells.³ The genome of HIV can nonspecifically integrate into the human genome, which may be problematic since insertional mutagenesis may lead to undesirable effects, such as malignant transformation.^{4,5} However, evidence indicates that episomal forms of the vector may also be harnessed for effective gene expression. Nonintegrating vectors retain the high transduction efficiency and broad tropism of conventional lentiviruses but avoid the potential problems associated with the nonspecific integration of the transgene.

Gene Targeting to Destroy Tumor Cells Directly

Tumor Suppressor Gene Therapy

A large number of oncogenes and tumor suppressor genes have been implicated in human cancers. Genes encoding retinoblastoma protein (pRB) and p53 transcription factor are the two most prominent tumor suppressor genes that are frequently mutated in human cancers.⁶ As a result, the cells that harbor the mutations proliferate without undergoing apoptosis, or have erroneous cell cycle entry or exit, leading to the development of tumors. Since mammalian cell proliferation is tightly regulated by an intricately woven network of cyclins, cyclin-dependent kinases, their inhibitors, and a large set of transcription factors, strategies that target critical components of the signaling pathways may have broad application for cancer therapeutic use.

As most tumors show defects in p53 and pRB, much enthusiasm has been directed at restoring the function of tumor suppressor genes for the treatment of hematolymphoid malignancies. Studies in the past decade have mainly focused on p53 replacement and it is the only tumor suppressor gene to be evaluated formally in clinical trials. Recombinant adenovirus-mediated gene expression has been explored in cell lines from a number of hematolymphoid malignancies.^{7–10} Studies using adenoviral vector expressing the wild-type p53 gene demonstrated efficient killing of the cell lines derived from human lymphoid malignancies, such as lymphoma, lymphocytic leukemia, and myeloma. Furthermore, it has been shown in vivo that adenovirus-mediated expression of wild-type p53 has correlated with apoptosis and necrosis in a nude mouse model carrying the t(2;5) translocation. The first clinical trial of p53 gene replacement in non-small-cell lung carcinoma in 1996 showed promise of this novel gene therapy-based strategy, but the results from the trials since have been disappointing.^{11–17} Possible reasons for the failure may be attributable to (1) low efficiency of gene transfer, (2) insufficient understanding of p53 biology, and/or (3) polymorphisms of the p53 gene. Indeed, many aspects of p53 biology remain to be defined.

Studies on replacement of Rb gene are far fewer than p53. Two reports suggested adenovirus-mediated Rb gene transfer may induce apoptosis in models of head and neck cancer and bladder cancer with minimal effects on normal cells.^{18,19} Additional tumor suppressor genes, such as p16^{INK4A} and p14^{Arf}, whose functions are closely intertwined with p53 and pRB, have emerged as potential targets for gene therapy. Research from one group has shown the efficacy of adenoviral vector-mediated expression of p16^{INK4A} in inducing apoptosis in a lymphoma model.²⁰

Taken together, progress has been made in assessing the therapeutic effect of viral vector-mediated expression of tumor suppressor genes in soliciting apoptosis in tumor cells. Although targeting tumor suppressor gene pathways is an attractive and logical strategy for cancer gene therapy, results from clinical trials have not met the success of pre-clinical studies. Greater understandings of the p53 and Rb tumor suppressor gene pathways are key to the improved gene therapy based on this strategy.

Suicide Gene Therapy

Suicide gene therapy is a strategy that has been extensively explored to treat cancers. The principle of this approach is the conversion of a nontoxic prodrug into an active toxic form by a suicide gene-encoded protein (frequently an enzyme), and the toxic metabolites then kill the tumor cells.²¹ Of the more than a dozen prodrug metabolizing enzyme (PDME) systems described to date, the most widely used suicide gene is herpes simplex thymidine kinase (*HSVtk*). *HSVtk* gene is transferred into tumor cells using retroviral vectors or adenoviral vectors, followed by the administration of prodrugs, ganciclovir (GCV) or acyclovir (ACV). GCV is efficiently converted by the *HSVtk* into a monophosphate form and subsequently phosphorylated by endogenous cellular kinases, producing toxic triphosphate derivatives that compete with normal nucleotides for DNA replication in host cells and leading to the death of actively dividing cells. Thus tumor cells that express *HSVtk* become sensitive to the toxic effect of GCV and may be eradicated in vivo by the administration of GCV.

One interesting feature of suicide gene therapy is the so-called “bystander” effect, which is a toxic effect on the adjacent tumor cells that lack the PDME gene modification.²² The bystander effect is most evident in tumor cells with gap junctions, indicative of transfer of a toxic metabolite or an apoptotic signal. Most hematolymphoid malignancies have a low number of gap junctions; therefore, the bystander effect should be less potent. One way to introduce a bystander effect independent of gap junctions is to link the *HSVtk* gene to the gene of another HSV protein, VP22.²³ Recent studies have shown that VP22 protein passes freely between cells by an unknown mechanism. After endogenous synthesis in a small subpopulation of cells, the protein spreads and forms a gradient in surrounding cells, making it a good candidate for efficient

transfer of cytotoxic molecules, and thus resulting in the bystander effect. Studies have shown the VP22-tk chimeric proteins retain the intercellular trafficking properties of VP22 and retain the thymidine kinase activity.²⁴ They spread between cells in sufficient quantities to induce cell death in response to GCV treatment, not only in the primary synthesizing cells but also in surrounding recipient cells, causing a bystander effect in cells devoid of gap junctions. Studies are underway using this system for the treatment of plasmacytoma and lymphomas in animal models.

There has been considerable interest in the donor lymphocyte infusion following hematopoietic stem cell transplantation to enhance antileukemic and antiviral effects. Transfer of *HSVtk*-engineered donor lymphocytes may potentially serve as a safety measure to control graft-versus-host disease (GvHD). If patients develop GvHD, the administration of GCV will selectively eliminate the infused donor lymphocytes. In a pilot clinical trial, eight patients who developed Epstein-Barr virus-induced lymphoma after T-cell-depleted bone marrow transplantation were treated with donor lymphocytes transduced with the *HSVtk* gene. The transduced lymphocytes survived for up to 12 months, resulting in anti-tumor activity in five patients. Three patients developed GvHD, which was effectively controlled by GCV-induced elimination of the transduced cells.²⁵ Similar results were observed from other studies.²⁶ These data show that genetic manipulation of donor lymphocytes may increase the efficacy and safety of allogeneic bone marrow transplantation.

Suicide gene technology may also be used to obtain tumor-free bone marrow or peripheral blood stem cell autografts for transplantation. Furthermore, suicide gene transfer into live tumor cell vaccines, allowing their selective removal with prodrug treatment, provides a safety measure as live, genetically modified tumor cell vaccines could sometimes maintain their intrinsic tumorigenic properties.²¹ Despite its promises, there are a number of limitations associated with the use of *HSVtk*.²⁷ First, it is most effective when used in combination with GCV, a prodrug that is widely used to treat Cytomegalovirus infection, thus precluding the use of *HSVtk* modified T cells. Second, GCV therapy is known to have myelosuppressive side effects that could be particularly relevant in the post-HSCT setting. Third, *HSVtk* is of nonhuman origin and therefore it is possible that an immune response may be elicited after repeated infusion of genetically modified T cells.²⁸ However, with the improvement of gene transfer processes, better timing of T-cell infusions and prodrug administration, and strategies to combine suicide genes with other immune-enhancing genes, suicide gene therapy may have therapeutic value in a wide range of hematolymphoid malignancies.

RNA Interference

RNA interference (RNAi) is a method for silencing gene expression. Studies have shown that certain specific, recurring chromosomal translocations are associated with particular

types of leukemia and lymphoma. Fusion transcripts encoding oncogenic proteins therefore represent potential targets for RNA-based therapeutic approaches.

Gene silencing based on RNAi has sparked great enthusiasm for its promising therapeutic potential. RNAi was first discovered in *C. elegans* as a response to double-stranded RNA (dsRNA) that resulted in sequence-specific gene silencing.²⁹ During RNAi, long double-stranded RNA molecules are processed to generate ~22-nucleotide small interfering RNAs (siRNAs), which are then incorporated into a multicomponent nuclease, RISC (RNA-induced silencing complex). The siRNA-nuclease complex then recognizes and destroys target mRNAs in a sequence-specific fashion.³⁰ siRNA has been intensely explored as a therapeutic approach for hematolymphoid malignancies.

The Philadelphia chromosome (Ph) translocation t(9;22) generates the *bcr/abl* fusion gene that encodes the chimeric BCR/ABL protein, characteristic of chronic myelogenous leukemia (CML) and Ph⁺ acute lymphoblastic leukemia (ALL).³¹ Studies using siRNA to target the fusion site of M-*bcr/abl* transcript in the CML cell line K562 have demonstrated an effective inhibitory effect of the M-*bcr/abl* transcript. Also observed with the downregulation of M-*bcr/abl* has been the induction of apoptosis in the Ph⁺-K562 cell line.³² In addition, when primary hematopoietic cells from CML patients are transfected with the siRNAs targeted against M-*bcr/abl*, the mRNA level of the oncogene is downregulated to levels between 50% and 70%.³³

siRNA strategy has also been tested in cell lines derived from patients with acute myeloid leukemia (AML). About 10–15% of all de novo AML patients have the t(8;21) translocation (see Chap. 34). The *AML1/ETO* fusion gene encodes the fusion product AML1/ETO, which functions as a constitutive repressor of transcription of several target genes normally involved in myeloid differentiation.^{34–37} Studies have shown reduction of *AML1/ETO* mRNA through fusion-specific siRNAs to 40–80% and that the block of myeloid differentiation may be overcome with the inhibition of *AML1/ETO* by siRNAs followed by stimulation with the differentiation-inducing agents TGFβ1 and Vitamin D3.³⁸

Interesting results have also been obtained using siRNAs against the fusion gene transcript at translocation t(2;5), which occurs in 75% of all pediatric cases of anaplastic large cell lymphoma (ALCL). The NPM/ALK fusion protein exhibits constitutive activation of the tyrosine kinase ALK, leading to cellular transformation through activation of various pathways critical for proliferation and cell survival.^{39,40} Studies using HeLa cells cotransfected with an NPM/ALK expression vector and siRNAs spanning the NPM/ALK fusion site have demonstrated impressive downregulation of NPM/ALK.⁴¹

Taken together, studies with siRNAs to date suggest that it is a promising therapeutic strategy for the inhibition of pathogenic gene expression. The establishment of murine models harboring the chromosomal translocations involved

in leukemia and lymphoma will undoubtedly facilitate studies of *in vivo* activity of siRNAs, and the experience gained from the successful treatment of such transgenic mice with fusion-site-specific siRNAs will serve as foundations for future human clinical trials.

Gene Transfer to Enhance Immune Responses to Tumor Cells

One of the most extensively studied strategies for gene therapy of hematolymphoid malignancies is aimed to enhance immune recognition of poorly immunogenic tumors by transducing tumor cells with immune-stimulatory genes and costimulatory molecules that activate cytotoxic T cells after engaging their surface ligands or counter receptors.⁴² Murine studies have shown augmented immunogenicity in tumor cell lines transduced with these molecules. Injection of neoplastic cells in doses that would normally establish a tumor instead recruits immune system effector cells and eradicates injected tumor cells. Often the animal is then resistant to challenges by further local injections of nontransduced parental tumor. The transduced tumor has, therefore, acted like a vaccine.

Gene Transfer of Immunostimulating Cytokines

The most advanced study using this approach to date has focused on chronic lymphocytic leukemia (CLL). CLL, a neoplasm of mature B lymphocytes that express surface immunoglobulin and B-cell-surface differentiation antigens, is the second most common type of leukemia. Genetic studies have revealed a variety of chromosomal abnormalities associated with CLL, such as trisomy 12 and deletions at 13q, 11q, or 17p, but there has not been any one gene – or genes – found responsible for development or progression of the disease.¹ That CLL cells have distinct expression profile of immunoglobulins, and that the commonly present chromosomal abnormalities in CLL may encode “altered-self” proteins that could serve as target antigens for immune recognition, makes CLL cells an ideal target against which to stimulate the patient’s immune system. The immune-stimulatory genes studied to date include immune cytokines, such as interleukin 2 (IL-2), interleukin 12 (IL-12), granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor- α (TNF- α).¹

Studies using a murine model have shown that plasmacytomas transduced to express IL-2 are better able to induce antitumor immunity than nontransduced cells.^{43,44} Coexpression of IL-2 and lymphotactic chemokine lymphotactin (Lptn) in murine B lymphoma cells more effectively induce antitumor immunity than nontransduced lymphoma cells or lymphoma cells transduced with IL-2 alone, suggesting there exists a synergy between chemokine and cytokine in enhancing antitumor immunity.⁴⁵ Human myeloid and lymphoid leukemia cell lines have been transduced *in vitro* by

retroviruses that encode IL-2,⁴⁶ but they have not been evaluated in clinical trials.

Studies using murine B-lymphoma cell line A20 transduced with IL-12 by replication-defective retrovirus have shown dramatic induction of T-cell-mediated antitumor immunity *in vivo*, compared with those transduced with a control vector.⁴⁷ Transfer of the IL-12 gene into A20 B-lymphoma cells resulted in the continuous production of IL-12 and caused abrogation of *in vivo* tumorigenicity.⁴⁸ Furthermore, tumor cells transfected with the IL-12 gene may effectively induce IL-12-activated killer cells (IL-12AK), IFN- γ production, and tumor-specific protective immunity. These results suggest that IL-12 is a promising cytokine for antitumor cytokine and gene therapy.

GM-CSF is a hematopoietic growth factor with the capability of inducing dendritic cell (DC) maturation and activation. The idea behind transducing tumor cells with GM-CSF is to stimulate activation and maturation of local DCs, thereby inducing a tumor-specific immune response. Studies with animal models for ALL and AML have demonstrated induction of tumor-specific immunity by leukemia cells transduced to express GM-CSF with nonviral vectors.^{49–51} More recent studies using adenovirus or lentivirus vectors have confirmed transduction and expression of GM-CSF in primary leukemia cells from patients with chronic myelogenous leukemia (CML), ALL, and AML.^{52,53} Reports from studies comparing the ability of different cytokines to enhance the immunogenicity of murine tumor cells have indicated GM-CSF as the most potent molecule for inducing antitumor immunity.^{54,55} This effect may be due to its ability to promote differentiation of the DCs, which are potent APCs for activating both class I- and class II-restricted T cells. These preclinical data suggest that GM-CSF gene immunotherapy may be of therapeutic value to be evaluated in clinical trials.

TNF- α plays a diverse role in biological processes such as cellular proliferation, differentiation, tumorigenesis, and viral replication. It is a key mediator of septic shock in response to infection. TNF- α may directly induce apoptosis of some tumors and can stimulate dendritic-cell maturation and function. As TNF- α is very toxic when administered systemically, gene therapy strategies have focused on local expression of TNF- α in the tumor microenvironment. In a murine model, when a myeloid cell line transduced with retrovirus to express TNF- α was administered as a vaccine, activation of dendritic cells and generation of tumor-specific cytotoxic T cells inhibited development and progression of leukemia.^{56–58} Furthermore, this strategy resulted in eradication of residual disease following chemotherapy and prevented relapse.⁵⁹

Gene Transfer of Costimulating Molecules

The expression of accessory molecules on the surface of T lymphocytes and antigen-presenting cells (APCs) plays critical roles in modulating both the initial T-cell responses

to an antigen, as well as differentiation into phenotypically distinct effector cells. T-cell recognition of antigen occurs through the interaction of T-cell receptor (TCR) with an antigen–MHC complex. Stimulation through the TCR induces rapid T-cell expression of CD40-ligand (CD154), which then binds CD40 on APCs and induces APC expression of costimulatory molecules, CD80 (B7–1) and CD86 (B7–2). These costimulatory molecules in turn bind to CD28 on activated T cells, providing a second signal to the T cells leading to their activation and proliferation.^{60,61}

B-cell neoplasms often show little or no expression of B7 antigens, making them a potential target for gene transfer of costimulatory molecules of B7–1 and B7–2 for induction of antitumor immune response. In a study using myeloma cell lines, recombinant adeno-associated virus vectors (rAAV) were used to induce transient expression of CD80 and CD86. Tumor cell expression of CD80 and CD86 was shown to activate T cells, resulting in high-level production of IL-2, INF- γ , and generation of tumor-specific cytotoxic T cells.⁶² Using retroviral vectors and lentiviral vectors, primary ALL B cells have been transduced to express CD80 alone or in combination with GM-CSF for use as vaccines.^{53,63} In vitro studies confirmed the ability of this whole-cell vaccine to stimulate T cells,^{64–66} and animal studies with transduced cell lines confirmed the in vivo activities.^{51,67} TRICOM, triad of costimulatory molecules, is a vaccine strategy using cells that are transduced to express immune costimulatory molecules and adhesion molecules, including CD80, intercellular adhesion molecule-1 (ICAM-1), and leukocyte-function-associated antigen-3 (LFA-3). In animal studies with B-lymphoma cells transduced to express the TRICOM surface molecules, the modified cells were shown to induce antitumor immune responses in vivo more effectively than non-modified lymphoma cells.⁶⁸ CLL cells transduced with a modified vaccinia virus vector carrying TRICOM more efficiently induced production of cytotoxic T lymphocytes against autologous CLL cells in vitro.^{1,69} Furthermore, the in vitro-derived cytotoxic T lymphocytes (CTLs) were reactive against uninfected, unmodified CLL cells. It is anticipated that this strategy will be evaluated in phase-I clinical trial for patients with CLL.

Advances in immunology have shed light on the mechanisms contributing to T-cell dysfunction in patients with CLL.⁷⁰ Despite expressing abundant quantity of class-II major histocompatibility antigens, CLL cells do not stimulate normal allogeneic T cells in mixed lymphocyte reactions, even in the presence of neutralizing antibodies to immunosuppressive cytokines, such as transforming growth factor β (TGF- β).⁷¹ CLL cells may also downregulate CD154 expressed on activated T cells, which leads to abrogation of expression of costimulatory molecules, such as CD80 and CD86 on CLL cells and thereby interferes with effective antigen presentation. This mechanism may account for the observed T-cell tolerance to the leukemia B cells in allogeneic and autologous mixed lymphocyte cultures. Therefore, it is critical to change the tolerance-inducing phenotype

of CLL cells into one that can stimulate T cells in vaccine development using whole leukemia cells.

In summary, studies have demonstrated that introduction of genes encoding diverse types of immuno-stimulatory molecules and costimulatory molecules into tumor cells may enhance the host's antitumor immunity. Tumors transduced with such molecules as vaccines have been evaluated in over 200 different clinical trials as of early 2000, but only few studies included patients with hematolymphoid malignancies. The major concerns in applying this type of tumor vaccines to hematolymphoid malignancies are (1) the primary malignant cells of many hematolymphoid malignancies are highly resistant to transduction by many available vectors; (2) the neoplastic cells show considerable phenotypic heterogeneity (i.e., vaccines made from a small fractions of the cells or obtained from one site may express only a limited number of antigens, not the full array present in the patient as a whole, which may render the vaccines ineffective). Development of viral vectors with higher transduction efficiency as well as strategies combining immunostimulatory genes introduced into the tumor based on a better understanding of the immune system will likely improve the therapeutic value of this approach.

Modification of T-Cell Function by Gene Transfer

Chimeric T-Cell Receptors

Chimeric T-cell receptors provide another strategy for targeting hematolymphoid malignancies. This approach combines the antitumor specificity of antibodies with the ability to activate lymphocytes.⁷² A chimeric receptor may be generated by joining the heavy- and light-chain variable regions of a monoclonal antibody comprising of a single-chain scFv to the ζ chain of the T-cell receptor (TCR- ζ). When transduced with these genes, the effector lymphocytes acquire an antibody-determined specificity. Since the recognition element is derived from an antibody V region, the redirected T cells are MHC independent. The chimeric receptor strategy renders the lymphocytes antitumor specificity while maintaining effector functions, thereby holding promise for cancer immunotherapy.

The chimeric receptor strategy may be potentially applied to a wide range of malignancies with a known cell surface antigen against which a monoclonal antibody exists. As the chimeric effector T cells function in an MHC-independent fashion, they may still recognize and destroy tumor cells that evade T-cell recognition through downregulation of HLA class I molecules and defective in antigen processing. Chimeric receptor-mediated effector functions may be more likely to eradicate tumor cells than humoral immune responses alone, because of the amplifying antitumor responses generated by the cytokine secretion, and the subsequent recruitment of

additional components of the immune system upon T-cell activation.⁴²

In a recent preclinical study, the activity of autologous T cells that were genetically engineered to target the immunoglobulin κ light chain of leukemia B cells was evaluated. Human T cells were transduced with retroviral vector carrying the gene encoding the recombinant receptor. The transduced T cells were found to be cytotoxic for human tumor cells expressing only κ -light chain. In addition, these T cells showed specific control of the growth of transplanted human κ -light-chain-expressing tumor cells in a xenogeneic mouse model system.¹

In another study, CD8⁺ T cells were transduced with a chimeric T-cell receptor specific for human CD20. The transduced T cells were found to be cytotoxic for tumor cells that expressed only CD20. Clinical trials are being considered for patients with CD20⁺ B-cell malignancies such as follicular lymphoma, small lymphocytic lymphoma, splenic marginal-zone lymphoma, diffuse large-B-cell lymphoma, and CLL.⁷³

Despite its numerous advantages over immunotherapies based on monoclonal antibodies or T lymphocytes alone, a number of limitations of chimeric receptor-expressing T cells have been reported from clinical studies. How to ensure long-term persistence of transferred T cells in the host remains a major challenge as modified T cells may be locally lysed by factors secreted by tumor cells. In addition, the functional capabilities of chimeric T cells in an environment where costimulation is limiting remain unclear. Furthermore, study is needed to determine the optimal binding affinity of the chimeric receptor for antigen recognition.

Gene Transfer of TCR for Retargeting

Another strategy for adoptive immunotherapy is to expand tumor-specific T cells *ex vivo* from patients followed by infusion through adoptive transfer. However, generating cytotoxic T cells (CTLs) directed to weak tumor antigens may prove ineffective due to an inability to expand selectively the low number of precursor cells specific for the desired target. This problem may be circumvented by transferring desired TCR specificity from donor T cells to recipient T cells via gene transfer, thereby extending the range of antigens for adoptive T-cell immunotherapy. Studies have shown that the specificity for a TCR is determined by its α - and β -chains, and transfer of these genes is sufficient to endow the recipient T cells with both antigen and MHC specificity of the donor T cells.^{74,75} Therefore, strategies transferring TCR genes of desired specificity have been used to generate tumor-specific T cells. It has been demonstrated that retroviral vectors carrying genes encoding the full-length cDNA for $\alpha\beta$ TCR, derived from MHC-restricted tumor antigen-specific T-cell clones, may efficiently transduce human peripheral blood lymphocytes (PBLs), and that the transduced PBLs may then recognize the tumor cells *in vitro*, producing cytotoxic T lymphocytes (CTLs) with antitumor reactivity.⁷⁶ Similar results have been observed from a number of studies

using gene transfer technology to redirect T-cell specificity to Epstein–Barr virus-encoded peptide^{77,78} and HIV-1.⁷⁹

One important limitation of this strategy is that the TCR-derived recognition domain interacts with antigen in an MHC-restricted manner. Tumors may escape from recognition by the modified T cells by downregulating surface MHC expression or by deficient antigen processing and presentation. Furthermore, the $\alpha\beta$ TCR do not usually target non-protein tumor-associated antigens, such as glycolipid and carbohydrate molecules, which limit the use of TCR gene-modified T cells to cancers with known tumor antigens.

Gene Transfer of Protective Genes

Many solid and hematolymphoid malignancies may actively evade the immune surveillance through transforming growth factor β (TGF- β), an immunosuppressive cytokine found at the site of most tumors. This effect may severely affect adoptively transferred, tumor-specific CTLs used in cellular immunotherapy. Transfer of protective genes into T cells may render the recipient cells protective from the immune-inhibitory tumor environment. Murine studies have demonstrated that T-cell-specific blockade of TGF- β signaling led to the generation of an immune response capable of eradicating tumors in mice challenged with live tumor cells.⁸⁰ In another study, when a dominant-negative TGF- β receptor was transduced into *ex vivo*-expanded human EBV-specific CTLs from patients with Hodgkin's lymphoma, TGF- β -mediated suppression of antitumor T-cell responses was diminished, and a strong survival advantage over nontransduced CTLs was observed.⁸¹ Therefore, gene transfer of transdominant-negative TGF- β receptor in tumor-specific CTLs may be of clinical value, although one potential concern is that lack of response to TGF- β may undesirably impair homeostasis of the tumor-specific CTLs.

Conclusion and Future Directions

Gene therapy holds great promise for treating hematolymphoid malignancies. It has already been used successfully to complement conventional therapies for malignant hematolymphoid disorders. To fully realize its therapeutic potential, many aspects of gene transfer process need to be improved, such as development of safer viral vectors, higher transduction efficiency, minimization of immunogenicity, and better control of viral vector behavior *in vivo*. Equally important is a better understanding of the molecular mechanisms underlying the malignancies, which will provide the basis for therapeutic designs that target tumors specifically with minimal disturbance to the surrounding normal tissues. Furthermore, as the immune system has been severely disturbed as a result of tumor formation, it is crucial to understand the intricate relationship between the host immune system and the tumor, so that gene therapy-based treatment will achieve eradication

of tumor and restoration of the function of the immune system. Gene therapy for hematolymphoid malignancies is still in its early developmental stage; past failures and successes will undoubtedly provide valuable lessons and insight for its future research.

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6

Chemical and Environmental Agents (Including Chemotherapeutic Agents and Immunosuppression)

Richard J.Q. McNally

Introduction

The etiology of leukemia and lymphoma is not clear. Both genetic and environmental factors are likely to be involved. At least two genetic or environmental events are likely to be needed to initiate a leukemia or lymphoma.¹ However, the final precipitating event is most likely to be environmental. Putative environmental exposures include ionizing radiation, electromagnetic fields, chemical exposures, smoking, diet, alcohol consumption, vitamin supplementation, contaminated drinking water, infections, and medications (including chemotherapeutic agents).

This chapter provides an overview of recent evidence concerning the role of environmental risk factors in the etiology of leukemia and lymphoma. There is a vast literature and PubMed (<http://www.ncbi.nlm.nih.gov/PubMed>) has been used to obtain relevant original references (primarily case-control, cohort and ecological studies). Reviews are also used to summarize important literature. Since there are known differences in putative risk factors and mechanisms, childhood and adult studies are (in most of this review) considered separately. Due to space limitations, it is not possible to include all references. The main findings are summarized and only key references are cited.

Ionizing Radiation

Early childhood exposure may be via direct residential exposure, parental occupational exposures, diagnostic exposures of a parent before conception or birth of the child, or diagnostic exposure of the child. These studies have been extensively reviewed.²⁻⁴ Adult exposure may be residential, occupational or diagnostic.

Childhood Studies

Background Radiation (Natural and Man-Made)

Most recent studies have found no associations with residential exposure to radon gas, gamma radiation, or with residential proximity to nuclear facilities.^{2,5,6}

One ecological study found decreased risk of acute lymphoblastic leukemia (ALL) with low levels of ground radon and another ecological study found increased risk of acute myeloid leukemia (AML) with greater in-door radon concentration.² However, care should be exercised when making general inferences from ecological studies. Since these are based on aggregated data, ecological findings may not represent individual cases. Area-level patterns may arise from similarly distributed confounding factors.⁷

One Swedish case-control study found a positive association between gamma radiation and increased risk of childhood leukemia (odds ratio (OR)=1.4; 95% confidence interval (CI): 1.0–1.9).² Two case-control studies found links with radiation from nuclear facilities. One of these studies (from the Ukraine) found a statistically significant increased risk of leukemia among males aged 0–20, whose estimated radiation exposure (from the Chernobyl accident) was higher than 10 mSv.⁸ The other study (from Germany) found a statistically significant OR of 2.19 (lower 95% confidence limit (CL): 1.51) associated with residential proximity of <5 km from a nuclear power plant. There was also a significant trend with distance, which remains unexplained.⁹

Parental Occupational Exposures

Recent studies have found the following positive associations for the child: maternal occupational exposure and increased risk of non-Hodgkin lymphoma (NHL) (OR=3.87; 95% CI: 1.54–9.75); paternal occupation in the nuclear industry and elevated risk of leukemia (OR=1.53; 95% CI: 0.85–2.76); employment as a radiation worker and increased risk of leukemia and NHL (relative risk (RR)=1.6 per 100 mSv exposure; 95% CI: 1.0–2.2). Other studies found no associations and a major review for the United Kingdom (UK) government concluded that there was no association between paternal radiation exposure and subsequent increased risk of leukemia and NHL in the child.²

Parental Diagnostic Exposures

Older studies found an increased risk of leukemia associated with intrauterine exposure to diagnostic radiation.⁴

One more recent study found an association between pre-conceptional paternal x-ray examinations and increased risk of leukemia in the child (OR = 1.33; 95% CI: 1.10–1.61). Other recent studies found no association with parental radiation,² including a case–control study (from the USA) of children with Down syndrome which found no association between maternal or paternal preconception exposure or maternal intrauterine exposure and risk of acute leukemia.¹⁰ Exposure to diagnostic x-rays during pregnancy has been radically reduced in more recent years, thus explaining a lack of positive associations in recent studies.

Childhood Diagnostic Exposures

One study found an increased risk of leukemia associated with >2 diagnostic x-rays of the child (OR = 1.48; 95% CI: 1.11–1.97). Other studies found no link with increased risk of leukemia.² The study of Down syndrome children also found no association with risk of acute leukemia.¹⁰

Adult Studies (Table 6.1)

Background Radiation (Natural and Man-Made)

One case–control study from Russia found increased risk of leukemia (excluding chronic lymphocytic leukemia (CLL)) associated with protracted exposure to ionizing radiation from a contaminated river.¹¹ Evidence from the USA suggests a weak excess of CLL linked with exposure to fallout from nuclear weapons.¹²

Occupational Exposures

Three case–control studies and one cohort study have found that occupational exposure to ionizing radiation is associated with increased risk of leukemia. The occupations involved in these studies included workers at five nuclear facilities (in the USA),¹² accident emergency workers (at Chernobyl),¹³ men who participated in the UK's atmospheric nuclear weapons tests and experimental programs¹⁴ and East German miners with a very long occupational career in underground uranium mining or uranium processing.¹⁵ Other studies have not shown consistent evidence linking risk of CLL with nuclear workers, but there was some evidence suggesting increased risk for uranium miners.¹² The evidence concerning NHL risk is unclear. One case–control study (from Canada) found increased risk of NHL associated with occupational exposure to radium,¹⁶ but other studies have found no associations.¹⁷

Diagnostic/Treatment Exposures

One nested case–control study from the USA found that radiotherapy for testicular cancer was associated with increased risk of subsequent leukemia in the patient.¹⁸ Increased risk of CLL has been associated with prolonged diagnostic exposures.¹² Another study found no evidence linking low dose diagnostic radiation with NHL, but there was increased risk of multiple myeloma (MM) in patients who were frequently exposed to x-rays.¹⁹

Electromagnetic Fields

Early childhood exposure may be through exposure to electrical appliances in the place of residence, electricity

TABLE 6.1. Studies showing an association between ionizing radiation and risk of adult leukemia and lymphoma (articles published post 1997).

Study reference	Disease	Place	Study design	Exposure	Risk or Risk estimate (95% CI)
<i>Background radiation</i>					
11	Leukemia (excluding CLL)	Russia	Case–control	Ionizing radiation from a contaminated river	Total dose: OR/Gy = 4.6 (1.7–12.3) External dose: OR/Gy = 7.2 (1.7–30.0) Internal dose: OR/Gy = 5.4 (1.1–27.2)
<i>Occupational exposures</i>					
12	CLL	USA	Nested case–control	Employment in nuclear facility	Non-significantly elevated risks
13	Leukemia (excluding CLL)	Chernobyl	Case–control	Accident emergency workers	Non-significantly elevated risks
14	All leukemia	UK	Cohort	Men who participated in nuclear weapons tests	All leukemia mortality: RR = 1.45 (0.96–2.17) All leukemia incidence: RR = 1.33 (0.97–1.84) Leukemia-CLL incidence: RR = 1.83 (1.15–2.93)
15	Leukemia	East Germany	Case–control	Very long career in underground uranium mining or uranium processing	Elevated risk of leukemia
16	NHL	Canada	Case–control	Radium	Increased risk of NHL
<i>Diagnostic/treatment exposures</i>					
18	Leukemia	USA	Case–control	Radiotherapy for testicular cancer	Increased risk of leukemia
19	MM	USA	Case–control	Frequent exposure to x-rays	Increased risk of MM

distribution near the residence, radio-frequency transmitters near the residence, and also in utero via parental exposures. Adult exposures may be residential or occupational.

Childhood Studies

Direct Childhood Exposures

Recent ecological studies have found increased risk of childhood leukemia associated with exposure to broadcast radiation. Other ecological studies have found increased risk of leukemia and hematological malignancies associated with proximity to high-voltage power lines.²

A number of studies, including a pooled analysis of nine studies, have demonstrated an association with estimated residential magnetic field exposures $\geq 0.4 \mu\text{T}$ and increased risk of childhood leukemia.^{2,20} Several studies have found increased risk of leukemia associated with residential proximity to overhead power lines.^{2,21} However, a number of studies that reported much lower exposures (via residential exposure and proximity to overhead power lines) did not find any associations.^{2,22}

Parental Occupational Exposures

Several studies have found statistically significant elevated risks for parental occupational exposure to magnetic fields $\geq 0.4 \mu\text{T}^2$.

Adult Studies (Table 6.2)

Residential Exposures

Three case-control studies have found that increased risk of hematological malignancies was associated with residential exposure to electro-magnetic fields (EMF).^{23–25} The first of these studies (from the UK) found an elevated risk of leukemia associated with residence of <25 m from an electricity sub-station.²³ The second study (from Tasmania) found that adults who had lived within 300 m of a power line during the first 15 years of life had a threefold increase in risk of a lymphoproliferative disorder (LPD) or a myeloproliferative disorder (MPD).²⁴ The third study (from Norway) revealed a non-significant excess risk for leukemia (all types combined) associated with residence near a high-voltage power line.²⁵

Some studies have shown increased risk of CLL associated with higher magnetic field frequencies.¹²

Occupational Exposures

A number of studies have found increased risk for leukemia or lymphoma associated with occupational exposures to EMF,^{26–32} while other studies found no associations.³³ The positive studies found the following associations: excess risk of leukemia for men employed as electrical engineers, technicians, power workers, and electrical workers in manufacturing industries (OR's of 1.1–1.5)²⁶; elevated risk of lymphomas in electric utility workers²⁷; increasing risk of CLL with increasing levels of exposure to EMF (lowest level: OR=1.1; 95% CI: 0.5–2.3; intermediate level: OR=2.2; 95% CI: 1.1–4.3; highest level: OR=3.0; 95% CI: 1.6–5.8)²⁸; increased risk of NHL and exposures above electric field intensities of 10 and 40 V/m in electric utility workers²⁹; increased risk of NHL and occupation as a radio operator³⁰; increased risk of acute leukemia in those who had ever worked in an electrical occupation³¹; and excess NHL risk for a number of occupations involving exposure to EMF.³²

It is possible that some of the differences between studies may be due to differences in diagnostic groups, study methodology, or in levels of exposure to EMF.

Chemical Exposures

Early childhood exposure may be through exposure in or around the place of residence and also in utero via parental exposures. Adult exposures may occur around the place of residence or as a result of occupation.

Childhood Studies

Pesticides and Fungicides

Ecological studies have suggested possible associations between childhood leukemia and agricultural pesticide use.²

Several older case-control studies have reported an increased risk of leukemia or lymphoma related to pesticides.² More recently, three case-control studies have found statistically

TABLE 6.2. Studies that show an association between EMF and the risk of adult leukemia and lymphoma (articles published post 1997).

Study reference	Disease	Place	Study design	Exposure	Risk or risk estimate (95% CI)
<i>Residential exposures</i>					
24	LPD or MPD	Tasmania	Case-control	Adults who had lived within 300 m of a power line during first 15 years of life	OR=3.23 (1.26–8.29)
25	All leukemia	Norway	Nested case-control	Residence near a high-voltage power line	Non-significant excess risk
<i>Occupational exposures</i>					
29	NHL	Canada	Case-control	Electric field intensities of 10 and 40 V/m	10 V/m: OR=3.05 (1.07–8.80) 40 V/m: OR=3.57 (1.30–9.80)
30	NHL	France	Case-control	Occupation as a radio operator	OR=3.1 (1.4–6.6)
31	Acute leukemia	New Zealand	Case-control	Ever worked in an electrical occupation	OR=1.9 (1.0–3.8)
32	NHL	British Columbia	Case-control	Occupations involving exposure to EMF	Excess risk

significant evidence of an association between exposure to pesticides or fungicides and increased risk of a leukemia or lymphoma in the child,^{34–36} while two case–control studies found no associations.^{37,38}

Specifically, one study (from Costa Rica) found that mothers' exposures to any pesticides during the year before conception and during the first and second trimesters were associated with increased risk (OR=2.4; 95% CI: 1.0–5.9; OR=22; 95% CI: 2.8–171.5; OR=4.5; 95% CI: 1.4–14.7, respectively). There was also an association with fathers' exposures during the second trimester (OR=1.5; 95% CI: 1.0–2.3).³⁴ Another study (from France) found that insecticide use during pregnancy was significantly associated with childhood acute leukemia (AL) (OR=2.1; 95% CI: 1.7–2.5), NHL (OR=1.8; 95% CI: 1.3–2.6), and mixed cellularity Hodgkin lymphoma (HL) (OR=4.1; 95% CI: 1.4–11.8). Paternal household use of pesticides was linked to AL (OR=1.5; 95% CI: 1.2–1.8) and NHL (OR=1.7; 95% CI: 1.2–2.6).³⁵ The third study (from Israel) found significantly increased risk of ALL in offspring of parents who had potential exposure to organic solvents or pesticides.³⁶

A study from the North of England found no association with preconception paternal occupational exposures to pesticides or herbicides.³⁷ Another study (from Texas) did not find any association between residence in counties that had moderate or high agricultural activity (taken as a proxy for exposure to pesticides) and risk of childhood leukemia or lymphoma.³⁸

Solvents, Benzene and Other Hydrocarbons

A number of older case–control studies have found statistically significant associations between the risk of childhood leukemia and exposure to solvents. The associations involved both direct exposure of the child and also maternal exposures during the preconception period and during pregnancy.² A number of other studies have also found increased risk of childhood leukemia and lymphoma associated with exposure to benzene and other hydrocarbons.² More recently, two case–control studies and one cohort study have found associations between parental exposure to solvents and risk of leukemia in the child.^{39–41}

The first recent case–control study (from the USA) found that there were significant associations between maternal exposure to solvents (OR=3.1; 95% CI: 1.0–9.7) and plastic materials (OR=6.9; 95% CI: 1.2–39.7) during pregnancy and plastic materials after pregnancy (OR=8.3; 95% CI: 1.4–48.8) and K-ras mutations, which have been implicated in the pathogenesis of leukemia.³⁹ The second recent case–control study (from Canada) found significant associations for maternal exposure to a number of occupational solvents and childhood leukemia, including toluene (OR=1.88; 95% CI: 1.01–3.47), mineral spirits (OR=1.82; 95% CI: 1.05–3.14), alkanes (OR=1.78; 95% CI: 1.11–2.86), and mononuclear hydrocarbons (OR=1.64; 95% CI: 1.12–2.41).⁴⁰ The cohort study

(from Taiwan) found an increased risk of childhood leukemia associated with potential maternal occupational exposure to organic solvents (RR=3.83; 95% CI: 1.17–12.55).⁴¹

Adult Studies (Table 6.3)

Pesticides and Fungicides

Thirteen recent studies (one cohort, ten case–control, one pooled analysis of two case–control studies and one meta analysis of 13 case–control studies) have found that an increased risk of leukemia or lymphoma (most notably NHL) was associated with exposure to pesticides or fungicides.^{42–55} Another study found no association with increased risk of NHL.⁵⁶ It is to be noted that for some of these studies the association was with prolonged or substantial exposure.⁵⁵

The studies reported the following associations: compared with flight instructors, aerial pesticide applicator pilots were at increased risk of leukemia⁴²; among people engaged in agriculture, those employed as tractor drivers and as “orchard, vineyard and related tree and shrub workers” appeared to be at increased risk for hematolymphoid malignancies⁴³; some specific classes of pesticides increased the risk of hematolymphoid malignancies: there was a (non-statistically significant) increased risk of NHL for subjects who were exposed to phenoxy herbicides and who were not using protective equipment and there was significantly increased risk for exposure to 2,4-dichlorophenoxy acetic acid (2,4-D)^{44,45}; risk of hematolymphoid malignancy was elevated in workers cultivating vegetables, increased risk of leukemia was associated with exposure to the pesticides mancozeb and toxaphene, and again increased NHL risk was associated with 2,4-D⁴⁶; persistent organochlorine chemical in plasma increased the risk of NHL⁴⁷; organochlorines in carpet dust elevated the risk of NHL⁴⁸; people whose homes were treated for termites had elevated NHL risk⁴⁹; compared to farmers who never used pesticides, the risk of t(14;18)-positive NHL was significantly elevated among farmers who used animal insecticides, crop insecticides, herbicides and fumigants⁵⁰; farmers exposed to non-arsenic pesticides were at increased risk of lymphoma⁵¹; sheep owners, exposed to hexachlorocyclohexane, were at increased risk of NHL⁵²; exposure to organochlorines increased the risk of NHL⁵³; and there was increased risk of NHL and hairy cell leukemia (HCL) for subjects exposed to herbicides, insecticides, fungicides, and impregnating agents.⁵⁴ The meta-analysis (of 13 case–control studies) showed that a long period of exposure (>10 years) increased the risk of all hematopoietic cancers combined and specifically, NHL.⁵⁵

Solvents, Benzene and Other Hydrocarbons

Eleven recent studies (one cohort, nine case–control and one meta analysis of 14 occupational cohort and four case–control

TABLE 6.3. Studies that show an association between chemical exposures and the risk of adult leukemia and lymphoma (articles published post 1997).

Study reference	Disease	Place	Study design	Exposure	Risk or risk estimate (95% CI)
<i>Pesticides and fungicides</i>					
42	Leukemia	USA	Cohort	Aerial pesticide applicator pilots	RR = 3.35
43	Hematolymphoid malignancies	Italy	Multicentre case-control	Employed as tractor drivers and orchard, vineyard and related tree and shrub workers	Increased risk
44,45	NHL and leukemia	Italy	Case-control	Some classes of pesticides	Non-significant elevated risks
46	Lymphohematopoietic cancer	California	Case-control	Workers cultivating vegetables	OR = 1.67 (1.12–2.48)
46	Leukemia	California	Case-control	Exposure to pesticides:	
	Leukemia			Mancozeb	OR = 2.35 (1.12–4.95)
	NHL			Toxaphene	OR = 2.20 (1.04–4.65)
	NHL			2,4-D	OR = 3.80 (1.85–7.81)
47	NHL	USA	Case-control	Persistent organochlorine chemical in plasma	Higher risk of NHL
48	NHL	USA	Case-control	Organochlorines in carpet dust	OR = 1.5 (1.2–2.0)
49	NHL	USA	Case-control	Homes treated for termites	OR = 1.3 (1.0–1.6)
50	t(14;18)-positive NHL	Nebraska	Case-control	Farmers who used:	
				Animal insecticides	OR = 2.6 (1.0–6.9)
				Crop insecticides	OR = 3.0 (1.1–8.2)
				Herbicides	OR = 2.9 (1.1–7.9)
				Fumigants	OR = 5.0 (1.7–14.5)
51	Lymphomas	Spain	Case-control	Farmers exposed to non-arsenic pesticides	OR = 1.8 (1.1–2)
52	NHL	Iceland	Nested case-control	Sheep owners exposed to hexachlorocyclohexane	OR = 3.86 (1.59–8.53)
53	NHL	Canada	Case-control	Exposure to organochlorines	OR = 1.83 (1.18–2.84) for strongest association
54	NHL & HCL	Sweden	Pooled analysis of two case-control studies	Herbicides	OR = 1.75 (1.26–2.42)
				Insecticides	OR = 1.43 (1.08–1.87)
				Fungicides	OR = 3.11 (1.56–6.27)
				Impregnating agents	OR = 1.48 (1.11–1.96)
55	All hematopoietic cancers	International	Meta analysis of 13 case-control studies	Long periods of exposure (>10 years)	OR = 2.18 (1.43–3.35)
	NHL				OR = 1.65 (1.08–2.51)
<i>Solvents, benzene and other hydrocarbons</i>					
57	Leukemia	France	Cohort	Estimated cumulative exposure to benzene ≥ 16.8 ppm-years	OR = 3.6 (1.1–11.7) with dose-response OR = 12 (1.0–1.5) per 10 ppm-years increase in exposure
58	NHL	Australia	Case-control	Exposure to any solvent	OR = 30
59	NHL (women)	USA	Case-control	Organic solvents	No overall effect, but a few high risk associations
60	NHL	Italy	Case-control	Occupational exposure to solvents (medium/high level):	
				Toluene	OR = 1.8 (1.1–2.8)
				xylene	OR = 1.7 (1.0–2.6)
				benzene	OR = 1.6 (1.0–2.4)
				technical solvents	OR = 2.7 (1.2–6.5)
				aliphatic solvents	OR = 2.7 (1.2–5.7)
61	NHL	Italy	Case-control	High levels of benzene	OR = 16.3 (0.8–321)
62	Malignant lymphoma	Germany	Case-control	Exposure to chlorinated hydrocarbons	OR = 2.1 (1.1–4.3)
63	HCL	Sweden	Case-control	Organic solvents	OR = 1.5 (0.99–2.3)
64	AML	UK	Case-control	Solvents	OR = 2.52 (1.45–4.39)
65	Leukemia	USA	Case-control	Possible exposure to solvents	Increased risk
66	Leukemia	China	Nested case-control	Working for 1 year in innertube department of tire factory	OR = 1.1 (1.00–1.24)
67	NHL	International	Meta-analysis of 14 occupational cohort and four case-control studies	Occupational trichloroethylene exposure	Modest evidence of an association

studies) have found that increased risk of leukemia or lymphoma (most notably NHL) was associated with exposure to solvents, benzene, or hydrocarbons.^{57–67} Another two studies found no association with increased risk of NHL, MM, or leukemia.^{68,69}

The following positive associations were found: risk of leukemia was increased in workers with an estimated cumulative exposure to benzene ≥ 16.8 ppm-years, with an indication of a dose–response relation⁵⁷; risk of NHL was increased by a factor of 30 for exposure to any solvent⁵⁸; no overall role for organic solvents in the development of NHL among women, but there were a few high risk associations⁵⁹; medium/high level of occupational exposure to solvents toluene, xylene, and benzene increased the risk of NHL and exposure to technical solvents and aliphatic solvents increased the risk for HL⁶⁰; high levels of benzene increased the risk of NHL⁶¹; high exposure to chlorinated hydrocarbons led to a higher risk of malignant lymphoma⁶²; exposure to organic solvents increased the risk of HCL⁶³; exposure to solvents increased the risk of AML⁶⁴; increased risk of leukemia among workers with possible exposure to solvents⁶⁵; and working for one year in the inner tube department of a tire factory was associated with increased risk of leukemia.⁶⁶

The evidence concerning occupational trichloroethylene exposure (from two meta analyses) suggests a modest association with NHL, but no association for MM or leukemia.^{67,68}

A case–control study from the USA found that hobbies with solvent exposure were not associated with increased risk of NHL.⁶⁹

Smoking

Exposure of the child mainly occurs via maternal (pre-conceptional or in utero) or paternal (pre-conceptional) smoking. Exposure of adults mostly occurs as a direct result of an individual smoking.

Childhood Studies

Parental Smoking

Older studies did not find any consistent association between paternal or maternal smoking and childhood leukemia.^{2,4} One more recent case–control study from France found no association with parental smoking (maternal or paternal) and increased risk of acute leukemia.⁷⁰ However, an interaction was observed in a case-only analysis with CYP1A1*2A variant allele (OR=2.2; 95% CI: 1.0–4.9) and with GSTM1 deletion (OR=2.3; 95% CI: 1.2–4.4).⁷¹ One large cohort study from Sweden found that maternal smoking was associated with increased risk of AML (hazard ratio (HR)=1.41; 95% CI: 0.74–2.67), but not ALL (HR=0.73; 95% CI: 0.58–0.91).⁷² One case–control study from

California found no association for maternal smoking, but paternal preconception smoking was significantly associated with an increased risk of AML (OR=3.84; 95% CI: 1.04–14.17) and there was a non-significant association for ALL with paternal preconception smoking (OR=1.32; 95% CI: 0.86–2.04).⁷³

Accurate assessment of parental smoking in childhood cancer studies is problematic. A recent study from the UK found that there was evidence of under-reporting among parents of case children.⁷⁴

Adult Studies (Table 6.4)

Only a few older studies found small increases of adult ALL associated with smoking, but much more pronounced associations have been found for adult AML.^{75–78}

More recently a cohort study found an increased risk of leukemia associated with smoking.⁷⁹ Another cohort study found elevated mortality from both lymphoblastic and myeloid leukemia in the group comprising both current and former smokers.⁸⁰ Four studies have also found a specific association for AML/acute non-lymphocytic leukemia (ANLL) among currently active smokers.^{81–84} One of these studies also showed that while chronic myeloid leukemia (CML) and ALL exhibited little association with smoking status, CLL showed a positive association in the periods ≥ 2 years prior to diagnosis.⁸⁴

Eight recent case–control studies have found that cigarette smoking increased the risk of HL.^{85–92} One study found no association.⁹³ Older studies of NHL risk and smoking were reviewed by Peach.⁹⁴ Only one out of five older cohort studies found a convincing association between NHL and smoking, while four out of five older cohort studies showed no association. Eight of 14 older case–control studies found no association, while six out of 14 reported a positive association. However, some studies had problems with their design. Seven recent studies have found an association between cigarette smoking and an increased risk of NHL.^{95–101} Some of these studies have found marked risks especially for certain sub-types.^{96–98,100,101} A further five studies (including a multi-centre case–control study from a number of European countries) found no associations for NHL.^{92,102–105} One cohort study (from the USA) found that current or former smokers had lower risk of follicular NHL.⁸⁹

One case–control study from Germany found a strong association between smoking and MM.⁸⁸

One case–control study from Spain found a dose-dependent increase in the risk of mycosis fungoides (MF) associated with increased smoking habits.¹⁰⁶

Overall, recent evidence suggests that cigarette smoking confers increased risks of AML, HL, and certain types of NHL among adults, although the evidence was not consistent over all studies. Again one explanation is that lack of consistency between studies may be due to difficulties in accurately reporting smoking status for cases.

TABLE 6.4. Studies that show an association between smoking and the risk of adult leukemia and lymphoma (articles published post 1997).

Study reference	Disease	Place	Study design	Exposure	Risk or risk estimate (95% CI)
79	Leukemia	Korea	Cohort	Smoking	Increased risk
80	Lymphatic and myeloid leukemia	UK	Cohort	Current &/or former smokers	Elevated mortality
81	AML	Canada	Case-control	Active smoking	OR = 1.5 (1.1–2.0)
82	AML	Sweden	Cohort	Current smokers	Incidence rate ratio (IRR) = 1.50 (1.06–2.11)
83	AML	USA	Cohort	Current smoking	RR = 3.47 (1.002–11.99)
84	ANLL	Germany	Case-control	Current smokers	OR = 1.65 (0.95–2.87)
85	HL	USA	Case-control	Current smokers	OR = 1.8 (1.3–2.9)
86	HL in women	California	Case-control	Cigarette smoking	Association for women
87	HL	Europe	Case-control	Smoking	OR = 2.35 (1.52–3.61)
88	HL in men	Germany	Case-control	Smoking	OR = 3.6 (1.7–7.5)
89	HL	USA	Cohort	Current smokers	RR = 2.25 (1.04–4.89)
				Recent quitters	RR = 4.20 (1.94–9.09)
90	HL	UK	Case-control	Ever-smokers	OR = 1.4 (1.1–1.9)
91	HL	Denmark/Sweden	Case-control	Current cigarette smokers	OR = 1.57 (1.22–2.03)
92	HL	Europe	Cohort	Smokers	HR = 2.14 (1.18–3.87)
95	NHL in women	France	Case-control	Current smokers	OR = 2.40 (1.19–4.84)
				Ever-smoked >30 years	OR = 5.04 (1.40–18.12)
96	Follicular lymphoma	CT, USA	Case-control	Women with cumulative lifetime exposure:	
				16–33 pack-years	OR = 1.5 (0.9–2.5)
				34 pack-years +	OR = 1.8 (1.1–3.2)
97	NHL	Italy	Case-control	Blond tobacco	OR = 1.4 (1.1–1.7)
	Follicular lymphoma				OR = 2.1 (1.4–3.2)
98	B-cell low grade (LG) NHL	Italy	Case-control	Current smokers (≥20 cigarettes per day)	OR = 1.14 (0.37–3.56)
	B-cell intermediate/high grade (I/HG) NHL				OR = 2.10 (0.94–4.67)
	T-cell NHL				OR = 25.84 (1.95–342.17)
99	NHL	International	Pooled case-control analysis	Current smokers	OR = 1.07 (1.00–1.15)
100	NHL overall	Denmark/Sweden	Case-control	Cigarette smoking	OR = 0.97 (0.87–1.08)
	Follicular lymphoma			Female smokers	OR = 1.41 (1.04–1.92)
	T-cell lymphoma			Men ever smoked	OR = 1.67 (1.11–2.51)
101	t(14;18)-negative NHL	Nebraska	Case-control	Women who ever smoked cigarettes	OR = 1.9 (1.1–3.3)
89	Follicular NHL	USA	Cohort	Current or former smokers	RR = 0.67 (0.52–0.86)
88	MM	Germany	Case-control	Smoking: men	OR = 2.4 (0.98–5.74)
				Smoking: women	OR = 2.9 (1.1–7.4)
106	MF	Spain	Case-control	Smoking	Dose-dependent increase in risk

Alcohol

Exposure of the child is mainly via parental pre-conceptional or maternal in utero alcohol consumption. Exposure of the adult is via direct consumption.

Childhood Studies

Parental Alcohol Consumption

Older studies found no association between paternal alcohol consumption and risk of childhood leukemia, but there was an association between maternal alcohol consumption and risk of childhood AML.^{2,4} One recent case-control study from Canada found lower risk of ALL associated with maternal alcohol consumption during pregnancy (OR = 0.7; 95% CI: 0.5–0.9), but there was an interaction with the GSTM1 null genotype

during the third trimester of pregnancy (OR = 2.4; 95% CI: 1.1–5.4) and with CYP2E1 variant G-1295C (or allele *5) during the nursing period (OR = 4.9; 95% CI: 1.5–16.7).¹⁰⁷

Two recent case-control studies have found increased risk of childhood leukemia associated with maternal alcohol consumption. The first study (from France) found that maternal alcohol consumption of more than one drink per day was related to ALL (OR = 2.8; 95% CI: 1.8–5.9).⁷⁰ The second study (from Canada) found that maternal alcohol consumption prior to conception (OR = 1.37; 95% CI: 0.99–1.90) and during pregnancy (OR = 1.39; 95% CI: 1.01–1.93) was associated with an excess risk of leukemia with a significant dose-response trend.¹⁰⁸

Adult Studies (Table 6.5)

A case-control study from the USA found a protective effect for acute leukemia for light and moderate beer intake and a

TABLE 6.5. Studies that show an association between alcohol consumption and the risk of adult leukemia and lymphoma (articles published post 1997).

Study reference	Disease	Place	Study design	Exposure	Risk or risk estimate (95% CI)
109	Acute leukemia	USA	Case-control	Light/moderate beer intake Moderate/heavy wine intake	RR=0.58 (0.44–0.76) RR=2.1 (1.2–3.8)
111	Malignant lymphoma	Japan	Case-control	Regular alcohol intake	Reduced risk
112	HL	Multi-centre	Multi-centre case-control	Alcohol amongst non-smokers	OR=0.46
89	NHL	USA	Cohort	>28 drinks/week	RR=0.77 (0.59–1.00) P for trend=0.02
104	NHL	Europe	Multi-centre case control	Alcohol drinking among men	OR=0.76 (0.62–0.93)
113	NHL	International	Pooled analysis of nine case-control studies	Alcohol	OR=0.83 (0.76–0.89) OR=0.51 (0.33–0.77)
114	Burkitt lymphoma (BL)	USA	Case-control	Men who consume ≥ 1 wine drink per day	OR=0.4 (0.2–0.9)
105	Follicular lymphoma	France	Case-control	Wine consumption Started age <20 >19 g/day	OR=2.19 (0.83–5.80) OR=4.04 (1.19–13.76) OR=4.37 (1.04–18.45)
127	NHL	Sweden	Case-control	>19.1 g of ethanol per day versus 0–2.2 g of ethanol per day	OR=1.2 (0.8–1.7)
115	NHL	Uruguay	Case-control	Beer drinkers	OR=5.5 (1.1–26.7)
116	NHL	USA	Case-control	Alcohol use in men with +ve family history of hematolymphoproliferative cancer: <13.7 g/day >13.7 g/day	OR=2.1 (1.0–4.7) OR=2.8 (1.3–5.9)
106	MF	Europe	Multi-centre case control	High alcohol intake >24 g/day	OR=3.02 (1.34–6.79)

positive association for moderate and heavy wine intake.¹⁰⁹ Another case-control study from Los Angeles county examining the effect of alcohol intake on risk of AML was inconclusive, but suffered from lack of power.¹¹⁰

A case-control study from Japan found that regular alcohol intake was associated with reduced risk of malignant lymphoma.¹¹¹ One multi-centre case-control study found a protective effect for HL for alcohol among non-smokers.¹¹² Another case-control study from the UK found no associations between HL and alcohol consumption.⁹⁰

A cohort study, a pooled analysis of nine case-control studies, and two other case-control studies found that alcohol was protective against NHL.^{89,104,113,114} Two other case-control studies found positive associations between alcohol intake and NHL.^{105,115} Another case-control study (from the USA) found that alcohol use was not associated with risk of NHL in men without a family history of hematolymphoid malignancy, but there was a risk with positive family history.¹¹⁶

A multi-centre European case-control study found a higher risk of mycosis fungoides (MF) associated with high alcohol intake of more than 24 g/day (OR=3.02; 95% CI: 1.34–6.79).¹⁰⁶

Diet, Vitamin, and Folate Supplementation

Childhood Studies

Case-control studies, that have been reviewed previously, have found increased risk of infant AML associated with maternal consumption of DNA topoisomerase 2 inhibitor-

containing foods and protective effects for childhood leukemia associated with maternal consumption of vegetables, fruits, protein sources, and related nutrients in the 12 months before pregnancy and protective effects for regular consumption of oranges or bananas and orange juice during the first 2 years of life. Four out of six case-control studies from the UK found an association between neonatal administration of vitamin K and subsequent increased risk of leukemia or lymphoma in the child. One case-control study found a protective association between iron or folate supplementation in pregnancy and risk of common ALL in the child.² A recent case-control study from the USA found that overall maternal consumption of fresh vegetables and fruits during pregnancy was associated with a decreased risk of infant leukemia, particularly MLL+, but for AML (MLL+) cases, maternal consumption of specific DNAt2 inhibitors increased risk.¹¹⁷ A case-control study from Greece found that the risk of ALL in the child was lower with maternal intake of fruits (OR=0.72; 95% CI: 0.57–0.91), vegetables (OR=0.76; 95% CI: 0.60–0.95) and fish and seafood (OR=0.72; 95% CI: 0.59–0.89) and higher with increased maternal intake of sugars and syrups (OR=1.32; 95% CI: 1.05–1.67) and meat and meat products (OR=1.25; 95% CI: 1.00–1.57).¹¹⁸ A case-control study of children with Down syndrome found no evidence of an association between children's regular multivitamin use and ALL (adjusted OR=0.94; 95% CI: 0.52–1.70) or AML (adjusted OR=1.90; 95% CI: 0.73–4.91). There was an indication of increased risk for AML associated with regular multivitamin use during the first year of life (OR=2.38; 95% CI: 0.94–5.76) or for an extended duration (OR=2.59; 95% CI: 1.02–6.59).¹¹⁹ One case-control study from France

TABLE 6.6. Studies that show an association between diet and the risk of adult leukemia and lymphoma (articles published post 1997).

Study reference	Disease	Place	Study design	Exposure	Risk or risk estimate (95% CI)
120	Leukemia NHL MM	Canada	Case-control	Dietary fish intake	OR=0.72 (0.58–0.89) OR=0.71 (0.60–0.85) OR=0.64 (0.45–0.90)
121	AML among women	Canada	Case-control	Milk intake Tea Beef	OR=0.25 (0.08–0.73) OR=0.50 (0.23–1.09) OR=4.78 (1.35–16.94)
115	NHL	Uruguay	Case-control	Red meat intake, barbequed meat and especially salted meat	OR=4.9 (1.4–17.7)

found that maternal coffee consumption was not related to risk of AL in the child. However, highest coffee intake of >3 cups per day during pregnancy was associated with AL in children whose mothers are non-smokers (OR=1.9; 95% CI: 1.0–3.5).⁷⁰

Adult Studies (Table 6.6)

Recent studies have found that dietary fish intake was protective for leukemia, NHL, and MM¹²⁰; AML risk was negatively associated with milk intake among women and tea and positively associated among women with beef¹²¹; increased risk of NHL was associated with red meat intake, barbecued meat, and especially salted meat¹¹⁵; but there was no significant association between NHL risk and intakes of folate, vitamin B2, vitamin B6, and methionine.¹²²

Drinking Water

Child Studies

Two older (pre-1998) case-control studies found a non-significant but elevated risk for leukemia for drinking water contaminated with solvents, including trichloroethylene, during pregnancy (with a significant dose-response relationship) and with high levels of trihalomethanes, chloroform, and zinc.²

Adult Studies

Two studies have identified increased risk of leukemia associated with chemical contamination of the drinking water supply. The first (a retrospective cohort study from Italy) found that high trihalomethane content of drinking water increased mortality from lymphocytic leukemia in females.¹²³ The second (a case-control study from Canada) found that increased risk of CML was associated with highest exposure duration to total trihalomethanes of more than 40 mg/l (OR=1.72; 95% CI: 1.01–3.08).¹²⁴

Immunosuppression and Viruses

There is a large body of evidence linking certain viruses to certain leukemias and lymphomas. Furthermore, immunosuppression also has a role to play in the development of some of these malignancies.

Viruses (Also See Chap. 7)

Child Studies

Although no specific agent has been consistently identified, an increased risk of childhood leukemia has been linked with greater affluence and situations of unusual population mixing, that would be consistent with delayed exposure to common infections. Protective effects have been found for immunization, early day care attendance, and breast feeding.^{2–4,125} Increased risk of HL has been associated with measles infection and protective effects with prolonged breast feeding. Although Epstein-Barr virus (EBV) viral DNA has been identified in the tumor cells of around 30% of HL cases, most cases in children are EBV-negative.¹²⁶ However, the evidence concerning NHL is less convincing,^{2–4} although EBV has been found in Burkitt lymphoma (BL) in parts of sub-Saharan Africa.¹²⁷ Recent studies have continued to investigate these putative risk factors.

One case-control study from Northern England found that there was increased risk of childhood leukemia and NHL in those children whose fathers had high levels of occupational contacts (OR=1.3; 95% CI: 1.0–1.5),¹²⁸ which may be considered as an unusual type of population mixing. Such situations have been postulated (by Kinlen) to lead to increased incidence of childhood leukemia.^{129,130} Another case-control study from the UK found a protective effect for childhood ALL associated with formal day care outside the home (OR=0.48; 95% CI: 0.37–0.62),¹³¹ which is consistent with a protective effect of early exposure to common infections as suggested by Greaves.¹³² A second analysis of this case-control study from the UK did not find any association between high levels of paternal occupational contact at birth (OR=1.02; 95% CI: 0.88–1.18) or diagnosis (OR=0.91; 95% CI: 0.79–1.06). This latter finding may reflect possible participation bias in the control group.¹³³ A third analysis found that more ALL cases were diagnosed with at least one infection in the neonatal period (OR=1.4; 95% CI: 1.1–1.9),¹³⁴ suggesting that the timing of an early infectious exposure may play an important role. Another case-control study (from New York State) found both weak positive and weak inverse associations between ALL and early infections.¹³⁵ This apparent inconsistency may be due to timing of exposures or the types of infection involved.

Recent case-control studies have considered specific agents and found the following positive associations between:

maternal infection with *mycoplasma pneumoniae* (OR=1.5; 95% CI: 0.9–2.4) or *H. pylori* (OR=2.8; 95% CI: 1.1–6.9) and childhood leukemia in the offspring¹³⁶; EBV and risk of ALL (OR=1.9; 95% CI: 1.2–3.0) and non-ALL (OR=4.5; 95% CI: 1.3–16) in the child¹³⁷; and prenatal adenovirus infection and subsequent development of ALL.¹³⁸

In contrast, one case–control study (from Sweden) found that human parvovirus B19 was not detected in Guthrie cards from children with ALL.¹³⁹

Adult Studies (Table 6.7)

Most adult studies have investigated direct (rather than indirect) evidence of infectious exposures as well as occupations that may lead to potential infectious exposures. For Adult T-cell leukemia/lymphoma (ATLL), there is causal evidence that ATLL is caused by the retrovirus Human T-cell leukemia/lymphoma virus type 1 (HTLV1). A small number of those infected will develop ATLL.¹⁴⁰ As discussed above, EBV has been found in a substantial proportion of mainly adult HL cases.¹²⁶ A meta-analysis of population-based cohort studies has shown that HIV infection led to increased risk of HL (standardized incidence ratio (SIR)=11.03; 95% CI: 8.43–14.4).¹⁴¹ Also, infection with HIV has been associated with greatly increased risk of NHL.^{142,143} A recent cohort study has found greatly increased risk of NHL in HIV positive individuals (SIR=62).¹⁴⁴

A case–control study from Italy found that later age at adenoidectomy and tonsillectomy (>10 years) increased the risk of lymphoblastic leukemia, indicating that late infection is a proliferative stimulus for B-cells.¹⁴⁵

The following positive associations were also found from case–control studies: tuberculosis, hepatitis, maternal tuberculosis (TB), paternal TB, and previous infectious mononucleosis

and increased risk of NHL¹⁴⁶; previous infectious mononucleosis and increased risk of HL¹⁴⁶; Ab_EBV positivity and increased risk of all lymphomas combined and also for CLL¹⁴⁷; human herpes virus type 8 (HHV-8) and increased risk for lymphoplasmacytic lymphoma and other low-grade lymphomas¹⁴⁸; high prevalence of hepatitis B virus (HBV) infection and increased risk of B-cell NHL¹⁴⁹; hepatitis C virus (HCV) prevalence and increased risk of NHL.¹⁵⁰ One cohort study from Sweden also found that increased risk of both NHL and MM were significantly related to >15 years of infection with HCV.¹⁵¹ Furthermore there is evidence suggesting that HCV infection may be associated with other lymphoid and myeloid malignancies.¹⁵²

Other studies did not find any positive associations between lymphomas or leukemias and HCV or were inconclusive.^{153,154} Another study (from South Africa) found no associations between six HHV's and HL, NHL, MM, or leukemia.¹⁵⁵

Transplantation

Older studies have reported an increase in the risk of lymphoma after solid organ transplantation.^{156,157} A large number of studies have found that lymphoma patients who receive autologous transplantation with high-dose conditioning regimens are at increased risk of developing myelodysplastic syndromes (MDS) or AML.¹⁵⁸ A recent multicentre case–control study has shown specifically that the type and intensity of pre-transplantation chemotherapy with alkylating agents are major risk factors for MDS or AML after autotransplantation.¹⁵⁹ Other recent studies have confirmed greatly increased risk of NHL associated with transplantation resulting from drug-related immunosuppression.^{141,144,160}

TABLE 6.7. Studies that show an association between infections and the risk of adult leukemia and lymphoma (articles published post 1997).

Study reference	Disease	Place	Study design	Exposure	Risk or risk estimate (95% CI)
145	Lymphocytic leukemia	Italy	Case–control	Later age at adenoidectomy & tonsillectomy (>10 years)	OR=4.2 (1.1–16.2)
146	NHL	Italy	Case–control	Tuberculosis (TB)	OR=1.6 (1.05–2.5)
				Hepatitis	OR=1.8 (1.4–2.3)
				Maternal TB	OR=2.8 (1.1–6.9)
				Paternal TB	OR=1.7 (0.7–3.9)
				Previous infectious mononucleosis (IM)	OR=4.0 (1.4–11.8)
				Previous IM	OR=4.4 (1.1–6.6)
147	HL	Europe	Case–control	Ab_EBV positivity	OR=1.42 (1.15–1.74)
	All lymphomas				OR=2.96 (2.22–3.95)
148	CLL				
148	Lymphoplasmacytic lymphomas	Spain	Case–control	HHV-8	OR=4.47 (1.34–14.85)
	Low-grade lymphoma/ lymphoma B-cell NOS				OR=5.82 (1.07–31.73)
149	B-cell NHL	Italy	Case–control	HBV infection	OR=3.67 (1.75–7.66)
150	NHL	Pooled international	Case–control	HCV	OR=1.78 (1.40–2.25)
151	NHL	Sweden	Cohort	>15 years infection with HCV	SIR=1.89 (1.10–3.03)
	MM				SIR=2.54 (1.11–5.69)
152	T-NHL, CLL, ALL, AML, CML	Italy	Case–control	HCV	Non-significant elevated associations

Arthritis

Older studies have found an increase in the incidence of lymphoma in rheumatoid arthritis patients, both in the presence and absence of immunosuppressive therapy (azothioprine or cyclophosphamide).^{161,162} However, a recent systematic review failed to confirm the association with treatment, suggesting that increased risk is conferred by the disease itself.¹⁶³

Chemotherapeutic Agents

Cytotoxic drugs have been clearly associated with therapy-related MDS and therapy-related AML.¹⁶⁴ It is most likely that this results as a direct consequence of mutational events induced by chemotherapy.¹⁶⁵ A number of recent studies have shown positive associations between chemotherapy and risk of leukemia. These include: platinum-based treatment for ovarian cancer and increased risk of leukemia (RR=4.0; 95% CI: 1.4–11.4)¹⁶⁶; cumulative dose of 650 mg cisplatin used for treating testicular cancer (RR=3.2; 95% CI: 1.5–8.4)¹⁸; increased risk of leukemia due to exposure to epipodophylotoxins and anthracyclines for treating a solid tumor (RR=7.0; 95% CI: 2.6–19, for patients who received high doses)¹⁶⁷; increased risk of AML/MDS after mitoxantrone-based chemotherapy (RR=15.6; 95% CI: 7.1–34.2) and for anthracycline-based chemotherapy (RR=2.7; 95% CI: 1.7–4.5) for breast cancer and¹⁶⁸; and derazoxane and increased risk of AML/MDS after treatment for pediatric HL.¹⁶⁹ Furthermore, another case–control found that the use of chemotherapy in a combined modality treatment program for HL significantly increased risk of leukemia compared with chemotherapy alone.¹⁷⁰ A recent review of the literature has also found that increased risk of therapy-related NHL is evidently associated with intensive exposure to chemotherapeutic agents.¹⁷¹

Discussion

Both genetic and environmental factors are likely to be involved in the etiology of leukemia and lymphoma. Recent studies have identified a number of environmental risk factors for childhood leukemia, including exposure to magnetic fields of more than 0.4 μ T, exposure to pesticides, solvents, benzene, and other hydrocarbons, maternal alcohol consumption (for specific genotypes), contaminated drinking water, and infections. Recent studies found little evidence for an association with ionizing radiation. Risk for childhood NHL included ionizing radiation, pesticides, maternal smoking (during pregnancy), and exposure to benzene and nitrogen dioxide. Breast feeding was protective for acute leukemia and HL. It should be noted that exposure to some of these risk factors is very rare.²

For adults, risk factors that have been identified for leukemia include ionizing radiation (occupational and treatment-related), exposure to EMF (residential and occu-

pational), pesticides, solvents, benzene, cigarette smoking, contaminated drinking water, and infections, consumption of beef, immunosuppression, transplantation-related treatment, and certain chemotherapeutic agents (for treating primary solid tumors). Risk factors for lymphoma include exposure to EMF (occupational exposures), pesticides, cigarette smoking, and infections, consumption of red meat, transplantation (due to drug-related immunosuppression), treatment for arthritis, and chemotherapy (for treating other malignancies). In contrast, certain types of alcohol consumption are protective against lymphoma.

In conclusion, the onset of a leukemia or lymphoma is likely to involve genetic factors together with at least one environmental exposure. The risk attributed to any single environmental factor is likely to be small. Future studies should seek to obtain better understanding of the underlying etiological mechanisms.

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Viral Oncogenesis

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Introduction

Evidence is accumulating that pathogens play a major role in lymphomagenesis. In some forms of lymphoma, the pathogen is localized within the malignant cells, implying a direct role in malignant transformation and/or in maintenance of the malignancy. Examples include human T-lymphotropic virus 1 (HTLV1) in acute T cell leukemia, human herpesvirus 8 (HHV8) in primary effusion lymphoma (PEL), and Epstein–Barr virus (EBV) in Burkitt, classical Hodgkin, nasal T/NK, PEL, and immunodeficiency-related lymphomas. The presence of the virus within all of the neoplastic cells of a given tumor implies that viral infection occurs prior to malignant transformation. Research continues in optimal ways to take advantage of the viral presence not only to measure tumor burden in clinical specimens but also more importantly to eradicate all virally infected cells as a means of eliminating the cancer.

In other forms of lymphoma, the pathogen is not localized to the neoplastic cells, but is required for (or at least strongly associated with) lymphoma development. Furthermore, eradication of the infection often leads to resolution of the lymphoma, implicating the pathogen as the primary driver of neoplastic cell proliferation.¹ Examples include *Helicobacter pylori*, *Campylobacter jejuni*, *Chlamydia psittaci*, *Borrelia burgdorferi*, and hepatitis C virus (HCV), linked to gastric MALT lymphoma, immunoproliferative small intestinal disease, ocular adnexal lymphoma, primary cutaneous B cell lymphoma, and splenic marginal zone lymphoma, respectively. Each of these five pathogens may establish a chronic infection with a persistent inflammatory infiltrate in a mucosal, epidermal, or hepatosplenic/nodal site, where the ground is set for a single marginal zone lymphocyte to expand clonally to form a low grade lymphoma. What drives this cell, from among all of the surrounding cells, to divide is the subject of ongoing research. Regardless, a remarkable observation is that the lymphoproliferation still depends on

the presence of the pathogen, at least initially. Eradication of the pathogen by antimicrobial therapy, in many instances, eliminates the neoplastic clone. Early diagnosis and intervention are desirable, since delay may result in transformation to high grade lymphoma, rendering the tumor resistant to antimicrobial therapy. Two likely contributors to high grade transformation are the reactive oxygen species, produced naturally at sites of inflammation and the environmental mutagens that are undoubtedly present at epithelial surfaces, each of which may induce secondary genetic defects.

Do In Vitro Models Reflect In Vivo Effects?

Viral oncogenesis is difficult to study in vivo, particularly for viruses that infect only human hosts. Most investigations of the effects of a given viral gene have been done using transfected cell lines, and much research remains to be done to show whether these effects extend to cancers arising naturally in association with infection by the intact pathogen. Moreover, whole virus infection in vitro bears little resemblance to natural infection in the presence of an immune system. Culture systems require infection of either normal cells or cells that are already malignant, both of which fail to recapitulate the multistep process of transformation to malignancy that seems to occur in vivo.

Recently, there has been progress in developing better mouse models for studying oncogenesis by replacing the mouse hematopoietic system with a human version.² This model allows controlled introduction and monitoring of viral infection in an animal that has a human immune system. This promising model may be useful for comprehensive and systematic evaluation of the role of viral infection in lymphomagenesis and, furthermore, it may allow testing of pharmacologic agents and preventive measures in a way that more closely reflects the human response.

Epstein–Barr Virus

EBV is best known for causing *infectious mononucleosis* (colloquially called mono), a common self-limiting febrile disease. The first proof that EBV was associated with cancer was provided in 1964 by Epstein et al, who studied Burkitt lymphoma. Since then, EBV has been linked to numerous malignancies, including classical Hodgkin lymphoma, several subtypes of non-Hodgkin lymphoma, including B lineage, T lineage, and NK/T lymphoma of nasal type, immunodeficiency-related lymphoproliferations, nasopharyngeal carcinoma, and gastric carcinoma.^{3,4} Indeed, EBV was the first virus to be associated with human cancer, and the spectrum of tumor types linked to EBV continues to grow (Table 7.1).

Most basic knowledge on the virus has come from the study of lymphoblastoid cell lines that represent normal B lymphocytes capable of growing in vitro following immortalization by EBV. Inside cells, viral infection occurs in one of two states: the *lytic* state in which it replicates itself to

form new virions, and the *latent* state in which the viral genome persists long-term as an episome tethered to human chromosomes. Naturally infected human tumors are latently infected, with three different forms of latency described based on the expression pattern of viral genes. The human immune system normally controls viral infection, although it is unable to completely eliminate the virus from the body, or to prevent it from replicating periodically. Indeed, the virus persists for the duration of life in its human host by cleverly combining latent infection of long-lived memory B lymphocytes with periodic reactivation to produce more virions that infect even more lymphocytes, or are shed in saliva to potentially infect additional humans.

X-linked lymphoproliferative disease (XLP) is a rare inherited immunodeficiency in which there is uncontrolled EBV infection, due to dysfunctional T cell and NK-cell immunity, as a consequence of mutation in the Src homology 2 domain protein 1A (SH2D1A) gene. XLP patients succumb to rampant EBV infection, or to EBV-associated lymphomas, and rarely survive beyond age 40 years. Stem cell transplantation is potentially curative.

Another potentially fatal EBV-related disease is post-transplant lymphoproliferative disorder (PTLD), in which EBV-driven B cells proliferate in patients who are iatrogenically immunosuppressed as a consequence of hematopoietic stem cell transplant or solid organ transplant (see Chap. 28). Regaining control of the EBV-driven B-cell proliferation is often achievable after immunosuppressive drugs are decreased or stopped, so that natural immunity is restored. Alternatively, cytotoxic T lymphocytes are infused after having expanded the cells in vitro to specifically recognize one of the expressed viral proteins. Anti-CD20 antibody therapy (e.g., rituximab) is widely used as a chemotherapeutic agent, although a CD20-negative subclone of tumor cells may escape monotherapy. Antiviral nucleoside analogs, such as acyclovir and ganciclovir, are not effective for inhibiting division of latently infected neoplastic cells since they thwart only active replication (lytic infection). Nonetheless, combining an antiviral agent with chemotherapy (or radiation) is a promising approach for synergistic killing of latently infected cells that are converted to lytic infection by the more traditional therapy.⁵

About half of PTLDs have crippling mutations of *IGH* variable regions, implying that EBV might rescue the defective cells from programmed cell death. In contrast, PTLDs occurring late after transplantation (>1 year out) tend to be EBV-negative and may have a pathogenesis more typical of sporadic lymphoma.

PTLD-like B cell lymphoproliferation may be seen in patients treated with immunosuppressive drugs, such as methotrexate. Withdrawal of the drug results in tumor regression, presumably as a consequence of restored ability to recognize and control EBV-driven B cell proliferation. The declining immunity of old age may predispose to similar tumors diagnosed as “age-related EBV-associated B cell

TABLE 7.1. Epstein–Barr virus-associated diseases.

Disease	Rate ^a
<i>Reactive infections:</i>	
Infectious mononucleosis	>99
Oral hairy leukoplakia	>95
Inflammatory pseudotumor	40
<i>Classical Hodgkin lymphoma:</i>	
Hodgkin lymphoma, mixed cellularity	70
Hodgkin lymphoma, nodular sclerosis	20
Hodgkin lymphoma, lymphocyte depleted	50
Hodgkin lymphoma, AIDS-related	>95
<i>Hodgkin lymphoma, lymph. predominant</i>	<5%
<i>Non-Hodgkin lymphomas and immunodeficiency-related neoplasms:</i>	
Non-Hodgkin lymphoma, all subtypes	5
Non-Hodgkin lymphoma, AIDS-related	40
Brain lymphoma, AIDS-related	95
Brain lymphoma, non-immunosuppressed	5
Post transplant lymphoproliferative disorder	95
Burkitt lymphoma, endemic	>95
Burkitt lymphoma, North American	20
Burkitt lymphoma, AIDS-related	30
Lymphomatoid granulomatosis	Most
Pyothorax associated lymphoma	90
<i>Carcinomas and soft tissue sarcomas:</i>	
Nasopharyngeal carcinoma, Asian	>95
Nasopharyngeal carcinoma, USA	75
Lymphoepithelioma-like carcinoma	Most
Gastric adenocarcinoma	7
Smooth muscle tumors in AIDS/transplant	>95
Follicular dendritic cell sarcoma	Most
Plasmablastic lymphoma, AIDS-related	60
Primary effusion lymphoma, AIDS-related (HHV8+)	70
Age-related B cell lymphoproliferative disorder	>95
Angioimmunoblastic T cell lymphoma (B cells infected)	Most
Peripheral T cell lymphoma	40
Extranodal NK/T cell lymphoma, nasal type	>95
Gamma-delta T cell lymphoma, non-hepatosplenic	Most

^aRate refers to proportion of cases EBV-related.

lymphoproliferative disorder.” Immunodeficient hosts are also prone to develop lymphomatoid granulomatosis of the lung and other extranodal sites, in which EBV-infected neoplastic B cells are far outnumbered by reactive T cells. In the setting of chronic pulmonary tuberculosis, a pleural-based B cell neoplasm (called pyothorax-associated lymphoma) is consistently EBV-infected.

EBV DNA is present within the tumor cells of about half of AIDS-related lymphomas. It is hypothesized that depletion of T cells by HIV-infection renders the immune system less potent in attacking EBV-infected B cells. A spectrum of histologies may be encountered, including immunoblastic, Burkitt, classical Hodgkin, and plasmablastic lymphoma. PEL is coinfecting with EBV and HHV8. Primary central nervous system lymphoma is a fairly common HIV-related malignancy and is virtually always EBV-related, such that diagnosis and monitoring of the lymphoma may be facilitated by measurement of EBV DNA in cerebrospinal fluid⁶ (see Chap. 29).

In those classical Hodgkin lymphomas harboring EBV within the pathognomic Reed-Sternberg/Hodgkin (RS/H) cells, EBV is hypothesized to contribute to cell proliferation and resistance to apoptosis, despite crippling mutations of the rearranged *IGH* gene. RS/H cells are particularly known for strong expression of the oncogenic latent membrane proteins, LMP1 and LMP2. This characteristic is being explored for potential targeted therapy using either an immunologic approach toward the viral proteins or else a biochemical approach to overcome downstream effects of viral gene expression (such as NFκB or TNFA receptor signaling).

EBV is detectable within the malignant RS/H cells in about half of all classical Hodgkin lymphoma cases, with considerable variation among histologic subtypes (Table 7.1). Furthermore, EBV presence confers a good prognosis in children, but a worse prognosis in adults over age 45 with nodular sclerosing histology.⁷ Variation in HLA class 1 type is thought to contribute to failed immune recognition of viral infection in affected patients (see Chap. 27).

Chromosomal translocation involving the *MYC* gene is the hallmark of Burkitt lymphoma, with *MYC* on chromosome 8 being dysregulated by juxtaposition with one of the immunoglobulin genes on chromosomes 2, 14, or 22 (see Chap. 23). EBV is present in about 20% of sporadic Burkitt lymphomas, 40% of immunodeficiency-related Burkitt lymphoma, and virtually all endemic Burkitt lymphoma. Few viral genes are expressed in this malignancy, in part because a largely intact immune system destroys any cells expressing foreign antigens, which in turn contributes to the debris-laden macrophages and starry-sky appearance of the tumor by light microscopy. Only two viral proteins are expressed at significant levels: EBNA1 has an internal glycine-alanine repeat, that prevents the entire protein from becoming available for proteasome digestion and eventual MHC presentation to T-cells; LMP2 is only weakly immunogenic but, nonetheless is a promising target for therapy using infused cytotoxic T cells that are LMP2-specific.

Histochemical Assays for EBV

The most consistently expressed viral gene in EBV-related neoplasia is EBV-encoded RNA (*EBER*). However, *EBER* is not translated into protein, so RNA-based detection methods are required to detect *EBER*. The most definitive laboratory test for proving that a neoplasm is EBV-related is *EBER* in situ hybridization because it localizes latent virus to particular cells within the histologic lesion. A number of commercial reagents and instruments are now available to facilitate routine clinical implementation. A control stain for a ubiquitous cellular RNA should be done to insure that RNA is well preserved and available for hybridization.

Other EBV gene products (such as the latency proteins EBNA1, EBNA2, LMP1, and LMP2 and the lytic proteins BHRF1, BZLF1, and BMRF1) may be visualized, using immunohistochemical methods to provide further information on the pattern of viral gene expression. These immunostains are not recommended for routine detection of EBV infection because technical and biologic factors limit their sensitivity and, in some cases, their specificity.⁸ The two exceptions are LMP1 immunostain that works as well as an *EBER* stain for detecting the virus in classical Hodgkin lymphoma tissue, and BZLF1 or BMRF1 immunostains to detect EBV in oral hairy leukoplakia, which represents a pure lytic infection of epithelial cells on the side of the tongue in AIDS patients.

Real-time PCR is typically used for EBV viral load measurement. A number of commercial reagents and instruments are available to facilitate DNA extraction and EBV quantitation.⁸ Levels of EBV DNA in plasma or whole blood tend to rise prior to and thus act as a harbinger of EBV-related neoplasia, while effective treatment is marked by a precipitous drop in EBV DNA levels (Figure 7.1). In high risk transplant recipients, EBV viral load guides preemptive therapy. Immunologic tests measuring the body's response to EBV may complement EBV viral load to maximize the utility of EBV as a tumor marker.⁸ In AIDS patients, EBV DNA detection in the cerebrospinal fluid is used as a marker of brain lymphoma that may not only facilitate diagnosis but also track efficacy of therapy.⁶ Vaccination against EBV is being explored as a means of building immunity prior to viral exposure in high risk transplant patients. It is also touted as a possible strategy to limit the severity of EBV infection and to prevent adverse sequelae, including lymphoma itself.

Human Herpes Virus 8/Kaposi's Sarcoma-Associated Herpes Virus

Human herpes virus 8 (HHV8), colloquially known as Kaposi's sarcoma-associated herpesvirus (KSHV), is a gamma herpesvirus that is closely related to EBV and likewise infects B lymphocytes. HHV8 has been localized to lesional tissue in virtually all cases of PEL, multicentric Castleman

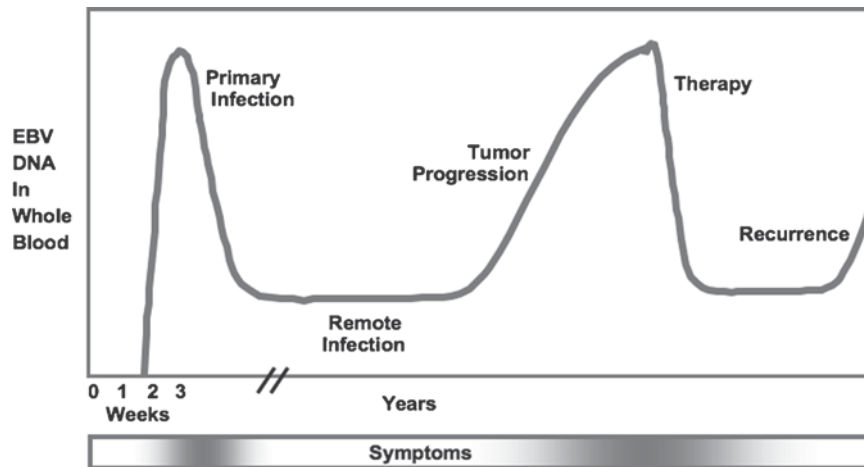


FIG. 7.1. EBV DNA levels in whole blood fluctuate in relation to the clinical setting. Levels soar soon after initial exposure to the virus, and then fall as the immune system controls the infection via interferon and lymphoid hyperplasia that produce symptoms typical of infectious mononucleosis. The virus lies latent in a small proportion of B lymphocytes for the duration of life. If the patient

later develops an EBV-related post transplant lymphoproliferation or nasopharyngeal carcinoma, levels typically rise before tumor diagnosis, suggesting that screening strategies may be effective in high risk individuals. Subsequent EBV viral load assays reflect tumor burden in a manner that allows one to monitor therapeutic efficacy and early recurrence.

disease (MCD), and Kaposi's sarcoma (KS).⁹ Although the virus was discovered relatively recently, there has been substantial progress in understanding its oncogenic effects and in devising potential therapeutic strategies to overcome these effects. For this reason, it is increasingly important to identify the virus in diseased individuals, so that targeted therapy may be considered and also, to take advantage of viral presence as a biomarker that facilitates monitoring of disease burden after therapy.

KS represents a malignant proliferation of endothelial cells, is one of the most common neoplasms in Africa, and is also the most prevalent tumor in HIV-infected patients.¹⁰ In KS lesions, HHV8 is localized to the spindle-shaped endothelial cells. PEL and MCD are considerably less common lymphoproliferative conditions in which HHV8 is localized to B lineage cells. HHV8 infection is necessary, but not sufficient, for the development of all three diseases. All three are more prevalent in HIV-infected patients and in certain other immunosuppressive states. PEL may be seen in nonimmunosuppressed elderly males, especially from the Mediterranean region, where HHV8 infection is prevalent. Cofactors beyond immunosuppression are poorly understood.

PEL often affects the pleural, peritoneal, and/or pericardial cavities although rarely it presents as a solid tumor. Cytologic examination of affected body cavity fluid or biopsy tissue reveals morphologic similarity to immunoblastic or anaplastic lymphoma with frequent evidence of plasma cell differentiation. Lack of the usual B- or T-cell markers may make it difficult to recognize the hematolymphoid nature of the malignancy, but CD45 antigen expression in almost all cases proves the hematolymphoid origin of PEL cells. Other frequently expressed markers are CD30, CD38, CD71, and

epithelial membrane antigen. Clonal *IGH* gene rearrangement with somatic mutation of the *IGH* variable region, along with focal or diffuse CD138 expression, imply a late stage of B cell development with a variable degree of plasma cell differentiation. Aberrant T cell receptor gene rearrangement has been described. Gene expression profiling indicates that PEL is indeed a unique form of lymphoma. Cytogenetic studies often reveal complex numerical abnormalities, and it is likely that secondary genetic events are essential to malignant progression of HHV8 infection.

Latency-associated nuclear protein (LANA, also called ORF73) is always expressed in latent HHV8 infection, including all HHV8-associated malignancies. In cell line models, LANA operates in part by binding to both TP53 and RB1 in a manner that promotes cell cycle progression while making apoptosis less likely to occur. LANA also upregulates beta catenin and, in mouse models, prolongs cell life and promotes tumorigenesis. Moreover, LANA is responsible for faithful partitioning of replicated HHV8 genomes to daughter cells upon mitosis. Other proteins consistently expressed by HHV8-related malignancies include viral cyclin D (v-cyclin) that inhibits cdk6 to promote cell cycle progression, vIRF3 that inhibits apoptosis, v-FLIP that activates NFkB, and vIL6 that activates the JAK-STAT and RAS-MAP signaling cascades and also induces VEGF. Some lytic cycle viral proteins are also implicated in tumorigenesis, even though the vast majority of lesional cells harbor latent infection rather than supporting lytic replication. One of these lytic viral factors, v-GPCR, is a chemokine receptor that triggers multiple downstream pathways, including AKT/mTOR and induction of VEGF. VEGF is hypothesized to contribute to effusion formation by increasing new vessel growth with enhanced vascular permeability.

Proving HHV8 infection in the neoplastic cells of a suspicious lesion is the *sine qua non* for diagnosing PEL and for differentiating it from pyothorax associated lymphoma or a secondary lymphoma that has spread to a body cavity. Immunohistochemical localization of LANA is the standard method for establishing HHV8 infection, although serology is an alternative reliable albeit indirect method of demonstrating infection. Since up to 15% of the general population is seropositive for HHV8 by ELISA, it is recommended that a positive ELISA result be followed up with molecular or immunohistochemical testing of the lesional tissue to confirm viral localization.

Molecular tests are now available to directly target HHV8 DNA in patient specimens. The virus has a 160-kb genome that may be extracted from neoplastic cells (i.e., body fluid, frozen or paraffin-embedded tissue) and PCR-amplified as proof of HHV8 infection. Typical assays target highly conserved viral sequences, such as the ORF26 gene. Quantitative PCR may be useful as a measure of tumor burden by monitoring viral load in peripheral blood.

The clinical presentation of a PEL patient reflects the effect of fluid overload in body cavities and also may reflect expansion of adjacent lymph nodes. On computed tomography (CT) scan, subtle thickening of the affected serous membranes is seen. Bone marrow staging often reveals the systemic nature of the malignancy. Prognosis is poor with a median survival of only 6 months.¹¹ In HIV patients, partial restoration of immunity by effective use of highly active antiretroviral therapy (HAART) helps complement the chemotherapeutic approach to managing PEL. Experimental therapies include cidofovir (an antiviral agent) and inhibitors of key virus-activated cellular factors, including mTOR (rapamycin), NFkB or proteasomes (bortezomib).¹² Anti-CD20 antibody therapy is considered in the rare CD20-expressing PEL.

Most PELs are coinfecting by EBV, particularly the PELs arising in HIV-positive patients vs. the rare HIV-negative PELs of elderly Mediterranean males. It is worth testing suspected PELs for EBV, since associated EBV DNA in blood or plasma represents a potential tumor marker by which to monitor tumor burden and efficacy of therapy. Circulating EBV DNA levels are typically measured by quantitative PCR. In paraffin-embedded tissue or cytologic cell blocks, EBV may be localized to PEL cells by in situ hybridization to *EBER*. EBV LMP1 protein is not expressed (see Chap. 29).

MCD is characterized by enlarged lymph nodes infiltrated by plasma cells with a prominent vascular proliferation resembling the plasma cell variant of Castleman disease. Clonality studies reveal a spectrum of B lineage lesions ranging from polyclonal plasmablasts to monotypic but polyclonal microlymphoma to monoclonal plasmablastic lymphoma. HHV8 is present in virtually all MCD arising in HIV patients and in about half of MCD arising in HIV-negative hosts. The infected plasmablasts express cytoplasmic IgM λ as well as aberrant immunophenotypic markers indicating their lineage is intermediate between B cells and plasma cells (positive

for IRF4 but negative for CD20 and CD138). Interestingly, they lack somatic mutation of *IGH*, implying that HHV8 infection of early B lymphocytes drove the cells to differentiate independent of germinal center maturation. The plasmablasts express the memory B cell marker, CD27, which is consistent with the virus' intention to remain in the human host long term. These infected cells tend to cluster in the mantle zone surrounding germinal centers.

MCD diagnosis is greatly facilitated by immunohistochemical demonstration of LANA expression. HHV8 viral load by quantitative PCR seems to rise in concert with vIL6 protein levels in serum to reflect the clinical status of MCD patients. By comparison, HHV8 DNA is amplifiable in circulating leukocytes from only about 10% of healthy seropositive individuals. HHV8 virions are periodically shed in saliva and at lower levels in genital secretions. Transmission by sexual contact is the major route of spread in low prevalence regions, while in high prevalence areas like the Mediterranean and Africa the virus seems to spread by an oral/salivary route. Once infected, the viral genome remains for life in a small subset of leukocytes (about one out of every 100,000 leukocytes), with the infected cells limited to the B lymphocyte subset. Unlike PEL, EBV is consistently absent in MCD lesions. Despite being polyclonal in most instances, MCD is an aggressive disease in HIV infected patients and is typically managed using chemotherapy (see Chap. 41).

A detailed list of the proposed mechanisms of HHV8-related disease pathogenesis was recently compiled by Du et al.¹³ It is clear that multiple cellular pathways are affected by HHV8 infection and that infection alone is insufficient for the development of disease. While secondary genetic hits are likely to contribute to KS and PEL, evasion of immune recognition might be an important factor in premalignant lesions such as MCD. HHV8 is known to evade the immune system by multiple strategies that probably depend, at least in part, on host genetics.¹⁴ For example, the vast majority of the 80 viral genes are not expressed in lesional tissue which limits immune recognition of infected cells. Even when selected viral genes are expressed, HLA type could affect degradation and presentation of viral proteins for MHC I exposure, and in any event HHV8 K3 and K5 proteins were found to downregulate MHC I presentation.¹⁴ Viral ORF45 and vIRFs inhibit interferon-mediated viral response, and the virus also encodes a factor thwarting complement-mediated cell lysis. Finally, inherited polymorphisms in cytokine genes appear to influence the outcome of infection.¹⁵ It is feasible that faulty immune control of HHV8, whether by inherited or acquired means, permits more active and abundant viral infection which increases the pool of infected cells at risk of secondary genetic events initiating cancer. Environmental mutagens may be critical in the multifactorial path to cancer.¹⁶ Furthermore, high levels of potent cytokines like vIL6, hIL6, and hIL10 in PEL and MCD patients implies that the virus effects are not limited to infected cells but rather are paracrine and thus systemic in effect.

Human T-Lymphotropic Virus

Human T-cell leukemia virus type 1 (HTLV1) is strongly associated with adult T-cell leukemia lymphoma (ATLL); whereas, HTLV2 is not associated with human malignancies. Regions of the world where the HTLV1 infection is prevalent are also the sites where ATLL incidence is high: Central Africa, the Caribbean, South America, Melanesia, the Middle East, and southwestern Japan. ATLL occurs in approximately 6% of infected males and 2% of females. HTLV1 infection appears to be necessary, but not sufficient, for ATLL development, and the cofactors for tumorigenesis remain obscure.

HTLV1 is a delta-type retrovirus that infects host cells mainly through cell-to-cell contact via three major routes of transmission: mother-to-child via infected lymphocytes in breast milk, parenteral exposure to infected blood cells, and sexual contact. Preventive measures include routine serologic testing of blood donors to minimize transfusion-mediated infection, and avoidance of breast-feeding by high-risk mothers to minimize vertical transmission. A typical virus carrier harbors the HTLV1 genome in 30% of circulating leukocytes, including 50% of CD4 T cells. The virus can enter helper T cells by the SLC2A1 (formerly called GLUT1) surface receptor. Other infectable cell types include CD8 T cells, B cells, monocytes, endothelial cells, and even basal epithelial cells of breast. Absence of cell-free virions and negligible expression of viral proteins may reflect tight control by the immune system, both humoral and T-cell mediated.

The virus seems to propagate itself by inducing proliferation of infected cells, or by transfer from cell to cell via synapses or exosomes. This route of propagation is unlike most other viruses, for which the typical strategy involves viral replication and virion production followed by infection of more host cells. The latent period from initial infection to onset of ATLL is about 40–60 years, implying that a rare second event must occur for tumorigenesis. Progression to ATLL occurs in about 1 in 1,000 carriers per year.

ATLL comprises a subset of peripheral T cell neoplasms. There are four clinical subtypes of ATLL, the most common of which is the prototypic *acute* type exhibiting high numbers of circulating tumor cells, frequent skin lesions, systemic lymphadenopathy, and hepatosplenomegaly. The *lymphomatous* type has prominent systemic lymphadenopathy with few (if any) abnormal cells in the peripheral blood. Both subtypes have a poor prognosis as they are aggressive and do not respond well to chemotherapy. The *chronic* type is characterized by a skin rash that usually progresses into one of the aggressive tumor types within a few years. The *smoldering* type is characterized by the presence of only a few ATLL cells, a low rate of progression, and longer survival.

Hypercalcemia is found in around 70% of all ATLL patients and exclusively in those patients with the two more aggressive clinical subtypes, making hypercalcemia one of

the most unique and striking features at presentation. The biologic basis for the high calcium levels is most likely an increased number of osteoclasts with accelerated bone resorption and lytic bone lesions. Osteoclast differentiation appears to be enhanced by tumor-derived secretion of TNFSF11 and macrophage colony stimulating factor.

The key pathologic findings are hyperlobulated medium to large lymphoid cells with clonal TCR gene rearrangement and expression of mature T cell surface antigens (typically CD2, CD3, CD4, CD5, and CD25 but usually lacking CD7). The rather striking multilobed nuclei of some neoplastic cells, also called “flower cells,” are critical for histologic consideration of ATLL in blood or biopsy tissue. Interestingly, Reed-Sternberg-like cells have also been described, but these are EBV-infected and are characteristically found in concert with an infiltration of smaller EBV-infected B lymphocytes, implying that they reflect diminished T cell immunity against EBV.

While skin is by far the most common extranodal site of involvement, other sites may include gastrointestinal, pulmonary, and even brain. Skin nodules often reveal Pautrier-like microabscesses of malignant cells in the aggressive forms of the disease, while the erythematous or papular skin rash of the smoldering disease patients is hyperkeratotic with a sparse dermal infiltrate of small neoplastic T cells with or without nuclear polylobation. Marrow involvement may be patchy or absent, implying that circulating cells may emanate from nodal or extranodal tumor sites.

FOXP3 or IL10 are expressed by about half of ATLLs, in which they may operate to suppress immunity. Patients with ATLL are often profoundly immunocompromised with frequent opportunistic infections such as pneumocystis, cytomegalovirus, varicella zoster, cryptococcus, strongyloides, or mycobacteria, indicating severely impaired cell-mediated immunity. These infections are a major factor in morbidity and mortality.¹⁷

Detection of HTLV1 is important in the workup of a suspected ATLL. Supporting a diagnosis of ATLL is demonstration of HTLV1 by either serologic or molecular methods. ELISA assays are typically used to screen for anti-HTLV1 antibodies in serum followed by confirmation using western blot or PCR.^{18,19} Serologic titers against viral structural proteins (e.g., env or gag) are generally higher in ATLL patients, compared with asymptomatic viral carriers, and rising titers are implicated as a risk factor for progression to cancer.¹⁹ In contrast, titers against the viral Tax protein are unusually low in ATLL patients, as are the numbers of cytotoxic T cells directed at Tax, implying that failure to express or recognize Tax is characteristic of ATLL patients. Tax is considered to be an oncoprotein because it is responsible, at least in part, for immortalizing T lymphocytes, and because it promotes cell growth by transactivating many other viral and cellular factors including the NFκB signaling pathway. A strong immune reaction to Tax in healthy viral carriers may be responsible for keeping HTLV1 infection in check, whereas

immune tolerance to Tax is a plausible route to cancer progression. Inability to respond adequately to Tax, either because host HLA type fails to recognize Tax or because Tax is mutated, suppressed, or epigenetically silenced, may result in poorly controlled, HTLV1-related cell proliferation and progression of tumor development.¹⁷ The mechanisms of HTLV1 oncogenesis include effects on cell cycle, inhibition of apoptosis, and immune evasion. Tax-mediated suppression of nucleotide excision repair may contribute to the accumulation of somatic mutations. Secondary genetic events in human oncogenes (like TP53 or less commonly RB1 or CDKN2A) are likely to contribute to malignant transformation.

PCR is typically used to quantitate HTLV1 proviral DNA in blood mononuclear cells. Primers commonly target the highly 3' conserved portion of the viral genome (formerly called the pX or X region and containing Tax among other coding sequences).^{19,20} Since reactive tissue could potentially contain HTLV1, particularly among people from endemic regions, it is reasonable to use a quantitative PCR approach to distinguish low level background infection from the high viral load associated with ATLL.^{19,21–23} Viral loads reportedly rise even before clinical onset of ATLL, and further research is warranted to define thresholds for distinguishing reactive from neoplastic infection. Southern blot analysis is a standard approach for demonstrating monoclonal viral genomic structure, but it is more expensive with a slower turn-around-time than quantitative PCR.

The tumor cells of ATLL are monoclonal with respect to the viral integration site within the human genome. Nevertheless, clonality does not appear to be entirely specific for malignancy, since reactive HTLV1 infections may also appear monoclonal. For example, HTLV1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a benign inflammatory neuropathy that is caused by HTLV1 and characterized by clonal integration of the virus into host chromosomal DNA.²⁴ The major mechanism of viral persistence is by division of previously infected cells, which propagates the integrated viral genome(s) to daughter cells and results in clonal lymphocytes.²⁴ HAM/TSP patients have a vigorous (yet anomalous) immune response to HTLV1 infection, in that viral loads are higher than in healthy carriers, while immune-mediated neurologic damage ensues.

Although most ATLLs have only one clonally integrated HTLV1 genome per malignant cell, occasional tumors may have more than one viral copy per cell.²⁴ The flanking human DNA is different in every patient's tumor and may be identified on a research basis using inverse polymerase chain reaction (PCR) followed by sequencing of the DNA adjacent to the virus' long terminal repeat sequences.^{24,25} This assay is not used clinically, since there is no evidence that knowledge of the integration site impacts on prognosis or therapy. Among ATLL tumors, the viral integration sites do not seem to favor any one chromosome over another.²⁴ Interestingly, however, integration tends to occur in coding sequences of

genes that are actively expressed in normal T cells, implying that integration is nonrandom.²⁴ In some instances, integration is accompanied by overexpression of adjacent human genes.²⁴ Integration may also lead to partial deletion of viral sequences, and it is hypothesized that such alteration leads to dysregulated viral gene expression, that may contribute to oncogenesis.

Once the integration site of a particular patient's tumor has been characterized, a patient-specific Q-PCR assay could potentially be designed to detect the uniquely fused human-viral junction from cells within the tumor clone. Further research is needed to determine if such an assay is specific for the tumor, in which case it could be used to monitor residual tumor burden. Soluble levels of interleukin 2 receptor have also been used as a marker of tumor burden and as an indicator of the efficacy of therapy.²⁰

Patients diagnosed with the aggressive subtypes of ATLL are treated promptly with chemotherapy and, if indicated, with marrow transplant; whereas, patients with the chronic subtypes of ATLL are often followed without treatment. Standard chemotherapy involves a combination of cyclophosphamide, hydroxydoxorubicin, vincristine, and prednisone (CHOP). The dismal prognosis is reflected by 3-year survival rates of only approximately 13%. On the horizon are targeted therapies such as NFkB inhibitors, or monoclonal antibodies against CD25. Vaccines are being explored as a preventive strategy.

Hepatitis C Virus

Investigation of the link between hepatitis C infection and lymphoproliferative disease has increased our understanding of how the immune system responds to chronic infection and the concomitant risk of autoimmunity and cancer. Around 5% of all chronic HCV patients eventually develop hepatocellular carcinoma, while about half get mixed cryoglobulinemia with or without frank lymphoma.²⁶

HCV is an RNA virus that is carried by 2% of humans worldwide. Millions of Americans are infected as blood supply screening was only introduced in 1990, and prior to that many transfusion-related infections took place. HCV transmission requires contact with contaminated blood, and spread occurs mainly via sexual contact and by dirty needles by intravenous drug users.^{26,27} Importantly, HCV is also transmitted to infants from 10% of infected mothers. Once infected, 70% of persons develop a chronic infection that places them at risk for hepatocellular carcinoma as well as cryoglobulinemia and lymphoproliferative disease. T cell immunity as well as antibody-mediated immunity are important in controlling and possibly clearing the virus. Mutations in the viral genome, particularly in the hypervariable-1 region of the E2 gene encoding envelope glycoprotein, influence the ability to clear the infection. When liver cancer occurs, HCV RNA is usually localized to

the malignant hepatocytes, whereas the virus is not localized to the malignant lymphocytes of lymphomas arising in HCV infected patients. A hit-and-run strategy has been proposed, whereby replicative HCV infection of B lymphocytes in patients with cryoglobulinemia is followed in some instances by oligo/monoclonal proliferation of B cells lacking the viral genome but selected for by viral antigen.^{26,27}

The hallmark of cryoglobulinemia is insoluble serum protein at temperatures below 37°C that resolubilize when warmed. The three major types of cryoglobulinemia are differentiated on the basis of their immunoglobulin class. Type I has a monoclonal make up, whereas type II and III show “mixed cryoglobulins” of several immunoglobulin classes with type II having polyclonal IgG and monoclonal rheumatoid factor (IgM), and type III having polyclonal IgG and rheumatoid factor (IgM). Mixed cryoglobulins appear to arise from a monoclonal yet not malignant-behaving expansion of B cells that give rise to a pathogenic IgM having rheumatoid factor activity.

In the setting of hepatitis C infection, mixed cryoglobulinemia (type II) is a strong risk factor for developing frank lymphoma, with an estimated 35-fold higher risk compared to the general population.²⁸ A spectrum of histopathologic subtypes of lymphoma are described, but the most common include splenic marginal zone lymphoma (formerly called splenic lymphoma with villous lymphocytes) as well as marginal zone lymphomas at nodal or extranodal sites, lymphoplasmacytic lymphoma, small lymphocytic lymphoma, and follicular lymphoma. These lymphomas arise in about 8%–10% of mixed cryoglobulinemia type II patients and usually occur only after many years of chronic infection by HCV.

HCV infection is particularly prevalent in patients with extranodal marginal zone lymphoma. Marginal zone lymphoma arises from lymphocytes having characteristics of both innate immunity and antigen-driven immunity, the latter being marked by somatic mutation of *IGH*. Of note, the antibody encoded by the rearranged *IGH* gene is not random but, rather, consistently has rheumatoid factor-like features, implying that autoimmune tolerance to antigen-driven B cell growth is involved in failure to eliminate the neoplastic clone.²⁷ HCV infection may drive B cell growth, at least in part, by the paracrine effects of the release of various inflammatory cytokines in infected hepatic tissue. Resolution of splenic marginal zone lymphoma upon antiviral therapy is evidence that neoplastic cell proliferation was driven by HCV infection.²⁹ Indeed, antiviral therapy (pegylated interferon alfa and ribavirin) is now considered upfront in management of lymphoma associated with HCV infection and mixed cryoglobulinemia. Combined anti-CD20 antibody therapy is effective in some patients. Clinical trials are needed to evaluate combination therapy in a controlled manner, and to evaluate next-generation antiviral agents, Toll-like receptor agonists, and antibody to “B cell activating factor belonging to the TNF family” (BAFF, now called TNFSF13B) to control viral infection as well as the associated cytokine-driven lymphoproliferation. Current strategies result in complete or partial regression of the lymphoma in approximately 75%

of patients.³⁰ Relapse of the lymphoma is marked by rising HCV viral load in plasma, further confirming the tight link between viral infection and tumor growth. Viral load is typically measured by commercial assays that rely on quantitative PCR, transcription-mediated amplification, or branched DNA signal amplification.

Antiviral strategies are even being explored for higher grade lymphomas arising in this clinical setting (typically diffuse large B cell lymphoma), although secondary genetic events may render their growth independent of underlying virologic/immunologic stimulus that seems to drive growth of the low grade lymphoma from which they probably transformed.²⁶ Reactive oxygen species produced at sites of chronic inflammation may contribute to genetic mutation. HCV may even have a more direct mutagenic potential through induction of nitric oxide synthase by viral C and NS3 proteins, and by viral E2 capsid protein interaction with CD81 on the surface of B cell that promotes somatic hypermutation. A greater understanding of the role of chronic infection and immunologic inducers of clonal cell growth will undoubtedly lead to even more progress in targeted therapy for lymphoma. Furthermore, cancer prevention is feasible by thwarting viral infection and its downstream effects or, better yet, by developing an effective vaccine that avoids chronic HCV infection. Efforts to develop a vaccine have been thwarted so far because of HCV’s high mutation rate.

Simian Vacuolating Virus 40

SV40 is a cancer-inducing virus of animals that was iatrogenically introduced into humans.^{31,32} Between 1955 and 1963, batches of polio-vaccine that were used to immunize many people around the world, including close to 100 million people in the US, were unknowingly contaminated with SV40. SV40 had been present in the monkey kidney cells used to produce the virus in vitro. Once the presence of and pathogenic effects of SV40 were discovered, the virus was removed from vaccines but only after it had been transmitted to many humans.

SV40, a DNA virus of the polyomavirus family, has been proven to cause several types of cancer in animals. Whether the virus leads to cancer in humans is still disputed. There are reports of SV40 being present in several types of cancer including non-Hodgkin lymphomas, but a causative relation is not established and the methods used to detect viral infection were not always reliable.^{33,34}

Measles Paramyxovirus

Recent studies have called into question earlier evidence of an association between measles virus and Hodgkin lymphoma.^{35,36} The conflicting data highlights the need to use well-validated assays to detect viral infection, preferably combining assays targeting protein and nucleic acid, and localizing the virus to lesional tissue using immunohistochemical or in situ hybridization methods.

Conclusion

HTLV1, HHV8/KSHV, EBV, and HCV have clearly been linked to several lymphomas and lymphoproliferative diseases. SV40 and measles remain suspects, but definitive proof is yet to be found. Nonetheless all pathogens deserve our ongoing attention to further elucidate the oncogenic pathways that highlight routes for intervention that may eventually improve patient diagnosis, treatment, and prognosis.

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Section II

Specific Techniques and Their Applications in Molecular Hematopathology

8

Techniques to Determine Clonality in Hematolymphoid Malignancies

Daniel E. Sabath

Introduction

Clonality testing is a well-established molecular diagnostic technique in the realm of hematopathology. Molecular techniques to diagnose hematological malignancies based on the detection of clonality have been with us since the mid-1980s, based on the seminal observation that lymphoid cells rearrange their antigen receptor genes and that recurrent genetic rearrangements are present in myeloid neoplasms. In this chapter, I review the history of clonality testing in cancer, describe the scientific principles on which clonality-based testing rest, and then describe the numerous techniques that are now available for diagnosing hematolymphoid malignancies by demonstrating their clonal nature.

Cancer as Clonal Process

It is now a scientific dogma that cancers are clonal processes. The assumption is that a single cell acquires one or more genetic alterations that give that cell a growth advantage over the other cells in the affected tissue. This assumption follows logically from Knudsen's seminal observation in 1971 that individuals with one mutated copy of the Rb gene on chromosome 13 had a much higher incidence of retinoblastoma than normal individuals. Furthermore, spontaneous retinoblastomas were found to have one mutated copy of the Rb gene with the other copy of Rb usually being deleted. This led Knudson to the hypothesis that for a tumor to develop, at least two genetic "hits" had to occur.¹ Since these genetic hits are rare events, it follows that the probability of two (or more) hits occurring in more than one cell at a given time and anatomical location is virtually impossible. Thus, a tumor represents the clonal expansion of a single cell with a unique genetic alteration. From a diagnostic standpoint, being able to detect the clone-specific genetic alteration(s) enables us to make cancer diagnoses by proving that a collection of cells is clonal.

X Inactivation

Even prior to the development of molecular genetic tests to demonstrate clonality, it was possible to demonstrate, at least in females, that cells were clonally related by virtue of nonrandom inactivation of the X chromosome. This concept came from the original observation by Lyon that only one of the two X chromosomes in the cells of female mammals was active and that the choice of which X chromosome was active was made early in embryonic development.² The presumption was that this inactivation occurred to prevent "overdosage" of the genes on the X chromosome. It was obvious that only one X chromosome was needed for normal cell functioning, since males get by with only one copy of this chromosome and XO females are viable (although not normal). Proof that only one X chromosome is active per cell came initially from studies by Beutler et al, showing that in females heterozygous for glucose-6-phosphate dehydrogenase (G6PD) deficiency, red cells were mosaic, i.e., the red cells were a mixture of G6PD-deficient and G6PD-normal cells.³ Similarly, Fialkow et al demonstrated that in individuals heterozygous for G6PD isoforms, only one allele of G6PD was expressed per cell.⁴

The site on the X chromosome required for chromosomal inactivation is termed the *XIC* locus, localized to the proximal long arm by Brown et al in 1991.⁵ This region contains the *XIST* gene, shown to be required for X chromosome inactivation. *XIST* encodes an untranslated RNA expressed by the inactive chromosome that coats the inactivated X chromosome.⁶ The chromatin of the inactive X chromosome is more condensed than that of the active chromosome, owing at least in part to various histone modifications (e.g., methylation, demethylation, and deacetylation). As the final result, the inactive X chromosome becomes heavily methylated at CpG sequences, resulting in the suppression of gene expression from the inactive chromosome. The realization that gene expression may be modulated by chemical modification was a novel concept and was the first example of epigenetics,

where the function of genes is based on factors other than the DNA sequence itself.

As mentioned above, the first demonstration of random X chromosome inactivation was the recognition that only one of two G6PD alleles was active in a given cell (in females). This observation was exploited to demonstrate the clonality of several types of tumors. Clonal X inactivation is still of some diagnostic utility in the setting of cancer, particularly where the malignant cells have no other clonal genetic marker that may be exploited. G6PD isotyping has been replaced now by examining monoallelic activation of the androgen receptor gene. The androgen receptor gene is highly polymorphic due to the presence of a trinucleotide repeat region in the gene.⁷ Thus, most human females have two different androgen receptor alleles with differing lengths of trinucleotide repeats.

The assay generally used (referred to as the HUMARA assay, for human androgen receptor) takes advantage of the fact that one of the two androgen receptor genes in a human female cell is methylated, and that for most female individuals, the two androgen receptor genes differ in the length of a polymorphic trinucleotide repeat region (Figure 8.1).⁸ DNA isolated from tissue of interest is subjected to digestion with restriction enzymes that cut only unmethylated DNA. Digested DNA is then subjected to polymerase chain reaction amplification across the trinucleotide repeat region, and the PCR products are compared to those obtained with undigested DNA. The undigested DNA will yield two different PCR products, representing each allele present. With the DNA digested with the methylation-sensitive enzyme, only the methylated (undigested) allele will be detected. The relative proportion of methylated DNA indicates the proportion of the tissue studied that is descended from a single clone.

Using the HUMARA assay to demonstrate clonality requires comparing the proportion of an X chromosome that is active in the tissue of interest to that of normal tissue obtained from the same individual. Interpretation is complicated by the fact that most neoplastic tissue contains a mixture of tumor (clonal) cells and normal (polyclonal) cells. Therefore, the ratio of the inactive X to the active X chromosome has to be outside a certain range to allow for the possibility of the normal tissue itself having nonrandom X inactivation.⁹

Lymphomas

Lymphomas are neoplasms of mature lymphoid cells, which have the property of having rearranged genes encoding antigen receptors. The biological property of lymphocytes makes demonstration of clonality a straightforward process.

Lymphoid Development

The function of lymphocytes, both B and T lymphocytes, is to recognize and respond to the entire foreign antigenic universe that an organism might encounter. In 1961, Burnet proposed

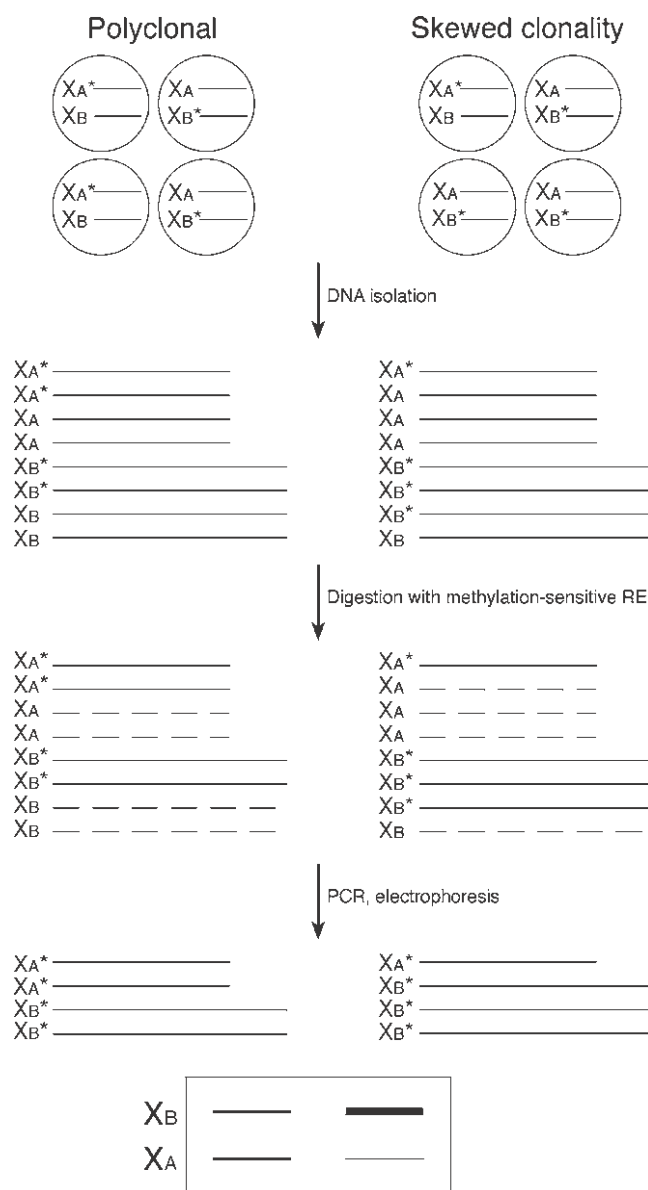


FIG. 8.1. Determination of clonality using the HUMARA assay.⁸ X_A and X_B represent the two X chromosomes in a female cell. The inactivated (methylated) allele is indicated by an asterisk. In the left-hand side of the figure, a polyclonal population is indicated, with equal numbers of each chromosome inactivated. When DNA is isolated and digested with a methylation-sensitive restriction enzyme, equal numbers of each chromosome are preserved. When amplified by PCR, two bands of equal intensity are observed, indicating random X inactivation. Two different sized bands are obtained due to the polymorphism of a CAG repeat in the amplified region. In the right-hand side of the figure, cells with an inactivated "B" allele are present at a 3-fold higher level than those with an inactivated "A" allele. Thus, a more intense band is observed after PCR, corresponding to the "B" allele. This indicates a skewing of X inactivation, which would support the presence of a clonal cell population.

that each lymphocyte has a distinct antigen specificity.¹⁰ Once the antigen receptor genes were cloned, the solution to this problem was revealed: the genes for the antigen

receptors (immunoglobulin in B cells, T cell antigen receptor in T cells) are assembled from modular elements that can be combined in numerous ways. An antigen receptor consists of one of many variable (V) segments, in some cases one of a number of diversity (D) segments, and one of a smaller number of joining (J) segments. In addition, random nucleotides are added by the enzyme terminal deoxynucleotidyl transferase at the V-D, D-J, and V-J junctions, resulting in additional diversity.

In B cells, a functional immunoglobulin receptor is formed by a combination of either a κ or λ light chain with an immunoglobulin heavy chain. In T cells, the antigen receptor is a dimer formed from either α and β or, less commonly, γ and δ chains. All these protein chains are encoded by rearranged antigen receptor genes. Because each lymphocyte has a unique antigen receptor, one may use this property to demonstrate the clonal diversity of a lymphocyte population.

B Cell Clonality

Light Chain Restriction

Even before molecular clonality determination methods were developed, it was possible to infer B cell clonality by the ratio of κ to λ light chains expressed on the B cell population. In humans, there is a slight preference for κ light chain expression to λ (typically the κ : λ ratio is approximately 1.4:1). However, in a B cell tumor, since all B cells are derived from the same precursor cells, all the malignant cells will express either κ or λ light chains, resulting in a marked skewing of the normal κ : λ ratio. The altered κ : λ ratio may be demonstrated by using antibodies specific for the κ and λ light chains.

If fresh tissue is available, light chain restriction may be easily demonstrated in a quantitative fashion by flow cytometry. Flow cytometry may be performed on peripheral blood, bone marrow, other body fluid specimens, or tissue biopsies in which cells have been dispersed into suspension. Most flow cytometry is performed to analyze molecules present on the surfaces of cells; however, cells can also be permeabilized to study intracellular antigens as well as nucleic acids.

For flow cytometry, cells are incubated with various antibodies that are fluorescently labeled. The most sophisticated instruments are currently capable of detecting 10 or more colors simultaneously. To demonstrate clonality by flow cytometry, one first identifies the B cells based on a lineage-specific cell surface marker, and then the κ : λ ratio is determined for this population. Any deviation from the normal ratio of 1.4 may be taken as evidence for B cell clonality. However, in the literature, a κ : λ ratio of $>3:1$ or $<1:2$ is generally considered evidence of B-cell clonality. Using multiparameter flow cytometry, one may demonstrate light chain restriction on small subpopulations of B cells that differ from the background population by expression of abnor-

mal cell surface proteins or even abnormal levels of normal proteins.¹¹

Demonstration of immunoglobulin light chain restriction may also be accomplished by immunohistochemistry, although light chain antibodies do not always react well with cell surface immunoglobulin in formalin-fixed paraffin-embedded tissue. This problem may be avoided by using frozen tissue, although with the availability of improved antibodies for the analysis of lymphocytes (other than κ and λ unfortunately), frozen section immunohistochemistry has largely been abandoned in favor of immunohistochemistry performed on paraffin-embedded tissue. The one situation in which light chain restriction can be demonstrated successfully in formalin-fixed tissue on a routine basis is for plasma cell disorders. Plasma cells have large amounts of cytoplasmic immunoglobulin that reacts well with the κ and λ antibodies used for immunohistochemistry in paraffin sections.

Finally, it is also possible to demonstrate light chain restriction in fixed sections by in situ hybridization for immunoglobulin light chain RNA. In this case, paraffin sections are hybridized to nucleic acid probes for κ and λ RNA, and hybridization is generally detected using an enzyme-linked color reaction. In situ hybridization may be used more successfully than immunohistochemistry to demonstrate light chain restriction in B cell neoplasms, other than plasma cell dyscrasias.¹²

Molecular Methods for Determining B Cell Clonality

The result of the molecular process that results in a rearranged antigen receptor gene provides an excellent molecular target for clonality determination. Since immunoglobulin gene rearrangement involves large-scale alteration in the structure of the immunoglobulin genes, DNA blot analysis can be used to detect this alteration in genetic structure. The immunoglobulin heavy chain has most commonly been used for this purpose, although the κ and λ light chain genes can also be used to demonstrate clonality.

For DNA blot analysis, high molecular weight genomic DNA is required. Therefore, fresh or frozen tissue must be available. Before the development of PCR-based clonality testing, it was frequently necessary to repeat biopsies to obtain fresh tissue if only fixed tissue was obtained in an initial biopsy later determined to be suspicious for lymphoma. The principle of using a DNA blot for clonality studies is that during the process of gene rearrangement, restriction sites that are present in the unrearranged (germline) DNA are deleted along with the DNA removed to assemble a functional immunoglobulin gene, and so the restriction map of the immunoglobulin locus changes. Thus, when a DNA blot is performed using a probe from the immunoglobulin locus, the probe will hybridize to different sized restriction fragments in a B cell that has undergone immunoglobulin gene rearrangement than in germline DNA. In a tissue sample with a polyclonal B cell population, each individual gene rearrangement is present at too low a frequency to be visible, so

generally the only visible restriction fragments correspond to the unrearranged genes in both the B cells present as well as the background non-B cells. However, if a significant fraction of the cells in a biopsy (typically 5–10%) is clonal, the combined signal from these clonal cells may be visualized above background, and one or more nongermline bands will be visible. The detection of these nongermline bands indicates the presence of a major B cell clone, which can be used to support a diagnosis of a B cell neoplasm.

Although DNA blot analysis is a robust method for determining B cell clonality, it has a number of disadvantages that have led most laboratories to abandon this method. First, DNA must be obtained from fresh or frozen tissue, which is not always available at the time of initial diagnostic biopsy. Second, a large amount of DNA, typically 30 μ (mu)g, is required for a DNA blot study. Third, performing a DNA blot is labor-intensive and the process requires up to a week to generate a result. Finally, most commonly used DNA blot techniques use radioactivity for probe detection, which requires the diagnostic laboratory to comply with additional levels of regulations and to pay for the cost of radioactive waste disposal.

In the early 1990s, techniques using the polymerase chain reaction were developed for detecting B cell clonality.

The principle behind these techniques was that when B cells rearrange their immunoglobulin genes, random numbers of nucleotides are inserted at the V-D, D-J, and V-J joins (so-called “N” region addition). Consequently, the distance from the V region to the J region in a population of B cells will vary from cell to cell. Using specific V and J region primers, a random population of B cells will generate a set of PCR products that has a roughly Gaussian distribution of sizes (Figure 8.2). In the case of lymphoma where all the cells belong to a single clone, PCR amplification using V-J primers will produce a single PCR product (two products if two alleles are rearranged). The PCR products produced in this manner are relatively small, typically 50–150 bp, and they may be resolved by agarose or polyacrylamide gel electrophoresis, denaturing gradient gel electrophoresis, heteroduplex analysis, or capillary electrophoresis.

For the first 13 years that PCR was used for B cell clonality determination, the most common target was the third complementarity determining region (CDR3) of the immunoglobulin heavy chain gene. This was the preferred target since it was relatively easy to design consensus V-region primers capable of annealing to the majority of V-region sequences in their framework 3 regions. Similarly, J_H primers that anneal to all six J_H regions were straightforward to design. In addition,

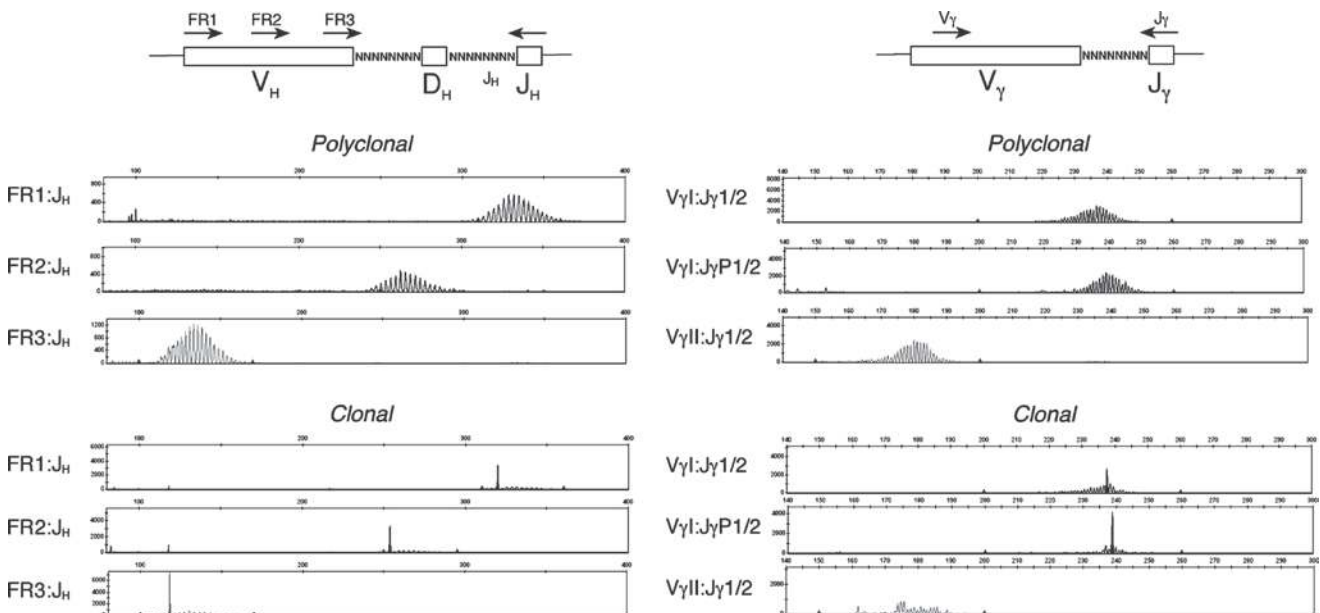


FIG. 8.2. Determination of B and T cell clonality by PCR. Consensus PCR primers flanking the regions where random numbers of nucleotides are added during antigen receptor rearrangement (“N regions”) produce a distribution of PCR product sizes with a polyclonal B or T cell population, but discrete PCR products when a clonal population is present. An example of B cell clonality testing using the BIOMED-2 multiplex immunoglobulin heavy chain primers¹⁴ is shown on the left-hand side of the figure. The V region primers (FR1, FR2, FR3) are distributed along the length of the V region, resulting in successively smaller PCR products.

With a B cell clone, each primer set produces a single PCR product of appropriate size. An example of T cell clonality testing using PCR primers for the T cell receptor γ chain gene¹⁹ is shown on the right-hand side of the figure. In this case, for the clonal T cell population, two distinct products are present, corresponding to rearrangement of each T cell receptor γ allele in the clone. A clonal population can generate at most two PCR products (one per rearranged allele), so if more than two distinct PCR products are detected, more than one T cell clone is present.

the PCR products obtained using such primers are relatively small, allowing the use of fixed tissue, which contains fragmented DNA. The main drawback of using CDRIII primers is that PCR frequently fails in B cells whose immunoglobulin genes have undergone somatic hypermutation, as occurs in a normal germinal center immune response. Approximately 30% of B cell lymphomas fail to be detected using CDRIII primers,¹³ and the failure rate is highest among lymphomas corresponding to post-germinal center B cells, including follicular lymphomas, marginal zone lymphomas, and plasma cell neoplasms.

Alternative strategies for working around the somatic hypermutation problem have included using primers from the other two framework regions of the V_H genes, although primer design is more difficult since there is less consensus among V_H segments in the framework 1 and 2 regions. A major step forward came with the work of the BIOMED-2 consortium in Europe. This group collaborated to design a large set of multiplex primers for the immunoglobulin heavy and light chain genes as well as for T cell receptor genes and common chromosomal translocations found in lymphomas.¹⁴ In principle, by using the BIOMED-2 primer sets, it is possible to detect essentially any B cell clone regardless of somatic hypermutation state.¹⁵ The main drawback of this approach is the expense of using a large number of primers and PCR reactions. It is still possible to detect more than 90% of B cell clones using a more limited set of BIOMED-2 heavy chain and κ light chain primers.¹⁶

Although PCR is generally considered to be a very sensitive technique for detecting small numbers of DNA copies, in the setting of clonality analysis, PCR is not much better at detecting small clonal populations than DNA blot techniques. This is because every B cell present in a diagnostic specimen will contribute to the signal observed. Thus, a clonal B cell population needs to represent 5–10% of the B cells present in a specimen to be detected above the polyclonal background, and these techniques are not suitable for the detection of minimal residual disease. The only difference between PCR and DNA blot to detect small clones is that only B cells contribute to the background in PCR-based methods, where any nucleated cell will contribute to the background signal with DNA blots.

T Cell Clonality

T Cell Receptor Restriction

T cells recognize antigen in the context of self-major histocompatibility antigens through the heterodimeric T cell receptor molecule. Like B cells, T cells rearrange their antigen receptor genes, generating a diverse set of antigen receptors capable of detecting the universe of foreign antigens. Unlike B cells, however, determination of T cell clonality on the basis of restricted expression of surface molecules is more complicated, since the structure of the T cell receptor is more variable than surface immunoglobulin. Specifically,

there is no κ/λ equivalent for the T cell receptor. It is possible to subdivide T cell receptor structures on the basis of the family into which the T cell receptor β chain belongs. There are 24 different $V\beta$ families, and these can be distinguished with specific antibodies. This makes determination of T cell receptor restriction more complicated than immunoglobulin restriction, since reagents of a higher order of magnitude are required for this determination, a complex and expensive proposition.

Flow cytometry can be used to demonstrate restriction of T cell antigen receptor expression by combining the $V\beta$ antibodies three at a time, where one antibody is labeled with fluorescein isothiocyanate (FITC), one is labeled with phycoerythrin (PE), and one is labeled with both. In this way, all 24 $V\beta$ families may be interrogated with eight flow cytometry tubes.¹⁷ An even more efficient approach using multiparameter flow cytometry is to combine these eight tubes into one, and to add an additional cocktail of six or more antibodies specific for various T cell antigens. The T cell population of interest is then identified based on a particular pattern of antigen expression, after which restricted T cell receptor expression is visualized by the presence of either only FITC, only PE, a dual FITC/PE signal, corresponding to a single $V\beta$ antibody. A polyclonal T cell population would contain cells with all FITC/PE combinations. This latter method is a rapid and relatively easy method for determining T cell clonality, although the reagents are expensive, and it requires a high level of technological expertise as well as flow cytometers capable of six or more color detection. In addition, this technique is limited to T cells expressing surface $\alpha\beta$ receptors, and so cannot be used for the rarer $\gamma\delta$ T cell neoplasms.

Molecular Methods for Determining T Cell Clonality

Molecular methods for determining T cell clonality by DNA blot analysis have been available since the late 1980s. DNA-based T cell clonality assays have been used more than B cell clonality assays owing to the relative difficulty in demonstrating T cell clonality at the protein level. The most common molecular target for T cell clonality determination by DNA blot is the T cell receptor β chain gene. The T cell receptor γ chain gene may also be used for DNA blot analysis, but it has the potential for false positive results due to the relatively limited diversity of this locus.¹⁸ DNA blot analysis is a robust method for determining T cell clonality, but it suffers from the same drawbacks as for B cell clonality determination, including the requirements for fresh or frozen tissue, the need for relatively large amounts of tissue, the labor intensity, and the (usual) need for radioactive detection methods. In addition, using a β chain probe will miss the small minority of T cell clones expressing $\gamma\delta$ T cell receptors.

PCR-based methods for T cell clonality determination are based on N-region addition during T cell receptor gene

rearrangement (Figure 8.2). The T cell receptor γ chain gene is most commonly used for T cell clonality determination, primarily because there are only 12 V γ region genes; so, it is easier to design primers capable of amplifying the complete assortment of rearrangements compared to the T cell receptor β chain gene. In addition, although 99% of human T cells express $\alpha\beta$ T cell receptors, the γ chain gene is rearranged first during T cell development, and so regardless of what type of receptor a T cell expresses, it has a rearranged γ chain gene in its genome. This makes the T cell receptor γ chain gene a “universal” target for clonality determination. In studies performed in our laboratory, T cell receptor γ chain clonality assays performed very favorably compared with DNA blot-based assays using β chain probes, detecting 96% of T cell clones also detected by DNA blot analysis.¹⁹ In addition, there were several cases in our series called “indeterminate” by DNA blot that were clearly clonal by PCR as well as one case of a $\gamma\delta$ T cell lymphoma that was negative by DNA blot and positive by TCR γ PCR. Similar to PCR for B cell clonality, a clonal T cell population needs to represent 5–10% of the T cells in a specimen to be detected above the polyclonal background.

The BIOMED-2 consortium has designed sets of multiplex PCR primers for T cell clonality determination that target all four T cell receptor genes (α , β , γ , δ), and can in principle therefore detect any T cell clone. As with B cell clonality determination, using the entire BIOMED-2 primer set can be very expensive, and for most clinical cases, a high level of detection can be achieved using a more restricted set of primers. Based on the literature describing T cell clonality methods, for the most part TCR γ PCR is capable of detecting close to 100% of T cell clones, so the BIOMED-2 primers may be reserved for those cases where there is a high index of suspicion for T cell clonality, but there is a negative TCR γ PCR result.

One of the most frequently encountered difficulties in using PCR for the determination of T cell clonality is the finding of apparent T cell clones in what is clinically most likely a reactive setting. One group has dubbed these “pseudo-spikes,” although they most likely represent real, although not neoplastic, T cell clones.²⁰ T cell clonality or restricted diversity appears to be more common in reactive conditions than B cell clonality, and thus caution should be used in interpreting the results of T cell clonality studies. A number of authors have proposed criteria for what one should call a “positive” T cell clonality test,^{19,21,22} although even using such criteria, it is not uncommon for a reactive T cell infiltrate to contain a single T cell clone or a very small number of clones. As with any diagnostic test, the results need to be considered in the context of other clinical and pathological information.

Natural Killer Cell Clonality

Natural killer (NK) cells comprise a subset of lymphocytes thought to be important in viral and tumor immunology. They recognize and kill cells that lack self-MHC class I molecules but express molecules that engage their “antigen receptors,”

the KIR and NKG2 molecules.²³ NK cell neoplasms are rare, but they need to be considered in the setting of what appear to be T cell malignancies that do not appear to be clonal otherwise. Unlike B and T cells, NK cells do not rearrange an antigen receptor, but the genome does encode several different KIR molecules, only one or a small number of which are expressed on the surface of an individual NK cell.²⁴ Thus, while molecular clonality studies are not easily performed on NK cells, it is possible to demonstrate KIR expression restriction using antibodies specific for the various KIR molecules and flow cytometry.²⁵

Demonstration of clonality of an NK cell process at the molecular level is difficult. If a patient is female, it may be possible to demonstrate clonality based on nonrandom inactivation of the X chromosome by any of the various assays described above. Alternatively, it may be possible to demonstrate a cytogenetic abnormality by conventional cytogenetics. Finally, since some NK cell neoplasms are associated with Epstein–Barr virus (EBV) infection, it may be possible to demonstrate a clonal EBV episome.

Other Clonal Markers in Lymphoid Malignancies

Translocations Resulting in Overexpression of Growth Regulatory Genes

In addition to having conveniently rearranged antigen receptor genes, many B and T cell malignancies also have characteristic chromosomal translocations that may also be used to demonstrate the clonal nature of the process. For many lymphomas, these translocations result in insertion of a growth-regulating gene into an immunoglobulin or T cell receptor locus, resulting in its inappropriate overexpression. Classical examples include overexpression of the *BCL2* gene in follicular lymphoma,²⁶ which prevents apoptosis, overexpression of the *cyclin D1* gene in mantle cell lymphoma resulting in dysregulated cell cycle progression,²⁷ and overexpression of *c-MYC* in Burkitt lymphoma, again resulting in an inappropriate proliferative drive.²⁸ These translocations can be detected by DNA blot analysis using probes for the translocated gene, PCR using primers that flank the translocation breakpoints, fluorescence in situ hybridization using probes for the translocated gene, the antigen receptor loci, or both, or standard visualization of metaphase chromosomes.

One great advantage of molecular techniques for demonstrating a translocation is the exquisite sensitivity of PCR. Unlike with B and T cell clonality methods based on rearrangement of antigen receptor genes, there are no normal cells with lymphoma-associated translocations (although some investigators have found these translocations in rare cells in normal individuals using unusually sensitive techniques). Thus, these assays are both specific for the disease processes they are designed to detect, and they may be used to detect minimal residual disease after therapy, the presence of malignant cells in stem cell products for autologous transplantation, and early disease relapse. Using 0.5 μ (mu)g of DNA in a reaction, translocation-specific PCR can

theoretically detect one malignant cell in approximately 100,000 normal cells.

Translocations Resulting in Production of a Novel Growth-Stimulating Protein

Some translocations can result in separate parts of two different genes being brought together on one chromosome. If the new gene created alters cell proliferation, lymphoma may result. This mechanism for malignant transformation is more common in leukemias, but there are several well-characterized examples that can cause lymphoma. In anaplastic large T cell lymphoma, a t(2;5) causes the *ALK* gene on chromosome 2 to form a fusion with the *NPM1* gene on chromosome 5, and the new fusion protein is an activated tyrosine kinase that presumably results in a strong proliferative signal to the affected T cells.²⁹ Less commonly, the *ALK* gene may also fuse with other partners with similar results.³⁰ The other common example in lymphoma is the t(11;18) translocation in mucosa-associated lymphoid tissue (MALT) lymphoma. In this translocation, the *MALT1* gene on chromosome 18 is fused to the *API2* gene on chromosome 11, and the resulting protein is thought to inhibit apoptosis.³¹

Clonal Viral Integration

As discussed in Chap. 7, Epstein–Barr virus is associated with several B cell malignancies, including posttransplantation lymphoproliferative disorders, endemic Burkitt lymphoma, other immunodeficiency-associated lymphomas, including those associated with HIV, and nasal-type NK/T cell lymphoma.³² The virus exists in infected cells as episomal DNA, and it is possible to demonstrate clonal integration by DNA blot analysis.³³ Since most tumors associated with EBV are of either B or T cell origin, clonality determination is usually based on clonal antigen receptor rearrangement. However, in the setting of EBV-associated NK cell malignancy, demonstration of viral clonality may be helpful in diagnosis. Demonstration of the presence of EBV (as opposed to clonality), is also useful in determining the pathophysiology of some lymphomas, particularly those associated with immunosuppression.

Also, as discussed in Chap. 7, the other clinically important virus in the setting of malignancy is human T cell lymphotropic virus (HTLV)-I, which is associated with adult T cell leukemia/lymphoma (ATLL). Although clonality may be determined by routine methods, demonstration of the presence of HTLV-I may be important to make the diagnosis of ATLL.³⁴

Myeloid Stem Cell Neoplasms

Determination of clonality for nonlymphocytic leukemias and other hematopoietic stem cell neoplasms depends primarily on demonstrating genetic abnormalities in the leukemic cells. Historically, this has been accomplished by cytogenetic analysis of metaphase chromosomes. However, as the molecular pathology associated with recurrent cyto-

netic abnormalities has been defined, it has become possible to detect these abnormalities using sequence-specific detection techniques, including DNA blot, PCR, including reverse transcriptase (RT)-PCR, and fluorescence in situ hybridization as well as direct sequence analysis. The specific molecular pathology associated with various stem cell disorders is covered in more detail in other chapters, so I will focus on the various techniques that may be used to demonstrate the clonal nature of leukemias.

Clonal Recurrent Chromosomal Translocations

In 1960, Nowell and Hungerford demonstrated that an abnormal small chromosome, termed the “Philadelphia chromosome,” was associated with chronic myeloid leukemia (CML).³⁵ Also see Chap. 30. This was the first demonstration that a human cancer might be caused by a genetic lesion. With the development of Giemsa banding techniques, the Philadelphia chromosome was found to be a reciprocal translocation between chromosomes 9 and 22,³⁶ and eventually the genes associated with this translocation were cloned.³⁷ Of interest, one of the genes, *ABL*, was previously known to be incorporated into the Abelson murine leukemia virus, an agent that causes lymphoid leukemia in mice. The other gene, *BCR* (for breakpoint cluster region), had not been previously characterized. These discoveries led directly to the development of molecular diagnostic techniques to detect the *BCR–ABL* fusion gene, first by DNA blot and later by fluorescence in situ hybridization. Molecular methods were also developed to detect the *BCR–ABL* RNA by RT-PCR. Since the advent of imatinib mesylate therapy for CML, quantitative RT-PCR methods have come into common use to monitor response to therapy and to detect early treatment failures.³⁸ This progression from scientific observation to diagnostic technique to therapy is one of the great success stories of modern molecular medicine.

Other recurrent chromosomal translocations are well-characterized in both myeloid and lymphoid malignancies, as detailed in the respective chapters. As with the Philadelphia chromosome, these translocations can be demonstrated by conventional metaphase cytogenetics, fluorescence in situ hybridization, and PCR techniques. See Chap. 9 on Techniques to Detect Chromosomal Translocations. Demonstration of one of these translocations is equivalent to demonstrating the clonal nature of a leukemic process. In addition, these PCR techniques (qualitative or quantitative) are exquisitely sensitive due to the absence of signal in normal cells, so they can be used for minimal residual disease detection.

Gene Mutations in Leukemia

(Also see Chap. 35 on Acute Myeloid Leukemias with Normal Cytogenetics)

Approximately 45% of acute myeloid leukemias (AMLs) have normal cytogenetics based on examination of metaphase chromosomes.^{39,40} This has led to efforts to identify

other clonal genetic lesions that are important pathophysiologically and may also be used for diagnosis. Approximately 25% of AML have a mutation of the FMS-like tyrosine kinase 3 (*FLT3*) gene.⁴¹ This is a tyrosine kinase, and the mutations that have been characterized result in an increase in the *FLT3* tyrosine kinase activity, presumably resulting in an uncontrolled growth signal in the leukemic cells. The most common *FLT3* mutation is a duplication of 5–51 amino acids in the juxtamembrane region of the receptor, referred to as an internal tandem duplication (ITD).⁴² ITD mutations have been shown to portend a worse prognosis in AML. Point mutations affecting the tyrosine kinase domain have also been described in approximately 8% of AML.⁴³ Until recently, they were of questionable clinical significance, but more recent evidence indicates that some point mutations are associated with worse prognosis.⁴⁴

FLT3 ITD mutations may easily be demonstrated by PCR using primers that flank the duplicated region to detect an abnormally large PCR product.⁴⁵ A number of techniques have been described for detecting point mutations of the tyrosine kinase domain, including allele-specific PCR with restriction digestion⁴⁵ and mutation detection using the LightCycler.⁴⁶

Another mutation found in approximately 25% of AML with normal cytogenetics is a small insertion mutation near the 3' end of the nucleophosmin (*NPM1*) gene on chromosome 5.⁴⁷ Most commonly, the mutation is an insertion of 4 bp of DNA sequence, resulting in a reading frame shift and the production of an *NPM1* protein with an altered carboxy terminus and relocation to the cell cytoplasm.⁴⁸ How (or if) this mutation contributes to the pathogenesis of AML is not clear at this point. The prognostic significance of *NPM1* mutations was initially controversial, but it seems clear now that, in combination with no *FLT3* mutation, the *NPM1* mutation is a positive prognostic indicator.⁴⁷ However, any AML with a *FLT3* mutation, regardless of *NPM1* status, has a less favorable prognosis and may be a candidate for more aggressive or experimental therapy.

The *NPM1* insertional mutation may be easily detected in genomic DNA by amplifying the mutated region by PCR and then analyzing the sizes of the PCR products obtained by capillary electrophoresis⁴⁹ or denaturing HPLC.⁵⁰ Sequence-specific PCR may also be performed to detect the most common mutations, but these methods will miss less common mutations.⁵¹ It is also possible to detect the mutant *NPM1* protein by immunohistochemistry based on its cytoplasmic localization.⁵²

A third commonly mutated gene in AML with normal cytogenetics is the *CEBPA* gene, which encodes a transcription factor on chromosome 19 that regulates hematopoiesis.⁵³ The *CEBPA* gene product is part of the core binding factor transcription regulator that regulates myelopoiesis. Most *CEBPA* mutations result in the production of a dominant negative form of the protein that inhibits myelopoiesis,⁵⁴ although in-frame insertion or deletion mutations are com-

mon too. Mutations in *CEBPA* are present in approximately 10% of AML, particularly AML M1 and M2 subtypes, and in the absence of negative prognostic factors (e.g., *FLT3* mutations), *CEBPA* mutations confer a more favorable prognosis.⁵⁵ AML with *CEBPA* mutations most likely represent a distinct biological entity based on their unique gene expression profile.⁵⁶ Detection of *CEBPA* mutations may be performed by PCR across the mutated regions and fragment size analysis to look for frameshift mutations. A recent publication describes a method using capillary electrophoresis that can detect 90% of *CEBPA* mutations in this way.⁵⁷ Direct sequencing may be required to identify mutations in which there is no insertion or deletion.

JAK2 Mutation in Myeloproliferative Neoplasms (Also see Chap. 31 on Non-CML Myeloproliferative Neoplasms)

JAK2 is a tyrosine kinase involved in signal transduction for non-tyrosine kinase hematopoietic growth factor receptors, such as the receptors for erythropoietin and thrombopoietin. Mutations in the *JAK2* gene have been described that result in constitutive tyrosine kinase activity and growth dysregulation.⁵⁸ The most common mutation is a valine to phenylalanine substitution at amino acid 617. This mutation is present in over 90% of polycythemia vera and in approximately 50% of essential thrombocythemia and primary myelofibrosis. Several different assays have been described to detect this mutation including direct sequencing, detection of a lost restriction enzyme site, and allele-specific PCR.⁵⁹

Array-Based Techniques that may Determine Clonality

Since all that is necessary to infer the clonality of a cell population is a genomic alteration of some kind, it is not necessary to know in advance what the genetic alteration is if a comprehensive mutation screen can be performed. With the availability of genomic-scale microarrays, it is now possible to scan the entire genome of a tumor for gains and losses of genetic material. Two types of arrays are available. Whole-genome single-nucleotide polymorphism (SNP) arrays can be used to look for deletions by virtue of loss of heterozygosity for a series of contiguous SNPs. Whole-genome arrays based on oligonucleotide or bacterial artificial chromosome probes can be used for comparative genomic hybridization (CGH). In CGH, genomic DNA from a tumor specimen labeled with one fluorochrome is hybridized to the array with DNA from a normal specimen labeled with a different fluorochrome. Loss of genetic material is detected by a decreased fluorescent signal from the tumor specimen; gain of genetic material is detected by an increased fluorescent signal. Neither of these techniques can detect balanced chromosomal rearrangements.

Summary

Cancer is a clonal process, and therefore demonstrating the clonal nature of a lesion thought to be cancerous may be useful for diagnosis. Techniques to demonstrate cell clonality have been available since the 1970s based on X chromosome inactivation, but these are limited by both technical consideration as well as their lack of utility in male patients. Demonstration of clonality in lymphoid malignancies is facilitated by the fact that lymphoid cells rearrange their DNA as part of normal development. Cytogenetic studies and molecular studies to demonstrate chromosomal translocations may be used to demonstrate clonality in many different malignancies, and disease-specific point mutations are now well-characterized for some stem cell disorders. The ability to perform whole genome analysis using microarrays will allow the detection of rare or novel genetic abnormalities and the discovery of new targets for molecular diagnosis.

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Techniques to Detect Defining Chromosomal Translocations/Abnormalities

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Introduction

There are multiple techniques to detect defining chromosomal translocations and other abnormalities, including conventional cytogenetic analysis, fluorescence in situ hybridization (FISH), spectral karyotyping (SKY), DNA microarray analysis, polymerase chain reaction (PCR) analysis, and immunohistochemical (IHC) analysis. These various techniques with their advantages and limitations will be discussed in this chapter.

Conventional (Routine) Cytogenetics

Cytogenetics involves the study of chromosomes in which both the number and morphology are analyzed. The domain of cytogenetics is inclusive of molecular cytogenetic techniques, specifically FISH, SKY, and microarray (includes array comparative genomic hybridization and oligonucleotide-based arrays). The discussion is limited to hematolymphoid malignancies.

Cytogenetic studies prior to treatment are a known predictor of outcome in many hematolymphoid malignancies, since the findings may identify the abnormality, assist with the diagnosis and prognosis, and aid in following the patient for minimal residual disease/relapse. Routine cytogenetic studies allow the detection of deletions, duplications and rearrangements, and identification of multiple cell lines and evolving clones and provide an unbiased assessment of abnormalities. Unlike PCR-based methodologies, cytogenetic analysis identifies all currently visible abnormalities and may distinguish between malignant clones that display mosaicism, as compared to a clone that is undergoing clonal evolution.

Chromosome analysis requires a source of dividing cells, which depends on the type of specimen and the malignancy. For leukemias and for tumors that are metastatic to the bone (e.g., neuroblastoma), 1–5 cc of fresh bone marrow aspirate or biopsy specimen should be placed into a sodium-heparin tube. If transferring from a syringe, the syringe must be coated with sodium heparin as well. Samples drawn into lithium-

heparin tubes are inadequate as lithium impedes cell division. For detection of acquired abnormalities in the peripheral blood, most laboratories feel that at least 20% blasts are required to insure an adequate number of dividing cells for analysis. Lymph node Scanty lymph node specimens (less than 2–3mm) may not be adequate for cytogenetic studies. They should be submitted in a suitable transport medium, as recommended by the laboratory performing the evaluation. Specimens are to be kept at room temperature and should never be frozen, as live cells are required for cytogenetic preparations and cell culture.¹

The study of chromosomes in hematologic malignancies generally involves a direct preparation and overnight growth of cells in at least two types of media followed by standard harvesting. The culture is arrested in mitosis with a mitotic spindle inhibitor, typically Colcemid™, which prevents the cells from entering anaphase. The cells are studied between prometaphase (the stage where the nuclear envelope disappears) and metaphase. The longer chromosomes are seen when entering prometaphase and the shortest chromosomes are seen when the chromosomes are lined up on the metaphase plate. The specimen is then treated with a hypotonic solution, which leads to swelling of the cells. The cell pellet obtained is washed with a fixative composed of acetic acid and methanol that makes the cell membrane fragile and removes hemoglobin and debris. The cell pellet is dropped onto slides, which ruptures the outer membrane and spills the cell contents onto the slide.²

Different methods of staining produce chromosomes with unique reproducible banding patterns, with each chromosome being composed of two sister chromatids. Typically, cells are treated with trypsin, a protease, which induces banding when stained with Giemsa or Wright stain (Figure 9.1a).²

Metaphase spreads are analyzed for abnormalities of number and structure. When studying malignancies, all metaphase cells are analyzed, with a minimum of three karyotypes, including at least one karyotype representing each abnormal cell line. A karyotype involves arrangement of the chromosome pairs in order so that abnormalities are readily identifiable (Figure 9.1b). The chromosomes are numbered

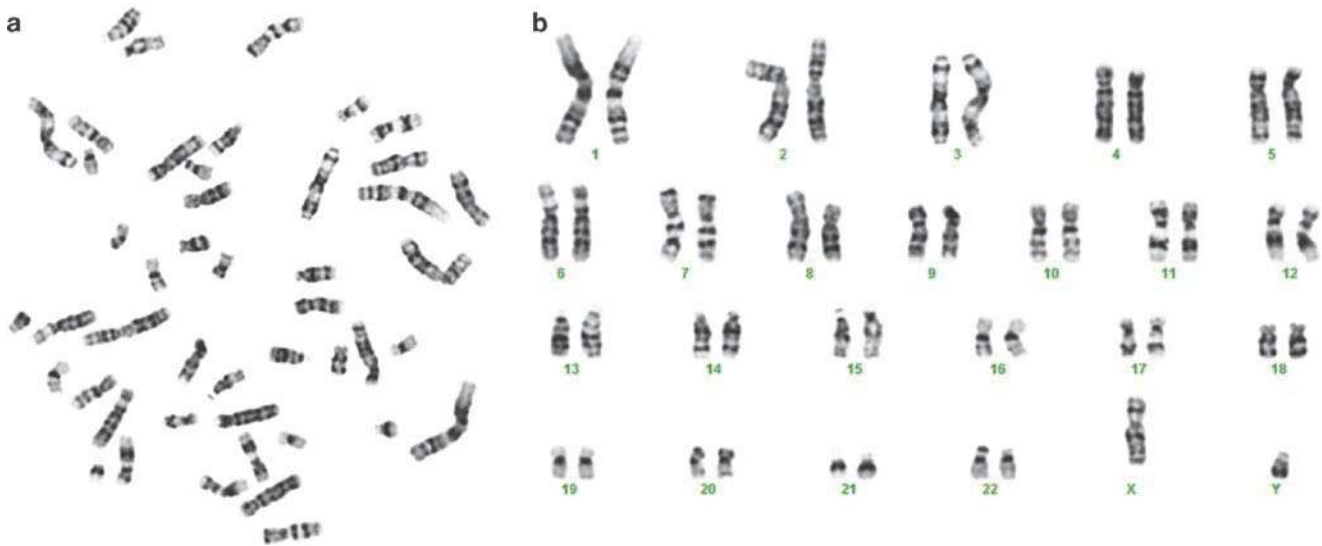


FIG. 9.1. (a) Banded metaphase spread from a bone marrow specimen. (b) Normal XY male karyotype with characteristic G-band pattern corresponding to the metaphase spread in (a).

based on size and position of their centromere, with the short arm of the chromosome named p for “*petite*” and the long arm named q. The bands are numbered independently on the long and short arms, with the band numbers increasing from centromere to telomere. The centromeres are named as p10 or q10, typically when representing either isochromosomes or whole arm fusions. For example, an isochromosome of the long arm of 17 is written i(17)(q10), representing a chromosome with two copies of the long arm and loss of the short arm of chromosome 17. A detailed internationally recognized nomenclature system is commonly used, called the International System for Human Cytogenetic Nomenclature (ISCN), which is routinely updated to include new techniques and increased banding resolution.³

Unlike in constitutional studies, mosaicism is common in hematological malignancies and is the reason for analyzing all cells studied. Mosaicism is defined as the presence of two or more cell lines differing in karyotype, based on number and/or structure. To identify an abnormal cell line, at least two cells must contain the same trisomy or structural chromosomal abnormality and three cells must have lost the same chromosome. A previously known abnormality or an abnormality clearly associated with the diagnosis, such as a single t(9;22) in a chronic myelogenous leukemia (CML) patient, is the sole exception.

Fluorescence In-Situ Hybridization (FISH)

FISH is a molecular cytogenetic technique that allows identification of specific cytogenetic abnormalities. FISH is often used as an adjunct to conventional cytogenetics for the identification of submicroscopic abnormalities and to monitor therapy response. Unlike conventional

cytogenetics, FISH studies identify only the abnormality specific for the designated probe set, thus a negative result is conditional. However, once an abnormality has been established, FISH is useful in the establishment of the percentage of abnormal cells before and throughout the treatment and can be useful in determining therapy response and minimal residual disease.

Most FISH probes are derived from bacterial artificial chromosomes (BACs) or PCR products that have been labeled with a fluorescent nucleotide and broken into 300–500 bp fragments by nick translation. When the probe and chromosomes are co-denatured, the probe will hybridize faster than the complementary strand of DNA, which can then be detected under a fluorescence microscope. There are four general approaches for FISH probe design: repetitive sequence probes, single gene probes, fusion-signal probes, and split-signal probes.

FISH can be used to study metaphase spreads (dividing cells) and interphase nuclei (nondividing cells). Both have advantages and disadvantages. When studying cells using interphase FISH (nuc ish), there is no reliance on dividing cells, which means that there is no selection for division in culture, allowing the study of a larger number of cells. The disadvantage is that, in looking at signals, it is not possible to determine whether the signal is specific or whether there is an uncommon rearrangement that can affect the prognosis (e.g., *BCR-ABL1* fusion on a different chromosome). FISH on metaphase spreads can confirm that a rearrangement is present on a specific chromosome. Figure 9.2a demonstrates an interphase cell which is *ETV6-RUNX1* (TEL-AML1) positive and deleted for the *ETV6* locus on the normal chromosome 12. Figure 9.2b is an apparently normal metaphase spread with the 12 and 21 chromosomes arrowed. The slide

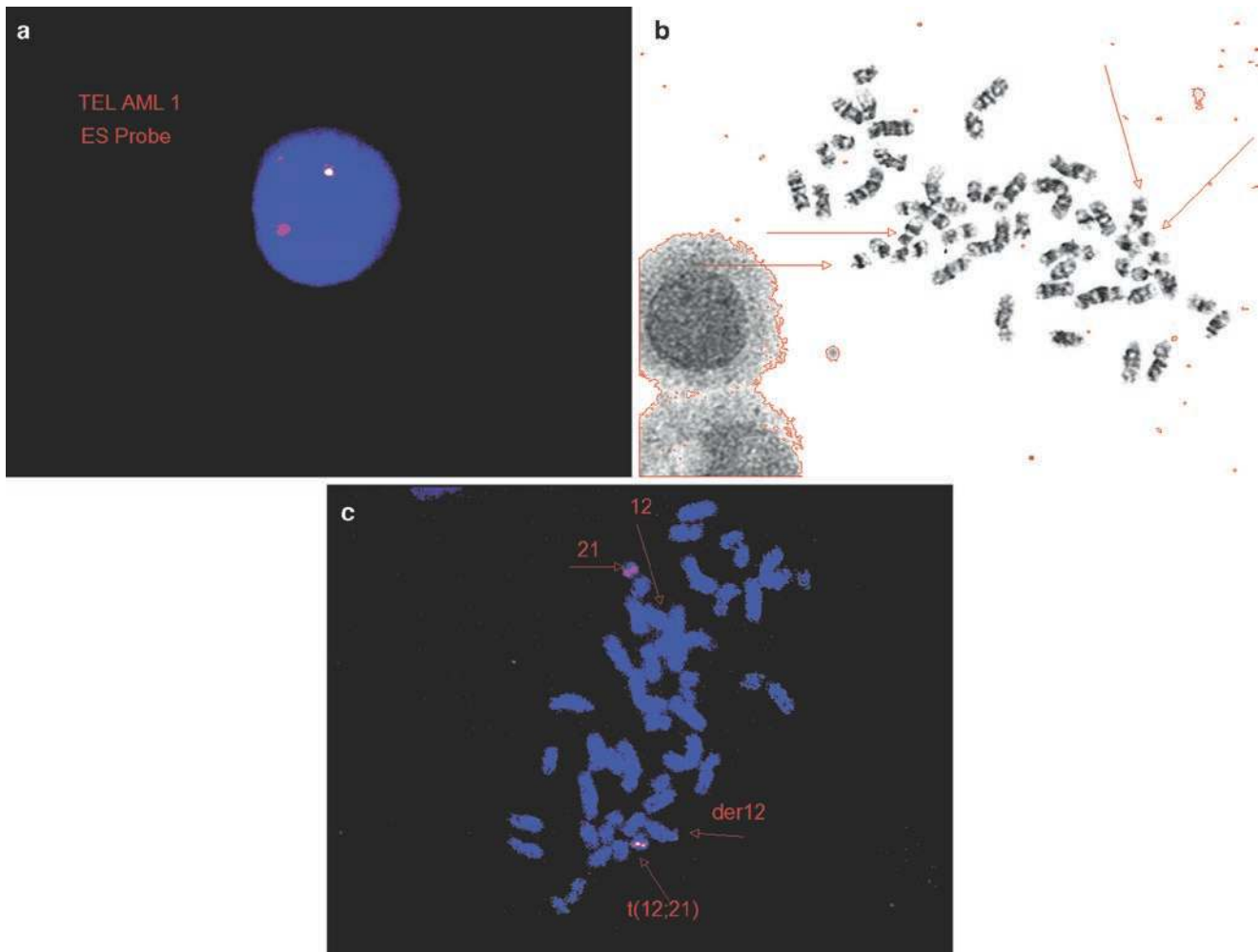


FIG. 9.2. Pediatric pre B-cell acute lymphoblastic leukemia patient on day 0 of treatment. (a) FISH for ETV6-RUNX1 rearrangement in interphase nuclei with fusion signal RUNX1 (arrowed), extra signal (small red signal), and normal AML (large red signal), but deleted for the normal ETV6 region. (b) Normal bone marrow

metaphase based on conventional cytogenetics, with normal 12 and 21 chromosomes and derivative 12 and 21 chromosomes arrowed. (c) FISH using the ETV6/RUNX1 probes on the previously G-banded metaphase from (b) identifying the location of the signals to their respective chromosomes.

was destained probed with the ETV6-RUNX1 FISH probes and the same metaphase spread was analyzed showing that ETV6 has translocated to the chromosome 21 (fusion signal) (Figure 9.2c). The disadvantage of metaphase FISH, frequently, is the paucity of abnormal metaphase cells present in the preparation.

Repetitive sequence probes are typically centromeric probes usually from the alpha-satellite sequences that comprise the pericentromeric region. These signals are often used for chromosome enumeration or as a control probe in combination with single gene or locus probes and provide a robust signal. An example would be the DXZ1 and DYZ3 probes that are used to enumerate donor and recipient cells following sex-mismatched bone marrow transplant (Figure 9.3).

Single gene or locus probes target genes either deleted or duplicated in malignancy. The probe will either contain the

gene or be within the gene. Often, there is a control probe on the same chromosome to differentiate between a deletion of a region and loss of the entire chromosome. An example of this methodology is the combination of D9Z1 and *p16/ink4* from 9p21 (Figure 9.4).

Fusion-signal FISH is used to detect a common translocation (such as the Philadelphia chromosome, as depicted in Figure 9.5a) that leads to two genes that are normally far apart and coming into close proximity to each other. The two probes are located in two genes, each labeled in different colors (typically red and green). In normal cells, there will be four signals, two green and two red signals. If the translocation has taken place, there will be three signals, the green and red signals that colocalize, thus appearing as a yellow signal, and the green and red signals of the unaffected genes. Fusion-signal FISH has the disadvantage of giving false positive results due to random signal colocalization and

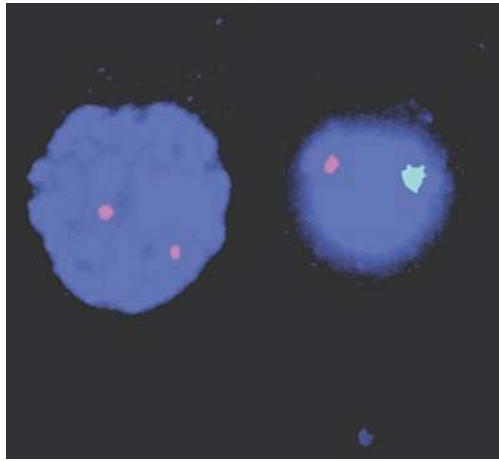


FIG. 9.3. Incomplete engraftment demonstrating chimerism for XX and XY cells. FISH for X and Y loci was used to assess bone marrow engraftment post-transplant from an opposite sex donor. In this patient, two cell types are present, with two red signals consistent with two X chromosomes and one red and one green signal consistent with one X and one Y chromosome.

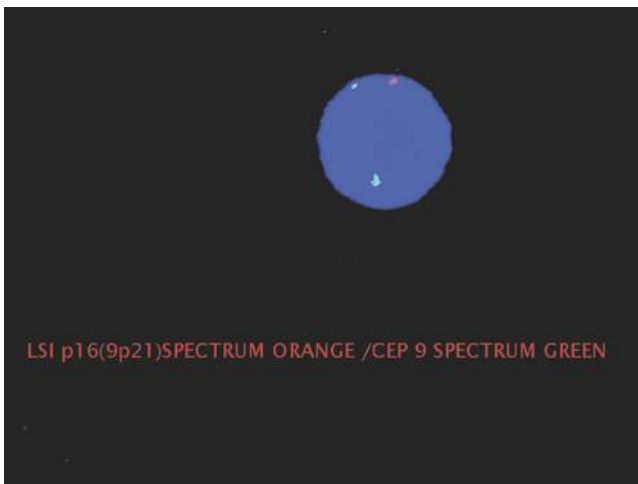


FIG. 9.4. Pediatric T-cell acute lymphoblastic leukemia patient with deletion of 9p21. FISH using a probe for the p16/ink4 locus on 9p21 identified a single red signal and two green signals representing the chromosome 9 centromeric region, indicating there are two 9 chromosomes present.

failure to detect alternative partners. Use of the extra signal probe, which combines the break-apart technology with the fusion gene methodology, can circumvent false positivity. An example of this methodology is the BCR-ABL1 extra-signal (ES) probe set in which the 9q34 probe is broken, leaving a smaller residual (extra) signal commonly used in the study of CML and ALL (Figure 9.5b).

In break-apart FISH methodology, also known as split-signal FISH, the gene being studied often has multiple translocation partners. In this case, the two probes are adjacent and labeled in different colors, either within a gene or

positioned at opposite sides of the breakpoint region in the gene, which are involved in the translocation. For example, the 5' end of the gene would be labeled in red and the 3' end of the gene in green. In normal cells, two yellow signals (adjacent red and green) will be present. In the event of a translocation, there would be one normal yellow signal from the unaffected allele and one green and one red signal, representing the separation of the 5' and 3' regions of the gene. An example of this methodology is the MLL break-apart probe, used in ALL and AML (Figure 9.6).

Break-apart probes have several advantages over the fusion FISH. The detection of involvement is not partner gene dependent, so it can be used to determine whether a common translocation partner is implicated. For example in multiple myeloma, use of the IGH probe can detect whether or not IGH is involved and can save time and reagents. Break-apart probes reduce false positives, since a fusion probe can be read as positive due to random colocalization. One disadvantage of break-apart probe methodology is that the translocation partner cannot be readily assessed unless metaphase spreads are available.

Break-apart FISH probes are often used when the gene is a promiscuous partner, but can only detect the partner gene when metaphase spreads are present. The value of this design compared to gene fusion FISH, is the lower rate of false positives. Chance "splitting apart" is rare, compared to chance co-localization using gene fusion probes.

Cytogenetic Abnormalities in Hematolymphoid Malignancies

Hematolymphoid malignancies may be classified according to the most recent iteration of the World Health Organization (WHO). In this classification, cytogenetic abnormalities are critical for the diagnosis in some disease categories, as will be discussed.

Acute Myeloid Leukemias (AML)

There are common numerical and structural chromosome abnormalities seen in patients with AML. This section will use the WHO classification to discuss the recurrent cytogenetic abnormalities used prognostically in AML.⁴ Conventional cytogenetic studies identify risk categories for individuals with AML that are used in clinical practice to assess prognosis and make treatment decisions in AML.

FISH is often performed as a second tier test to identify underlying abnormalities in a normal karyotype. Cytogenetic abnormalities in AML are highly significant and correlate well with interphase FISH analysis.⁵ A comprehensive FISH panel for AML may include -5/5q, -7/7q, +8, t(8;21), t(9;22), t(11;variable partner), t(15;17), and inv(16)/t(16;16), either as a second tier test series or supplemental at the time of diagnosis.

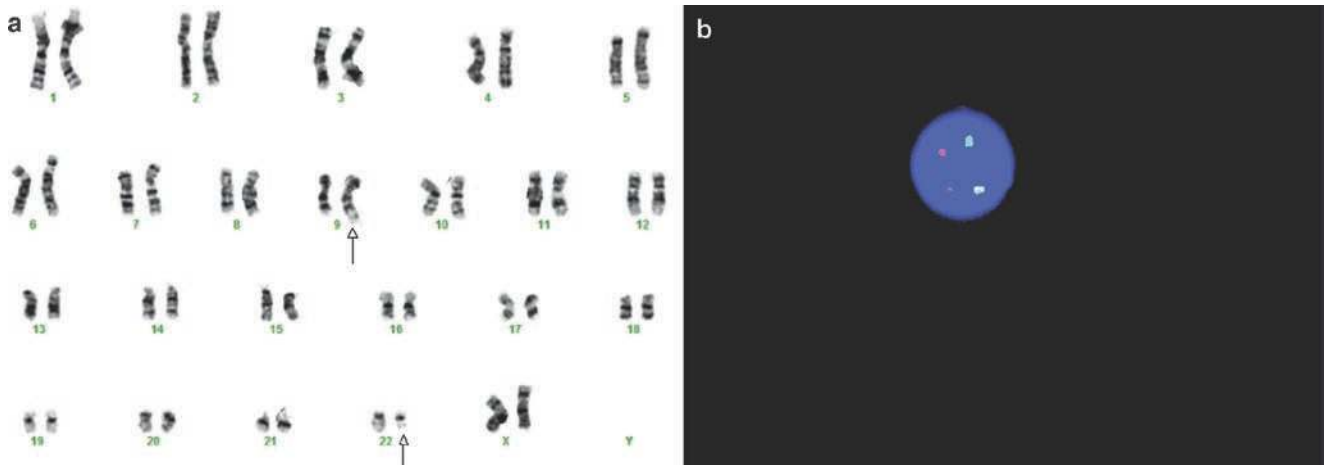


FIG. 9.5. A 9;22 translocation identifying the BCR-ABL fusion. (a) G-banded chromosomes with the translocated chromosomes 9 and 22 arrowed. The derivative chromosome 22 is often called the Philadelphia chromosome (Ph+); (b) Detection of the BCR-ABL

fusion using the BCR/ABL extra signal (ES) probe, with one *green* (BCR), one *red* (ABL), one small *red* (residual extra signal on 9q34), and the BCR-ABL fusion (*arrowed*).

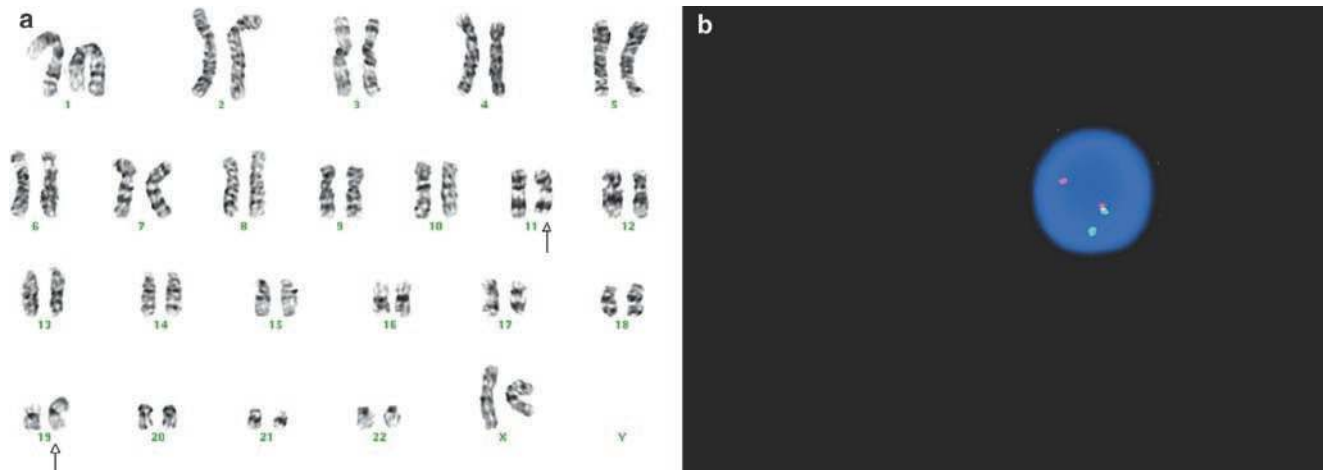


FIG. 9.6. MLL rearrangement in an infant presenting with pre B-ALL. (a) Conventional cytogenetics identified an 11;19 translocation in all cells studied. (b) FISH using the break-apart MLL probe identified a split signal. Probes are on the 5' and 3' end of

the MLL gene on 11q23. A normal signal pattern is two adjacent signals appearing *red-green* or *yellow*. In this case, one pair of probes is split apart signifying a rearrangement involving the MLL locus.

World Health Organization (WHO): AML with Recurrent Cytogenetic Translocations

t(8;21)

Translocations involving the *ETO* gene on chromosome 8q22 and the *AML1* gene on 21q22 are found in 5–12% of AML (Figure 9.7a).⁴ Among the non-random chromosomal aberrations observed in AML, t(8;21)(q22;q22) usually correlates with FAB AML M2 but some AML M1 and AML M4 cases showing the same translocation have been

reported. t(8;21) is considered a good prognostic indicator. The t(8;21) translocation in FAB AML M2 is more commonly identified in children than adults. Some patients demonstrate additional chromosome abnormalities, including loss of a sex chromosome or deletion of 9q22, which is why conventional cytogenetics should be performed.

FISH for t(8;21)

This translocation is commonly seen in AML M2 and is analyzed using a dual color dual fusion probe. In a normal cell, two



FIG. 9.7. ETO-AML1 rearrangement in a pediatric patient. (a) Conventional cytogenetics identified an 8;21 translocation. (b) Dual-color, dual-fusion ETO-AML1 FISH probes identified chromosome

8 in red, chromosome 21 in green, and the two derivative chromosomes 8 and 21 with fusion signals.

red and two green signals would be present, encompassing the *ETO* gene on chromosome 8q22 regions and the *AML1* gene on 21q22. When a translocation occurs, the *ETO* gene breaks within introns 1a or 1b, and the *AML1* breaks within intron 5, splitting these probes. The resulting abnormal cell will appear as two fusions (yellow) since both derivative (translocated) chromosomes contain pieces from each gene, and the one red and one green signal representing the unaffected chromosomes (Figure 9.7b). This probe set can identify other abnormalities of chromosomes 8 and 21, though additional probes must be used to determine whether there is a numerical abnormality or an alternative translocation involving one of these genes.

t(15;17)(q22;q21)

Sensitivity of FAB AML M3 cells to retinoic acid helped identify the critical rearrangement involving the retinoic acid receptor alpha (*RARα*) gene on 17q21 fusing with the zinc finger binding transcription factor on 15q22 (*PML*) gene, giving rise to a *PML-RARα* fusion gene product. The *PML-RARα*(alpha) fusion is seen in at least 70% of cases.⁴ Some chromosomal variants that involve *RARα*(alpha) but not *PML* have been identified. Detection of these variants is significant since the cells with the t(11;17) show decreased sensitivity to retinoic acid treatment, but the cells with the t(5;17) remain sensitive.⁴

FISH for t(15;17)

Suspicion of AML M3 is considered a cytogenetic emergency and should be carried out with same day or next-day turnaround time to allow for immediate treatment. The 15;17 translocation is commonly seen in AML M3 and can be analyzed using either a dual-color single-fusion or dual-color dual-fusion probe. The dual-color, dual-fusion probe

has the advantage of decreasing false positivity and can help identifying alternative translocations. The dual-color, dual-fusion probe design typically encompasses the *PML* gene in one color. The breakpoint on chromosome 17 is within the large (17 kb) intron 2 within *RARα*(alpha) and the probe encompasses the entire gene. Thus, when there is a *PML-RARα*(alpha) translocation, there are two fusion signals representing the two derivative chromosomes, one red and one green, representing the normal chromosomes 15 and 17. If a variant translocation occurs involving *RARα*(alpha) instead of fusion signals, there will be two red and three green signals, representing the break within *RARα*(alpha). Either identification of the signals on metaphase chromosomes or alternative probes must be used to differentiate between trisomy 17 and a translocation variant. Use of a *RARα* break-apart probe can differentiate between these events.

inv(16)(p13q22)/del(16)(q22)/t(16;16)(p13;q22)

Abnormalities involving chromosome 16 are commonly associated with the AML-M4Eo in the FAB classification, and is considered a good prognostic indicator. Both the inverted chromosome 16 and the t(16;16) lead to fusion of the *CBFB* gene at 16q22 to the smooth muscle myosin heavy chain (*MYH11*) at 16p13.⁴ Patients with a terminal deletion of chromosome 16q22 do not respond as well to therapy as those with chromosome 16 rearrangements.

FISH for Chromosome 16 Rearrangements

This inversion or translocation is commonly seen in AML-M4Eo and may be analyzed using the *CBFB* break-apart probe. The probe is designed with the 5' end of the *CBFB* gene labeled in one color (e.g., red) and the 3' end of the *CBFB* gene labeled in a different color (e.g., green), the

common breakpoint present between the probes. In a cell where there is no *CBFB* abnormality, there are two fusion (yellow) signals. In cells with a *CBFB* abnormality, the *CBFB* gene is broken in intron 5 and the 3' end of the gene either inverts and forms a fusion gene with *MYCH11* on 16p13.1, or translocates to the other chromosome 16p13.1 homolog, forming the *MYCH11-CBFB* fusion gene. To differentiate between these events metaphase cells must be analyzed.

11q23 Rearrangements

Rearrangements of the *MLL* gene confer an intermediate risk regardless of age. *MLL* rearrangements occur in approximately 65% of infant AML, decreasing regressively to age to 4–7% of adults. There is a strong association between FAB AML M5/M4 and deletion and translocations involving 11q23. The most common translocations observed in childhood AML are the t(9;11)(p21;q23) and the t(11;19)(q23;p13.1). *MLL* is involved in many other translocations; at least 50 different partner-chromosomes have been identified. A partial tandem duplication of *MLL* gene has also been reported in the majority of adult patients whose leukemic blast cells have a trisomy 11 and in some with normal karyotype.

FISH for 11q23 (*MLL*)

FISH to identify *MLL* rearrangements can be useful, since there are many translocation partners, some of which are subtle and can be missed with poor metaphase quality. Due to the many *MLL*-partner genes a break-apart probe strategy is employed. The probe is designed with the 5' end of the *MLL* gene labeled in one color (e.g., green) and the 3' end of the *MLL* gene labeled in a different color (e.g., red). In a cell where there is no *MLL* translocation, there are two fusion (yellow) signals. In cells with an *MLL* translocation, the 5' end of *MLL* remains on chromosome 11, and the 3' end of the gene translocates to another chromosome. This probe can also identify terminal deletions of 11q23 with either a single fusion signal or one fusion and one green signal.

Abnormalities Seen in AML with Multi-Lineage Dysplasia

Chromosome abnormalities associated with this group are similar to those seen in MDS and include $-5/\text{del}5q$, $-7/\text{del}7q$, $+8$, $+9$, $+11$, $+19$, and $+21$. These are discussed in further detail in the section on MDS. Abnormalities involving 3q26, including the inv(3)(q21q26), t(3;3)(q21;q26), and ins(3;3)(q21;q26) are also seen in this subtype of AML.⁴

Secondary or Therapy-Related AML

Cytogenetic abnormalities are commonly seen in therapy-related AML secondary to chemical exposures. The most

common abnormalities seen secondary to alkylating agents are deletions of 5q and 7q, which may be seen individually, together, or as part of a more complex rearrangement.⁴

Secondary AML has been shown to develop in some patients after treatment with DNA-topoisomerase II inhibitors, leading to an increased risk for balanced translocations and rearrangements, such as inv(3)(q21q26). The most common translocations seen in secondary AML associated with the *MLL* gene include t(9;11) and the t(11;19), but other common rearrangements are also seen, such as the t(8;21) and t(15;17).

Chronic Myeloid Leukemia (CML): t(9;22)(q34;q11.2)

CML is the first malignancy in which a chromosome abnormality, the Philadelphia chromosome, was seen as reported by Peter Nowell and David Hungerford in 1960.⁶ It originally referred to a small marker chromosome, now known to be the derivative 22. The abnormality was later identified as a balanced translocation between chromosomes 9q34 and 22q11.2 leading to the translocation of the *ABL1* gene on 9q34 to the *BCR* gene on 22q11.2 (Figure 9.5b).⁷

CML is a triphasic disease with different chromosome abnormalities seen, dependent on the phase. During the chronic phase, the *BCR-ABL1* fusion is present in 100% of cases often as the sole abnormality and is a diagnostic requirement in the WHO classification. The *BCR-ABL1* fusion is usually seen in the form of a t(9;22), which is found in 90–95% of cases. In the remaining instances, there is a cryptic translocation or a complex rearrangement requiring FISH or q-RT-PCR to identify.⁸ These atypical rearrangements are often associated with a poor prognosis. In up to 15% of cases, cryptic deletions have been identified at the 9;22 breakpoints. This is further discussed below, in the FISH section covering the 9;22 translocation.

As the disease advances, additional chromosome abnormalities are frequently seen, with trisomy 8 being one of the most common anomalies seen in addition to the t(9;22). Other cytogenetic markers of progression include trisomy 19, an isochromosome of the long arm of chromosome 17, leading to loss of the TP53 locus on 17p13, and additional copies of the Philadelphia chromosome, typically seen as a supernumerary +der(22)t(9;22). These can be seen as a unique additional anomaly or with several anomalies in cases with clonal evolution.

FISH studies for the t(9;22) may reveal additional information, that would be missed by conventional cytogenetic analysis. In approximately 5% of CML cases, the *BCR-ABL1* fusion may be cryptic (masked Philadelphia chromosome) or present on a marker chromosome. Detection of a masked *BCR-ABL1* fusion must be identified on metaphase chromosomes. A common deletion of 9q34 that is proximal to the *ABL1* gene and inclusive of the arginino-succinate

synthetase (ASS) gene can be predictive of interferon response.⁹

There are many commercially available FISH probes, with the most common being: dual-color, dual fusion FISH probe, a tricolor dual fusion probe that includes the proximal ASS gene in a third color, and the dual color, single fusion extra-signal probe, in which the ASS gene and ABL1 gene are included on the same probe.¹⁰

Myelodysplastic Syndromes (MDS)

Over 50% of newly diagnosed patients, and up to 80% of therapy-related MDS patients have chromosome abnormalities.¹¹ Identification of cytogenetic abnormalities at diagnosis is critical, since the cytogenetic findings often determine the therapeutic approach.^{12,13} A normal karyotype is associated with good prognosis. Loss or gain of genetic material can be the result of unbalanced translocations, which are frequently observed in MDS with multiple abnormalities. In MDS, chromosomal abnormalities are predominantly characterized by deletions or monosomy and gains or trisomy with a paucity of balanced rearrangements, in contrast to AML.¹³ The most common chromosomal abnormalities seen in MDS include $-5/\text{del}(5q)$, $-7/\text{del}(7q)$, $\text{del}(11q)$, $\text{del}(12p)$, $\text{del}(20)(q12)$, $-Y$, and $+8$.

Conventional cytogenetic analysis is the preferred manner to study patients with MDS and related disorders. Based on literature data, comparative analysis of conventional cytogenetics and interphase FISH both detected abnormalities in 35–40% of specimens, without improvement of detection using FISH.^{14,15} In other case series, interphase FISH abnormalities were more likely to be detected when the bone marrow contained blasts. These patients ran a greater risk of progression to AML.¹⁶

FISH probes used for MDS include the regions commonly seen by conventional cytogenetics: EGR1/D5S23 ($-5/5q-$), D7Z1/D7S522 ($-7/7q-$), MLL (11q23 rearrangements), D8Z2 (trisomy 8), D13S319 (deletion 13q14.3), and D20S108 (deletion 20q12). In clinical practice, conventional chromosome studies are preferable to interphase FISH in both low and high-risk patients with MDS. However, FISH should be employed whenever conventional chromosome studies are unsuccessful, as they can detect the common abnormalities.

5q– Syndrome

Deletions involving the long arm of chromosome 5 are relatively common in MDS, accounting for approximately 15% of abnormalities.¹⁷ There is a variety of breakpoints seen on 5q, most frequently between 5q13 and 5q33 [$\text{del}(5)(q13q33)$], with the critical region inclusive of 5q31.¹⁸

5q deletion-patients can be categorized into three karyotypically defined subsets: isolated $\text{del}(5q)$, including patients with the 5q– syndrome, $\text{del}(5q)$ with one additional

chromosome abnormality, and $\text{del}(5q)$ with two or more cytogenetic abnormalities and a complex karyotype. Overall survival is dependent on karyotypic presentation, with a complex karyotype portending a poor prognosis. 5q– syndrome is characterized by an isolated 5q deletion and no additional karyotypic abnormalities, and $<5\%$ blasts in the bone marrow.^{18,19}

The drug Lenalidomide has been used with success to obtain a durable clinical response in the subset of patients with a 5q31.1 deletion.²⁰ It was shown that the response rate varied by cytogenetic pattern and was highest among patients with a chromosome 5q31.1 deletion by conventional cytogenetics (83%), compared with patients who had normal karyotypes (57%), or other chromosomal abnormalities (12%).

Monosomy 7/7q–

Monosomy and deletions of chromosome 7 are seen in both the pediatric and adult populations and can be associated with MDS, AML, and AML. Deletions of 7q cluster between 7q11 (proximal) and 7q36 (distal), with the critical deleted sections in 7q22 and in q32–34.¹¹ Monosomy 7 or $\text{del}(7q)$ is a frequent finding in therapy-related MDS and AML following treatments with alkylating agents. Treatment response and outcome are poor in these patients.

Other Abnormalities in MDS

Deletion of the long arm of chromosome 20, typically 20q12 through 20qter is seen in 4–5% of MDS patients, and can also be seen in AML. These patients often present with thrombocytopenia and/or anemia. Deletion 20q is associated with a favorable prognosis when seen as the sole cytogenetic abnormality.

Of note is that in polycythemia vera (PV), a dominant negative missense mutation in the JAK2 gene (V617F) has been identified as a clonal abnormality in more than 80% of patients.²¹ Molecular testing is necessary for identification of this abnormality and is not detectable cytogenetically.

Acute Lymphoblastic Leukemia (ALL)

Acute lymphoblastic leukemia is a malignancy characterized by immature lymphocytes that are committed to either the B or T cell lineage or their precursors. When morphology and flow cytometry identify greater than 25% lymphoblasts in the bone marrow aspirate, the diagnosis of acute lymphoblastic leukemia is considered established.²² B and T cell-ALL are seen in both the pediatric and adult populations.

Cytogenetic Abnormalities Seen in T-ALL

T-ALL represents 15% of childhood and 14–28% of adult ALL. Data of cytogenetic characterization have been more difficult to obtain than those of B-ALL as fewer than 50% of

cells demonstrate cytogenetic abnormalities, and the cytogenetic correlation with outcome is unknown. The majority of abnormalities involve the T-cell receptor (*TCR*) genes, with *TCR* α (alpha) and δ (delta) present on 14q11, *TCR* β (beta) on 7q35, and *TCR* γ (gamma) on 7p15.²² Some of the more common translocations includes the *TAL1* gene on 1p32, and t(5;14)(q35.1;q32.2), a cryptic translocation that involves the *RANBP17* and *TLX3* genes. Significantly, *ABL1* amplification is seen in 6% of cases, usually on an episome. In this case, the *ABL1* gene forms a fusion gene with *NUP214*. Cells containing the fusion product of *NUP214-ABL1* are Imatinib responsive.²³ FISH using the BCR-*ABL1* probe can identify *NUP214-ABL1* amplification, and should be performed on all pediatric T-ALL patients.

Cytogenetic Abnormalities Seen in Pre B-ALL

There are well-defined groups of cytogenetic abnormalities in precursor B-cell ALL, which correlate well with prognosis.^{24,25} Numerical abnormalities can be classified as near-haploid, hypodiploid, hyperdiploid with fewer than 50 chromosomes, hyperdiploid with more than 50 chromosomes, and near triploid/tetraploid. There are many structural aberrations seen in pre B-ALL, including translocations, deletions or duplications. Remission rates are lowest in patients with hypodiploidy/near-haploidy, t(9;22), t(4;11), t(8;14) and rearrangements involving 14q32 (IGH rearrangements, 14q+).

Numerical Abnormalities in Pre-B-ALL

High hyperdiploidy is seen in approximately 25% of pediatric patients at diagnosis²⁴ and 10% of adults with ALL, and is considered a good prognostic indicator. Trisomy for chromosomes 4, 10, and 17, which are of prognostic significance, in addition to chromosomes X, 6, 14, 18, and 21, with chromosome 21 often tetrasomic, is a common pattern.²⁶ The modal number of chromosomes is greater than 50, with a median around 55 (chromosome numbers range between 51 and 68).²⁶ High hyperdiploidy is less common in adults, generally seen in 2–11% of cases, and is associated with younger age and a normal white blood cell count.

Low hyperdiploidy is distinct from high hyperdiploidy both in chromosome pattern and in clinical features. These moderately hyperdiploid cells have chromosome numbers between 47 and 50, with most demonstrating 47 or 48 chromosomes.²⁶ This type of chromosome abnormality is most common in young children (20–30%), and is rarely seen in infant or adults.²⁶

Hypodiploidy is much less common than hyperdiploidy, with only 6% of cases presenting with less than 46 chromosomes. The majority (85%) of these patients have 45 chromosomes, with most commonly the loss of the X chromosome. The next most common monosomies are 13, Y, 7, 18, 21, and 22, which are approximately equally represented. Of these, monosomy for chromosome 7 is the most significant, since it portends a poor response to therapy.

Individuals with a near-haploid karyotype have a modal number varying between 26 and 28 chromosomes. Structural abnormalities are rarely seen in hypodiploid cases. There is usually one copy of each chromosome, with the exception of chromosomes 21, 10, 14, 18, and/or a sex chromosome.²⁷ These cell lines can be mistaken for hyperdiploid cell lines, due to an endoreduplication event leading to a “high” hyperdiploid cell line, but retaining the poor prognostic significance. The pattern of chromosomes differs from true hyperdiploidy in that there is generally only 2 or 4 of each chromosome.

Structural Rearrangements

Translocations Associated with Pre B ALL

t(12;21)(p13;q22)

The *ETV6/RUNX1*, formally known as *TEL/AML1*, gene rearrangement results from a balanced, reciprocal t(12;21)(p13;q22), and occurs in as many as 25% of children with precursor B cell acute lymphoblastic leukemia.²⁸ This is a cryptic rearrangement and is discussed in the FISH section below. In over 50% of the cells positive for *ETV6-RUNX1*, additional abnormalities have been identified, the most common of which were deletion of the other allele of 12p13, deletion of 9p21 (p16/Ink4), and trisomy 21. This underlines again the importance of conventional cytogenetics in addition to FISH studies since additional cytogenetic abnormalities can modify the prognostic significance.

FISH for the *ETV6/RUNX1* (*TEL/AML1*) translocation is essential for identification of this cryptic rearrangement²⁸ and is a requirement for all Children’s Oncology Group (COG) pre B-cell ALL patients. The *ETV6-RUNX1* fusion product is present on the derivative chromosome 21.²⁹ Expression of the *ETV6/RUNX1* fusion product defines a distinct subgroup of patients as low-risk with excellent prognosis.³⁰ The FISH strategy that many laboratories employ relies on a dual color probe set, with a fusion marking the translocation and an extra signal representing the other derivative chromosome (Figure 9.2). Use of this probe can also identify whether the other *ETV6* allele is deleted, and can identify *RUNX1* amplification, a poor prognostic indicator.^{31,32}

Myeloid Lymphoid Leukemia (MLL) Rearrangements

The *MLL* gene (myeloid/lymphoid or mixed lineage leukemia) is located on 11q23, a region frequently involved in translocation in acute leukemia (both ALL and AML), as previously mentioned in the AML section. The 5’ end of the *MLL* gene fuses with a partner gene leading to leukogenesis.³³ There have been over 73 different translocations with 54 known partner-genes described.^{34,35} *MLL* rearrangements are seen in 67–80% of infants with ALL, 8 % of children, and 7% of adults.²⁵ *MLL* gene rearrangements associated

with infant ALL predicts a poor prognosis, which worsens with earlier age of presentation.³⁶

t(4;11)(q21;q23)

There are many translocation partners for the *MLL* gene, with the most common being the t(4;11)(q21;q23), seen in 2–3% of patients. This translocation leads to the fusion of the *AF4* gene on 4q21 with *MLL*. Patients presenting with the t(4;11) commonly display clonal evolution with additional cytogenetic abnormalities acquired during disease progression. The prognosis of t(4;11) in all ALL patients, and the rearrangement of *MLL* in infants portend a poor outcome.

FISH for *MLL* is performed to detect rearrangements of the *MLL* gene. A dual color break-apart probe is used due to the high number of translocation partners. Identification of the partner gene requires metaphase cells for localization of the translocated probe.

t(1;19)(q23;p13.3)

The t(1;19) translocation fuses the *PBX1* on 1q23 with the *E2A* gene on 19p13.3. Both balanced and unbalanced translocations are seen, with the unbalanced translocation retaining the derivative 19 chromosome, in the presence of two normal chromosome 1's. The t(1;19) translocation is seen in 3–6% of pediatric pre B-ALL and is considered an adverse prognostic factor.

t(9;22)(q34;q11.2)

The t(9;22) is the most common recurring abnormality in adult ALL, occurring in 11–29% of patients, both B- and T-ALL included.²⁶ This translocation involves the *BCR* locus on chromosome 22q11.2 and the *ABL1* locus on 9q34 as previously mentioned in the section on CML. 97% of BCR-ABL1 fusions occur on the derivative chromosome 22, with only 3% being variant translocations, located on a different chromosome. Therapy response is worse for variant BCR-ABL1 fusions than with the standard translocation. Abnormalities in addition to the Philadelphia chromosome are common at diagnosis and with disease progression, necessitating conventional cytogenetics in addition to FISH.

Pediatric ALL

In childhood acute lymphoblastic leukemia, numerous good and high-risk cytogenetic subgroups have been identified, which are regularly used to stratify patients to particular therapies. Currently, COG stratifies patients into risk categories.³⁷ The low-risk group is seen in approximately 27% of cases and includes hyperdiploidy with the “triple trisomy” of 4, 10, and 17, and the ETV6/RUNX1 (TEL/AML1) translocation involving 12p13 and 21q22. The very high-risk group included t(9;22), hypodiploidy (defined as less than 45 chromosomes), monosomy 7 *MLL* rearrangements in infants, and individuals with poor induction response.

Adult ALL

Many adult ALL cytogenetic studies are centered on the presence or absence of the Philadelphia chromosome (often written as Ph+ or Ph¹), seen in approximately 25% of cases.³⁸ The Ph+ usually arises from a reciprocal translocation t(9;22)(q34;q11.2) and results in *BCR-ABL1* fusion, as discussed in the section on CML. High hyperdiploidy can be seen in leukemias with or without the Philadelphia chromosome and is a good prognostic indicator.

Non-Hodgkin Lymphomas (NHL)

Follicular Lymphoma and Diffuse Large B-Cell Lymphomas with t(14;18)

Follicular lymphoma is a common NHL, seen in 35% of adult cases but is very rare in children in whom it has a better prognosis.³⁹ The most common cytogenetic abnormality in adult follicular lymphoma is the t(14;18)(q32;q21), in which the *BCL2* gene is under the expression control of the *IGH* promoter on 14q32. This translocation is seen in 75–90% of the cases. However, it is also seen in 30% of DLCL and thus is not diagnostic. Little is known about the cytogenetic abnormalities in childhood follicular lymphoma.

t(14,18)(q32;q21) Follicular and Diffuse Large B-Cell Lymphomas

This translocation is commonly seen in 80–90% of follicular lymphomas and in 30% of diffuse B-cell lymphomas. This translocation involves the juxtaposition of the *BCL2* gene on 18q21 and the *IGH* promoter on 14q32. Related translocations are seen involving the *IGV* locus on 22q11.2 and the *IGK* locus on 2p12. A dual-color, dual fusion FISH probe can be used to detect the common translocation. When there is an *IGH-BCL2* translocation, there are two fusion signals representing the two derivative chromosomes, and one red and one green signal, representing the normal chromosomes 14 and 18. If a variant translocation occurs, involving *BCL2* instead of fusion signals, there will be three *BCL2* signals representing the break within *BCL2* with an unknown partner. Although trisomy 18 is uncommon, identification of the signals on metaphase chromosomes or alternative probes must be used to differentiate between trisomy 18 and a translocation variant.

Mantle Cell Lymphoma with t(11;14)

Mantle cell lymphoma (MCL) is an aggressive B-cell malignancy seen in 3–10% of NHL and often characterized by the t(11;14)(q13;q32), found in 50–70% of cases.³⁹ The genes involved are *BCL1* (cyclin D1) on 11q13 and the *IGH* locus on 14q32. The critical event lies in the translocation of *BCL1* to 14q32, leading to over-expression of *BCL1* due to the *IGH* enhancer. This translocation is usually seen in combination

with other anomalies, often as a complex karyotype, and less frequently in other lymphoid malignancies.

FISH for t(11;14)(q13;q32) Mantle Cell Lymphoma

The translocation can be identified in virtually all cases by interphase FISH. In mantle cell lymphoma, other abnormalities are seen in up to 80% of cases,⁴⁰ which makes conventional cytogenetics a better choice. However, if cytogenetics fails to detect an abnormality, dual-color, dual fusion FISH studies of interphase nuclei are warranted. FISH for other common abnormalities seen in mantle cell lymphoma include 17p13.1 (*TP53*, 40%), 13q14.3 (D13S319, 40%), D12Z1 (chromosome 12 centromere), 6q21 and 11q23 (*MLL*, 25%).⁴⁰

Burkitt Lymphoma with t(8;14) and Other Burkitt Translocations

Burkitt's lymphoma (BL) is a B-cell lymphoma characterized by chromosomal translocations involving the *MYC* gene, the most common of which is the t(8;14)(q24;q32) translocation observed in approximately 80% of patients. The other common translocations seen in BL are t(2;8)(p11.2;q24) placing *MYC* under the influence of the immunoglobulin kappa light chain on 2p11.2 or the t(8;22)(q24;q11.2) placing *MYC* under the influence of the lambda light chain on 22q11.2. In all 3 instances, the result is deregulation of *MYC* expression. Other abnormalities seen include trisomy for the long arms of chromosome 1, 7, and 12, and deletion of 17p13⁴¹ (inclusive of the *TP53* locus), and abnormalities of band 13q34. These additional abnormalities are thought to portend a poor prognosis⁴¹ and may be associated with tumor progression, especially in adults.

FISH for t(8;14) and Other Burkitt Translocations

Burkitt lymphoma is predominantly characterized by translocations involving the c-myc (*MYC*) gene on 8q24 and *IGH* on 14q32. The most common translocation is t(8;14)(q24;q32) seen in 60–70% of cases. However, two variant translocations are also seen, involving the *IGL* on 22q11.2, t(8;22)(q24;q11) observed in 10–15% of cases, and the *IGK* locus on 2p12 t(2;8)(p12;q24) seen in 2–5% of cases. Interphase FISH may be performed using either a break-apart FISH probe that will identify all three translocations, or, for enumeration, a tricolored, dual-fusion probe that is inclusive of the centromeric region of chromosome 8, D8Z2. The break-apart probe can identify a partner only if metaphase cells are available for study.

Anaplastic Large Cell Lymphoma

Anaplastic large cell lymphoma (ALCL) is associated with the t(2;5)(p23;q35) translocation. This balanced translocation leads to a novel fusion protein consisting of the nucleophosmin gene (*NPM*) on chromosome 5q35 joined with the

anaplastic lymphoma kinase (*ALK*) on chromosome 2. There are several variant translocation seen involving the *ALK* gene.

FISH for ALK Rearrangements

In ALCL, the common translocation is t(2;5)(p23;q35). However, there are several variant translocations seen, including t(1;2)(q25;p23), t(2;3)(p23;q35), and an inversion of chromosome 2. Thus, the FISH probe design used is a break-apart probe, such that any rearrangements involving the *ALK* locus can be identified. Metaphase cells are necessary for identification of the partner chromosome in a positive interphase FISH.

Splenic Marginal Zone Lymphoma

Deletions of the long arm of chromosome 7q21-q32 have been described in approximately 40% of cases.³⁹ Trisomy 3 is seen in up to 17% of cases, and *BCL2* rearrangements, specifically t(14;18)(q32;q21) may be seen.

Mucosa-Associated Lymphoid Tissue Lymphoma (MALT)

There are several cytogenetic abnormalities commonly associated with MALT, including trisomy 3, in 60% of cases, and t(11;18)(q21;q21), in 25–50% of cases.

Multiple Myeloma (Plasma cell myeloma)

Chromosome abnormalities are among the most important prognostic parameters for patients with multiple myeloma (MM). Conventional cytogenetic studies have been hampered by the slow growth of MM cells in culture, and chromosomal abnormalities are often missed by this technique, with 50–70% of cases appearing cytogenetically normal.^{42–44}

Numerical abnormalities are the most commonly identified in MM by conventional cytogenetics. They are broken into the hyperdiploid (47–74 chromosomes) and non-hyperdiploid.^{45,46} Hyperdiploidy in MM is characterized by specific trisomies for chromosomes 6, 9, and 17 and is a good prognostic indicator seen in 45% of patients. The non-hyperdiploid group is composed of numerical and structural abnormalities, and groups hypodiploid (44–45 chromosomes or less), pseudodiploid (45–47 chromosomes), and near-tetraploid (greater than 75 chromosomes) karyotypes.

The non-hyperdiploid group is characterized by a high prevalence of IgH translocations (>85%), whereas such translocations are less common in the hyperdiploid MM (<30%). There are several translocations that are commonly seen in non-hyperdiploid MM that involve the *IGH* locus on 14q32 and lead to the up-regulation of oncogenes. The most common include the cyclin D1 gene (*CCDN1*) at 11q13 and the *MMSET* (and in some cases *FGFR3*) genes at 4p16, both of which are seen in 15% of cases. Translocations involving

the 16q23 and 6p21 loci leading to over-expression of *c-maf* and cyclin D3 are seen in 6% and 4% of cases, respectively. The t(4;14)(p16.3;q32) and t(14;16)(q32;q23) are associated with a very poor prognosis.^{47,48} Being cryptic, they must be detected by FISH.

Deletions of 13q14.3 and monosomy 13 are common abnormalities seen in MM and are more prevalent in non-hyperdiploid cases, with monosomy 13 being the most common (85%), and are associated with an adverse prognosis. Chromosome 13 abnormalities are seen in the same cells as t(4;14)(p16.3;q32) in approximately 90% of cases.⁴⁹

FISH Studies for Multiple Myeloma

Identification of abnormal cells by interphase FISH is often preferred in MM, since division of the abnormal cells is unnecessary. The interphase FISH can be used to detect the most common genomic abnormalities, including deletion of 13q14.3 or monosomy 13, deletion of *TP53*, and translocations involving *IGH*, including t(4;14)(p16;q32), t(14;16)(q32;q23), and t(11;14)(q13;q32).⁴⁹ Interphase cytogenetic panels are often employed, since abnormalities correlate well with prognosis.⁵⁰ The t(4;14)(p16.3;q32) is karyotypically cryptic and is seen by interphase FISH in 15–20% of MM cases. The t(14;16)(q32;q23) is detectable in 2–10% of patients with MM by FISH.

The most common chromosome 13 abnormality in MM is monosomy 13, so when two signals are seen, this is often interpreted as a normal result. However, most near tetraploid karyotypes will have lost two copies of chromosome 13 and therefore give an apparently normal result, so interpretation of the chromosome 13 probe should be done cautiously.

Deletions involving 17p13.1 inclusive of the p53 locus are seen in about 10% of MM cases and portend a poor prognosis.⁵¹ Use of this probe can identify either a deletion of 17p13.1 or trisomy 17 associated with hyperdiploidy. The rec-

ommended FISH panel for MM includes a 4 probe approach including *IGH/CCND1* to identify the t(11;14), D13S319 or *RBI* (deletion 13q14.3), IgH break-apart probe, and p53. If the IgH break-apart probe is positive, identification of the translocation partner should be performed to determine whether a high risk translocation is present [t(4;14) or t(14;16)].

Spectral Karyotyping

Spectral Karyotyping (SKY) is a fluorescent molecular cytogenetic technique in which all the chromosomes can be simultaneously visualized, each in a different color, in a single experiment.⁵² Through the use of combinatorial fluorescent nucleotide incorporation each chromosome probe generates a different spectral pattern. These probes are hybridized onto metaphase spreads and computer software generates pseudo-colors for each spectral pattern, with each chromosome having a unique color signature. This method allows for identification of marker chromosomes and additional material of unknown origin, in addition to rearrangements.

Spectral karyotyping is not widely available, and in most settings is only performed on a research basis. Figure 9.8a demonstrates the partial karyotype with a marker chromosome, chromosome 11 pairs, and a derivative X chromosome. Figure 9.8b demonstrates the pseudo-color image identifying the marker chromosome as derived from chromosome 1, and detecting a balanced translocation that was unidentifiable cytogenetically.

DNA Microarray

There are multiple types of arrays currently available for the analysis of DNA copy number, mostly falling into either comparative genomic hybridization (CGH) or

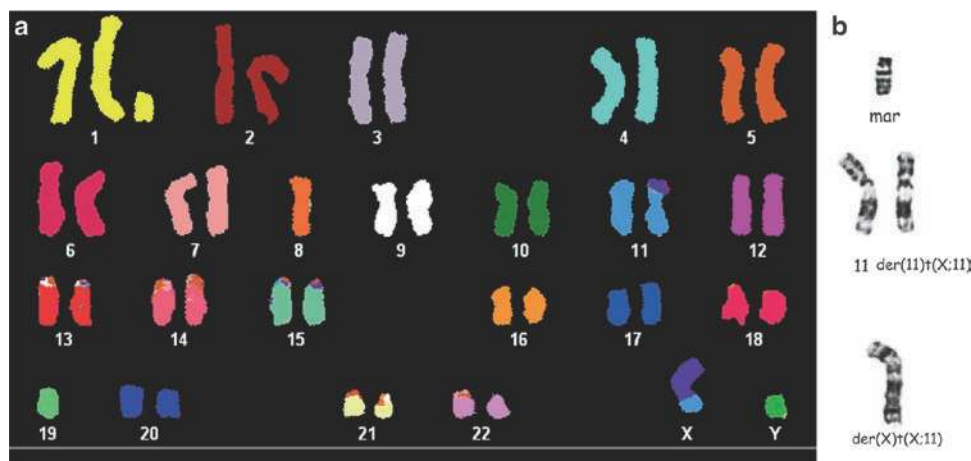


FIG. 9.8. Bone marrow from a 3 year old male with a diagnosis of bilineage leukemia. (a) Chromosome analysis revealed a karyotype of 47,XY,+mar. (b) SKY analysis revealed the origin of the marker to be entirely from chromosome 1 and also identified an

apparently balanced translocation between chromosomes X and 11 that had gone undetected in the G-band analysis. (Courtesy of Dr. Constance Griffin and the Molecular Pathology Cytogenetics Laboratory, John Hopkins University, Baltimore.)

oligonucleotide arrays. The oligonucleotide arrays may be further broken into those that contain single nucleotide polymorphisms (SNP) and those that do not.

Currently most arrays are being used to detect constitutional abnormalities, but array based technology for hematolymphoid malignancies and solid tumor analysis is beginning to be utilized for diagnostic and prognostic purposes.

Comparative genomic hybridization (CGH) is a molecular cytogenetic technique that allows detection of copy number changes throughout the genome in a single hybridization.⁵³ Essentially this is a giant FISH experiment in which patient DNA is extracted and labeled in one color, and a reference (normal) DNA is labeled in a different color. The patient and reference DNAs are co-hybridized to a slide containing known pieces of human DNA, either from bacterial artificial chromosomes (BACs) or oligonucleotides. Unbound pieces are washed off the slide and the image is captured and analyzed. If equal amounts of patient and reference DNA are present at a given spot, there is a 1:1 ratio. If a duplication occurs in the patient (trisomy), there is a 3:2 ratio. If there is a deletion in the patient (monosomy), there is a 1:2 ratio. Figure 9.9 shows the CGH profile of a patient with pre B-ALL and hyperdiploidy.

SNP arrays were initially developed for genotyping purposes and generally contain greater than 500,000 SNPs. Clinically, these arrays may yield both genotype and copy number (intensity) information.⁵⁴ The genotyping data indicates the presence of either an A or B allele, from a known SNP. This technique can thus identify loss of heterozygosity, as seen in many cancers. Intensity information is generated by comparison of the patient DNA to a pool of controls, with a brighter signal representing increased copy number than controls and a dimmer signal representing a decreased copy number than in controls. When intensity shows normal copy number, but there are no heterozygous SNPs this indicates loss of heterozygosity (LOH).

Polymerase Chain Reaction

Increased understanding of the molecular events involved in hematolymphoid malignancies has both advanced the knowledge of pathogenesis and led to increased ability to diagnose, and in some cases, treat disorders by targeting the specific molecular events. Detection of specific molecular translocations associated with different subsets of leukemia has become a defining feature of certain leukemias, for example *BCR-ABL1* in CML and *PML-RAR α* (alpha) in acute promyelocytic leukemia. PCR detection of *BCR-ABL1* and *PML-RAR α* (alpha) fusion products has pioneered the use of molecular methods to detect translocations for diagnosis and to monitor response to therapy. The examples listed below are illustrative of some of the major important concepts regarding PCR testing for translocations; other details of the significance of detecting translocations associated with specific subtypes of hematolymphoid neoplasms may be found in the chapter for that subtype.

PCR is used routinely for diagnosis and monitoring of several hematolymphoid malignancies that have a defining molecular translocation. PCR is more sensitive than routine cytogenetics or FISH analysis. The sensitivity of PCR exceeds 0.1% and real-time fluorescent PCR techniques can typically detect 1 malignant cell in a background of 10^4 to 10^6 normal cells. PCR is useful for diagnosis, detection of minimal residual disease, and monitoring response to therapy by real-time quantitative PCR (qPCR).

Chromosomal translocations may be detected by PCR if the DNA sequence of the genes involved in the translocation is known. PCR primers specific to the two juxtaposed genes are designed to span the translocation breakpoints, which results in production of a specific PCR product only if the translocation is present. Primers must be designed that are

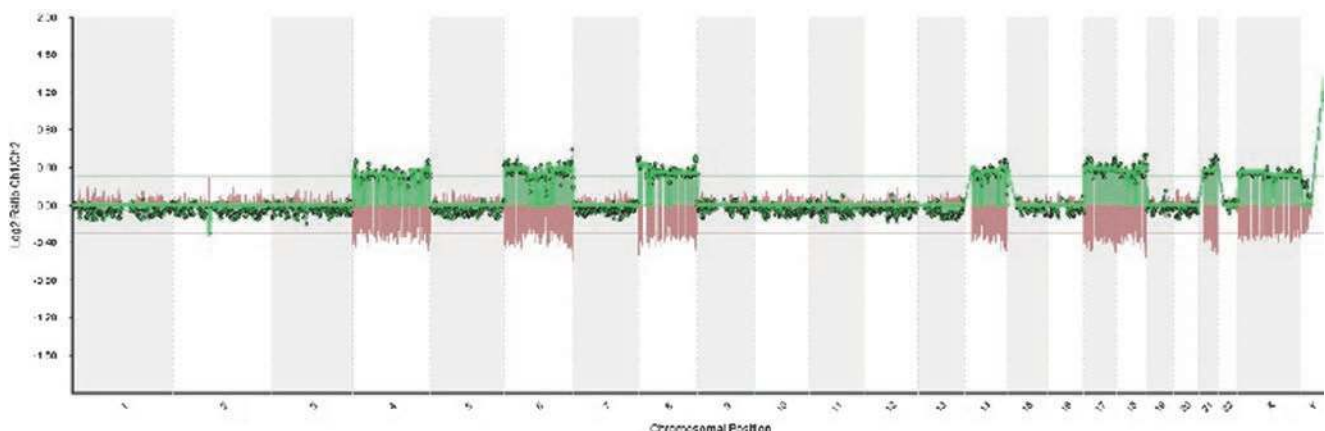


FIG. 9.9. Array CGH analysis of a patient with pre B-ALL. DNA was extracted from the residual bone marrow and run on the Blue Gnome CytoChip using CGH. The ratio of patient DNA to reference DNA is plotted using dye-swap methodology, seen by

mirror images in the plot. CGH analysis confirmed this patient was a high-hyperdiploid with trisomy for chromosomes 4, 6, 8, 14, 17, 18, 21, and X, with no other deletions or duplications. (Courtesy of BlueGnome Ltd, Cambridge, UK.)

specific to each breakpoint of interest, so prior knowledge of the translocation breakpoints is necessary. PCR and FISH are complementary techniques and often either or both may be used for diagnosis and to monitor response to therapy. The advantages of PCR are greater sensitivity and the ability to detect rare cryptic translocations that do not involve a large enough DNA region to be detected by FISH. The advantages of FISH are detection of gene amplification and detection of translocations that occur outside the typical breakpoints. PCR techniques will only detect the specific translocation breakpoints interrogated by specific primers, whereas the comparatively larger DNA regions interrogated by FISH probes are more forgiving of alternative breakpoints. Both PCR and FISH require prior knowledge of the genes or chromosomal regions of interest and the use of specific primers or probes.

Reverse Transcriptase PCR (RT-PCR)

In many cases, reverse transcriptase PCR (RT-PCR) is used, in which mRNA is used as the starting material instead of DNA. The mRNA is converted to complementary DNA (cDNA) using reverse transcriptase and the cDNA is then used as the PCR template. One of the advantages of RT-PCR is that it allows for PCR amplification across large breakpoint regions within introns (Figure 9.10). One of the limitations of PCR

is that amplification of more than 1 kilobase is not as efficient as amplification of shorter DNA regions. Use of mRNA as starting material, in which the introns are spliced out, brings exons of genes involved in the translocation closer together and more readily amplified. The major disadvantage of RNA based assays is that RNA is labile and subject to degradation by ubiquitous RNases, so controls of RNA quality must be incorporated and appropriate sample collection and storage is of paramount importance.

Real-Time Quantitative PCR

Real-time quantitative PCR (qPCR or Q-PCR) is a technical advance that has revolutionized monitoring of disease and response to therapy in patients with translocations. Incorporation of a fluorescent molecule into the PCR product, typically with use of a fluorescent probe specific to the gene of interest, both increases the sensitivity of PCR and allows for quantitation. Detection of fluorescence during the early cycles of PCR amplification allows for real-time measurement of the amount of PCR product generated during the log-linear phase of PCR, during which the increase in PCR product is proportional to the amount of starting template DNA. The amount of starting nucleic acid (DNA or RNA) can be quantified by comparison to a standard curve or to an endogenous gene or transcript (Figure 9.11). Real-time qPCR techniques have a broad linear range exceeding five orders of magnitude. The precision of qPCR is sufficient for clinical applications. Since each PCR cycle results in a doubling of DNA molecules, a measured twofold quantitative difference is due to only a one-cycle difference and is within the variability of most real-time PCR assays. Typically, five to ten-fold quantitative differences can be measured precisely by qPCR.

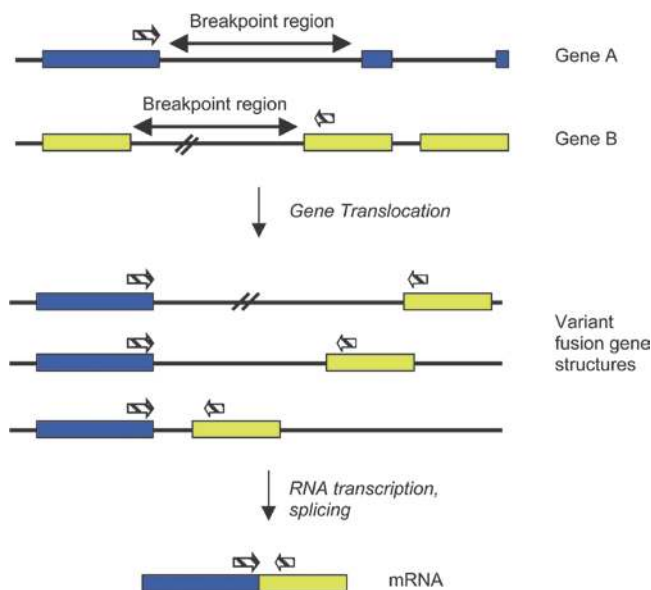


FIG. 9.10. RT-PCR amplification across large translocation breakpoint regions. The top panel is a schematic of the genomic DNA structure of two genes involved in translocation. Variant translocation breakpoints within large intronic regions of DNA can result in various gene fusion products (*middle panel*). RNA transcription and splicing results in a fusion coding mRNA with introns removed (*bottom panel*). This allows for PCR amplification using a single set of primers (*block arrows*) to detect all the variant fusion genes, which encode similar RNA. Coding exons are represented by boxes and noncoding introns by connecting lines.

BCR-ABL1 [t(9;22)]

RT-PCR is routinely used for detection of the *BCR-ABL1* fusion gene associated with t(9;22) in CML and Philadelphia chromosome (Ph¹)-positive ALL. Virtually all cases of CML have the *BCR-ABL1* gene fusion, which results in production of an aberrant BCR-ABL protein with constitutive tyrosine kinase activity. Cases of CML that are Philadelphia chromosome negative by cytogenetics may have a cryptic translocation resulting in fusion of the *BCR* and *ABL1* (*c-abl*) genes that can be detected by RT-PCR. Patients with Ph¹ negative CML who have *BCR-ABL1* fusion at the typical molecular breakpoints are clinically and morphologically indistinguishable from those with Ph¹ positive CML.⁵⁵

Several different translocation breakpoints in the *BCR* gene have been defined (Figure 9.12). Translocation at the major breakpoint cluster region (M-bcr) of the *BCR* gene results in a BCR-ABL1 fusion protein of 210 kDa (p210) and is associated with most cases of CML and ~30% of Ph¹ positive ALL. Translocation at the *BCR* minor breakpoint

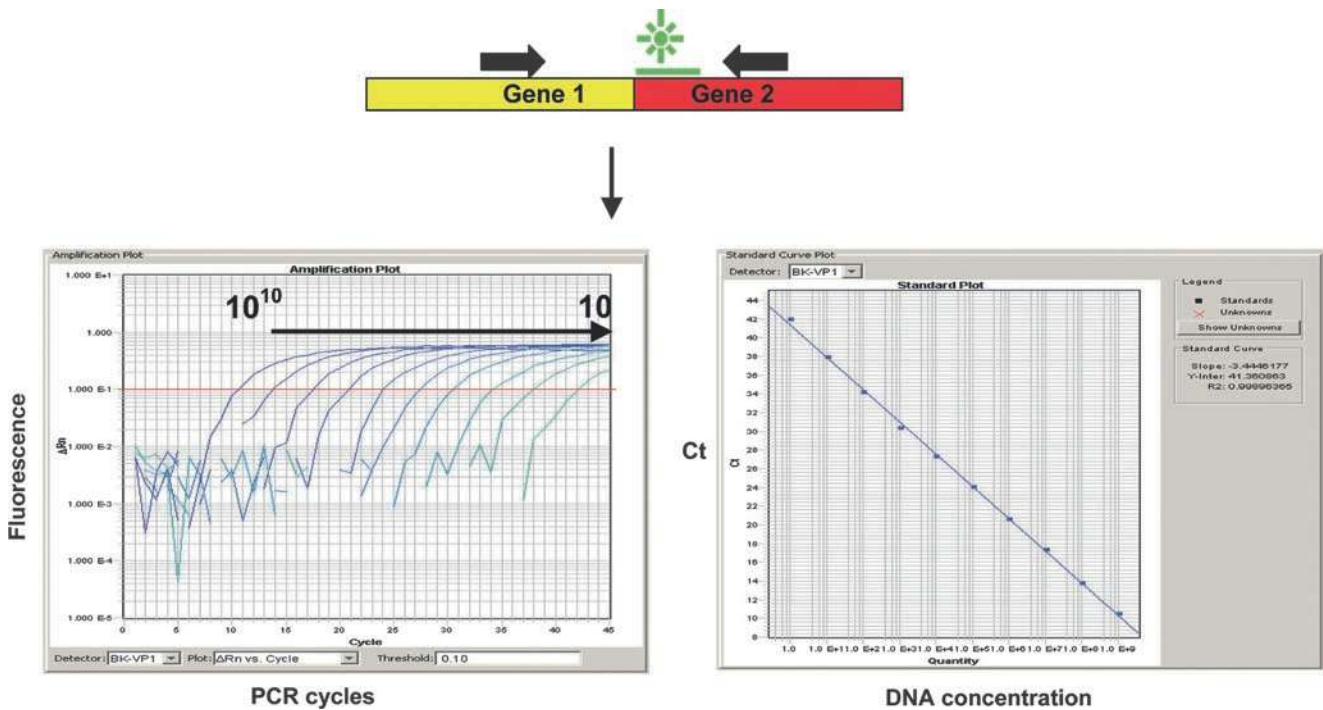


FIG. 9.11. Real-time quantitative PCR. Shown is an example of real-time qPCR using a fluorescent probe. At the top is a schematic showing the PCR primers (block arrows) and fluorescent probe (green bar) specific to the translocated genes of interest. On the bottom left is an example of amplification of a serial dilution of

standard controls ranging from 10 to 10^{10} DNA copies showing the amount of fluorescence produced during increasing cycles of PCR. The red line depicts the crossing threshold (Ct). On the bottom right is the standard curve generated from the Ct of the standard controls.

cluster region (m-bcr) results in a BCR-ABL1 fusion protein of 190 kDa (p190) and is associated with ~70% of Ph¹ positive ALL.⁵⁶ The relative distribution of M-bcr and m-BCR differs in adult versus pediatric ALL. The m-bcr is seen in ~80% of childhood Ph1- positive ALL but only ~50% of adult ALL, with the remainder utilizing the M-bcr.⁵⁷ An alternative downstream breakpoint, called the μ (micro)-BCR, results in a 230 kDa fusion protein (p230) and is associated with a neutrophilic variant of CML.⁵⁸ The translocation breakpoint in the *ABL1* gene can occur over a 300 kb region spanning from exon 1b to exon 2.^{57,59} During mRNA transcription, *ABL1* exons 1b and 1a are spliced out, resulting in a chimeric BCR-ABL1 mRNA with fusion of a portion of the *BCR* gene to exon 2 of the *ABL1* gene.

Use of RT-PCR allows for detection of all the *ABL1* breakpoints using a downstream primer homologous to *ABL1* exon 2. A different upstream PCR primer is required for detection of each of the variant *BCR* breakpoint regions (Figure 9.12). It is possible to design a multiplex PCR assay with multiple primers that can detect the presence of more than one breakpoint in a single reaction. However, most RT-PCR assays in widespread use in clinical laboratories are designed to detect either the M-bcr or m-bcr and will not detect the μ (mu)-BCR. As with other PCR-based translocation assays, testing requires knowledge of the breakpoint of interest prior to analysis. For example, in a patient with newly diagnosed

ALL, RT-PCR assays for both the major and minor breakpoints should be performed, since either breakpoint can be seen in ALL. Once the specific breakpoint associated with a patient's disease is identified, RT-PCR can be performed to follow response to therapy and for detection of minimal residual disease in that patient. It is important to document RT-PCR positivity prior to using RT-PCR to follow a particular patient in order to ensure the correct assay is used for disease monitoring and to establish a baseline quantitative result. Rare alternative breakpoints in either *BCR* or *ABL1* that fall outside the regions amplified will not be detected by PCR.

Real-time quantitative RT-PCR (qRT-PCR or RQ-PCR) has become standard practice for monitoring of disease and response to therapy in patients with BCR-ABL1 translocation. Serial qRT-PCR testing has clinical utility for monitoring response to therapy, prediction of relapse, and detection of resistance to targeted therapies such as Gleevec (imatinib mesylate) (see Chap. 30). It is now possible to measure molecular remission, defined as RT-PCR negativity. However, due to the exquisite sensitivity of PCR, it is possible to detect very low levels of BCR-ABL1 fusion which may not be clinically relevant. Low levels of BCR-ABL1 fusion RNA detectable by RT-PCR have been described in normal individuals, particularly in elderly individuals.^{60,61} This has been described primarily using

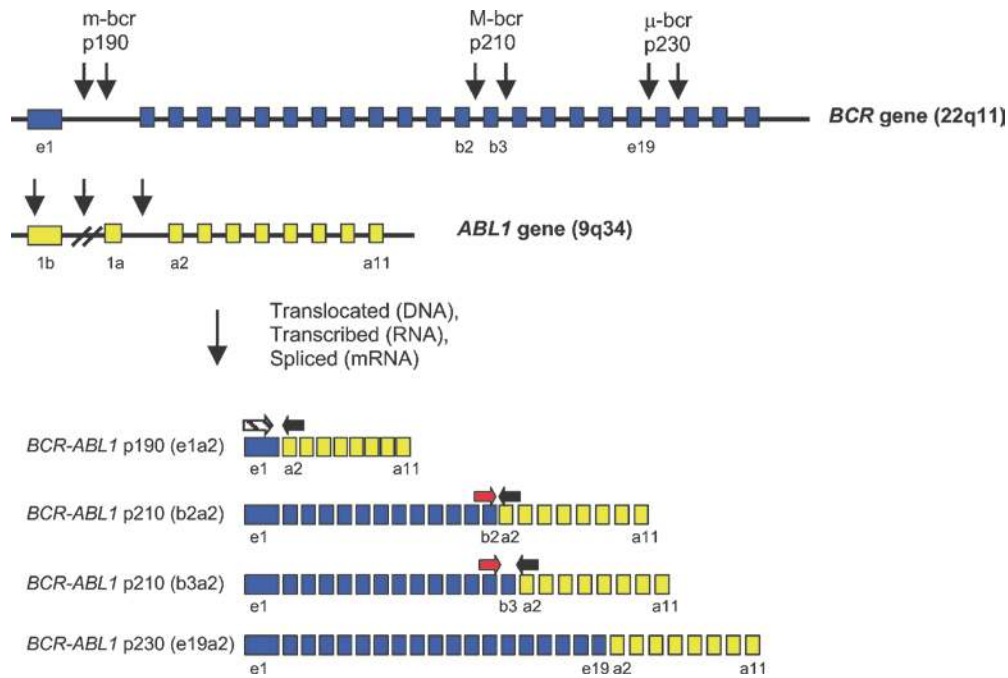


FIG. 9.12. *BCR-ABL1* RT-PCR. Shown in the top panel are the genomic DNA structures of the *BCR* and *ABL1* genes with relevant exons numbered. The location of the typical translocation breakpoints are depicted by vertical arrows. Exons are depicted as boxes and introns as horizontal lines. Shown in the bottom panel are the mRNA structures of the various translocated *BCR-ABL1* fusion gene products. The different PCR primer locations used to detect the major (p210) and minor (p190) breakpoint RNAs are shown by horizontal block arrows. Translocation at the *BCR* minor breakpoint cluster region (m-bcr) results in an mRNA transcript

with the *BCR* exon e1 fused to *ABL1* exon a2, encoding a 190 kDa protein. Translocation at the *BCR* major breakpoint cluster region (M-bcr) results in a longer mRNA transcript with either the *BCR* exon b2 (e13) or b3 (e14) fused to *ABL1* exon a2, encoding for a 210 kDa protein. Both the b2a2 and b3a2 major breakpoint RNA products can be detected with the same PCR primer pair, resulting in different sized PCR products. Translocation at the alternative micro breakpoint (μ (mu)-bcr) results in an mRNA transcript with the *BCR* e19 exon fused to the *abl* a2 exon, which encodes a 230 kDa protein.

very sensitive nested PCR techniques and is not common with real-time quantitative RT-PCR, using a single set of primers. However, the possibility of false positive results in normal patients must be kept in mind when interpreting PCR results. Because of differences in the sensitivity of RT-PCR assays and the lack of standardization between different labs, serial quantitative RT-PCR monitoring is of greater clinical value than a single negative or positive quantitative result.⁶² Efforts to standardize *BCR-ABL1* qRT-PCR assays so that values can be compared between different laboratories are underway.⁶³

PML-RARA [t(15;17)]

RT-PCR to detect fusion of the promyelocytic leukemia gene (*PML*) on chromosome 15q22 and the retinoic acid receptor alpha gene (*RAR α*) on chromosome 17q21 associated with acute promyelocytic leukemia (AML M3) is useful for diagnosis, to predict response to all-trans retinoic acid (ATRA), and to monitor response to therapy by qRT-PCR (see Chap. 34). Three common translocation breakpoints occur within the *PML* gene, resulting in the formation of three major

forms of the *PML-RAR α* (α) fusion gene (Figure 9.13). The breakpoint in the *RAR α* (α) gene almost always occurs within intron 2 upstream of *RAR α* (α) exon 3. RT-PCR assays to detect the different RNA forms typically utilize a downstream primer within exon 3 of the *RAR α* (α) gene and two different upstream primers within exons 3 and 6 of the *PML* gene, either as a multiplex or as two separate RT-PCR reactions. Use of both *PML* primers facilitates optimal detection of the three common fusion RNAs. The *PML* exon 6 primer will amplify the long (bcr1) and variant (bcr2) forms, and the exon 3 primer will amplify the short form (bcr3) (Figure 9.13). FISH probes that detect the fusion recognize all three forms of *PML-RAR α* (α) in addition to rarer breakpoints that can occur at other regions within the *PML* or *RAR α* (α) genes. RT-PCR for *PML-RAR α* (α) will not detect fusion of the *RAR α* (α) gene with variant translocation partners associated with acute promyelocytic leukemia, such as the *PLZF* gene associated with t(11;17)(q23;q21), the *NUMA* gene associated with t(11;17)(q13;q21), or the *NPM* gene associated with t(5;17)(q23;q21). Specific RT-PCR assays to detect these rarer variant translocations can be performed.

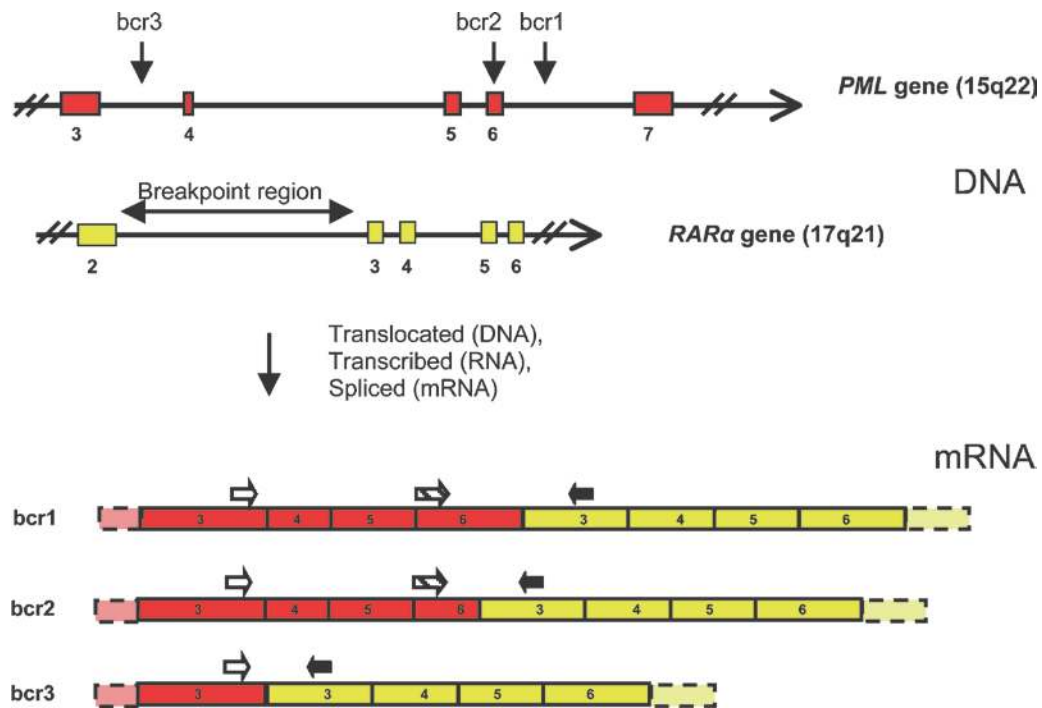


FIG. 9.13. *PML-RARα(α)* RT-PCR. Shown at the top of the figure is a schematic of the partial genomic DNA structures of the *PML* and *RARα(α)* genes indicating the relevant breakpoint regions. Exons are numbered and depicted by boxes, introns are depicted by horizontal lines. The *PML* breakpoint regions are indicated with vertical arrows (bcr1, bcr2, bcr3). The typical breakpoints in *RARα(α)* occur within intron 2 between exons 2 and 3. Shown at the bottom are the mRNA structures of the various *PML-RARα(α)* fusion gene products. PCR primers used to amplify the fusion breakpoints are indicated by horizontal block arrows. Use of a downstream PCR primer in exon 3 of *RARα(α)* and a single upstream primer within

exon 3 of *PML* can amplify all three forms of fusion RNA. However, amplification of the long (bcr1) and variant (bcr2) forms with an exon 3 *PML* primer is not as efficient as with an exon 6 *PML* primer. In addition, multiple RNA splice variants of bcr1 and bcr2 occur in vivo, resulting in multiple different sized PCR products with use of a *PML* exon 3 primer. Use of a *PML* primer within exon 6 to amplify the bcr1 and bcr2 forms results in a smaller discrete PCR product that is amplified more efficiently and can make interpretation more straightforward. Including an exon 3 *PML* primer is necessary to detect the short (bcr3) form (seen in ~50% of APL), as exon 6 is not included in the bcr3 fusion gene.

BCL2-IGH [t(14;18)]

Translocation of the *BCL2* gene from chromosome 18 to the immunoglobulin heavy chain gene (IgH) locus on chromosome 14 results in the *BCL2-IGH* fusion gene associated with follicular lymphoma. Most *BCL2* breakpoints occur in the major breakpoint cluster region (mbr) within exon 3 or minor breakpoint cluster region (mcr) further downstream.⁵⁵ Intermediate *BCL2* breakpoints can also occur. The IgH gene breakpoint is within the joining region (JH). PCR assays may be performed using an upstream primer specific for each of the *BCL2* breakpoints and a downstream JH primer. Because each of the breakpoints is located within a short segment of DNA, RT-PCR is not necessary and PCR analysis can be performed using genomic DNA as the template. A major advantage of using DNA as starting material is that it is more stable than RNA and not as susceptible to degradation.

MLL (11q23)

RT-PCR assays have been developed to detect *MLL* translocation with several different translocation partners.⁶⁴

However, because multiple translocation partners are associated with *MLL* gene translocation, use of PCR is not as practical for detection as with translocations involving two consistent genes. Southern blot or FISH analysis using a probe specific to the *MLL* gene can detect any gene rearrangement or translocation involving *MLL*, but does not identify the translocation partner gene. Southern blot analysis can be used to detect *MLL* gene involvement at diagnosis, but is not as useful to follow response to therapy or for detection of minimal residual disease because it is not quantitative or as sensitive as PCR.

Other Translocations

PCR can be used to detect any chromosomal translocation as long as the genes and breakpoint regions are known. PCR-based assays have been developed for detection of many other translocations associated with hematolymphoid disorders including *BCL1-IGH*, *FIP1L1-PDGFRα*, *AML-ETO*, *CBFB-MYH11*, *TEL-AML1 (ETV6-RUNX1)*, *E2A-PBX1*, and *NPM1-ALK*. The College of American Pathologists (CAP) proficiency testing (PT) program includes many of these

translocations as part of the molecular oncology proficiency survey, indicating their growing use in clinical molecular laboratories. Identification of the presence of a translocation by molecular testing can be useful for diagnosis, to help define the molecular subtype of lymphoma or leukemia, and in some cases as a prognostic indicator. Quantitative PCR to monitor response to therapy is not frequently used for these other translocations. However, this may change as some of the genes involved in translocations become molecular targets of therapy.

Immunohistochemistry

Immunohistochemistry or IHC refers to the process of localizing proteins in cells of a tissue section, exploiting the principle of antibodies binding specifically to antigens in biological tissues. Such IHC applications in the diagnosis of hematolymphoid malignancies are described in the following sections.

Acute Myeloid Leukemias

PAX 5 [t(8;21)]

As previously mentioned, AML with t(8;21)(q22;q22) is most often classified as AML with maturation (AML-M2), according to the FAB system. Immunophenotypically, the blasts express myeloid antigens.^{65–70} However, the B-cell antigen, CD19, is reported to be positive in 50–80% of cases. The biologic significance of CD19 expression by the blasts in these tumors has been considered unknown, possibly representing merely an isolated aberrant marker, or signifying evidence of persistent B-cell differentiation. To this end, it has been demonstrated by immunohistochemical (IHC) methods, and confirmed by Western blot methods, that B cell-specific activator protein (BSAP) is expressed in every case of AML with t(8;21).⁷¹ BSAP has an important role in B-cell development and lineage commitment by targeting the activation of B cell-related genes, including CD19 and CD79a. BSAP expression has been detected by IHC evaluation for PAX5, a member of the paired domain family of transcription factor genes that encodes for BSAP.⁷² Valbuena et al also showed that BSAP expression is limited largely to AML with t(8;21), and its expression correlated with the level of CD19 in these cases. In most cases, however, the staining intensity is weaker than that of the mature B cells. In addition, rare cases of AML-M0 (CD19–) and precursor T-cell lymphoblastic leukemia (CD19+) have also demonstrated expression of BSAP. However, in the context of morphology and other IHC markers (i.e., TdT, MPO, etc.), and when flow cytometric analysis and cytogenetic analysis are not available, PAX5 may serve as a reliable surrogate marker for the t(8;21) in AML.

CBFβ(beta)-SMMHC Protein [inv(16)(p13q22)]

As previously mentioned, the inv(16)(p13q22) or, less commonly the t(16;16)(p13;q22), is characteristic of AML with abnormal bone marrow eosinophils, also known as AML-M4Eo.^{73–76} Although detection of this abnormality is most commonly done by cytogenetic or molecular methods, a recent study by Zhao et al determined the utility of IHC and immunofluorescence (IF) methods using a rabbit polyclonal antibody (AH107) against the C-terminus of the CBFβ-SMMHC chimeric protein for a diagnosis of AML-M4Eo.⁷⁷ Immunohistochemical analysis of routinely processed paraffin-embedded bone marrow sections showed that CBFβ-SMMHC staining is predominantly nuclear in all cases of AML-M4Eo and is not nuclear in other AML types. Indirect IF analysis of fresh bone marrow aspirate smears showed that AML-M4Eo blasts have a distinct nuclear microgranular or fine-speckled pattern of staining, with or without faint cytoplasmic staining. By contrast, other types of AML and normal bone marrow specimens were either negative or had a nonspecific pattern of staining. In summary, immunostaining for CBFβ-SMMHC using either IHC or IF analysis as described in this study reveals a distinctive pattern of staining for AML-M4Eo. This approach is a specific, reliable, and convenient alternative to cytogenetic and molecular methods for the diagnosis of AML-M4Eo and may be particularly helpful in cases with indeterminate histologic features or in cases in which cytogenetic and molecular studies are either uninformative or not available.

Non-Hodgkin Lymphomas

BCL-2 [t(14;18)]

Follicular hyperplasia may generally be distinguished from follicular lymphoma (FL) with the use of BCL-2 IHC. BCL-2 is non-reactive in reactive germinal centers and is typically strongly reactive in the malignant nodules of FL. However, it should be noted that there are rare cases of FL, which are composed of BCL-2– malignant nodules. BCL-2+ reactive germinal centers have not been described.⁷⁸

A further discussion of BCL-2 IHC is warranted at this time. Small lymphocytes show cytoplasmic staining for BCL-2 in peripheral blood, interfollicular areas, and mantle zones of lymph nodes and the thymic medulla.⁷⁹ In addition, BCL-2 has also been shown to be consistently expressed by reactive marginal zone B-cells of the spleen, abdominal lymph nodes, and ileal lymphoid tissue; thus, BCL-2 expression by IHC should not be used as a criterion for discriminating between benign and malignant marginal zone B-cell proliferations involving these sites.⁸⁰

Although expression of the BCL-2 protein is associated with the t(14;18) chromosome translocation and it is expressed on a significantly higher percentage of FLs associated with this translocation, expression of the BCL-2

oncogene protein is not specific for the t(14;18) chromosomal translocation.^{78,81,82} BCL-2 protein expression may be detected in a substantial number of B-cell as well as T-cell lymphoproliferative disorders not associated with the t(14;18).^{83,84} A study by Wheaton et al revealed BCL-2 expression in 100% of small lymphocytic lymphomas, 80% of FLs, 38% of diffuse large cell lymphomas (DLCLs), 33% of high-grade B-cell Burkitt's-like lymphomas, 0% of Burkitt's lymphomas (BLs), and 0% of B-cell lymphoblastic lymphomas.⁸³ Thus, the significant difference in BCL-2 expression between Burkitt-like high-grade BCL and BL was suggested as an additional use of BCL-2. BCL-2 positivity by IHC may rarely occur in BL and has been described when there is a coexistent t(14;18) and Burkitt's translocation. In addition, although marginal zone hyperplasias in the spleen, abdominal lymph nodes, and ileal lymphoid tissues may reveal BCL-2 expression, BCL-2 may represent a useful marker for distinguishing reactive monocytoid B-cell hyperplasia from marginal zone lymphoma in other sites. Although T-cell lymphoproliferative disorders had a significantly lower BCL-2 expression than B-cell disorders, peripheral T-cell lymphoma, including anaplastic CD30+ LCL and angioimmunoblastic-type, and lymphoblastic lymphomas may reveal expression of the BCL-2 protein.

Cyclin D1 [t(11;14)]

As eluded to previously, mantle cell lymphoma (MCL) is a distinctive naïve B-cell lymphoma with cyclin D1 over-expression being the key pathogenetic event. Using IHC techniques, cyclin D1 may be demonstrated in 70–100% of cases of MCL, with the larger series usually reporting a positivity rate >90%.^{85–92} A recent study has demonstrated a highly sensitive probe for identifying MCL (SP4 – a novel anti-cyclin D1 rabbit monoclonal antibody).⁹³ Since normal and reactive lymphoid cells as well as other small lymphoma types are consistently negative, immunoreactivity for cyclin D1 may serve as a convenient defining marker for MCL.⁹⁴

The main applications of cyclin D1 antibody by IHC in diagnostic hematopathology include: (1) classification of small B-cell lymphomas, a common diagnostic problem resulting from overlapping morphological features. [Mantle cell lymphoma is important to recognize because of its very poor response to therapy and unfavorable prognosis. In fact, it has the worst 5-year failure-free survival (11%) among all lymphoma types according to the Non-Hodgkin's Lymphoma Classification Project study],⁹⁵ (2) exclusion of MCL in lymphoid proliferations predominated by small cells, and (3) ascertaining the presence of subtle involvement in staging biopsies or in diagnosing early relapse in patients with known MCL.

C-myc (Burkitt translocations)

C-myc encodes two nuclear phosphoproteins, of which p62 constitutes its main protein product.⁹⁶ The detection of c-myc

expression in various tissues may be performed by means of IHC, which is of value in studying the c-myc p62 distribution in neoplastic and reactive cells. C-myc over-expression may reflect either a structural genetic abnormality or merely an increased rate of cell proliferation (i.e., it may be a consequence rather than a cause of tumorigenesis).⁹⁷

The detection of c-myc by IHC may be considered in distinguishing Burkitt lymphoma (BL) and diffuse large B-cell lymphoma (DLBCL). BL and DLBCL usually may be distinguished easily at the morphologic level.⁹⁸ However, there is a subset of cases with a spectrum of morphologic features that are intermediate between BL and DLBCL (i.e., B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL). It is this latter group that can cause diagnostic difficulty, especially in small biopsy specimens showing artifactual distortion, and these cases often require additional studies for an accurate diagnosis. Various studies have suggested that there are immunophenotypic differences that might aid in the distinction of these entities, and shown that the IHC expressions of 6 proteins (c-myc, CD10, bcl-6, bcl-2, CD138, and MIB-1) are useful in this differential diagnosis.^{98–102} In a study by Frost et al, expression of c-myc differed significantly between BL and DLBCL with 30 (91%) of 33 cases of BL showing staining in the majority of cells (≥50%), while only 2 (10%) of 20 DLBCL cases showed a similar degree of staining ($P < 0.0001$). Absent or minimal staining for c-myc (≤25% of tumor cells stained) was seen in only 3 cases of BL (9%), but this low level of staining was noted in the majority of DLBCL cases (11/20 [55%]). However, substantial overlap in the expression of c-myc was noted between BL and DLBCL, severely limiting its use as a single discriminatory marker.

As alluded to previously, the differential diagnosis of BL includes DLBCL with morphological high-grade features, including those with a c-myc rearrangement. Ki-67 (MIB-1) has been claimed as a useful marker by paraffin IHC to distinguish BL from DLBCL with morphological high-grade features. The MIB-1 index should be greater than 98% and not less than 95% in BL. In contrast, the MIB-1 index in DLBCL with c-myc rearrangement ranges between 48 and 90% (mean, 66%); a MIB-1 index greater than 95% was not observed in this group. Another immunophenotypic difference is the presence of BCL-2 in a higher percentage (75%) of these cases.¹⁰³

Akasaka et al recently explored molecular features of DLBCL with c-myc/IGH fusion and the impact of this genetic abnormality on clinical outcome of DLBCL. They found that the c-myc/IGH fusion gene of DLCL is identical to that of the sporadic type of BL. In addition, DLBCL with c-myc/IGH also shares clinical features with BL, but is characterized further by an older age distribution.¹⁰⁴ C-myc expression has also been analyzed in primary central nervous system (CNS) DLBCL, among immunocompetent individuals. In a study by Chang et al, expression of p53, c-myc, or bcl-6 correlated with poorer overall survival (p53, c-myc,

and bcl-6) and an increased mortality rate (p53, c-myc, and bcl-6).¹⁰⁵ In addition, this same group of authors reported expression of p53 or c-myc in non-CNS DLBCL correlated with an adverse clinical outcome.¹⁰⁶

ALK-1 [t(2;5)]

As mentioned previously, ALCL is commonly associated with the t(2;5)(p23;q35). The 2;5 translocation results in the expression of a chimeric 80-kD protein, NPM-ALK or p80, consisting of the N-terminal portion of NPM (comprising 40% of the molecule) linked to the entire intracellular portion of ALK.¹⁰⁷ IHC staining for ALK-1 is highly sensitive and the most informative technique for detecting 2p23 rearrangements associated with a deregulation of the ALK gene.¹⁰⁸ In fact, although ALK-1+ cases by immunostaining and negativity for the NPM-ALK fusion gene by RT-PCR have been observed (likely due to a cryptic or variant translocation involving 2p23), the reverse pattern (RT-PCR positive, ALK-1 negative immunostaining) has not been observed. ALK-1 positivity by immunostaining does not equate solely with the presence of the t(2;5), indicating that a chromosomal abnormality involving the ALK gene on 2p23 with no simultaneous involvement of the NPM gene at 5q35 may also be pathogenetic in, and diagnostic of, this lymphoma. These variant or cryptic translocations result in differences in ALK-1 immunostaining patterns. In the majority of cases, ALK-1 immunostaining demonstrates both a cytoplasmic and nuclear staining pattern; however, in cryptic or variant translocations involving 2p23, ALK-1 staining is usually restricted to the cytoplasm.¹⁰⁵ The cytoplasmic and nuclear staining with ALK-1 associated with the t(2;5) appears to be due to oligomer formation with wild-type NPM and subsequent transport from the cytoplasm to the nucleus directed by nuclear localization signals in the NPM molecule.¹⁰⁹

The main applications of ALK-1 antibody in diagnostic hematopathology include:

- Recognition of ALK-1+, CD30+ ALCL which may be morphologically difficult and present as small cell, lymphohistiocytic, giant-cell, Hodgkin-like, and sarcomatoid variants,¹¹⁰
- differentiation from classical Hodgkin lymphoma (cHL) (ALK-1 is not expressed in cHL¹¹¹),
- prediction of prognosis in systemic ALCL and primary ALCL of the central nervous system (ALK-1 positivity is associated with younger age and more favorable prognosis^{112–115}),
- distinguishing primary cutaneous from systemic ALCL (the primary cutaneous form of ALCL is characteristically ALK-negative and has a more favorable prognosis than systemic ALCL; since systemic ALCL may be ALK– and primary cutaneous ALCL is characteristically ALK+, there may be occasional cases in which the distinction of these two forms is unclear), and
- staging of bone marrows for involvement by ALK-1+, CD30+ ALCL, since single cells may infiltrate the bone marrow and be difficult to detect by routine histological examination.

In addition, one should be aware that ALK overexpression likely contributes to the pathogenesis of two additional otherwise unrelated neoplasms including, “ALK-positive diffuse large B-cell lymphoma” and inflammatory myofibroblastic tumor.^{116–118} These entities should be included in the differential diagnosis of ALK-1+, CD30+ ALCL, especially the sarcomatoid variant of ALCL in the latter differential diagnosis.

Multiple Myeloma

Cyclin D1

The t(11;14)(q13;q32), as discussed previously, is the hallmark of MCL, may also be recurrently found in multiple myeloma (MM). Cyclin D1 over-expression by IHC methods has thus been observed in MM, occurring in all cases bearing this translocation, but also observed in cases with other abnormalities of chromosome 11, or in the absence of any chromosome 11 abnormality, implying that upregulation of the cyclin D1 protein might be the result of mechanisms other than the t(11;14).^{119–123}

There have been varying reports in the literature the prognostic significance of cyclin D1 positivity by immunostaining in MM. Athanasiou et al correlated cyclin D1 over-expression by IHC and in-situ hybridization (ISH) methods with higher histologic grade and stage in MM.¹²⁴ Later studies have shown no correlation of cyclin D1 over-expression by IHC methods with clinical parameters, extent of bone marrow infiltration, histologic grade, proliferative activity index, response to therapy, or overall survival in MM patients.^{123,125} To add to the confusion of the prognostic significance of cyclin D1 over-expression in MM, another recent study correlated strong or weak cyclin D1 positivity by IHC methods with improved survival.¹²⁶

The main applications of cyclin D1 in evaluating plasma cell proliferations include: (1) detecting strong cyclin D1 over-expression in MM, which highly correlates with the presence of t(11;14),¹²⁶ (2) distinguishing MM from lymphoplasmacytic lymphoma (LPL) (cyclin D1 over-expression has not been observed in LPL¹²⁴), and (3) distinguishing MM from reactive plasmacytoses (cyclin D1 over-expression has not been observed in normal plasma cells¹¹⁹). It is important to recognize that the “lymphoid” or “small cell” variant of MM is commonly associated with cyclin D1 over-expression, in order to correctly diagnose this disorder, and not incorrectly diagnose as MCL.¹²⁶

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Molecular Techniques to Detect Disease and Response to Therapy: Minimal Residual Disease

Marie E. Beckner and Jeffrey A. Kant

Overview

Evaluation for the presence of neoplastic cells or karyotypic abnormalities has traditionally been performed to monitor therapeutic response of hematolymphoid neoplasms. The application of multicolor flow cytometry and nucleic acid amplification techniques has extended evaluable markers and lowered limits of detection, thus leading to the term “minimal residual disease” (MRD). The ability to monitor MRD has, in turn, led to new concepts in the definition of disease “remission” and early relapse and opportunities for personalized therapy. This chapter focuses on the principles of the molecular assessment of MRD.

Ballpark sensitivities of techniques to monitor residual hematolymphoid neoplasia are indicated in Table 10.1. Inherent in being able to measure MRD is the ability to define tumor burden with reasonable precision. Methods such as end-point PCR amplification, which can detect low levels of disease, particularly when performed in a “nested” fashion, do not accurately enumerate actual levels of a tumor marker. In Figure 10.1, samples with both low and high levels of target subjected to PCR amplification yield the same end level of product (line B), although the sample with higher signal amplifies considerably earlier (line A). To address this deficit, affordable quantitative methods have been developed. These will be discussed in later sections.

Tests for MRD should ideally detect one tumor cell in a background of 10^5 – 10^6 (or more) normal cells. However, detection at lower sensitivities, even down to 1 part tumor in 10^3 parts normal, is often useful. It is important to recall that patients newly diagnosed with leukemia have 10^{12} (or more) leukemic cells.^{1–3} Thus, a sensitive assay detecting 1 leukemic cell in 10^5 normal cells in a sample of peripheral blood or bone marrow would still be negative in a patient with a significant reservoir of residual leukemia,^{2,4} and treatment protocols are implemented with the understanding that negative results of even the most sensitive tests available do not guarantee absence of disease.

Molecular Approaches to MRD Monitoring

Patient Samples

It may be extremely useful to establish the presence of a molecularly evaluable marker in a patient’s cancer process. This is most easily done on a diagnostic sample with abundant disease. Sometimes, this is not “medically necessary,” if other diagnostic techniques are performed (i.e., karyotypic or FISH analysis for the *BCR-ABL1* translocation in chronic myeloid leukemia [CML]). It is always useful to have stored nucleic acid from a diagnostic sample because if MRD assays are subsequently requested and negative, it may be important to establish that the negative result reflects a decrease in neoplastic cells versus an unusual case that lacks an evaluable molecular marker. In our experience, diagnostic samples are not always sent for molecular testing or nucleic acid preparation and storage.

Targets

A disease-associated DNA or RNA MRD target may be identified in most patients with certain disorders [i.e., CML and acute lymphoblastic leukemia (ALL)], but less commonly in others [30–50% of patients with acute myeloid leukemia (AML)].⁵ Tumor-specific molecular markers are also present in many neoplasms of lymphoid origin. Eighteen European studies and one United States consensus meeting are mentioned in a recent review.⁶

Antigen Receptor Rearrangements in the Immunoglobulin Super Family

Somatic rearrangement associated with the processing of germline gene sequences in lymphocytes leads to a diversity of immunoglobulin heavy and light chain genes, as well as T-cell receptor genes (*TCRs*) in patients with reactive

TABLE 10.1. Comparison of methods for minimal residual disease assessment.

Method	Sensitivity
Cytogenetics, conventional	3–10%
Southern blot	1–10%
Cytogenetics, FISH	0.2–10%
Flow cytometry	0.01–2%
PCR/RT-PCR, quantitative	1 in 10 ³ –10 ⁵
PCR/RT-PCR, nested	1 in 10 ⁵ –10 ⁶

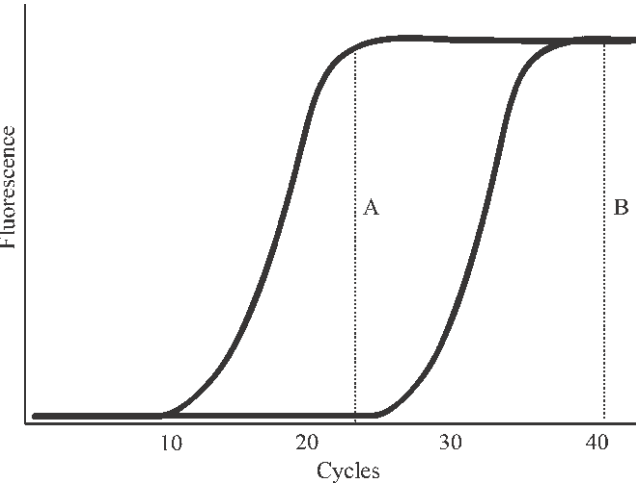


FIG. 10.1. PCR amplification schematic. Idealized amplification curves are depicted for two samples amplifying earlier and later, respectively, due to different amounts of target. The Y axis depicts signal due to product amplification and the X axis PCR cycle number. Note the earlier sample has achieved near-maximum amplification at a time when the later-amplifying sample has not yet begun to demonstrate exponential product accumulation (line A, ~23 cycles). Both samples have attained maximal levels at a later time point (line B, ~40 cycles).

responses. In lymphoid neoplasms, which arise from a single progenitor cell, one or more of these changes will predominate and may usually be determined by Southern blot or PCR analysis using isolated DNA. PCR is most convenient, and approaches employing single or multiplex mixtures of consensus primers allow detection of residual neoplasm in 1–5% of total cells. Allele-specific oligonucleotide PCR (ASO-PCR), using one or more patient-specific primers associated with *IgH* and *TCR* gene rearrangements in leukemia and lymphoma patients, may detect MRD at useful levels (one positive cell in 10⁵–10⁶ benign leukocytes).^{7–10} Studies have focused on B-ALL, using PCR targets for MRD in the following order of priority: *IGH*>*IGK*(*Vk-Kde*)>*TCRD*>*TCRG* and *IGK*(*intron-Kde*). In T-ALL, preferential order for MRD detection is as follows: *SIL-TAL*>*TCR*δ(delta)/*TCR*β(beta)>*IGH*(*D_H-J_H*)>*TCR*γ(gamma).¹¹ Rearrangements have also been followed for MRD in multiple myeloma,^{12,13} although secondary *Ig/TCR* rearrangements, which occur in 8%, 4%, and 2% of patients with myeloma, follicular lymphoma, and CLL, respectively,

may cause false negatives. Recommendations are to monitor two or more *Ig/TCR* targets.⁵ Patient- and clone-specific PCR approaches have not been broadly adopted in the clinical laboratories of USA because of resource demands.

Chromosomal Translocations Resulting in Fusion-Gene Transcripts Followed by RNA Analysis

Stable hybrid gene transcripts arising from chromosomal translocations are widely used to monitor MRD in leukemias and offer an important advantage over translocations assessed by assaying DNA (discussed in the next section). Regardless of the location of a translocation breakpoint in an intron, subsequent splicing yields mature transcripts directly apposing junctional exons from the involved genes. This permits the design of assays that target relatively short RT-PCR products. Translocations are specific to the leukemic process (unlike the more general processes of *IGH* and *TCR* gene rearrangement), so MRD levels may be routinely followed sensitively at 1 part tumor in 10⁴–10⁶ parts normal.

Current clinical targets include the major and minor breakpoint transcripts of *BCR-ABL1* associated with the Philadelphia (Ph) chromosome in >95% of CMLs, a portion of ALLs, and rare cases of AML. Fusion transcripts involving the *PML* and *RAR*α(alpha) genes are almost universal in patients with acute promyelocytic leukemia. Other rearrangements considered for MRD testing in acute leukemias include those involving core binding factor (CBF) subunits (CBFbeta-MYB11, CBFalpha2(RUNX1 or AML1)-ETO, etc.), t(1;19)(q23;p13) resulting in the *E2A-PBX1* fusion gene, t(4;11)(q21;q23) resulting in the *MLL-AF4* fusion gene, t(12;21)(p13;q22) resulting in the *TEL-AML1* fusion gene, and intrachromosomal microdeletion on 1p32 resulting in the *SIL-TAL1* fusion gene. These have been characterized within the European BIOMED-1 and Europe Against Cancer (EAC) networks.⁸ The fusion gene transcript *NPM-ALK*, associated with t(2;5) in anaplastic large cell lymphoma, may also be tested.^{5,14,15}

Chromosomal Aberrations Resulting in a Fusion Gene Followed with DNA

While breakpoint regions differ among patients, each region of chromosomal fusion is unique and represents a potential tumor-specific MRD target to follow with quantitative PCR. The *IGH-BCL2* translocation, t(14;18)(q32;q21), in large numbers of patients with follicular lymphoma is an example. Translocations involving the *BCL1* and *IGH* genes t(11;14) exhibit breakpoints clustered within a restricted area (MTC region) in 30–40% of patients with mantle cell lymphomas. Submicroscopic 1p32 (*TAL1*) deletions in 5–15% of T-ALL patients also result in patient-specific breakpoints, which are useful to monitor MRD.⁵

Additional Considerations in the Use of RNA or DNA

DNA is more stable in samples, and the presence of one target per cell simplifies quantitation (unless there is gene

amplification). Multiple RNA transcripts per cell facilitate detection, but do not always allow quantification of the number of malignant cells. RNA markers may also be influenced by environmental selection and epigenetic factors, such as methylation. Stabilization agents to prevent RNA degradation soon after collection are recommended by some,¹⁶ although many labs do not feel this is necessary. Normalization of RNA target results against an endogenous “housekeeping” RNA target adjusts for degradation (see below).

“Point” Mutations

Certain molecular changes (not associated with translocations) lend themselves to MRD assessment using mutation-specific primers. Three variants of *NPM1* represent 90% of all AML cases with a normal karyotype.¹⁷ Additional nucleotides, randomly inserted when the *FLT3* gene, undergo internal tandem duplication (ITD) and may be used as patient-specific MRD markers, although such changes may be unstable.⁵ *MLL-PTD* and *CEBPA* have also been considered for following MRD in AML.¹⁷

Aberrant Gene Expression

Genes that are highly overexpressed in malignancy may be useful to follow MRD, although background expression in normal cells prevents these levels from becoming “undetectable.” *WT1*, a potent repressor of several growth factors, including insulin-like growth factor II and CSF1, is aberrantly expressed in acute leukemias and myelodysplastic syndromes.^{6,18} A cryptic translocation, t(5;14) involving *HOX11L2*, is highly expressed in 20–35% of T-ALLs. Increased expression of the *CCND1* and *PRAME* genes have been used as MRD-PCR targets.⁵ In addition to mutational changes in DNA (discussed above), *NPM1* RNA overexpression may be used for assessment of MRD in some cases of AML.¹⁷ Similarly, *EVII* expression in 20% of AML cases may be a useful MRD marker, often associated with rearrangements at its chromosomal location, 3q26.¹⁷

Other Techniques to Monitor MRD

Karyotype and FISH

Conventional karyotype studies remain useful to detect evolving chromosomal abnormalities in patients with modest to significant tumor burdens. These have limited sensitivity and require viable cells to analyze metaphases as well as specialized personnel.

Interphase FISH offers better sensitivity over conventional karyotyping for a known abnormality, by analyzing larger numbers of cells (up to 500), but is less sensitive than PCR. Dual-fusion FISH permits analysis of up to 6,000 nuclei.¹⁹ Early studies comparing PCR and FISH have shown concordance with superior sensitivity for PCR.^{20–22} Consensus has been reached that FISH cannot reliably distinguish patients

in complete cytogenetic remission (who have also achieved an adequate molecular response), and that FISH is insufficiently sensitive to detect impending relapse in patients with low (but rising) levels of *BCR/ABL1* transcripts.²⁰ FISH may be useful to monitor disease levels in some patients with AML, but it lacks the sensitivity of multiparameter flow cytometry and PCR techniques for evaluating MRD.²³

Flow Cytometry

Multiparameter flow cytometric immunophenotyping (FCI) has been used to follow MRD in several types of leukemia. One advantage over PCR is that with broad panels of markers and attention to detail, an aberrant immunophenotype may be identified for up to 80+% of cases.⁶ Of course, this advantage over PCR requires that the neoplastic cells demonstrate an aberrant immunophenotype, which is not always the case. Panels of antibodies have been suggested by national and international consensus groups (i.e., ELN, www.leukemia-net.org).¹⁷ Multiparameter FCI, at the end of induction and consolidation therapy, may be used to predict relapse in AML,^{3,17,23–25} and has also been developed for monitoring MRD in ALL.⁸ Additional advantages of flow are that results are not influenced by RNA degradation or inhibitors of PCR. Disadvantages include immunophenotypic shifts over time, by chance or in response to therapy, and confusion of markers that are shared by leukemic and regenerating hematology cells. By contrast, most PCR-based targets for MRD remain stable over the course of disease.

Multiparameter FCI is more sensitive than PCR assays, which employ consensus (but not patient or clone-specific) primers for immunoglobulin family gene rearrangements. Excellent sensitivity for detection of residual MRD in CLL has also been demonstrated.^{26,27} High costs and effort are barriers to common use of multiparameter FCI, plus standardization of PCR assays may be easier to attain.^{6,8,17}

Microarrays

Although expression profiling via DNA microarrays has been investigated for prediction of MRD status in ALL,²⁸ careful comparisons have revealed few gene signatures predictive of relapse⁸ or just a small number of genes that differ significantly between diagnosis and relapse.²⁹ Further investigations are needed to determine the utility of expression profiling, as well as array CHG, SNP, and copy number arrays in predicting MRD status in leukemias.

Transcription-Mediated Amplification (TMA)-Hybridization Protection Assay (HPA)

The TMA-HPA is a quantitative technique using reverse transcriptase and RNA polymerase. It does not require a thermal cycler and demonstrates good detection sensitivity of one *BCR-ABL1* positive cell among 10⁴–10⁵ *BCR-ABL1* negative cells.³⁰

Quantitative PCR and RT-PCR

Quantitative PCR Techniques (Nested, Competitive, Real-Time)

As effective treatments have been developed for hematolymphoid malignancies, sensitive techniques to monitor MRD have become an important expectation of clinical laboratories. For CML, PML, and select other disorders, sensitive RT-PCR-based real-time quantitative techniques with wide dynamic ranges have largely replaced qualitative and semiquantitative techniques. Results facilitate alteration of dose, decision to change therapeutic agents, prediction of impending relapse, and investigation of resistant clones using additional molecular tests.

The progression of techniques developed for monitoring *BCR-ABL1* as a marker for MRD has been reviewed.²⁰ The presence (or absence) of *BCR-ABL1* transcripts was initially detected qualitatively by single or two-step ("nested") PCR^{31–33} and later, as competitive PCR quantified transcript numbers per microgram of RNA³⁴ or as a log-based ratio of *BCR-ABL1/ABL1*.³⁵ Real-time PCR followed and supplanted previous methods. Numerous studies have favored real-time quantitative PCR (RQ-PCR) for monitoring MRD in CML, ALL, and AML.^{3,5,8,16,20,36–40}

Results were initially reported as a log-based ratio^{41–46} and then as log₁₀ reduction from standardized baseline levels seen in untreated patients.^{4,20} Nested PCR is highly sensitive, but end-point PCR results are only qualitative. Nested PCR may have value in testing for residual disease, when RQ-PCR is negative and is a critical therapeutic or clinical trial goal.^{47–49} RQ-PCR has been reported to be less sensitive than nested PCR.^{17,42,50,51} However, others have reported that real-time PCR may be more sensitive than nested RT-PCR.^{6,52} Repeat testing of a patient's sample also increases the chance of amplifying a rare transcript or DNA molecule with RQ-PCR, when stochastic distributions are considered.^{53–55} Regardless of the sensitivity issues, inclusion of internal controls with RQ-PCR is more reliable than nested PCR, since there is compensation for specimen degradation and variations in amplification. Unlike nested PCR, competitive PCR is quantitative, but both are end-point assays. There is a consensus that RQ-PCR is preferred over the other PCR techniques for several reasons: (1) evaluation of PCR products during amplification, rather than at an end-point, does not require coamplification of a competitor or post-PCR procedures, (2) assay sensitivity and dynamic range of assessment is improved by assessing fluorescence during the exponential phase of PCR,³⁶ and (3) RQ-PCR as an automated method requires less time, labor, and expertise, and has diminished likelihood of contamination from postamplification procedures. RQ-PCR is also more amenable to standardization for sequential comparisons in individual patients and among different laboratories.^{5,6,11,20,36,38}

RQ-PCR Assay Design

Most markers followed for MRD in hematolymphoid neoplasia are RNA transcripts. Following RNA isolation and reverse transcription, PCR amplification is performed, and data is interpreted and reported. General considerations for DNA markers are similar. There are currently no FDA-cleared (IVD) MRD assays in the United States for hematopoietic targets. Tests are laboratory-developed from scratch, or internal validation of "research use only"-labeled kits from manufacturers. Each laboratory determines its sample requirements and performance characteristics for accuracy, precision, analytic sensitivity and specificity, as well as reportable range. Good communication with oncologists and hematopathologists using results for patient management is important for optimal patient care.

Samples

Peripheral blood or bone marrow are commonly assessed. EDTA or ACD are recommended anticoagulants; heparin may interfere with PCR amplification. Transcript levels from peripheral blood and bone marrow follow similar trends during therapy; some studies claim levels in bone marrow are more sensitive.^{20,48,56,57} Monitoring a consistent sample type is recommended in CML. Bone marrow may be followed more commonly in other types of leukemia,^{58–60} but blood with sufficient numbers of mononuclear cells may be used.¹¹ Typically, 5–10 mL of peripheral blood are collected and RNA is extracted from 1 mL or greater. Volumes of 5–10 mL (sometimes 20 mL or more) have been suggested with the goal of analyzing at least $1–2 \times 10^7$ nucleated cells. Samples (and standards) are typically assayed in duplicate (or triplicate) to detect variability. Duplicates are generally reproducible, except at very low levels of transcript.

RNA Extraction

RNA quality is an important determinant of reproducibility and significance.^{16,61} Peripheral blood, anticoagulated with EDTA and stored at ambient temperature 24–48 h, loses 20–50% of *BCR-ABL1* transcripts,¹⁶ presumably due to nucleases. Thus, it is useful to chill (not freeze) and transport samples to the laboratory for processing within 24 h of collection and/or to consider adding RNA stabilization agents.^{16,20,21,47–50,62,63} Care should be taken to prepare samples under RNase-free conditions. RNA is quantified spectrophotometrically; integrity may be assessed by intact bands of 18S and 28S ribosomal RNAs or, more commonly, via expected activity of an internal housekeeping gene. Prepared RNA may be stored at -80°C for stability.

Reverse Transcription (RT)

The amount of starting RNA varies, but may be as little as 1 μg . Choice of RT enzyme and the primer type may affect the yield of cDNA.^{16,64} Good sensitivity has been obtained

with MMLV or Superscript enzymes. Random primers (hexamers or nonamers) appear to give more efficient cDNA synthesis than oligo-dT priming.^{16,38,65,66} A standardized RT protocol is provided in a study by the EAC group.³⁸

RQ-PCR Amplification

Targets are assayed on an RQ-PCR instrument, using appropriate leukemia or patient-specific hydrolysis (TaqMan), or hybridization (LightCycler) probes. An intercalating dye, such as SYBR Green (which binds the minor groove of double stranded DNA) may be used, if there is minimal nonspecific amplification. Assays using hydrolysis and hybridization probes yield comparable results.^{20,67} Primers and probes should be designed to not bind other areas of genomic DNA or pseudogenes, using BLAST searches as well as commercial or public domain primer design software to minimize primer dimers, polymorphic nucleotides in regions targeted by primers and especially probes. For RNA targets, primers (and even probes) may be designed to cross exon–exon junctions so that amplicons are not generated efficiently from contaminating genomic DNA. For example, a polymorphic site in *BCR* exon 13 and a small intron between *ABL1* exons 2 and 3 were specifically avoided when designing primers and probes for *BCR-ABL1* amplification.²⁰ Rare translocation breakpoints in *ABL1* introns 2 and 3 may be accommodated by placing PCR primers in *ABL1* exon 4.

Standardized primers and probes for *BCR-ABL1* CML breakpoints, as well as a number of other hybrid chromosome transcripts, have been designed and validated in EAC studies.³⁸ The forward primer for the major breakpoint region is placed in *BCR* exon 13 (aka exon b2) such that it amplifies both e13a2 (b3a2) and e14a2 (b2a2) *BCR-ABL1* junction transcripts with breakpoints in *BCR* introns 13 or 14.^{36,38} The reverse primer and detection probe are placed in *ABL1* exon 2. Primers and probes for an endogenous control gene include *BCR*, *ABL1*, *GUSB*, *G6PD*, and *G3PD*. Reagents for the endogenous control are ideally incorporated into a multiplex reaction, but may be assayed independently if there is significant competition.^{68,69}

A negative control, often normal RNA from a cell line lacking the *BCR-ABL1* translocation, as well as no-template (water, saline) controls for both the RT and PCR steps are important to ensure the absence of contamination. “No-amplification” controls lacking *Taq polymerase* may also be used. Standard precautions to prevent contamination are important to include “clean” areas where reagents are prepared, other clean areas for sample preparation and assay set-up, and “dirty” areas for dilution of positive controls (i.e., K562 RNA or plasmids) and RT and PCR reactions. Other standard precautions in evaluating MRD with RQ-PCR for *BCR-ABL1* have also been described.²⁰

Early RQ-PCR studies relied on absolute quantification, but relative quantification is often used now to leverage internal “housekeeping” RNAs, which correct for slight

variations among samples for RNA content, RNA degradation, reverse transcription, quantitative amplification, and possible inhibitors. There are two major types of relative quantification methods yielding equivalent results. One uses a normalized ratio of target to the “housekeeping” control gene copy numbers based on standard curves. The other uses the crossing thresholds ($C_{T,s}$) of target and control amplification curves without a standard curve, known as $\Delta\Delta C_T$. There are no absolute units of measure for relative quantitation results, and target/control ratios track the level of disease-specific hybrid transcripts in a patient. Variations on relative quantitation methods also avoid standard curves through software programs, using complex mathematics to analyze amplification curves and $C_{T,s}$.⁷⁰

Quantitation, Standard Curve Method

Dilutions of a reference cell line (or plasmid containing the target and endogenous controls) are amplified in the same assay to generate the necessary standard curves. Crossing thresholds, $C_{T,s}$, are placed on the *Y* axis and dilutions of total RNA (or DNA) on the *X* axis. Crossing thresholds are defined as 10 SD above baseline values of preamplification cycles in RQ-PCR.⁷¹ The relative expression level is “normalized” by dividing target and control levels (log dilution of reference RNA or DNA); units of measure cancel. The number of levels tested may vary, but it should include target levels at the desired laboratory-defined minimum limit of detection. At least three standard dilutions of reference material should be included for target and control.⁶⁵ Some individuals like DNA standards for reasons of stability; although the authors’ experience has been that properly stored K562 RNA does not suffer degradation. Plasmids (containing both target and control sequences) are also preferred by some individuals, in order to limit variability.^{4,36} The advantage of the standard curve method is that the efficiencies of amplification for target and endogenous control may differ without affecting accuracy, and the method may be easier to conceptualize. In a recent interlaboratory comparison study of MRD in CML, 36 of 38 laboratories generated standard curves for use in normalizing their assays of *BCR-ABL1*.⁷²

Quantitation, $\Delta\Delta C_T$ Method

If amplification efficiencies of the target and housekeeping gene are approximately equal, and amplicons are relatively small (≤ 150 bp), standard curves are not necessary for relative quantification by calculations involving C_T values. The amount of target normalized to the endogenous control, relative to a calibrator target sample is $2^{-\Delta\Delta C_T}$. For example, for *BCR-ABL1*: $\Delta C_T = C_T(\text{BCR-ABL1}) - C_T(\text{endogenous control})$ and $\Delta\Delta C_T = \Delta C_T(\text{patient sample}) - \Delta C_T(\text{reference sample})$.⁷² The derivation of the formulas for $2^{-\Delta\Delta C_T}$ has been described.⁷³ Validation studies must be performed to establish equivalent amplification efficiencies of target and control genes, and the absolute value of the log input amount versus ΔC_T

should be <0.01 .⁷³ If amplification efficiencies are not equal, primers may be redesigned. Positive controls, which serve as calibrators at high, medium, and low levels, may be monitored for performance by comparing CTs over serial assays. In a recent interlaboratory comparison of MRD analysis using *BCR-ABL1*, results from laboratories using the $\Delta\Delta C_T$ method were consistent with those from laboratories relying on standard curves.⁷² The $\Delta\Delta C_T$ method may save on costs, through avoidance of multiple wells or tubes for standard curves.

Data Analysis and Reporting of Results

An example of *BCR-ABL1* RQ-PCR by the $\Delta\Delta C_T$ method is presented in Figure 10.2. Calibrators of total K562 RNA at neat and at 1:100 and 1:10,000 dilutions are tested along with total RNA from a Jurkat T-cell leukemia cell line (as a negative control). Ct values of the calibrators must fall within predetermined ranges of suitability confirming stability, acceptable linearity, and sufficient sensitivity. Negative controls must be

free of *BCR-ABL1* signal. From averages of the replicate Ct values determined from the measurement threshold for each sample, $\Delta\Delta C_T$ relative expression levels are calculated by the instrument software normalized to the undiluted (neat) calibrator sample of K562 RNA.

The resulting relative percentage values of 100% K562 are reported both as absolute percentage values and also as a log reduction from the laboratory's "baseline" value, representing a mean level in patients with newly diagnosed and untreated disease. For our lab, that value is 30% of the K562 level. Hence, a level of 0.03% would be 3.0 log reduction from the baseline. Samples where replicates give a low positive and an undetectable signal are reported as "positive at a low level in one of two replicates," and the value of the single positive replicate is listed in a comment along with a notation that high levels of confidence should not be placed in such values.

Labs must set their own criteria for what change in relative expression level is significant. For most labs, a 3–5-fold change in log reduction or gain is compatible with a significant downward (or upward) change, and most certainly a 1 log change.

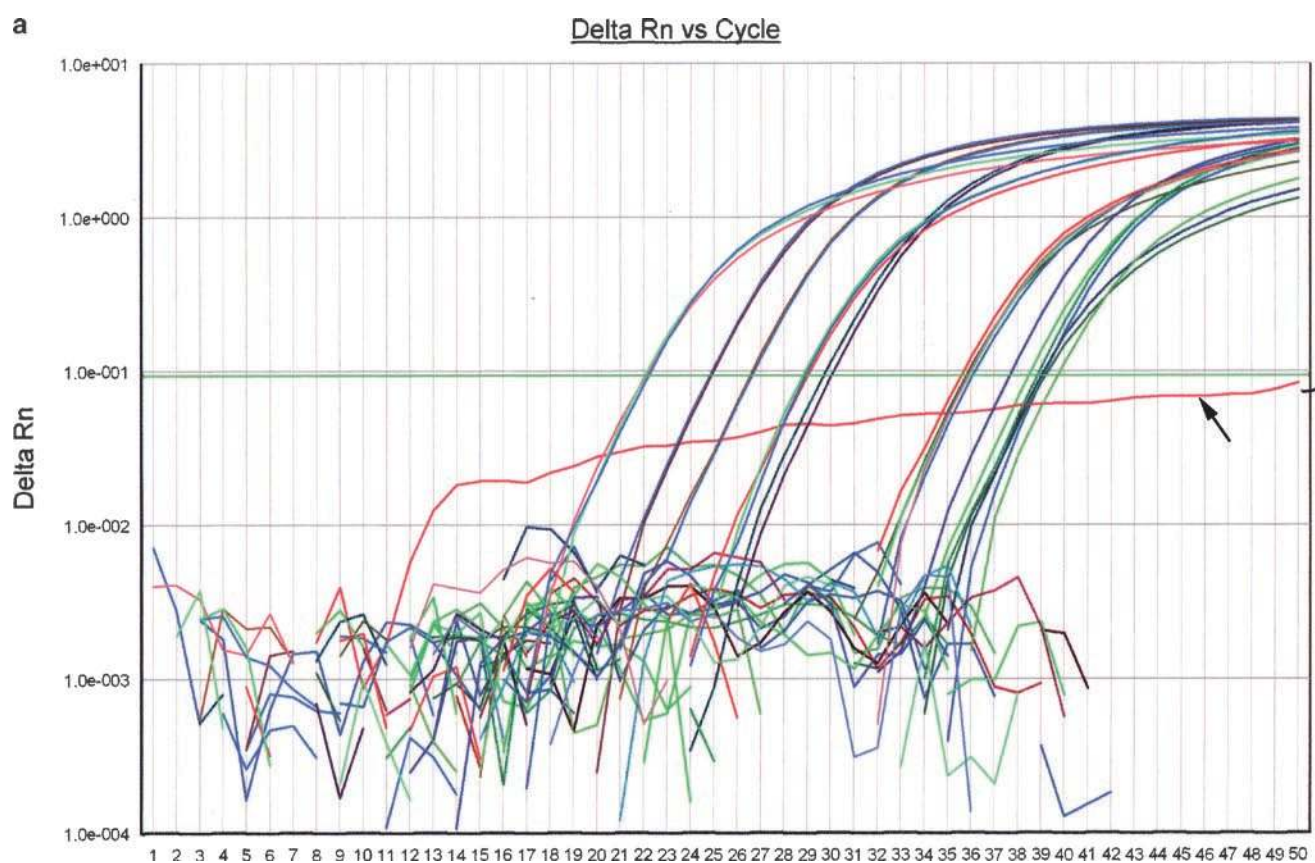


FIG. 10.2. *BCR-ABL1* quantitative RT-PCR assay run (printout from ABI 7500 instrument). PCR cycle number is depicted on the X axis versus target signal accumulation on the Y axis ($\log_{10}$ scale); low level erratic lines represent background noise. Positive and negative control RNA samples (isolated from K562 cells) were tested in triplicate, patient samples in duplicate. (a) *BCR-ABL1* target data. A range of early to late-amplifying positive samples and controls as

well as negative samples and controls are seen. Note that product accumulation curves are parallel for all positive samples. The "threshold" for measurements (horizontal line just below 0.1) passes through the exponential phase of product accumulation for all samples and is set just above a replicate from one sample which demonstrates low level nonspecific "creeping" accumulation of product (arrow). This sample is interpreted as negative.

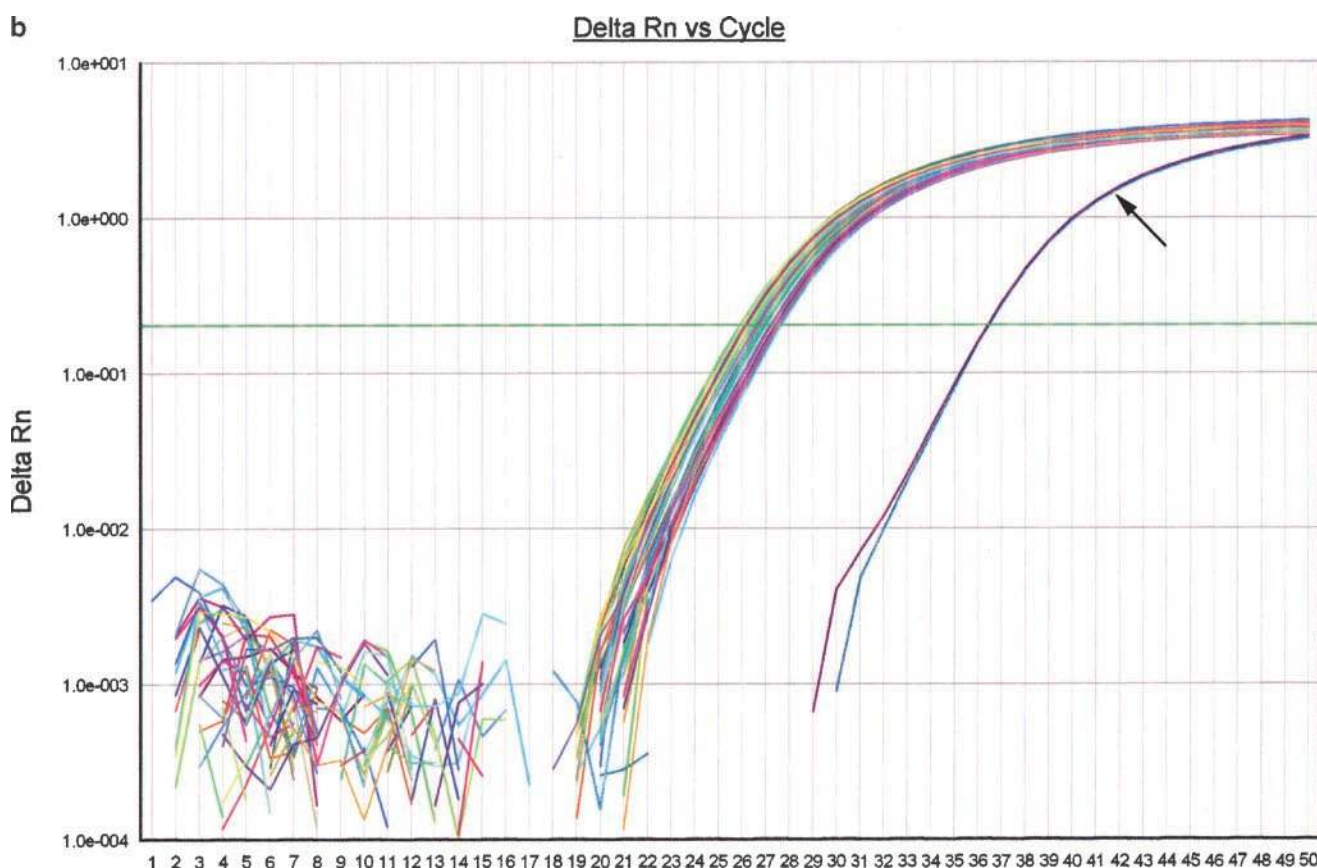


FIG. 10.2. (continued) **(b)** Housekeeping Beta glucuronidase (*GUSB*) gene data. *GUSB* expression allows assessment of isolated sample RNA integrity and suitability for analysis as well as normalization of *BCR-ABL1* expression levels for modest differences in RNA quality. Note virtually that all *GUSB* curves are closely bunched in a satisfactory range (threshold ~26–27.5 cycles). A single sample (arrow) shows

significantly reduced quality ~10 cycles below others in both replicates tested. This sample gave no *BCR-ABL1* signal and is interpreted as technically unsatisfactory with a recommendation that another sample be submitted if clinically indicated. Even were *BCR-ABL1* signal positive from this sample, confidence in the relative value normalized to *GUSB* would be reduced because of compromised RNA quality.

Consistently rising relative percentage expression, measured by MRD assays, supports impending clinical relapse. A small percentage of patients on treatment attain true molecular remissions in which the target is consistently absent, and it is common for others to be relatively stable at intermediate (1–3 log reductions from baseline) or low (>3 log reductions) levels of *BCR-ABL1* transcript. False positive results may arise from low-level contamination or theoretically from transcripts of unclear relevance, which have been reported in asymptomatic individuals,^{74–76} although we are not aware of reports of the latter.

Units of Measurement and Assay Standardization

Results for monitoring MRD have evolved as procedures have changed. Absolute quantification of marker from a standard curve was common in early competitive PCR studies. The number of *BCR/ABL1* transcripts were quantified per μg of leukocyte RNA or as a log ratio of *BCR/ABL1* to *ABL1*.^{20,34,35}

Transcripts per lymphocyte or per μL of bone marrow were also used. Relative quantification has been previously described.⁷⁷ The concept of reporting $\log_{10}$ reduction from a standardized baseline for untreated patients was introduced in the International Randomized Study of Interferon versus STI571 (IRIS study) as reported by Hughes et al.^{4,20} A group of 30 or more patients with untreated disease is recommended to establish the baseline. Some clinicians find this a more user-friendly unit of measurement than direct reporting of the ratio of target/control expressed as a percentage.²⁰ Many laboratories report both values.

A proposed International Scale of measurement, which applies laboratory-specific conversion factors derived from assay of patient samples at a reference laboratory, has indicated that there may be accurate interlaboratory comparisons of molecular response rates based on relative *BCR-ABL1* transcript levels.⁷⁸ Work is in progress to develop a validated quantitative control material on a large scale, which may be purchased by clinical laboratories to determine a local

conversion factor for standardizing their baseline levels to an international standard, similar to what has been achieved for monitoring levels of hepatitis C and hepatitis B viruses.

Sensitivity, Specificity, and Precision

Some authors have suggested that analytic sensitivity of 10^{-4} to 10^{-6} (1 malignant cell in 10^4 to 10^6 normal cells) is preferred for MRD assessment, but a sensitivity of 10^{-3} or better is still useful.⁶ RQ-PCR assays generally do not have difficulties attaining levels of 10^{-3} to 10^{-5} .

European laboratories collaborated in a program, BIOMED-1 Concerted Action, to develop standardized nested RT-PCR assays with sensitivities of at least 1 in 10^4 normal transcripts (RNA diluted into RNA) for diagnosis and monitoring of MRD for 15 different leukemia fusion transcripts. The cell lines used along with primer and probe sequences are published.^{15,38} Using centrally prepared RNA (or cDNA) from cell lines or patient samples diluted in normal patient RNA or E. Coli rRNA, overall false negative rates ranged from 1.7–4.6% at a 10^{-4} dilution, although one target was more problematic. Overall, false positive rates were 2.1–7.5% with values from zero to as high as 10–20% for certain individual targets. A false-negative result was defined as a known positive sample yielding positive results in <50% of three replicates; a false-positive was a known negative with positive results in at least 50% of three replicates. Sensitivity should be established for each assay, and patient samples should be reanalyzed when equivocal results are obtained, such as inconsistent replicates. Results should always be considered in the clinical context of the patient.²⁰

Stochastic influences impact sensitivity and precision at low target levels.^{53–55} Distinct RNA and DNA molecules are measured in low quantities in monitoring MRD, so the Poisson distribution should be kept in mind. This relationship predicts probabilities of 36.8, 36.8, 18.4, 6.1, 1.5, 0.3, and 0.1%, respectively, for the presence of 6, 5, 4, 3, 2, 1, and 0 molecules to actually be distributed to a PCR tube.⁵⁵ Such considerations strongly support the use of replicates in RQ-PCR for monitoring low level MRD.⁵³

Detection sensitivity varies according to the disease marker being followed for MRD. Overexpressed genes, which are measured against a background of normal transcripts, are least sensitive with sensitivity as low as 1 malignant cell in 100 normal cells. Leukemia or patient-specific fusion transcripts (or mutations) typically demonstrate the best sensitivities. Sensitivity for standard curve and $\Delta\Delta C_T$ methods are calculated differently but should be similar.⁶⁵ The standard curve method requires linear amplification curves at varying dilutions as reflected by correlation coefficient (R^2) determination in each assay. Slopes between -3.0 and -3.9 will likely be acceptable as long as R^2 is >0.95 .^{5,16} The $\Delta\Delta C_T$ method requires that amplification efficiencies of the target and control/normalizing genes to be approximately equal, which is typically demonstrated during assay validation

and are not repeated for each assay. Relative amplification efficiencies may be compared from tenfold cDNA dilutions.¹⁶ Software programs for the $\Delta\Delta C_T$ method may be able to correct for differing amplification efficiencies.⁷⁰ Attention to C_T values of positive RNA or cDNA standards (“calibrators”) allows assessment of assay deterioration.³⁸ Precision may be expressed as the coefficient of variation of replicate ratios.¹⁶

Controls/Normalizing Genes

The importance of positive and negative controls, including no template and no enzyme controls, has been alluded to previously. Control genes appropriate for normalizing RQ-PCR assays should show stable expression in all nucleated cells and not be affected by treatments. Control genes (which lack pseudogenes) are preferred. Variations in a control gene should reflect variations in quality, quantity, or efficiency of sample RNA for reverse transcription, as well as the presence of inhibitors.⁶⁵

The issue of an optimal control gene has stimulated much investigation. *ABL1* has been used extensively for monitoring levels of *BCR/ABL1* fusion transcripts in a number of RQ-PCR studies for CML.^{21,44,63,79–81} *Glucose-6 phosphate dehydrogenase (G6PD)* has shown greater heterogeneity than *ABL1* in normalized ratios during the course of CML (in some but not all studies).^{48,50} The *BCR* gene has also been used in CML.⁸² Fourteen control genes were evaluated in a large collaborative study conducted by EAC.⁶⁵ Seven candidates (*ABL1*, *ACTB*, *B2M*, *GAPDH*, *PBGD*, *TBP*, and *18S rRNA*) had previously been investigated (references in⁶⁵). Additional candidates included *PO*, *GUSB*, *CYC*, *HPRT*, *PGK*, *PBGD2*, and *TFRC*.^{38,65} Criteria for selection of control genes included location other than the X chromosome, absence of pseudogenes, medium levels of expression, similar expression in peripheral blood and bone marrow, normal and leukemic (various) samples, and noncell-cycle-dependent expression. Although *ABL1* was selected, known issues included some amplification of genomic DNA and inaccuracy due to competition in samples with high levels of *BCRABL1* fusion transcripts.⁶⁵ In a recent interlaboratory comparison monitoring MRD in CML, *ABL1* performed less well than *GAPDH*, *BCR*, *G6PD*, and *B2M* with log reductions consistently lower than expected.⁷² Another study using cell line RNA showed the lowest variability among eleven candidate genes with *18S rRNA*, *GUSB*, and *ACTB* and highest variability with *GAPDH*.⁸³ Albumin has also been used as a control gene in RQ-PCR studies of MRD.^{5,11,37,57,84–86} Accurate normalization of RQ-PCR data by averaging multiple internal control genes has also been examined.⁸⁷

Assay Validation

Necessary aspects of analytic validation have been alluded to previously to include sensitivity and analytic limit of detection, specificity, efficiency of amplification (particularly for

the $\Delta\Delta C_T$ method), intra and interrater variation, and reagent stability. Justification for and performance characteristics of a control/normalizing gene should also be documented. In early studies, PCR methods for monitoring *BCR/ABL1* as a MRD marker in leukemia were validated by comparisons with FISH and conventional cytogenetics, which have much lower sensitivities. Once RQ-PCR emerged as the quantitative technique of preference, sensitivity was verified among PCR methods.^{44,63,79,88,89}

The clinical validity of most MRD assays is fairly self-evident. The laboratory must also decide on criteria for result interpretation and reporting. A written standard protocol explaining performance and interpretation of the assay must be prepared. Validation data should be kept separately in an organized binder (or packet) suitable for review by a laboratory inspector.

Clinical utility of the assay should be considered in the laboratory protocol. Utility for various assays is dependent on management options, such as alternative treatments or discontinuation of therapy for patients with particular disorders. Serial monitoring of *BCR/ABL1* quantitative values has been clearly validated as a useful indicator of treatment response and predictor of hematologic and clinical relapse in CML.^{21,45,80}

Working with Clinicians

Clinicians vary in comfort with percentage ratios that often yield very small numbers, with results expressed using scientific notation, and occasionally with the concepts of log reduction or increase. We encourage laboratorians to have group meetings with clinicians who will be using an MRD assay prior to its introduction to review validation studies and the types of reports that will be issued on patient samples. Clinicians may also not appreciate necessary levels of disease that must be present to perform ancillary studies, such as mutation analysis of the *ABL1* kinase in patients who are not responding as expected.

Ensuring Quality

Important quality measures have been mentioned in previous sections to include necessary positive and negative controls with attention to contamination control and monitoring of expression to verify reagent quality and satisfactory sample preparation.

Regular proficiency testing is also important. External quality assessment is offered in the USA through the College of American Pathologists MRD Survey, which provides three challenge samples twice a year. Laboratories may wish to supplement formal PT surveys with interlaboratory exchange programs and/or blind repeat analysis of previously assayed samples. Informal sample exchanges have also been conducted among member laboratories by groups, such as EAC and the Association for Molecular Pathology (AMP).⁷² Those studies have highlighted areas for potential improvement,

for which the aforementioned international standard allowing better standardization among laboratories using different assays would represent a major achievement.

Future Directions

The standardization of assays to detect and follow *BCR-ABL1* as a marker for MRD is an ongoing focus that will require considerable interaction with the laboratory community through an organizational discipline. Detection of *BCR-ABL1* in plasma from CML patients followed for MRD may be more sensitive than detection in cellular samples and a useful parameter to follow.⁹⁰ In a recent study, at every time point after treatment, median levels of *BCR-ABL1* mRNA in plasma were greater than those in peripheral blood cells, but the difference was only significant at 3 months. Also, as treatments are further improved and longer term results of treatments for CML and other hematolymphoid disorders emerge, there may be greater insight in what constitutes a "molecular remission" or even criteria for a "cure."

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Detection of Resistance to Therapy in Hematolymphoid Neoplasms

Karen Weck

Introduction

In the past several decades, treatment of hematolymphoid disorders has made dramatic strides. However, the presence or development of resistance to various chemotherapeutic agents is an important problem that affects response to therapy. Drug resistance can be either intrinsic to cancer cells or acquired while on therapy. In this chapter, the major molecular mechanisms of resistance to therapy in hematolymphoid disorders are discussed and techniques that are used to detect resistance to therapy are described. General markers of resistance to therapy, such as cytogenetic markers of resistance or functional assays to measure response to therapy, are not described in this chapter. Rather, this chapter focuses on the detection of specific molecular mechanisms of drug resistance due to the presence or alteration of a specific gene product.

Resistance to Targeted Tyrosine Kinase Inhibitors

Resistance to Inhibitors of BCR-ABL

The development of imatinib mesylate (Gleevec, STI-571) and other tyrosine kinase inhibitors (TKIs) as targeted therapy for the BCR-ABL tyrosine kinase has dramatically improved the treatment for chronic myeloid leukemia (CML). The success of imatinib heralds the development of a new generation of anticancer drugs targeted to the molecular mechanisms of oncogenesis. Unfortunately, the rapid replication and mutation rate of cancerous cells facilitates the development and outgrowth of mutations that confer drug resistance. Shortly after clinical use of imatinib began, the development of imatinib resistance was reported, and resistance is now well recognized as a significant factor affecting treatment response. The development of resistance to imatinib has prompted the development of second generation TKIs, such as dasatinib and nilotinib. However, the use of these newer TKIs is also associated with the development of resistance.

Resistance may be either intrinsic (primary resistance), demonstrating a lack of efficacy from the onset of treatment, or acquired while on therapy (secondary resistance), demonstrating a loss of efficacy over time. Secondary resistance is more common and affects a significant percentage of CML patients on long-term therapy with TKIs. The development of imatinib resistance occurs more rapidly in acute lymphoblastic leukemia (ALL) and in accelerated and blast phases of CML than in chronic phase of CML, likely due to the more rapid replication rate in these processes, which favors the development and outgrowth of resistance mutations.^{1–3}

Resistance to imatinib therapy can occur through several mechanisms. The most common mechanism of resistance is due to mutations in the tyrosine kinase domain of the *ABL* (*ABL1*) gene; other mechanisms of imatinib resistance include BCR-ABL over-expression due to gene amplification, decreased bioavailability of imatinib due to drug efflux or altered transport, and activation of alternative or downstream pathways of oncogenesis.^{1–8} The clinical hallmark of secondary resistance to TKIs is the demonstration of hematologic, cytogenetic, or molecular relapse while on TKI therapy. Thus, routine assays used to monitor response to therapy may indicate the presence of resistance and trigger the use of specific tests to identify the mechanism of drug resistance.

Mutations in the ABL Tyrosine Kinase Domain

BCR-ABL mutational analysis has become a routine practice in monitoring drug resistance in patients on TKI therapy. Detection of mutations in the BCR-ABL tyrosine kinase domain associated with resistance to imatinib and other TKIs is useful for diagnosis of resistance and to direct further therapy. Resistance mutations occur throughout the tyrosine kinase domain of the *ABL* gene, with over 70 imatinib resistance mutations described.⁴ Resistance mutations cluster to four functional domains within the *ABL* tyrosine kinase domain: (1) the ATP-binding P-loop (amino acids 248–256), (2) the imatinib binding region (amino acids 315–317), (3) the catalytic domain (amino acids 350–363), and (4) the activation (A)-loop (amino acids 381–402).

A small number of amino acid substitutions account for the majority of resistance, with 7 frequently mutated codons accounting for 60–70% of imatinib resistance and 15 mutations accounting for 80–90% of imatinib resistance.⁸

The degree of imatinib resistance conferred varies depending on the mutation, with some mutations conferring low-level resistance (e.g. F317, M351T), and others conferring high-level resistance (e.g. T315I, Y253H, E255K). Identification of the mutation may help direct therapy, as some low-level resistance mutations may respond to an increase in imatinib dosage, while other mutations with high-level resistance to imatinib require alternative therapy, such as the use of other tyrosine kinase inhibitors (e.g. nilotinib, dasatinib). Some mutations confer cross-resistance to other tyrosine kinase inhibitors, and their presence may dictate alternative therapeutic approaches. For example, the T315I mutation, which disrupts the imatinib binding residue, confers pan-resistance to all currently available ABL tyrosine kinase inhibitors. Development of third generation TKIs which may circumvent resistance to T315I is underway.^{9,10} Other mutations show more specificity, with some mutations conferring high-level resistance to either dasatinib or nilotinib but lower-level resistance to imatinib.^{1,4,8,11–18}

There are several different methods of mutation detection that have been developed. The most commonly used method is dideoxy DNA sequencing following RT-PCR amplification of the BCR-ABL fusion RNA. Other methods are targeted to the detection of specific mutations. The advantages of sequencing are that the entire tyrosine kinase domain may be interrogated, allowing for the detection of all resistance mutations, including novel resistance mutations. Since the description of the first mutation associated with imatinib resistance, it was soon apparent that multiple mutations may confer drug resistance and the number of resistance mutations identified continues to increase. Some targeted methods of mutation detection have better sensitivity, but will only detect those mutations specifically interrogated.

The major disadvantage of sequencing is its decreased sensitivity, compared to some targeted methods of detection. Dideoxy DNA sequencing has the ability to detect a mutation at a level of ~20% of the population, but may not detect the presence of low-level mutations below this threshold. However, the clinical relevance of the presence of a low-level mutation is unclear. Some reports indicate that the presence of a low-level mutation prior to the start of therapy may predict the development of resistance; other studies have shown that there is poor correlation between the presence of a low-level mutation and development of clinical resistance.^{19,20} Because the clinical significance of low-level mutations is unclear, DNA sequencing has been recommended by an international consensus panel as the gold standard method for BCR-ABL resistance mutation detection.²¹

Targeted methods of BCR-ABL resistance mutation detection include allele-specific PCR (ASO-PCR), targeted microarrays, and bead arrays using Luminex technology.

Quantitative targeted mutation detection methods, such as pyrosequencing, mutation-specific quantitative PCR, the polymerase colony (polony) method, and a nanofluidic chip-based method have also been developed, which may assess the proportion of mutated clone.^{22–25} These targeted methods of mutation detection are more sensitive than sequencing, and some may reach sensitivities of <1% presence of a mutant clone, but are limited by their inability to detect all relevant mutations. Several clinical laboratories direct detection at the T315I mutation, which is the most common BCR-ABL resistance mutation, confers pan-resistance to all the available TKIs, and dictates the use of alternative treatment strategies.

Other methods to detect resistance mutations include mutation scanning methods to screen for the presence of mutations prior to DNA sequencing, such as denaturing high pressure liquid chromatography (D-HPLC).^{26,27} *In vitro* functional assays, which entail cloning of a specific mutation and measuring kinase activity or growth of mutant expressing cell lines in the presence of drug, are useful for determining the degree of resistance of a particular mutation, but are not feasible for routine clinical use.

Amplification of the BCR-ABL Fusion Gene

Gene amplification of the BCR-ABL fusion gene results in over-expression of the oncogenic BCR-ABL protein. This causes drug resistance due to the inability of the drug to effectively inhibit BCR-ABL at physiologic concentrations. Gene amplification is associated with resistance to imatinib and to other BCR-ABL-targeted TKIs. Resistance due to gene amplification may respond to increasing drug dosage. BCR-ABL gene amplification may be detected by fluorescent *in situ* hybridization (FISH), which elucidates the number of BCR-ABL gene fusion copies per cell.

BCR-ABL Independent Resistance: Downstream Targets

Failure of imatinib treatment has been described in patients who do not have BCR-ABL mutations or amplification. Activation of downstream targets of BCR-ABL, such as LYN and other SRC family kinases, has been recognized as an alternative mechanism of resistance to TKI inhibitors and may circumvent the requirement for BCR-ABL in maintenance of oncogenesis.^{28–32} Patients with constitutive activation of LYN kinase may respond to dasatinib, which is an inhibitor of all SRC family kinases.^{28,29} Development of alternative drugs to target downstream or alternate pathways to BCR-ABL is underway.³³

Constitutive activation of LYN kinase has been measured in cell lines derived from patients with imatinib resistance by *in vitro* phosphorylation assays, cell survival analyses, and analysis of apoptosis. Assays to measure LYN kinase activity are largely experimental at this time and used for discovery, not routine clinical use.

KIT Mutation and Imatinib Response

Imatinib mesylate inhibits several tyrosine kinases in addition to BCR-ABL, including KIT (c-kit) and platelet derived growth factor receptor alpha (PDGFRA). Recent evidence indicates that the presence of activating mutations in the *KIT* gene is predictive of response to imatinib in melanoma.^{34–37} Thus, melanomas with KIT activating mutations are better candidates for treatment with KIT tyrosine kinase inhibitors such as imatinib. This is similar to the association of KIT activating mutations with imatinib response in gastrointestinal stromal tumors (GISTs). Activating KIT mutations resulting in ligand-independent kinase activity have been reported in 10–20% of melanomas and in 75–90% of gastrointestinal stromal tumors (GISTs).^{35–39} KIT activating mutations have also been described in 30–50% of core binding factor (CBF)-AML. KIT and its ligand stem-cell factor are important in hematopoiesis and in melanocyte development, and expression of KIT (CD117) is common in early stage melanoma. KIT activating mutations occur more frequently in a subset of melanomas arising on mucosal and acral sites and on skin with chronic sun damage than in melanomas arising on skin without chronic sun damage.³⁶

Testing for KIT activating mutations may become an important clinical tool to direct the use of imatinib and other KIT TKIs in melanoma and AML. Molecular assays to detect activating mutations have been developed, most involving direct DNA sequencing of the exons in which mutations occur. In melanomas, KIT activating mutations have been seen in the juxtamembrane and kinase domains and can be associated with *KIT* gene amplification.^{34–37} Studies in GISTs indicate that ~70% of KIT activating mutations occur in the juxtamembrane domain (exon 11), ~15% in the extracellular domain (exon 9), and <5% in the kinase domains (exons 13 and 17).³⁸ In GISTs, KIT mutations in exon 11 are associated with better response to imatinib than are activating mutations in other domains.³⁸ Further research in melanoma and AML is necessary to correlate the prevalence and significance of specific KIT activating mutations with TKI response.

The development of KIT resistance mutations would be predicted to occur in TKI-treated melanoma and AML similar to that seen in GISTs. Most patients with GISTs who initially respond to imatinib become resistant due to the selection of secondary mutations in the kinase domains of KIT or PDGFRA.^{38,39} Molecular testing for secondary resistance mutations in the KIT kinase domains may become important to monitor resistance in melanoma and AML, especially as TKI therapy is used more frequently to treat patients with KIT activating mutations.

FLT3 IN AML

The FMS-related tyrosine kinase 3 gene (FLT3) encodes a class III receptor tyrosine kinase (CD135) that is expressed in early hematopoietic progenitor cells. Activating mutations in FLT3 that result in constitutive, ligand-independent

tyrosine kinase activity have been associated with 25–35% of cytogenetically normal AML.^{40,41} FLT3 activating mutation has also been identified in other subtypes of leukemia, including the different cytogenetic risk groups of AML, and is associated with a worsened prognosis.^{42,43}

The most common activating mutation of FLT3 is internal tandem duplication (ITD) of the juxtamembrane domain of the gene. ITD mutations involve in frame duplications that range in size from 15 to 231 repeats.⁴⁴ The inserted sequence is thought to lead to constitutive activation of the kinase domain by disrupting the auto-inhibitory interaction between the juxtamembrane domain and the FLT3 activation loop. Activating point mutations of amino acid codons 835 or 836 involving the activation loop of the FLT3 protein have also been described in up to 7% of AML.^{40–42} The presence of a FLT3 activating mutation in patients with AML is associated with a worsened prognosis and with poor response to chemotherapy.⁴¹ Detection of FLT3 ITD, using molecular tests designed to detect the size of the duplicated region, are currently used clinically for prognostic information and to direct therapy in cytogenetically normal AML.

Targeting of FLT3 with specific inhibitors is a treatment strategy that is currently being actively explored in clinical trials.^{45,46} In the near future, molecular testing for the presence of FLT3 activating mutations is likely to accompany clinical treatment with targeted inhibitors of FLT3. The rapid development of resistance toward FLT3 targeted inhibitors has been a major challenge. Resistance can be due to increased drug clearance, inherent resistance, and/or the acquisition of resistance mutations, similar to imatinib resistant BCR-ABL mutations.^{46,47} Several acquired mutations have been described which displace FLT3 inhibitors from blocking the activating site.^{46,48,49} Molecular tests to predict response to FLT3 targeted inhibitors and to detect the presence of resistance mutations will likely become important companion diagnostic tests for the use of FLT3 small molecular inhibitors.

Multidrug Resistance

P-glycoprotein (MDR-1)

One of the most important mechanisms of resistance to chemotherapeutic agents is the presence of multidrug resistance (MDR) due to over-expression of P-glycoprotein (P-gp), a plasma membrane efflux pump that actively transports a variety of drugs and other compounds out of the cell in an energy-dependent manner. P-gp is a member of the ATP-binding cassette (ABC) superfamily of membrane transport proteins. The physiological role of ABC pumps is to transport endogenous metabolites and exogenous toxins out of cells.⁵⁰ P-gp is encoded by the *MDR1* (*ABCB1*) gene and is normally expressed in a variety of tissues, including bone marrow stem cells and lymphocytes.^{51–54}

Over-expression of *MDR1* is associated with multidrug resistance in numerous cancers.

P-gp transports a large number of exogenous drugs that are structurally and mechanistically unrelated, including many anticancer drugs. The physiological substrates of P-gp are not known, but are thought to include phospholipids, steroids, beta-amyloid peptides, and interleukins.⁵⁰ The known substrates of P-gp are all amphipathic, lipid-soluble compounds that are usually positively charged and often have aromatic rings. Anticancer drug substrates of P-gp include vinca alkaloids (vinblastine and vincristine), anthracyclines (doxorubicin and daunorubicin), taxanes, epipodophyllotoxins, and anthracenes, as well as tyrosine kinase inhibitors such as imatinib mesylate.

Detection of MDR in Hematologic Malignancies

Multidrug resistance has been seen in many hematologic malignancies, including AML, ALL, lymphomas, multiple myeloma, and CML.⁵⁵⁻⁶¹ In hematologic malignancies, low levels of *MDR1* expression may be seen prior to therapy and may be markedly increased after chemotherapy treatment.⁵⁰ There is a good correlation between *MDR1* expression levels and response to chemotherapy in adult AML, but not in pediatric AML.⁵⁵ In addition, *MDR1* expression has been reported to be an independent poor prognostic factor in adult AML.^{56,58} *MDR1* expression has also been correlated as an adverse prognostic factor in adult, but not in pediatric, ALL.⁵⁶

Detection of multidrug resistance is generally targeted at detection of the P-gp protein by immunohistochemistry or flow cytometry or detection of *MDR1* RNA by Northern blot, slot blot, or RT-PCR. In addition to techniques designed to detect expression of *MDR1*, functional assays that measure intracellular retention of labeled drugs such as daunorubicin or fluorescent dyes such as rhodamine have been used. Some of the variability in association of these genes with drug resistance may be due to the lack of standardization and correlation of different methodologies. Consensus recommendations have been developed in an attempt to standardize immunophenotypic and functional assays for use in clinical samples.⁶² None of these assays are commonly used clinically. However, as drugs targeted to inhibit multidrug resistance are developed, testing to detect *MDR1* may become more useful to identify subsets of cancer most likely to respond and to follow drug efficacy.

Other Multidrug Resistance Proteins

Other proteins associated with multidrug resistance in leukemia include multidrug resistance-associated protein (MRP1, MRP, ABCC1), breast cancer resistance protein (BCRP, ABCG2, MXR), and lung resistance protein (LRP).^{58,63-65} These are also ABC pumps and important secondary pathways of multidrug resistance that may circumvent drugs designed to inhibit P-gp. They have all been detected in

patients with AML and ALL.^{58,66} Similar methods of detection have been used for these markers as for P-gp, including detection of RNA by RT-PCR, of protein levels by flow cytometry, and functional assays that measure intracellular retention of fluorescent dyes.^{63,64,67}

Other Molecular Mechanisms of Resistance

Resistance to cytarabine (ara-C) in acute leukemias has been associated with the deficiency of deoxycytidine kinase (dCK) in some studies. Deoxycytidine kinase is essential for phosphorylation of cytarabine, a deoxycytidine analog. Loss of dCK expression in leukemia may be due to *DCK* gene rearrangement or mutation.⁶⁸ Expression of dCK has been measured using immunocytochemistry or RT-PCR in research studies, but is not commonly used in clinical care.^{68,69}

Abnormalities in apoptosis may also play a role in the development of drug resistance by leukemic cells. Flow cytometry and immunohistochemistry have been used to detect proteins involved in apoptosis such as Bcl-2, Bcl-x(L), Mcl-1, and Bax in leukemic cells.⁷⁰⁻⁷³ Experimental drugs targeting antiapoptotic molecules have been developed and are currently under investigation in clinical trials.⁷⁴

Microarray Analysis

Despite our increased understanding of the molecular mechanisms of drug resistance, a significant proportion of resistance to anticancer therapies is not well understood. Gene expression profiling techniques, such as cDNA microarrays and SAGE (serial analysis of gene expression), have been employed to identify genes with increased expression in resistant leukemia cells.^{67,75-77} High resolution array CGH (comparative genomic hybridization) has also been used to identify loss or gain of genes in drug resistant melanoma cells.⁷⁸ These gene discovery techniques may identify novel mechanisms of drug resistance which may become potential targets of drug development.

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Monitoring Engraftment of Bone Marrow Transplant by DNA Fingerprinting

Jessica K. Booker

Introduction

Allogeneic bone marrow transplantation is used to treat a large number of inherited and acquired diseases. Inherited diseases that have been successfully treated by bone marrow transplantation (BMT) include lysosomal storage diseases, immunodeficiencies, autoimmunity diseases, and hemoglobinopathies. Acquired diseases that have responded to BMT include hematolymphoid neoplasms and solid tumors.¹

Many protocols have been developed for both the conditioning of the patient as well as the treatment of the harvested bone marrow prior to the transplant. Bone marrow is not the only source of allogeneic hematopoietic stem cells and may be replaced by cord blood or stem cells enriched from peripheral blood. Regardless of the source and conditioning of the transplanted allogeneic stem cells, the purpose of the transplant, or the conditioning of the patient prior to transplant, there is a need for monitoring engraftment once the transplant has been performed. At this time, the most common method used for monitoring hematopoietic stem cell transplant (HSCT) recipients is DNA fingerprinting.

DNA fingerprinting uses polymorphic DNA sequences to create a unique signature that can distinguish one individual from the other. There is an expanding breadth of applications for DNA fingerprinting from identity testing in forensics and medicine to genotyping used in agriculture and paleontology. Both the applications and the methodology have evolved since the first description almost 25 years ago and will certainly continue to do so in the future.

A Brief History of DNA Fingerprinting Methods

Past

DNA fingerprinting was discovered by Sir Alec Jeffreys as an unexpected application in his search for variable regions of DNA in humans that might help with gene mapping. The potential for the identification and, by extension, the

relationship of individuals were immediately appreciated with applications that included forensics, paternity testing, and conservation biology.² The first practical use of DNA fingerprinting was in the resolution of parentage in an immigration case in 1985. The method required endonuclease restriction digestion of DNA that was then electrophoresed and subjected to Southern blot hybridization using multiple radiolabeled minisatellite probes.³ Minisatellites are tandem repeats from 6 to 100 nucleotides (also known as VNTRs for variable number of tandem repeats). In 1986, DNA profiling was used in the first forensic application to prove the innocence of a man who had falsely confessed to murder.^{4,5} The limitations of these earliest methods included the requirement for large amounts of DNA, labor intensity, time consumption, and expense. When methodology began to shift away from Southern blotting to the use of the polymerase chain reaction (PCR), an additional disadvantage in the use of minisatellites was the large variation in repeat numbers of these markers that could lead to preferential amplification of shorter alleles. When there is preferential amplification of shorter repeat alleles, quantitative calculations may be less accurate.

Present

For monitoring BMT engraftment, the most widely used method utilizes PCR to amplify microsatellite markers (also known as STRs for short tandem repeats). Development of this method has been driven by the field of forensics, but it is used for a broad range of applications. The United States Federal Bureau of Investigation utilizes a standard panel of thirteen microsatellite markers. To accommodate the forensic requirements, there are currently several commercially available kits that utilize multiplex PCR amplification of a panel of microsatellite markers with fluorescent tags, which may be detected by capillary gel electrophoresis and then analyzed by computer software programs. Primers may also be developed, or purchased for individual STR markers. These methods require very small amounts of DNA, are relatively easy to use, have a rapid turn-around-time, and are quantitative.

While the assay is relatively inexpensive to run, it does require investment in thermocyclers and expensive capillary instrumentation. The ability to obtain quantitative results for the relative contribution of mixed populations is critically important when monitoring engraftment in BMT recipients. SNP (single nucleotide polymorphism) analysis by quantitative real-time PCR may be used as a more sensitive method of monitoring engraftment,⁶ but it is not as widely used as STR analysis at this time.

Future

The two areas of identity testing that may change are (1) the targets and (2) the methods, used in analysis. The most likely targets for future identity testing are SNPs and the most likely methods are microarrays. SNP microarrays are already widely used in many areas of biological research.

Monitoring Engraftment of Bone Marrow Transplant

The optimal method for monitoring engraftment in HSCT recipients is one that is highly sensitive, meaning that it is able to detect low levels of cells of recipient origin in a sample that is predominantly composed of cells of donor origin. Fluorescent in situ hybridization (FISH) may be used for sex-mismatched transplants, and FISH as well as other molecular methods may be used for the detection of tumor markers.⁷ The following discussion focuses on the use of DNA fingerprinting, which is currently the most widely used method for monitoring engraftment in HSCT recipients.

Microsatellite Markers

Microsatellite markers (also known as STRs for short tandem repeats) are polymorphic DNA loci consisting of tandem repeats of 2–7 nucleotides. The repeat number differs for each marker, but there are typically fewer than twelve alleles for any given marker (ranging from 10 to 30 repeats).

Commercial kits are available for the amplification of STRs, either as single markers or as multiplex assays (combining up to sixteen markers). For the multiplex assays, multiple fluorescent dyes are used, when necessary to eliminate overlap between the sizes of the amplified markers that are labeled with any given fluorescent label. Included in many of these kits are primers for the amelogenin gene. This gene is present on both the X and Y chromosomes, but the gene on the Y chromosome has a small deletion enabling the differentiation of the two amelogenin alleles based on size of the amplified product for gender determination.⁸ Once amplification has been performed, the products are separated

by capillary gel electrophoresis. The alleles for each marker are determined based on size and comparison to an allelic ladder available for each marker.

Technical Considerations

Identification of Informative Markers

For the monitoring of HSCT engraftment, it is important to have a donor sample and a pretransplant recipient sample. DNA from these samples is run with a panel of STR markers used by the laboratory to identify the DNA fingerprint of the donor and recipient. Based on the donor and recipient fingerprints, specific markers are selected as being optimal for distinguishing cells of donor from those of recipient origin. It is much easier to find informative markers when the donor and recipient are unrelated, as is the case when donors come from the National Marrow Donor Program. In the case of closely related (first degree) relatives, there are usually several suitable markers that may be used, if selection is made from an appropriately extensive panel of markers.

In cases where a donor or pretransplant recipient sample is not available, a buccal swab from the transplant recipient may be used. While these samples should contain endothelial cells, and therefore exhibit the pretransplant profile,⁹ they are often a mixture of cells of donor and recipient origin, presumably from lymphocyte infiltration. When mouthwash is used, the cells are of donor origin.¹⁰ Comparison of these samples may be utilized to determine the donor and pretransplant recipient DNA fingerprints.

A complicating factor that must be taken into consideration when identifying informative markers is the phenomenon of stutter. Stutter is a PCR artifact that may occur when amplifying across repetitive sequences, given the propensity for the polymerase to slip and produce a stutter product that is typically one repeat shorter than the true repeat size.¹¹ These stutter products are typically less than 10% of the full size product, and may interfere with the interpretation of quantitative analysis. Take (for example) a donor and recipient pair in which, for a given marker, the donor has an eight repeat allele and a ten repeat allele, and the recipient has a seven repeat allele and a nine repeat allele. Interpretation of low levels of seven and nine repeats with high levels of eight and ten repeats from a posttransplant sample would be uninformative because of the inability to determine whether the seven and nine repeats are from the recipient or stutter from the donor's eight and ten repeat alleles.

Informative markers are selected for each donor and recipient pair, with avoidance of markers in which differentiation between recipient allele and stutter from donor allele is not possible. It is desirable to select at least three informative markers for patient monitoring, so quantitative results from each marker may be calculated and then

reported as an average. Averaging of the percent chimerism using multiple markers reduces the variability inherent in individual markers.¹²

Quantitative Calculations

When the number of PCR cycles is kept relatively low, there is a linear relationship between the amount of the allele present and the peak area of that allele when the fluorescently labeled PCR products are analyzed by capillary gel electrophoresis.¹³ When chimerism is present, the relative contribution of donor and recipient alleles may be calculated using the area of the peaks of informative markers.¹⁴ Quantitative results are typically reported as an average of the percentages of donor and recipient populations. The greatest clinical utility for quantitative monitoring of chimerism is with longitudinal testing to assess relative changes over time.¹²

Sensitivity

Sensitivity is determined by each laboratory using the kit or home brew assay of their choice, during assay validation. The reported sensitivity should be demonstrated every time posttransplant samples are analyzed and may either be done with a single control, or individual controls may be used for each patient. In either case, if a sensitivity of 5% is reported, the ability to detect recipient cells in a mixture of 5% recipient and 95% donor must be demonstrated for each run, in order to be able to report the sensitivity of a posttransplant sample when recipient cells are not detected. The ability to detect a minor population is generally between 1 and 10%, and the limits of detection should be stated in the report.

Clinical Considerations

Enriched Populations

The most significant advances in monitoring BMT recipients in recent years has been in the ability to enrich for specific populations and determine the relative contributions of donor and recipient cells within these populations. Enrichment may be accomplished with the use of monoclonal antibodies bound to magnetic beads. By enriching for a specific lineage, the sensitivity is greatly increased compared to the sensitivity of unfractionated peripheral blood. It is particularly important to monitor lineage-specific engraftment when pretransplant conditioning has either been nonmyeloablative or of reduced intensity.¹ Monitoring of T-cell chimerism may provide early indications of graft versus host disease, relapse, and responsiveness to donor lymphocyte infusions.¹² Monitoring of natural killer (NK) cell chimerism may provide unique prognostic information.¹⁵

Chromosomal Abnormalities

Bone marrow transplants are frequently done as part of the treatment of hematolymphoid neoplasms, which may harbor chromosomal abnormalities or may be chromosomally unstable.¹⁶ If initial treatment is unsuccessful or there is a relapse, the chromosomal abnormalities may impact quantitative monitoring of chimerism. For STR markers that are on chromosomes for which there is aneuploidy, the calculations will be inaccurate. This is one of the reasons it is important to use multiple markers from multiple chromosomes when monitoring engraftment.

Impact on Future Genetic Testing

Once a patient has received an HSCT, provided they become fully engrafted, it is important to consider that any future molecular testing done on peripheral blood or bone marrow will reveal the genotype of the donor rather than the recipient.

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Gene Expression Profiling

Cherie H. Dunphy

Introduction

Gene expression (GE) analyses by use of microarrays (MAs) have become an important part of biomedical and clinical research and the resulting data may provide important information regarding pathogenesis and be extrapolated for use in diagnosing/prognosticating lymphomas and leukemias. This chapter will first review the various techniques used in gene expression profiling (GEP), and then present the pertinent practical applications of the data acquired by GEP in diagnostic hematopathology, as summarized in various tables, referenced throughout the text.

Techniques of Gene Expression Profiling

MAs contain precisely positioned DNA probes designed to specifically monitor the expression levels of genes in parallel. Data mining often utilizes mathematical techniques traditionally employed to identify patterns in complex data. Supervised and unsupervised approaches may be used.

Supervised Gene Expression Profiling

Typically, in order to correlate array data directly to clinical, cytomorphological, or cytogenetic features, the application of a supervised analysis requires the grouping of patients according to predefined characteristics. After detecting differential GE, it is often necessary to accurately classify samples into known groups.¹ In supervised machine learning methods, the observer first “trains” or derives a GE profile on a training set of cases and then subsequently “tests” the predictive power of this GEP on a set of previously unanalyzed “test” cases. Many of these techniques, particularly when “leave one out” cross-validation is performed on large data sets, require extensive parametric studies or the solution of large matrix problems that can only be done using parallel computers. Thus, these methods require carefully designed cohorts, cross-validation, and statistical analyses. It is best

to confirm preliminary results on independent data sets. Willman et al have developed and applied several different learning methods for class prediction in leukemia cohorts, including Bayesian networks and Support Vector Machines (SVMs).² A Bayesian network is a graph-based model for representing probabilistic relationships between random variables. A Bayesian net asserts that each node is statistically independent of all its nondescendants, once the values of its parents (immediate ancestors) in the graph are known. This makes Bayesian nets an attractive framework for GE analysis, since they can methodically hypothesize and test gene regulatory models (and other relationships) using the rigorous methods of classical probability theory and statistics. SVM attempts to define the maximal hyperplane (or corridor) between 2 parameters (such as long-term remission versus failure) in a GE data set. This corridor or hyperplane may be linear or nonlinear. Genes marking the boundaries of this hyperplane are the most discriminating. A first step in most classification models is the application of feature selection techniques to identify those unique and robust gene sets that best discriminate among the classes of interest. Recursive feature elimination (RFE) is an SVM-based method for feature selection in binary classification problems RFE searches through the given gene space of approximately 12,000 genes to find the optimal hyperplane separating the 2 classes.

Unsupervised Gene Expression Profiling

By contrast, clustering is an unsupervised method for organizing expression data into groups with similar signatures. Unsupervised clustering can be used to reduce the complexity of the matrix-like data and to visualize it in a more understandable way, but also to predict the categorization of unknown samples and for “class discovery”: the discovery of intrinsic biologic groups of patients (pts) based on shared patterns of GE.² Patterns are discovered solely from the data itself, without assumptions of previous knowledge or grouping of the data. Many mathematical algorithms may be used for class discovery, including hierarchical

clustering (HC), K-means, self organizing maps (SOMs), and principal component analysis (PCA). Two-dimensional HC sorts both patients and genes according to similarities, and leads to a tree-structured dendrogram that may easily be viewed and explored. This hierarchical structure provides potentially useful information about the relationship between adjacent clusters. Common crossing points represent similar patient characteristics, as well as similarities with regard to the co-expression of distinct genes. By use of a detailed gene annotation, functional groupings of genes based on their similarity may be discovered among the GEPs. Such information may offer insights into physiological pathways and may also help to characterize genes of uncertain function. However, with the exception of PCA, most unsupervised HC algorithms are not multidimensional enough or stable enough to resolve multiple clusters in very large data sets. Willman et al have developed “higher order” multidimensional clustering algorithms such as VxInsight, which has the capacity to cluster patients or genes, using all of the GE data without having to select smaller subsets of genes for actual clustering, in a novel and intuitive way. Similar genes are clustered together spatially and represented in a 3 dimensional terrain map, where large mountains represent large clusters of similar genes, and smaller hills represent clusters with fewer genes. Clusters that are the most similar (genes or patients) are also sited nearer to each other and farther away from less similar clusters.

Usefulness of Ready-Made Macroarrays

Dales et al determined the usefulness of ready-made macroarrays as routine diagnostic tools by analyzing lymph node (LN) biopsies [4 of follicular lymphoma (FL), 2 of diffuse large B-cell lymphoma (DLBCL), 3 of benign LNs].³ In the clustered array data, purified cells sorted from samples sharing common histological lesions were grouped together, whereas the array/histology correlation was less satisfactory for tissues. The GEPs of both the array and immunohistochemical (IHC) methods correlated for most caspases and samples, suggesting that, in the future, pathologists might be able to analyze, by IHC methods, potential markers of interest previously

identified by array technology. The reliability of the array analysis on tissues remains questionable because even non-Hodgkin lymphoma (NHL) samples of the same type are intrinsically heterogeneous regarding their neoplastic and reactive cell content. The correlation between HC and histological features is better for purified cells than for tissues. This point could possibly hamper the use of the method for routine diagnostic purposes because the preparation of the sorted cells is technically more complex.

Likewise, Staal et al reviewed the considerations and infrastructure for MA experiments.⁴ These considerations were illustrated via a MA-based comparison of GEPs of paired diagnosis-relapse samples from patients with precursor-B lymphoblastic leukemia (LL). Initial experiments showed that several seemingly differentially expressed genes were actually derived from contaminating non-leukemic cells, particularly myeloid cells and T-lymphocytes. In addition, extra RNA amplification led to skewing of particular gene transcripts. These technical aspects should be considered when applying these techniques to lymphoma and leukemia diagnosis/prognosis.

Practical Applications in Diagnostic Hematopathology

Prognostic Factors in Chronic Lymphocytic Leukemia and Correlation with Practical Clinical Applicability (See Table 13.1)

IgVH Mutational Status

In B-CLL, the immunoglobulin heavy-chain variable (IgVH) region may be mutated (Ig-mutated CLL) or unmutated (Ig-unmutated CLL); and the presence or absence of somatic mutations in this region of CLL cells distinguishes 2 clinically distinct forms. Ig-unmutated CLL pts have a much worse overall survival (OS) and a relatively shorter median survival (79 and 119 months) than Ig-mutated CLL pts, who have a median survival reaching 293 months and many of whom never require treatment.⁵⁻⁷ IgVH mutational status

TABLE 13.1. Prognostic factors in chronic lymphocytic leukemia (CLL): correlation with practical clinical applicability.^{5-9,11-14}

Prognostic factor	Affect on outcome	Correlation with practical clinical measurement
Mutational status of IgVH ^a	Mutated IgVH, associated with better OS	No detection of ZAP-70 expression
	Unmutated IgVH, associated with worse OS	Unmutated IgVH with up to 93% concordance with ZAP-70 expression No detection of ZAP-70 expression due to rare biological occurrence
HTERT	Increased expression associated with advanced stage and shorter OS	NA
ZAP-70 ^b	ZAP-70 expression, associated with worse OS and up to 93% concordance with unmutated IgVH	May be detected by IF, FC, IHC, RT-PCR techniques

IgVH immunoglobulin heavy chain variable region; *HTERT* human telomerase reverse transcriptase; *IF* immunofluorescence; *FC* flow cytometry; *OS* overall survival; *NA* not applicable; *IHC* immunohistochemistry; *RT-PCR* real-time polymerase chain reaction.

^a Considered single most informative stage-independent prognostic factor.

^b Considered best CLL subtype distinction gene.

has been considered as the single most informative stage-independent prognostic factor in CLL.⁷

Of note, human telomerase reverse transcriptase (hTERT) has been associated with disease aggressiveness in human cancers. Quantitation of hTERT, using real-time polymerase chain reaction (RT-PCR), and analysis for somatic mutations in IgVH genes in CLL cells has shown detection of the hTERT gene in 59% of pts.⁸ The level of expression increased with advancing CLL stage ($p=0.0064$); hTERT-positive pts showed significantly shorter survival than hTERT-negative pts ($p=0.000034$), irrespective of the disease stage. On average, the level of hTERT mRNA expression was 7× higher in the poor prognosis (Ig-unmutated) CLL group, than in the Ig-mutated group ($p<10^{-7}$), and the level of hTERT expression discriminated the 2 groups in 89% of cases.

Interestingly, the set of CLL subtype (mutated versus unmutated IgVH) distinction genes showed enhanced expression of genes related to B cell activation through the B cell antigen receptor (BCR). Genes up-regulated during BCR stimulation were more highly expressed in Ig-unmutated CLL, and genes down-regulated during BCR stimulation were expressed at lower levels in this group. This finding suggests that stimulation through the BCR may play a role in the pathogenesis of CLL. The CLL subtype distinction genes further suggest that ongoing BCR stimulation in the Ig-unmutated CLL subtype contributes to the more progressive clinical course of these patients.⁹

Rosenwald et al found more than 100 genes differentially expressed between the Ig-mutated and Ig-unmutated CLL subtypes with high statistical significance.¹⁰ The most differentially expressed gene was ZAP-70, which encodes a tyrosine kinase. It was expressed in all Ig-unmutated CLL samples and in some B-cell lymphoma cell lines but in none of the Ig-mutated CLL samples. Another study has shown samples of purified B lymphocytes from healthy individuals all showed ZAP70 values of less than 10% (normal B-cells do not express ZAP70).¹¹

ZAP70

Subsequently, Ferrer found, by GEP of Ig-unmutated and Ig-mutated CLLs and sequencing the VH gene mutation status, that the greatest variances between the unmutated and the mutated groups were in expressions of ZAP70, RAF1, PAX5, TCF1, CD44, SF1, S100A12, NUP214, DAF, GLVR1, MKK6, AF4, CX3CR1, NAFTC1, and HEX.¹² ZAP70 was significantly more expressed in the Ig-unmutated CLLs; whereas, expression of all the other genes was higher in the Ig-mutated cases. This study confirmed that ZAP70 expression could predict the VH mutation status and suggested that RAF1, PAX5, and other differentially expressed genes may offer good markers for differentiating these 2 groups and serve as prognostic markers.

Weistner et al further profiled an expanded cohort of CLLs and confirmed that ZAP-70 was the single best

gene distinguishing the unmutated and mutated subtypes.¹³ Ig-unmutated CLLs expressed ZAP-70 5.54× more highly than Ig-mutated CLLs ($p<0.001$). ZAP-70 expression correctly predicted IgVH mutational status in 93% of patients. ZAP-70 expression and IgVH mutation status were comparable in their ability to predict time to requirement for therapy following diagnosis.

Methods of Detection of ZAP70 Expression

ZAP70 expression may be detected at the mRNA level and by various techniques, including immunofluorescence (IF), flow cytometry (FC), and immunohistochemistry (IHC).¹¹ The sensitivity and specificity of ZAP70 by IF detection have been reported as 91 and 100%, respectively.⁸ The detection of ZAP70 expression by these various techniques has been compared with the IgVH mutational status and has revealed occasional discordant results between ZAP70 expression and mutational status.

Discordance Between IgVH Mutational Status and ZAP70 Expression

In a minority of patients (7/107), Weistner et al showed discordant results in ZAP-70 expression and IgVH mutational status (i.e., 4 Ig-mutated CLLs had high ZAP-70 expression and 3 Ig-unmutated CLLs had low ZAP-70 expression). Among these ZAP-70 “outliers,” those with Ig-mutated CLL had clinical features uncharacteristic of this CLL subtype: 2 required early treatment and 2 had a mutated VH3-21 gene (i.e., an IgVH gene that has been associated with progressive disease). In addition, this study developed RT-PCR and IHC assays for ZAP-70 expression that could be applied clinically and determined that ZAP-70 was the best CLL subtype distinction gene. Based on their findings, this group suggested that perhaps CLL should be considered a continuum of diseases rather than 2 discrete subtypes and stressed that while IgVH mutational status and ZAP-70 expression can define prognostically distinct subtypes of CLL, some Ig-mutated CLLs may have progressive disease and some Ig-unmutated CLLs may remain stable for long periods or show only minimal disease progression.¹¹

Due to these discordant cases, a subsequent study by Orchard et al clarified whether the IgVH gene mutational status best predicted prognosis in CLL and assessed the usefulness of ZAP70 as a prognostic marker in CLL.¹¹ They developed a FC assay for ZAP70 protein expression and investigated its concordance with ZAP70 mRNA expression, IgVH gene mutational status, and clinical outcome in CLL pts. This study showed high concordance between ZAP70 protein expression and ZAP70 mRNA expression (97%) and IgVH gene mutations. Sixty-five percent of pts had mutated IgVH genes and were ZAP70 negative; 28% had unmutated IgVH genes and were ZAP70+. Findings were discordant in 8% (13/167) pts: 6 had mutated IgVH genes but were ZAP70+ and 7 had unmutated genes and were ZAP70-negative. Median survival was

24.4 years in ZAP70-negative pts and 9.3 years in ZAP70+ pts. In regard to the discordant cases, 5/6 pts who expressed ZAP70, but had mutated IgVH genes had 97% homology to the germline sequence and 1 had 96% homology. These pts would be judged to have a poor outlook in studies in which either 95% or 97% homology is used to define prognostic subgroups. All pts with less than 95% homology failed to express ZAP70, emphasizing the correlation between ZAP70 expression and low mutational load rather than absence of mutations. The 7 pts with $\geq 98\%$ homology to the germline sequence and ZAP70 negativity are of even more biologic interest. Crespo et al also identified 3 similar pts, indicating these pts represent a biological occurrence rather than a technical difficulty in ZAP70 measurement.¹⁴ Longer follow-up of numerous discordant cases is necessary to determine if ZAP70 expression or IgVH gene mutational status is the better predictor prognosis.

Diagnosis of Mantle Cell Lymphoma

Mantle cell lymphoma (MCL) is known to be associated with the t(11;14)(q13;q32), resulting in deregulated cyclin D1 expression.¹⁵ High levels of cyclin D1 are associated with higher proliferation and poorer survival (Table 13.2).¹⁶ Determination of t(11;14) and/or cyclin D1 protein over-expression are important for a diagnosis of MCL and have conventionally been detected by conventional cytogenetic and fluorescent-in-situ hybridization (FISH) techniques and IHC, respectively.

More recently, a GEP study of lymphomas morphologically consistent with MCL revealed 92/101 cases with high expression of cyclin D1 mRNA by quantitative RT-PCR.¹⁷ More than 1,000 genes were significantly differentially expressed between cyclin D1+ MCLs and other lymphoma subtypes. A GE-based predictor of MCL was fashioned from 42 of the most discriminatory genes, yielding a “MCL signature.” Of note, cyclin D1 was excluded to test whether cyclin D1-negative MCLs could be identified by the predictor. The predictor correctly classified 98% of cyclin D1+ MCLs. More interestingly, of the 9 cyclin D1-negative MCLs, 7 were classified as MCL by their expression of the “MCL signature” genes. Of interest, 3 of these tumors expressed high levels of cyclin D3 or cyclin D2, suggesting that these proteins may

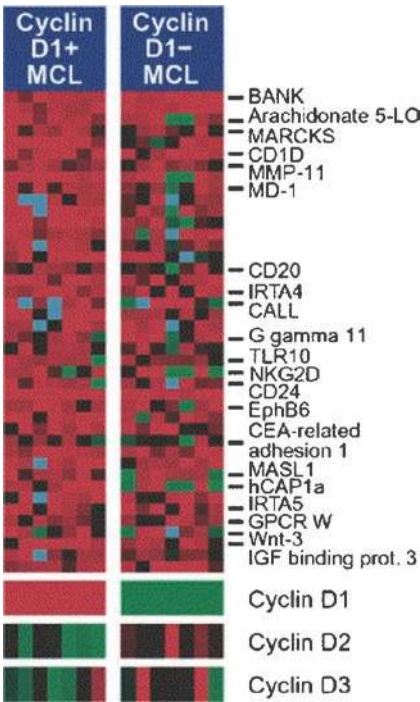


FIG. 13.1. Expression of MCL signature genes in seven cyclin D1-positive and seven cyclin D1-negative lymphoma cases. Cyclin D1-negative cases had MCL morphology and immunophenotype and were classified as MCL based on their gene expression profile. Shown is the relative gene expression of cyclin D1 (as measured by quantitative RT-PCR) and cyclins D2 and D3 (as measured by DNA microarray analysis). (From Rosenwald et al¹⁷ Used with permission.)

functionally substitute for cyclin D1 and have a pathogenetic role in these cases (Figure 13.1). These findings were later confirmed in an additional study.¹⁶

Diagnosis of Hairy Cell Leukemia

Several genes have been specifically identified in HCL by GEP, and their related protein expression has been confirmed by IHC analysis.¹⁸ Some of these genes were already known to be expressed in HCL (i.e., cyclin D1, FGF2, and IL-3R α). The expression of only 7 genes was found decreased in HCL cells compared with all other samples (including chemokine receptor CXCR5 involved in B-cell homing, the TNF receptor-associated factor 5 involved in the signal transduction of TNF-type receptors, including CD40 and CD27). Among recently identified genes over-expressed in HCL were: (a) GAS7, a growth arrest-specific (Gas) gene that is essential for neurite outgrowth in cultured cerebellar neurons (over-expression of GAS7 in HCL may explain the characteristic projections of the hairy cells), (b) the FGFR1 receptor that with over-expression of its ligand suggests the presence in HCL of an autocrine loop, (c) the receptor tyrosine kinase FLT3, which functions as a growth factor receptor for hematopoietic stem

TABLE 13.2. Immunohistochemical stains of practical use in non-CLL cases based on GEP data.

Type of small B-cell malignancy	IHC marker	Clinical use/indication
MCL	Cyclin D1	Diagnosis/associated with worst prognosis ¹⁶
HCL	Annexin A1	Diagnosis: specific to HCL ¹⁹
FL	CD68	Increased number associated with better prognosis ²²

MCL mantle cell lymphoma; HCL hairy cell leukemia; FL follicular lymphoma.

cell and/or progenitor cells, and (d) 3 different inhibitors of matrix metalloproteinases: TIMP1 and TIMP4, 2 secreted tissue inhibitors of metalloproteinases, and RECK, a membrane-anchored glycoprotein that represses synthesis and secretion of metalloproteinases. The up-regulated mRNA expression in HCL cells was confirmed for the following proteins by IHC analysis: FGF2, annexin 1, CD135 (FLT3), NA+ CP type I (SCN1B), CD63, Syndecan-3, TIMP1, IL-3R α , cyclin D1, FGFR1, GAS7, EPB4.1L2, β -actin, CPVL, β -arrestin-2, insulin-like growth factor binding protein (IGFBP3), MYF6, protein tyrosine phosphatase receptor μ , synaptotagmin 1, plexin-C1, TIMP4, and β -2-microglobulin. A subsequent study stained 500 B-cell tumors with anti-annexin A1 (ANXA1) and showed that ANXA1 protein expression was specific to HCL (Table 13.2).¹⁹ It was especially useful in differentiating HCL from SLVL and HCLv (2 entities not infrequently in the differential diagnosis of HCL). The results of these studies with detection of these proteins have biological implications relevant to the pathogenesis of HCL as well as clinical implications for its diagnosis and therapy.^{18,19}

Grade and Disease Aggressiveness in Follicular Lymphoma

Supervised classification of paired follicular lymphomas (FLs) with indolent and aggressive disease courses has established a GEP of 81 genes, that accurately classified 93% of FLs in an independent validation set. Most importantly, in a 3rd series of FLs with ambiguous histologic grading, this 81 GEP showed a classification accuracy of 94%. Genes significantly up-regulated in the aggressive phase of the disease include those involved in cell cycle control (CCNE2, CCNA2, CDK2, CHEK1, MCM7) and DNA synthesis (TOP2A, POLD3A, HMGA1, POLE2, GMP5, CTPS), as well as those reflecting increased metabolism (FRSB, RARS, HK2, LDHA) and activation of several signaling pathways (FRZB, HCFR1, PIK4CA, MAPK1). Genes derived from the reactive infiltrate of T cells and macrophages (CD3D, CXCL12, TM4SF2) were up-regulated in the indolent phase of the disease.²⁰

Of interest, Dave et al determined the prognostic significance of the “non-malignant” components of the tumor microenvironment in FL.²¹ By whole-genome MA analysis of untreated FLs, they further defined 2 GE signatures of tumor-infiltrating immune cells that by multivariate analysis predicted survival in FL. Genes associated with favorable prognosis had an “immune-response 1 signature”, and genes associated with unfavorable prognosis had an “immune-response 2 signature.” The “immune-response 1 signature” included genes encoding T-cell markers (i.e., CD7, CD8B1, ITK, LEF1, and STAT4) and genes that are highly expressed in macrophages (i.e., ACTN1 and TNFSF13B). The “immune-response 2 signature” included genes known

to be preferentially expressed in macrophages, dendritic cells, or both (i.e., TLR5, FCGR1A, SEPT10, LGMN, and C3AR1). A more recent study by Farinha et al has supported that the lymphoma-associated macrophage (LAM) content, detected by CD68 immunohistochemistry, within FLs is an independent predictor of OS (Table 13.2).²² FLs with >15 CD68+ macrophages/high power field (hpf) had a better OS than those FLs with <15 CD68+ macrophages/hpf, independent of the International Prognostic Index (IPI) score.

Subgrouping Diffuse Large B-Cell Lymphoma and Correlation with Immunohistochemical Markers

The sentinel study by Alizadeh et al revealed that the GEPs of DLBCLs were largely distinct from those of CLL and FL and showed additional biological complexity.²³ The genes that defined germinal center B-cell (GCB)-like DLBCL were highly expressed in normal GC B-cells. In contrast, most of the genes that defined activated B-like (ABC) DLBCL were not expressed in normal GC B cells. Known markers of GC differentiation included CD10, CD38, the nuclear factor A-myb, the DNA repair protein 8-oxoguanine DNA glycosylase (OGG1), bcl-6, and a host of new genes (bcl-7A and LM02). The ABC-DLBCL signature also included a gene translocated in lymphoid malignancies, IRF4 (MUM1/LSIRF). Another notable feature of the GEP of ABC-DLBCLs was the expression of two genes whose products inhibit programmed cell death: FLIP (FLICE-like inhibitory protein, which can block apoptosis) and the key anti-apoptotic gene BCL-2 (which is 4 $\times$ higher than in GC B-cells). Of note, this BCL-2 over-expression did not correlate with the bcl-2 translocation.

Importantly, GCB-like and ABC DLBCLs were associated with statistically significant differences in OS ($p < 0.01$) and event-free survival (EFS). 76% of GCB-like DLBCL pts were still alive after 5 years, as compared with only 16% of ABC DLBCL pts. This difference held up when compared within the IPI score. Thus, the GEP of DLBCL and the IPI apparently identify different features influencing survival.

Linderoth et al applied IHC markers (Bcl-6, CD10, BCL-2, bax, CD138, CD40, and CD23) to DLBCLs to differentiate histogenetic origin and prognosis, as have been described by GEP.²⁴ Bcl-6, CD10, and CD40 were considered markers of GC phenotype; CD23, as pre/early GC origin; and CD138, as post-germinal center origin. BCL-2 and bax were considered as apoptotic regulators. There was no prognostic significance of CD10, bcl-6, or CD138 IHC results. CD40 was expressed in 76% of cases, and this group was associated with superior time to treatment failure ($p = 0.027$) and OS ($p = 0.0068$), independent of IPI. CD23 was expressed in 16% of cases (all CD5-negative and all CD40-positive). This group showed a strong tendency for better OS ($p = 0.033$).

TABLE 13.3. Immunohistochemical markers associated with prediction of outcome in DLBCL based on GEP data.²⁴

Expression pattern of DLBCL	IHC markers expressed	Association with prognosis
(A) GCB pattern	CD10+ and/or bcl-6+	Best OS
(B) "Activated" GCB pattern	At least 1 GCB marker (CD10, bcl-6) and 1 activation marker (mum1, CD138)	Worse OS than pattern A
(C) Activated non-GCB pattern	mum1 and/or CD138	Worse OS than pattern A

GCB germinal center B-cell.

CD40 expression correlated with bax but not with BCL-2 expression.

A later IHC study evaluated a panel of GC B-cell (CD10 and Bcl-6) and activation (MUM1/IRF4 and CD138) markers to define prognosis in de novo DLBCL pts.²⁵ They were classified into 3 expression patterns: (A) GC B-cell pattern expressing CD10 and/or bcl-6 but not activation markers, (B) activated GC B-cell pattern expressing at least one of the GC B-cell markers and one of the activation markers, and (C) activated non-GC B-cell pattern, expressing MUM1/IRF4 and/or CD138 but not GC B-cell markers. Patients with pattern A had a much better OS than those with the other 2 patterns ($p < 0.008$). The IPI scores and the expression pattern of these markers were independent prognostic markers, and thus, these IHC markers may be practically applied (Table 13.3).

Differentiation of Classical Hodgkin Lymphoma from Lymphocyte-Predominant Hodgkin Lymphoma and From Diffuse Large B-Cell Lymphoma and Correlation with Immunohistochemical Markers

Marafioti et al investigated the expression of 5 intracellular signaling molecules found in B cells in cHL and found that the Src family kinase Syk, the B-cell adaptor protein BLNK, and phospholipase C (PLC)- γ^2 were consistently absent from R-S cells; whereas, 2 other Src kinases (Lyn and Fyn) were heterogeneously expressed in a proportion of cases (12 and 42%, respectively).²⁶ In contrast, the tumor cells in all Lymphocyte-Predominant Hodgkin Lymphoma (LPHLs) were positive for Syk, BLNK, PLC- γ^2 , and Fyn; Lyn was positive in a minority. Thus, in R-S cells, the defect in B-lineage marker expression apparently includes a spectrum of molecules involved in intracellular signaling, and the difference in expression of these signaling proteins between cHL and LPHL may be diagnostically useful.

Browne et al studied the expression of B cell-specific activator protein (BSAP), Octamer-binding transcription factor 2 (OCT-2), and B-cell Oct-binding protein 1 (BOB.1) in cHLs, nodular LPHLs, DLBCLs, including T-cell-rich large

TABLE 13.4. IHC markers useful in differentiation of cHL, NLPHL, and DLBCL based on GEP data.²⁶

Lymphoma subtype	IHC pattern
cHL	BSAP+ and OCT-2-/BOB.1- was predictive of cHL (75% of cases) BSAP-/OCT-2-/BOB.1- was seen only in cHL BSAP+/OCT-2+/BOB.1+ was seen in only 18% of cHL OCT-2 and BOB.1 staining was weak to moderate and focal in R-S cells No cHL cases expressed all 3 pan B-cell markers (CD20, CD22, and CD79a)
NLPHL and DLBCL	BSAP+/OCT-2+/BOB.1+ was predictive of NLPHL and DLBCL OCT-2 and BOB.1 staining was uniform and strong in NLPHL and in 96% of DLBCL cases All NLPHL cases and 91% of DLBCL cases expressed all 3 pan B-cell markers (CD20, CD22, and CD79a)

IHC immunohistochemical; cHL classical Hodgkin lymphoma; NLPHL nodular lymphocyte-predominant Hodgkin lymphoma; DLBCL diffuse large B-cell lymphoma.

B-cell lymphoma (TCRLBCL) and T/null-cell anaplastic large cell lymphomas (ALCL).²⁷ BSAP+ and either Oct-2 or BOB.1-negativity was predictive of cHL (75% of cases). All BSAP-negative cHL cases were also negative for Oct-2 and BOB.1. BSAP+/OCT-2+/BOB.1+ was predictive of NLPHL or DLBCL; however, 18% of cHL were also positive for all 3. However, the staining of cHL with Oct-2 or BOB.1 tended to be focal, weak-moderate in the R-S cells; whereas, these 2 stains were uniformly, strongly positive in the NLPHLs and in 96% of the DLBCLs. None of the cHLs expressed all 3 pan-B-cell markers (i.e., CD20, CD22, and CD79a); whereas, 91% of DLBCLs and all NLPHLs had expression of all 3 pan-B-cell markers (Table 13.4).

Class Assignment of Pediatric Precursor B Lymphoblastic Leukemia

Gene expression profiling of a large number of pediatric LLs, by using oligonucleotide MAs with HC and HD oligonucleotide MAs, representing most of the identified genes in the human genome, have identified discriminator genes for the subgroups, achieving an overall diagnostic accuracy of 97%.^{28,29} These unbiased clustering algorithms demonstrated that pediatric LLs clustered primarily into 7 major subgroups: precursor T-LL and 6 subtypes of precursor B-LL corresponding to (1) rearrangement in the MLL gene, (2) t(1;19)(E2A-PBX1), (3) hyperdiploid with >50 chromosomes, (4) t(9;22)(BCR-ABL), (5) t(12;21)(TEL-AML1), and (6) a subgroup with a distinct GEP (high expression of a group of genes, including the receptor phosphatase PTPRM and LHFPL2), including cases with normal, pseudodiploid, or hyperdiploid karyotypes, and lacking any consistent cytogenetic abnormality (Table 13.5).

TABLE 13.5. Class assignment of pediatric precursor-B all based on GEP (97% accuracy).^{28,29}

7 Classes	Correlation with known markers	Correlation with prognosis
(1) Rearrangement of MLL gene	Low/undetectable expression of CD10	Unfavorable
(2) E2A-PBX [t(1;19)]	Intermediate expression of CD10	Unfavorable
(3) Hyperdiploid (>50 chromosomes) ^a		Favorable
(4) BCR-ABL [t(9;22)]		Unfavorable
(5) TEL-AML1 [t(12;21)] ^b	High expression of CD10	Favorable
(6) Cases with a distinct GEP (high expression of receptor phosphatase, PTRM, and LHFPL2) with no consistent cytogenetic abnormality (including normal, pseudodiploidy, or hyperdiploidy)		Unknown
(7) Heterogeneous group lacking any defined genetic lesions and not clustering with class #6		Unknown

^aPrecursor B-ALL with more than one abnormality (i.e., BCR-ABL and hyperdiploidy of >50 chromosomes) may only be assigned to 1 class (i.e., hyperdiploidy by GEP).

^bFISH analysis has been shown to be more sensitive than RT-PCR analysis for detection of TEL-AML1.

In addition, a heterogeneous group of B-lineage cases was identified that lacked any of the defined genetic lesions, failed to cluster in the novel subgroup, and were left unassigned (Figure 13.2).

It should be noted that 100% diagnostic accuracy was achieved for only 2 of the leukemia subtypes (precursor T-LL and TEL-AML1). The top 5- class-discriminating genes were used in a supervised learning algorithm and resulted in 97% accuracy of class assignment. Although the number of genes required for optimal class assignment varied between classes, the overall diagnostic accuracy was essentially the same using either the top 20 or top 50 genes per class.

Interestingly, of the rare misclassification errors, 2 were BCR-ABL-expressing ALLs that by GE analysis were classified as hyperdiploid with >50 chromosomes. The GEP thus correctly identified the presence of the hyperdiploid with >50 chromosomes class; however, since each case is assigned to only a single class, the algorithm failed to correctly identify the presence of BCR-ABL. Nevertheless, the data demonstrated the exceptional accuracy of this single platform for the diagnosis of the prognostically important subtypes of pediatric LL.

Distinctive Gene Expression Signatures of Precursor T-Cell Lymphoblastic Leukemia and Correlation with Stage of Differentiation, Markers, Cytogenetic Findings, and Prognosis

Several genes have been shown to play a role in precursor T-LL: HOX11, SCL, LM01, LM02, and LYL1. Ferrando et al studied precursor T-LLs and showed distinct GEPs strongly associated with specific oncogenic transcription factors.³⁰ Gene expression profiling showed 5 different T cell oncogenes (HOX11, TAL1, LYL1, LM01, and LM02) that were often aberrantly expressed in a much larger fraction of T-LLs than those actually harboring the activating

chromosomal translocations. Using oligonucleotide MAs, this study identified several GE signatures that were indicative of leukemic arrests, due to T-cell oncogene interference, at specific stages of normal thymocyte development: LYL1+ signature (pro-T), HOX11+ (early cortical thymocyte), and TAL1+ (late cortical thymocyte) (Figure 13.3). HC analysis of GE signatures grouped samples according to their shared oncogenic pathways and identified HOX11L2 activation as a novel event in T cell leukemogenesis. This group subsequently identified 5 different multistep pathways leading to precursor T-LL, involving activation of different T-ALL oncogenes: (1) HOX11, (2) HOX11L2, (3) TAL1 plus LM01/2, (4) LYL1 plus LM02, and (5) MLL-ENL.³¹ HOX11 activation was significantly associated with a favorable prognosis in pts treated with modern combination therapy; while expression of TAL1, LYL1, or, surprisingly, HOX11L2 confers a much worse response to treatment and a high risk of early failure. The precursor T-LL oncogenes and their correlation with stage of differentiation, expressed known markers, cytogenetic findings, and prognosis are outlined in Table 13.6.

Association of Distinct Gene Expression Profiles with Acute Myeloid Leukemias with Recurring and Complex Abnormalities and Correlation with Commercially Available Molecular Markers by Polymerase Chain Reaction Analysis and with Prognosis

Studies have shown a unique correlation between AML with specific cytogenetic aberrations and distinct GEPs (Table 13.7).^{32,33} MA analyses on BMs of newly diagnosed adult AMLs, all representing one of the distinct subtypes [i.e., AML M2 with t(8;21), AML M3/M3v with t(15;17), AML M4eo with inv (16), or AML with 11q23/MLL] have revealed

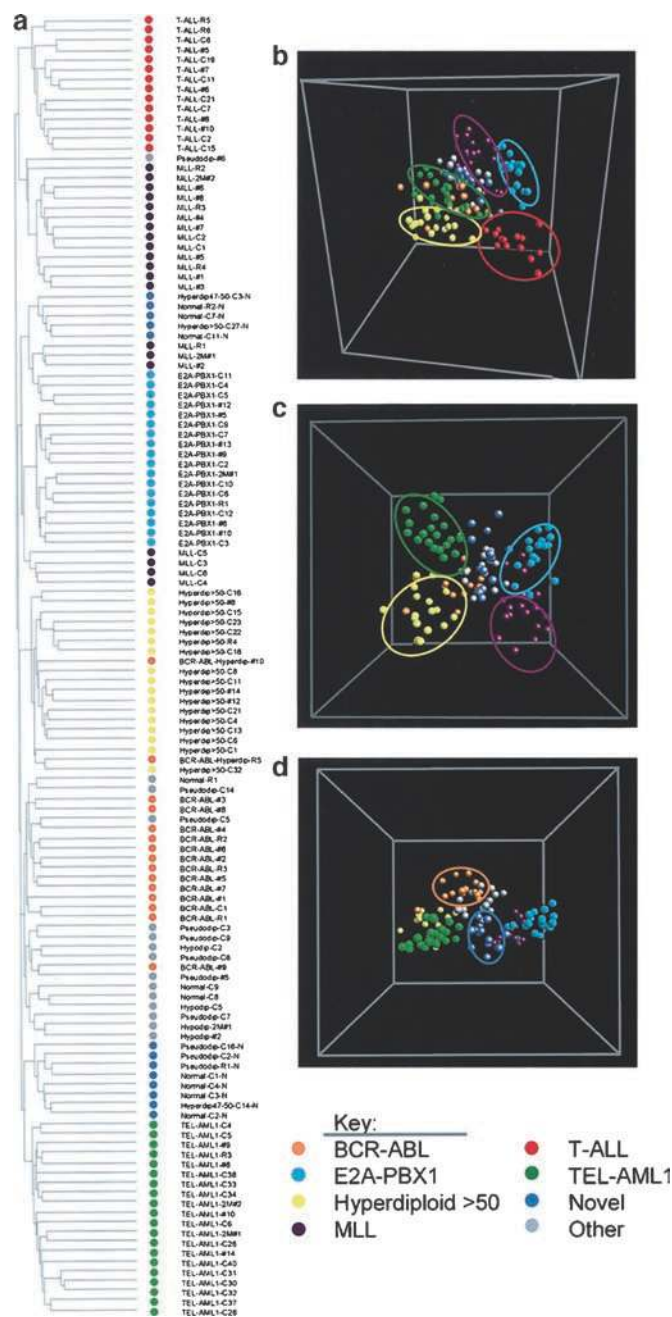


FIG. 13.2. Distinct leukemia subtypes can be defined based exclusively on their expression profiles. Expression profiles were obtained on leukemic blasts from 132 diagnostic bone marrow aspirates and the data analyzed using (a) an unsupervised 2-dimensional clustering algorithm and (b-d) principle component analysis (PCA). In this analysis the cases in the training and test sets were combined, and the analysis was performed with the 26 825 genes from the Affymetrix U133A and B microarrays that varied in their expression across this dataset. (a) A 2-dimensional hierarchic clustering was performed using Pearson correlation coefficient and unweighted pair group method using arithmetic averages. (b) Multidimensional scaling plot of all cases using PCA.

(c) Multidimensional scaling plot of B-lineage ALL cases ($n=118$). (d) The identical multidimensional scaling plot as shown in panel C except the plot was rotated 90 degrees. Each case is represented by a sphere and is color coded to indicate the genetic subgroup to which it belongs: *BCR-ABL* (orange), *E2A-PBX1* (aqua), hyperdiploid with more than 50 chromosomes (yellow), *MLL* (purple), *T-ALL* (red), *TEL-AML1* (green), novel cases (blue), and unclassified cases (gray). (This research was originally published in Blood. Ross ME, Zhou X, Song G, et al Classification of pediatric acute lymphoblastic leukemia by gene expression profiling. *Blood*. 2003;102:2951-2959. Copyright the American Society of Hematology.)

FIG. 13.3. (continued) by RT-PCR. The genes depicted were chosen from the top 200 nearest neighbors of each major oncogene (boldface type) on the basis of their potential functional relevance and then were grouped according to their involvement in T cell differentiation, apoptosis, cell proliferation, or chemotherapy response. Express-

sion levels for each gene were normalized across the samples; levels greater than or less than the mean (by as much as three standard deviations) are shown in shades of red or blue, respectively. Numbers at the bottom correspond to the case numbers of the samples in the study. (From Ferrando et al³⁰ Used with permission.)

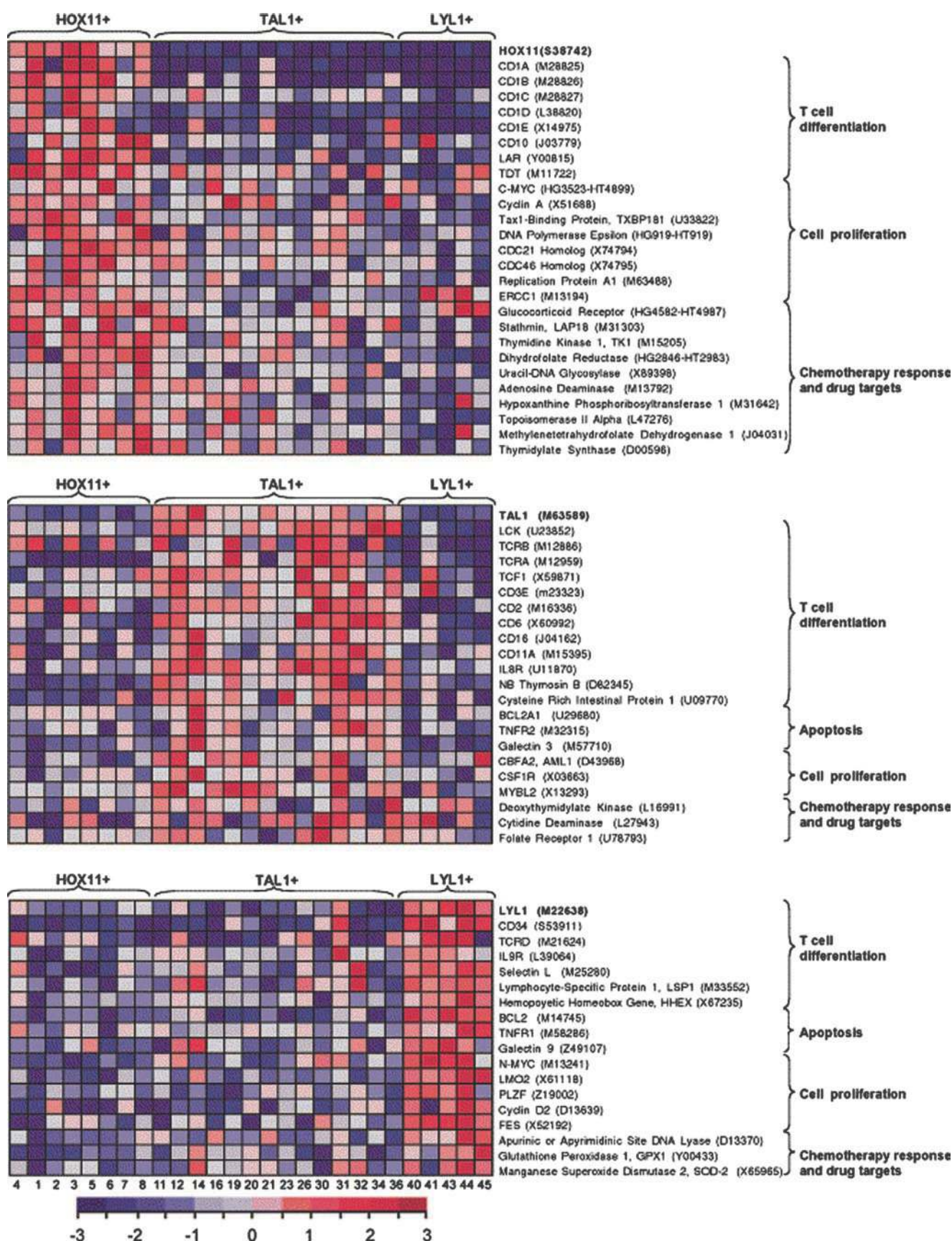


FIG. 13.3. *HOX11*⁺, *TAL1*⁺, and *LYL1*⁺ nearest neighbor analysis. Each row of squares shows the expression pattern of a particu-

lar gene selected by nearest neighbor analysis, while each column represents 1 of the 27 samples positive for *HOX11*, *TAL1*, or *LYL1*

TABLE 13.6. Distinctive GE signatures of precursor T-ALL: correlation with stage of differentiation, markers, cytogenetic findings, and prognosis.^{30,31}

T-cell oncogenes expressed	Stage of differentiation	Correlation with known markers	Correlation with cytogenetic findings	Prognosis
LYL1+	Undifferentiated/ prothymocyte	CD34+	5q- and 13q- deletions	Unfavorable
(LMO2+ and LMO1-) ^a		BCL-2+		
HOX11+	Early cortical thymo- cyte	Myeloid markers+ CD1+, CD10+/-, CD4+, CD8+, CD3-	t(10;14)(q24;q11) or t(7;10) (q35;q24)	Favorable
TAL1+	Late cortical thymo- cyte	CD2+, CD3e+	Recurrent translocations of chro- mosome band 1p32	Unfavorable
(LMO1+ and/or LMO2+) ^b		Bcl-2A1+		
HOX11L2	N/A	Not described	Chromosome 5q abnormality	Unfavorable
MLL-ENL ^c	N/A	Not described	t(11;19)(q23;p13.3)	Not necessarily unfavorable

^aThe LYL1+ GE signature was associated with high expression of LM02, but not LM01.

^bThe TAL1+ GE signature was associated with expression of LM01 and/or LM02.

^cVery rare cases of precursor T-ALL reveal MLL-ENL by RT-PCR analysis; none of these rare patients have died.

TABLE 13.7. AMLs with recurring and complex abnormalities associated with distinct GEPS: correlation with commercially-available molecular markers by PCR analysis and with prognosis.³⁴

Cytogenetic abnormality	Molecular marker (PCR)	Prognosis
t(8;21)	AML1/ETO	Favorable
t(15;17)	PML-RAR	Favorable
inv(16)	CBFB/MYH11	Favorable
11q23	MLL	Unfavorable
Complex	None	Unfavorable

that by GEP, a minimum set of 39 genes was sufficient to distinguish normal BMs and AMLs with 1 of the aberrations with 100% accuracy by a leave-one-out cross-validation.

The potential of GEP to predict AML subtypes and assign AMLs to specific cytogenetic groups [t(8;21), inv(16), t(15;17), MLL, complex karyotype, normal karyotype] was validated with high accuracy (ranging from 86 to 100%) (Table 13.7).³⁴ The lowest accuracy (86%) was seen in the normal karyotype group, indicating a high degree of heterogeneity in this AML subtype.

Comparison of AMLs with a complex aberrant karyotype to AMLs with 8;21, inv 16, rearrangement of the MLL gene, sole trisomy 8 abnormality, and a normal karyotype revealed that only 1 to 7 genes were necessary to discriminate AML with complex aberrant karyotype from every other subgroup with 100% accuracy as assessed by leave-one-out crossvalidation (Figure 13.4).³⁵ The expression of both HOXA9 and HOXA7 was discriminative between AML with complex aberrant karyotype (both HOX genes expressed) and AMLs with 8;21, 15;17, and inv 16 (no or very low expression of these HOX genes). Compared to all other AML subtypes, AML with complex aberrant karyotype had a significantly higher expression (1.7-fold; $p=0.0001$) of an apoptotic gene involved in double-stranded break repair, RAD21, as well as significantly elevated expression (1.5–3-fold) of the

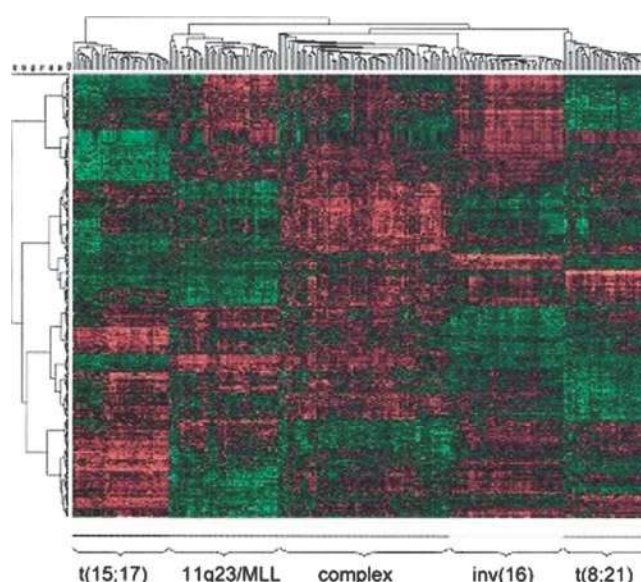


FIG. 13.4. Hierarchical cluster analysis of adult AML samples using a combination of 1,130 genes (rows) identified to separate specific cytogenetic subgroups. Normalized expression value of each gene is color-coded: red indicates high expression; green indicates low expression. The adult AML samples comprise T(11q23)/MLL ($n=44$), t(8;21) ($n=38$), t(15;17) ($n=42$), inv(16) ($n=44$), and complex aberrant karyotypes ($n=76$). The hierarchical clustering was performed by use of the Euclidean distance metric and the Ward clustering algorithm as implemented in GeneMaths software, version 2.01 (Applied Maths BVBA, Sint-Martens-Latern, Belgium). (From Schoch C, Kern W, Kohlmann A, et al Acute myeloid leukemia with a complex aberrant karyotype is a distinct biological entity characterized by genomic imbalances and a specific gene expression profile. *Genes Chromosomes Cancer*. 2005;43:227-238. Copyright 2005, Wiley-Liss, Inc. Reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.)

genes involved in DNA repair and DNA damage-induced checkpoint signaling, RAD1, RAD9, RAD23B, PIR51 (RAD51 interacting protein), NBS1, MSH6, UBL1, and ADPRTL2. The high expression of these genes may play an important role in resistance to chemotherapeutic agents which cause DNA damage.

Comparison of the GEPs of AMLs with rearrangements of the MLL gene and different partner genes to AMLs with trisomy 8, AMLs with complex aberrant karyotype, and normal BMs revealed all groups could be classified robustly with 100% accuracy (leave-one-out cross-validation), based on distinct patterns of differentially expressed genes. Within the MLL leukemia samples, a cluster of HOXA family members, including HOXA7, HOXA9, and HOXA10, and TALE family genes, including PBX3 and MEIS1, were highly expressed.

Similarly, an independent comparison of the GEPs using MAs of AMLs with 8;21, 15;17, inv 16, and 11q23 aberrations and AMLs with normal cytogenetics revealed many discriminating genes.³⁶ Interestingly, the expression status of specific genes correlated with 11q23 as well as with AML of normal karyotype. The latter group was characterized by distinctive up-regulation of members of the class I homeobox A and B gene families, implying a common underlying genetic lesion for AMLs with a normal karyotype.

More recent studies of AMLs revealed the possibility for a comprehensive unsupervised clustering of the disease.^{37–39} Bullinger et al demonstrated tight separate clusterings of AML with translocations 15;17 and 8;21, and inv 16, respectively.³⁸ Valk et al used a Pearson correlation coefficient analysis to sharply classify the prognostically important subgroups of AML (i.e., 8;21, 15;17, and inv 16). They were able to predict these 3 cytogenetic classes within a large and diverse cohort of AML, according to the expression levels of only 5 genes which were tightly linked to the fusion genes of the recurrent translocation (i.e., ETO in AML with 8;21 and MYH11, AML with inv 16). Moreover, AMLs with 11q23 abnormalities or FLT3 or CEBPA mutations aggregated in other particular signature clusters.³⁷ Ross et al also demonstrated, by unsupervised 2-D HC analyses of pediatric AMLs, recognizable clusters characterized by the AML subtypes with the 15;17, 8;21, and MLL chimeric fusion genes, as well as FAB M7.³⁹

Interestingly, GEP proved to identify cases that had escaped standard cytogenetic detection. Among homogeneous expression clusters linked to inv 16 or t(15;17), Valk et al observed cases which had not presented with the corresponding cytogenetic abnormality; however, additional molecular analysis of these cases revealed the presence of these molecular abnormalities.³⁷ Moreover, in this same study, within clusters of pts carrying mutations in the CEBPA gene, cases were present without mutations in this gene, suggesting that these AMLs share a common underlying molecular theme. In addition, AML with FLT3-ITD was precisely discriminated within the

t(15;17) cluster, demonstrating the power of GEP to disclose the molecular heterogeneity within pre-established subsets of leukemia.

AMLs with FLT3 Length Mutation

The most common molecular abnormality in AML is the internal tandem duplication in the *fms*-like tyrosine kinase-3 gene (FLT3), a hematopoietic growth factor receptor, which occurs in 23% of all cases and in 40% of AMLs with a normal karyotype.^{40–42} Comparisons of the GEPs of AMLs with a normal karyotype and AMLs with FLT3 length mutation (FLT3-LM) with GEPs of normal BMs, AMLs with t(8;21), inv 16, t(15;17), t(MLL), trisomy 8, and complex aberrant karyotype have revealed discrimination of the FLT3-LM group from trisomy 8 with 97% accuracy and from all karyotypically aberrant AML groups with 100% accuracy; the confidence was 0.85 for comparison to AML with complex aberrant karyotype and 1.0 for all other comparisons.⁴³ Although separation of AMLs with normal karyotype and FLT3-LM (27 cases) and those without FLT3-LM (21 cases) was not possible, the same analysis within each FAB subgroup resulted in a clear distinction between FLT3-LM+ and FLT3-LM-negative cases. The 20 top genes found to be discriminatively expressed in each analysis varied substantially between the FAB subtypes, although many were downstream target genes of the FLT3 pathway.

AML with FLT3-ITD is generally identified as a poor prognostic disease. Recently Lacayo et al demonstrated that GEP could distinguish a subset with relatively good outcome among the AMLs with FLT3 mutations.⁴⁴ Analysis of DNA MAs have identified GEPs related to FLT3 status and outcome in childhood AML. Based on 81 diagnostic specimens, 42 were FLT3-MU+ and predictive analysis of MAs of these FLT3-MU+ cases identified 128 genes correlating with clinical outcome. Event free survival in FLT3-MU patients with a “favorable” signature was 45% versus 5% for those with an “unfavorable” signature ($p=0.018$). Among FLT3-MU specimens, high expression of the RUNX3 gene and low expression of the ATRX gene were associated with inferior outcome. The ratio of RUNX3 to ATRX expression classified FLT3-MU cases into 3 EFS groups: 70%, 37%, and 0% for low, intermediate, and high ratios, respectively ($p<0.0001$). Thus, GEP identified FLT3-MU+ AMLs with divergent prognoses and the RUNX3 to ATRX expression ratio should be a useful prognostic indicator in these AMLs.

Of interest, some AMLs are associated with a partial tandem duplication within the MLL gene (MLL-PTD) (approximately 6% of AMLs); these cases do not show chromosomal rearrangements by banding analysis and have been associated with an unfavorable prognosis.⁴⁵ Discrimination of MLL-PTD from AMLs with a normal karyotype and without this aberration by GEP using MAs has not been possible since a specific

GEP differentiating the MLL-PTD+ from PTD-negative cases has not been able to be defined. However, a specific GEP has been found for the various AMLs with t(MLL) as compared to the MLL-PTD and normal karyotype groups. Similarities were found between MLL-PTD and t(MLL) in HOX gene expression pattern (HOXA7, HOXA9, HOXA4, HOXA5, HOXA10), although HOXB2, HOXB5, HOXB6, and HOXB7 were expressed at lower levels in t(MLL). These data suggest that in t(MLL) and MLL-PTD+ cases, HOX gene regulation is altered in a common way, but the overall GEP is markedly different between the 2 groups. Since the MLL-PTD pattern cannot be well differentiated from that of AML with normal karyotype, these 2 groups may be more closely related to each other than MLL-PTD to t(MLL). Ross et al similarly showed in a combined pediatric data set of 130 AMLs and 137 ALLs that AMLs containing partial tandem duplications of MLL failed to cluster with MLL chimeric fusion gene cases, suggesting a significant difference in their underlying mechanism of transformation.³⁹

Summary

The findings by GEP of leukemias and lymphomas aid in diagnosis and prognostication of these diseases. The extrapolation of these findings to more timely, efficient, and cost-effective methods, such as flow cytometry and immunohistochemistry have and will continue to result in better diagnostic tools to manage these diseases. At this time, there are flow cytometric and immunohistochemical applications of the information gained from GEP in chronic lymphocytic leukemia (CLL) (i.e., mutational status of IgVH, HTER, and ZAP-70) as well as other low-grade B-cell lymphomas/leukemias (i.e., cyclin D1 in mantle cell lymphoma; annexin A1 in hairy cell leukemia; CD68 in follicular lymphoma), diffuse large B-cell lymphoma (i.e., germinal center B-cell (GCB); "activated" GCB pattern; activated non-GCB pattern), and nodular lymphocyte-predominant and classical Hodgkin lymphoma (cHL) (i.e., immunohistochemical reactivity patterns of BSAP, OCT-2, and BOB.1). For practical clinical applications, GEP profiling of precursor B- and precursor T lymphoblastic leukemia (LL) has mainly supported the information obtained by cytogenetic and molecular studies (i.e., MLL; t(1;19); hyperdiploidy of >50 chromosomes; t(9;22); t(12;21) in precursor B LL and undifferentiated/prothymocyte stage associated with 5q- and 13q-; early cortical thymocyte stage associated with t(10;14)(q24;q11) or t(7;10)(q35;q24); late cortical thymocyte stage associated with recurrent translocations of chromosome band 1p32; a HOX11L2+ GEP associated with a chromosome 5q abnormality; and a MLL-ENL+ GEP associated with t(11;19)(q23;p13.3) in precursor-ALL). Likewise in AML, GEP has supported the distinction of AMLs with recurring cytogenetic and complex cytogenetic abnormalities by cytogenetic and molecular techniques (i.e., t(8;21)-AML1/ETO; t(15;17)-PML-RAR α ;

inversion 16-CBF β /MYH11; and 11q23 abnormality-MLL). However, of particular interest has been the identification of a distinct GEP in AMLs with FLT3 length mutations, which occur in 40% of AMLs with a normal karyotype. This finding is important since FLT3 mutations may be detected by molecular analysis and has been generally associated with a poor prognosis. However, GEP has also identified a subset of AML with FLT3 mutations with a relatively good outcome, and thus, continued extrapolation of GEP data to more practical techniques is greatly anticipated in the management of these diseases.

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Proteomics of Human Malignant Lymphoma

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Introduction

The proteome represents the total complement of proteins present in a complex, an organelle, a cell, tissue, or an organism.¹ Proteomics encompass the multifaceted study of protein expression, interactions, posttranslational modification, and function at the cellular level. Mass spectrometry offers significant opportunities for the analysis of single proteins and the unbiased large-scale analysis of proteins in complex mixtures. The ability to conduct large-scale investigation of proteins in an unbiased fashion dramatically improves the opportunities for biological discovery and is relevant for the elucidation of novel biological insights into physiology and disease. In this regard, mass spectrometry is considered a key technology that will drive the achievement of several milestones in the identification of key proteins involved in disease detection and treatment. This chapter provides a synopsis of the principles of the techniques employed in the current state-of-the-art proteomics and the opportunities that this suite of technologies offers in biological discovery as it relates to human lymphomas. Advances in mass spectrometry-based proteomics have shifted the paradigm of translational cancer research (for a review of background on proteomics and mass spectrometry see²⁻⁴). The achievement of the ultimate goals of identifying biomarkers for diagnosis and prognosis and the development of novel agents for therapy will require significant effort in understanding the basic protein building blocks and the global proteomic circuitry.

Biological Samples for Proteomics

The sample material from which proteins for proteomics studies may be extracted includes fresh or snap frozen cells from varied sources such as biological fluids (i.e., serum, urine, or plasma) or solid tissue material (as with biopsy specimens). Moreover, proteins isolated from ethanol-fixed paraffin-embedded tissues may be utilized for mass spectrometry analysis.⁵ Furthermore, protocols for the identification

of proteins from formalin-fixed paraffin embedded (FFPE) tissue material have been recently developed,^{6,7} including laser capture micro-dissected tissues.^{7,8} FFPE material is the most common form of biopsy archiving that is utilized worldwide, and this development represents an important advancement for the large-scale interrogation of proteins in archival patient-derived material.

General Strategy for Proteomics

While recent developments in protein/peptide array technology hold promise for widespread future applications in proteomics, mass spectrometry is currently the principal technology for the high throughput analysis of peptides and proteins. Mass spectrometry requires separation of the proteins/peptides, which may be performed via a variety of technologies.

Protein Microarrays

Protein microarrays consist of large (hundreds to thousands) arrays of immobilized peptides or proteins on a solid matrix. Incubation with biologic fluids (such as serum), followed by a visualization step, leads to the identification of proteins/antibodies that are reactive with the spotted peptide/protein. Protein microarrays may be spotted with peptides, proteins, antibodies, or cell/tissue lysates. They represent sensitive, high throughput methods for large-scale screening of disease-related antigenic proteins in biologic fluids or tissue extracts. Protein microarrays are ideal for studying receptor–ligand interactions, enzyme activity, and antibody–antigen interactions in a high throughput manner. They have the potential to detect a protein with a sensitivity 1,000-fold greater than an enzyme-linked immunosorbent assay and do not require a mass spectrometer.

Reverse phase protein arrays (RPPA)⁹ are a variation of protein microarray, in which cell or tissue lysates are spotted onto a solid matrix. Antibodies that recognize native or

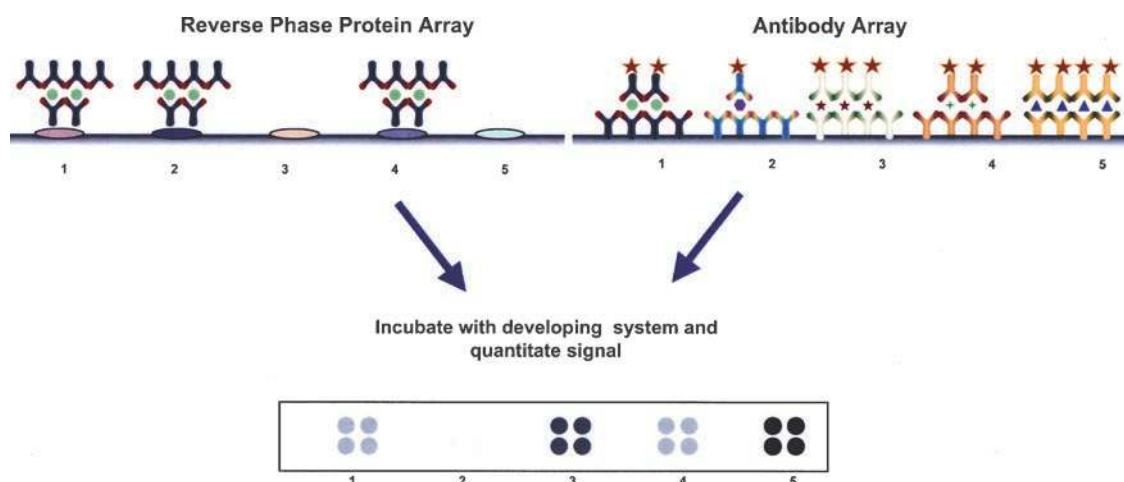


FIG. 14.1. A schematic representation of protein microarrays. Protein microarrays can be either spotted with antibodies/proteins/peptides or cell lysates (reverse phase protein arrays). In antibody arrays, various antibodies are coated on the surface and each antibody is

used to detect the presence of its immunoreactive antigen. Reverse phase protein arrays are a variation of protein arrays in which cell or tissue lysates are spotted onto a solid matrix.

posttranslationally modified forms, such as phosphorylated proteins, are used to interrogate the microarrays. They represent a sensitive, high throughput functional technique to evaluate differential expression of active and parental proteins in a quantitative fashion, using very small amounts of cell lysates (picogram to femtograms). Although RPPA facilitate assessment of protein modifications and activation of broad networks of biologically relevant pathways, one limitation is that only known proteins/targets are identified. See Figure 14.1 for a schematic diagram of protein microarrays.

Mass Spectrometry-Based Proteomics

There are several different modalities used for the separation of proteins from complex mixtures. These include one-dimensional gel electrophoresis, which achieves resolution of proteins based on molecular weight; two-dimensional gel electrophoresis, which involves separation of proteins based on isoelectric point followed by separation based on molecular weight; high performance liquid chromatography; ion exchange; and different types of affinity chromatography.

Mass spectrometers measure the mass-to-charge ratio of the smallest molecules with high accuracy, and have the ability to detect low-abundance proteins at sub-picomolar concentrations. In essence, peptide sequence-based protein identification by tandem mass spectrometry (MS/MS) centers on the fact that peptide sequences of 6–30 amino acid residues or greater may be sufficiently unique to their parent proteins of origin. The proteins are identified by matching them with those in databases that contain genomic sequences translated to their protein counterparts. In bottom-up proteomics, the sample is initially digested using a proteolytic enzyme such as trypsin. The resulting peptides are separated

by chromatography, and then analyzed by mass spectrometry (MS). The source proteins are identified by matching the experimental tandem mass spectra with those from theoretical tandem mass spectra of translated genomic databases subjected to *in silico* cleavage using specific enzymes.¹⁰

Peptide Sequencing by MS/MS

Tandem mass spectrometry (MS/MS) has emerged as a reliable approach for identification of proteins from multiple sources including complex mixtures. In MS/MS performed in ion-trap mass spectrometers, peptide ions undergo fragmentation upon collision with neutral gas molecules in the collision chamber of the mass analyzer. The collisional-induced dissociation of the peptide ions occurs along the peptide backbone, and the most frequently observed cleavage site is at the amide bond between the amide nitrogen and the carbonyl oxygen. Matching of multiple MS/MS spectra to peptide sequences within the same protein increases the confidence of protein identification. MS/MS based protein identification is applicable to EST databases with reliable matches (Figure 14.2). The experimental tandem mass spectra are matched against theoretical MS/MS spectra and cross correlation scores are calculated, based on the extent to which the predicted and experimental spectra overlap.¹¹

Quantitative Proteomics

While methods for absolute quantitation of peptides/proteins are available, most quantitative proteomic studies are designed to determine the “relative” proteomic differences between one cellular state and another. Quantitative protein expression profiling may be performed using multiplex ELISA, peptide/protein arrays, 2 dimensional gel electrophoresis

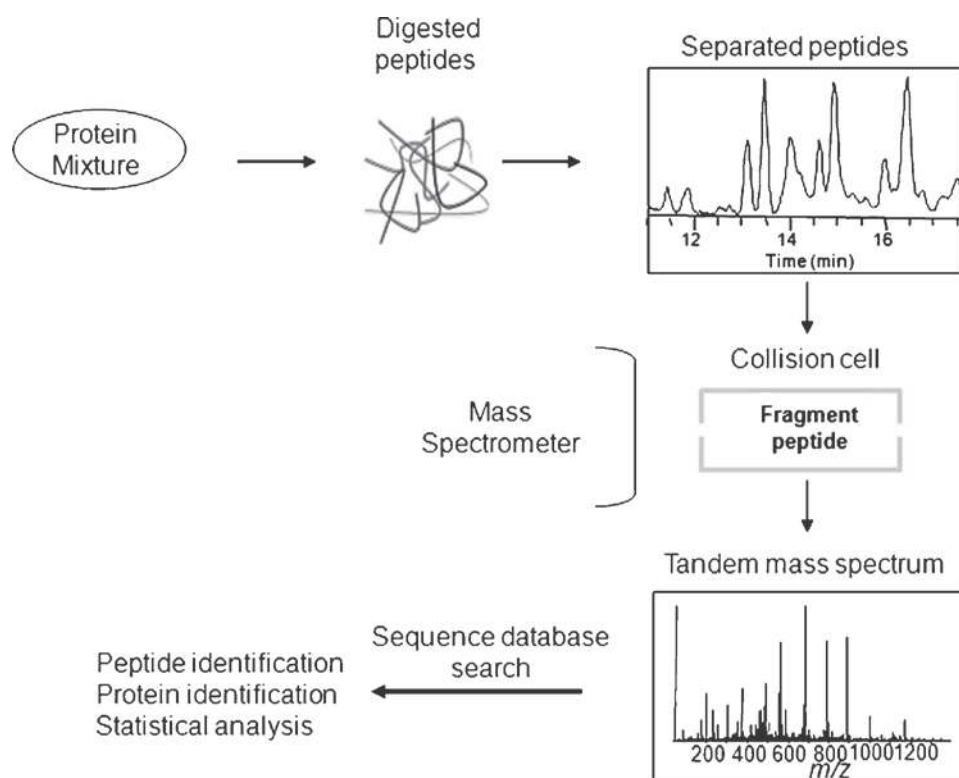


FIG. 14.2. Tandem mass spectrometry as a reliable approach for protein identification from complex mixtures. Proteins are digested into peptides in the “bottom-up” approach before analysis in an ion-trap mass spectrometer. Peptide ions undergo fragmentation upon

collision with a neutral gas. The tandem mass spectra that are generated from collision induced dissociation is used to match with those in the sequence databases.

(2D-PAGE), and mass spectrometry-based approaches. Each platform has its advantages and disadvantages with regard to analytical parameters, such as sensitivity, specificity, dynamic range of quantification, precision, and accuracy. For discovery studies, two-dimensional gel electrophoresis has been utilized extensively with great success, although it is a relatively low throughput approach which requires a large amount of starting material. To overcome the limitations of gel-to-gel reproducibility, differential gel electrophoresis (DIGE) has been developed, in which up to three different samples may be analyzed by labeling the proteins with different fluorescent dyes, such as Cy2, Cy3, and Cy5.¹² Mass spectrometry-based approaches involve labeling, using stable compounds, containing stable isotopes of elements in all biologic samples (i.e., C, H, O). Relative quantification is achieved by comparison of the peak heights or areas of the isotope/tag pairs for each peptide distinguished by the mass difference of the isotope or tag. Isotope-coded affinity tags (ICAT)^{13,14} involve chemical labeling of specific functional groups in peptides, and is limited to pair-wise comparison, while isobaric tags for relative and absolute quantitation (iTRAQ).¹⁴ may be used for multiple comparisons (up to 8). The advantage of the stable isotope labeling approaches is that any peptide mixture may be analyzed, including frozen or fixed samples. In contrast, metabolic labeling methods,

such as stable isotope labeling by amino acids in cell culture SILAC,¹⁵ require the use of viable cells in culture passaged for 5–8 cycles in the presence of medium supplemented with labeled amino acids (i.e., lysine or arginine containing ¹³C or ¹⁵N). See Figure 14.3 for an outline of general experimental strategies that have been used for mass spectrometry-based proteomic studies of lymphoma.

Proteomic Studies of Human Malignant Lymphoma

The following sections describe recent proteomic studies of malignant lymphoma. The approaches described include identification of interacting proteins, large scale quantitative protein profiling of secreted proteins, proteomic consequences of small molecular inhibition, and protein identification from formalin-fixed archived cells.

Proteomic Studies of B-Cell Lymphomas

The majority of B-cell lymphomas are associated with specific chromosomal translocations, but these are not considered the sole genetic event responsible for malignant transformation.

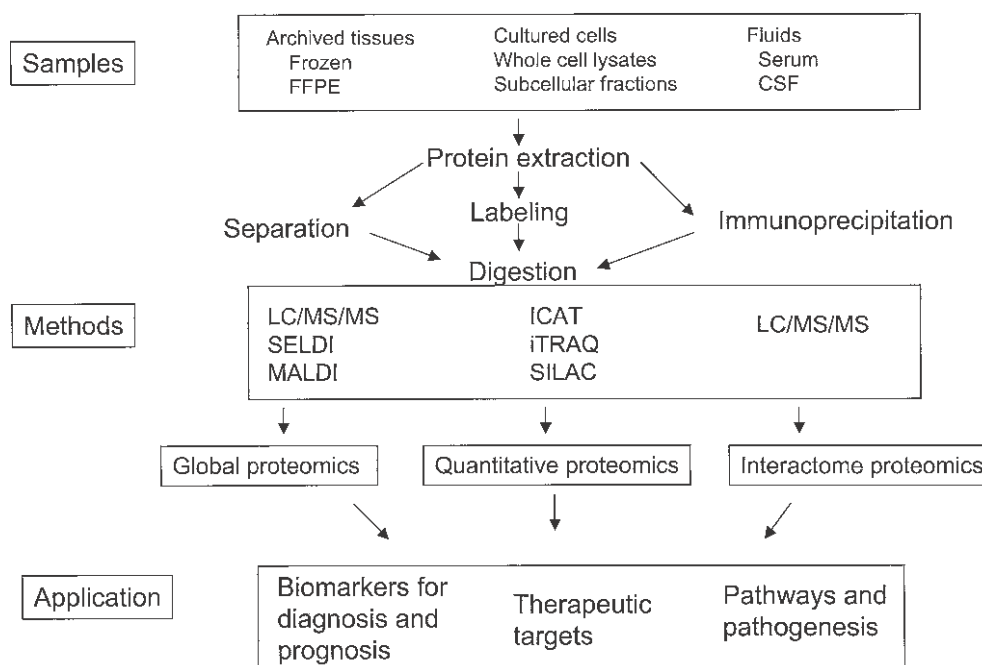


FIG. 14.3. General strategies of mass spectrometry-based proteomic analysis used for the study of human malignant lymphoma. Abbreviations: FFPE, formal-fixed, paraffin-embedded; LC/MS/MS, liquid chromatography, tandem mass spectrometry; SELDI, surface-enhanced laser

desorption/ionization; MALDI, matrix-assisted laser desorption/ionization; ICAT, isotope coded affinity tags; iTRAQ, isobaric tags for relative and absolute quantitation; SILAC, stable isotope labeling by amino acids in cell culture.

It is anticipated that molecular and proteomic characterization of lymphomas will reveal insights regarding pathogenetic mechanisms of lymphomagenesis. The following sections describe recent proteomic studies of B-cell non-Hodgkin and Hodgkin lymphoma (HL) using mass spectrometry (MS)-based approaches for protein identification. The approaches described include identification of interacting proteins of key B-cell lymphoma oncoproteins (i.e., BCL6 and TCL-1); large scale protein profiling of lymphoma cells; quantitative differential proteome profiling of lymphoma cells; and analysis of secreted proteins for identification of lymphoma biomarkers in serum and other body fluids.

Analysis of Interacting Partners of B-Cell Lymphoma Oncogenes

Proteins do not function in isolation but as members of multiprotein complexes. Such interactions serve to stabilize enzymes and their substrates, such as a kinase with its phosphorylation target in a signaling cascade, or to form and regulate the functional complexes for transcription or translation. Direct protein interactions are also required for regulatory functions, such as steric inhibition or covalent modification. Characterization of interacting partners provides insight into function, and the initial understanding of a protein's cellular role is often gained through observation of interactions with proteins whose function is better understood.

Traditional methods for the study of protein–protein interactions include yeast two-hybrid studies, but these are limited to the characterization of direct interactions between only two proteins at a time. Immunoprecipitation may be used to pull down a complex of proteins, but this is traditionally followed by a western blot using an antibody targeted at only one member of the complex. Mass spectrometry-based proteomic studies, on the other hand, allow the unbiased identification of multiple components of a complex mixture of proteins in a single experiment. Thus, using mass spectrometry-based protein identification in conjunction with immunoprecipitation allows the characterization of numerous constituents of large protein complexes within a single experiment. This approach has been applied to characterize the interacting proteins, or interactomes, of key proteins related to B-cell lymphomagenesis.

B-cell lymphoma 6 (BCL6) is a transcriptional repressor whose function is required for germinal center formation and T-helper 2-mediated antibody response.^{16–18} BCL6 is the most frequently altered gene in de novo diffuse large B-cell lymphomas. BCL6 contains a carboxyl-terminal zinc finger region that mediates sequence-specific DNA binding¹⁹ as well as protein–protein interactions.²⁰ The amino-terminal region has a Pox virus zinc finger/bric-a-brac, tramtrack, broad complex (POZ/BTB) domain, which mediates BCL6 homo- and heterodimerization.²¹ Through these interaction domains, BCL6 recruits corepressor proteins to assemble

into a large corepressor complex. Transcriptional repression may be mediated at BCL6 DNA binding sites, or at the DNA binding sites of BCL6 interacting proteins. Thus, the identification of BCL6 interacting proteins may further characterize the BCL6 repressor complex, identify novel transcription factor interacting partners and repression targets, and identify novel BCL6 regulatory proteins.

BCL6-interacting proteins were identified using liquid chromatography tandem mass spectrometry (LCMS/MS) peptide sequencing, following enrichment using immunoprecipitation (IP). To decrease the complexity of the protein mixture presented to the mass spectrometer, proteins in the BCL6 immunocomplex were size-fractionated using 1-dimensional gel electrophoresis, and then resolved using high performance liquid chromatography (HPLC).

Through this approach, the list of BCL6-interacting proteins was expanded to include additional transcription regulation factors, including ATF-7, early B-cell factor, Elf-1, heat shock factor protein 4, hepatocyte nuclear factor-1 α (alpha), and YY1.²² Although BCL6 is known to interact with transcription factors and play a role in chromatin remodeling, this study identified proteins with more diverse cellular functions within the BCL6 immunocomplex. These included proteins with signal transducer activity, such as NF- κ B-repressing factor, LPA2, P2Y9/LPA4, EphB6, and Smoothed homolog. The kinases identified could potentially participate in regulation of BCL6, similar to mitogen-activated protein kinase, which phosphorylates and targets BCL6 for proteosomal degradation. Additional studies will be required to determine if any of these novel BCL6 interacting kinases function in a similar manner to regulate BCL6. Figure 14.4 demonstrates the complexity of the BCL6 interactome.

Proteomic Analysis of Follicular Lymphoma Transformation

Follicular lymphoma (FL) is a common low-grade B-cell lymphoma, and the majority of patients experience a protracted disease course. A subset of patients undergo histologic transformation into aggressive diffuse large B-cell lymphoma (DLBCL), which is associated with significant mortality. There are currently no biologic predictors to determine which patients are at risk for transformation. Furthermore, the pathogenetic mechanisms involved in the transformation process itself are poorly understood especially at the proteomic level. Application of MS-based proteomics to the study of this clinically important process is limited. SELDI-TOF/MS has been used to identify differentially expressed proteins potentially involved in follicular lymphoma (FL) transformation.²³ The approach involved direct comparison of transformed FL (DLBCL) to the preceding and clonally related FL from an earlier biopsy of the same patient. The SELDI/TOF approach utilizes ProteinChip Array System (Ciphergen Biosystems) that takes advantage of matrices with distinctive chromatographic properties such

as hydrophobic, hydrophilic, ion exchange, and immobilized metallic ion matrices to separate proteins based on physical properties. Minimal amounts of tissue are required, thus the technology may be applied to the study of protein extracts from small tissue biopsy samples.

Comparison of the mass spectral profiles of matched pairs of low-grade FL and DLBCL identified consistent patterns, corresponding to differentially expressed proteins. Incorporation of molecular weight information, the pI, and chip binding characteristics identified upregulation of cyclin D3 (32.5 kDa) and downregulation of caspase 3 (11.8 kDa). The upregulation of cyclins, which promote cell cycle progression, is a common mechanism implicated in tumorigenesis. Similarly, the downregulation of caspases, which mediate apoptosis, is also a common mechanism exploited in tumorigenesis.^{24–26} This study not only demonstrated the application of MS-based proteomics to patient tissues, but also showed how the technology may be applied to a biological problem, with the identification of proteins whose expression levels may be altered in lymphoma progression.

Proteomic Consequences of Signaling Pathway Inhibition

The mitogen-activated protein kinase (MAPK) p38 is upregulated in the transformation of FL to DLBCL.²⁷ Lin et al²⁸ used a quantitative differential proteomic analysis to evaluate the cellular consequences of MAPK pathway inhibition.²⁸ The t(14;18)-positive transformed follicular lymphoma-derived cell line OCI Ly-1 was exposed to the selective p38MAPK inhibitor SB203580. Proteins were harvested and labeled in vitro with a stable isotope-coded affinity tag (ICAT). Untreated control cells were labeled with a different isotope coded tag, and the specimens were then mixed and subjected to LC/MS/MS. The relative abundance of an identified protein corresponds to the relative peak heights of the “heavy” and “light” isotopically labeled peptides. Two hundred seventy-seven differentially expressed proteins (1.5-fold increase or decrease) were identified after 3 h of SB203580 and 350 were identified to be differentially expressed (1.5-fold) after 24 h. The majority of differentially expressed proteins was downregulated and included predominantly those related to cell growth, cell signaling, and transcriptional regulation, providing support for the notion that p38 MAPK is an important mediator growth signaling in these cell lines.

Global Protein Profiling Studies

Burkitt Lymphoma

Although uncommon in adults, Burkitt lymphoma accounts for approximately one-third of pediatric non-Hodgkin lymphomas.²⁹ This aggressive neoplasm leads to rapid demise if treatment is not initiated in a timely manner, and a subset of patients succumb even with aggressive therapy.

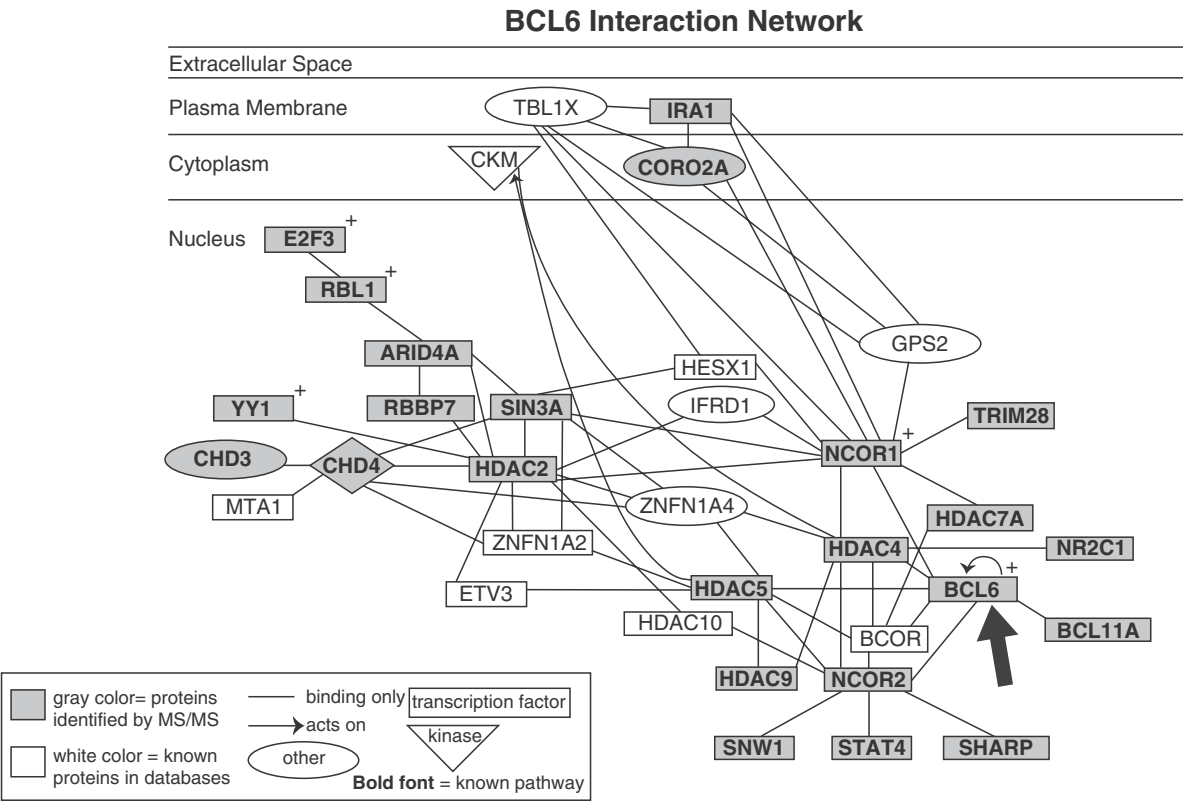


FIG. 14.4. Pathway analysis of BCL6 interaction proteins identified by tandem MS. Multilayered data analysis software was used to generate a protein interaction network for BCL6 (arrow) including cell location.

Red shading indicates proteins identified in the BCL6 immunocomplex by tandem mass spectrometry.

To further understand the pathogenesis of this disease, Henrich et al have characterized the nuclear proteome of the Burkitt lymphoma-derived cell line Raji through two complementary approaches.³⁰ These approaches allowed the investigators to identify nuclear proteins, which in whole cell lysates are often poorly represented due to the relative abundance of cytoplasmic proteins. Proteins extracted from nuclear fractions obtained by sucrose gradient centrifugation were resolved by two-dimensional gel electrophoresis. Protein spots visible with Coomassie blue staining were subjected to MALDI-TOF MS analysis for protein identification. This approach identified 124 proteins with marked enrichment for known nuclear proteins. In the second portion of their study, DNA binding proteins were selected using DNA-agarose affinity purification prior to electrophoresis and MS. This approach identified 131 proteins with enrichment for known DNA- and RNA-interacting proteins. While there was overlap in the proteins identified by the two approaches, the whole nuclear proteome study identified 78 proteins not found in the DNA-binding fraction; the DNA-binding fraction contained 85 proteins that were not detectable in the whole nuclear proteome. Overall, a total of 209 unique proteins were identified from the two fractions. This study

demonstrates the utility of both nuclear compartment and DNA-binding enrichment for the identification of nuclear proteins in a lymphoma cell line.

Hodgkin Lymphoma

Two studies have characterized proteins expressed by the Reed-Sternberg cells of HL-derived cell lines in an attempt to identify potential biomarkers.^{31,32} In addition to collecting proteins from the culture media, Wallentine et al utilized a subcellular proteomic approach by harvesting proteins from cytoplasmic, nuclear, and membrane cell fractions. After separation by one-dimensional SDS-PAGE, proteins were subjected to enzymatic digestion and analyzed by LC-MS/MS. A total of 1945 proteins (i.e., 785 from the cytosolic fraction, 305 from the membrane fraction, 441 from the nuclear fraction, and 414 released proteins) were identified, using a minimum of two peptide identifications per protein and an error rate cutoff of <5.0%. Proteins of particular interest are those involved in (or associated with) cell signaling and proliferation. Multiple proteins within the list directly involved within the NF- κ B, Notch, and Ras/Raf/MAPK/ERK signaling pathways were identified. These signaling

pathways have been previously implicated in the pathogenesis of HL. To validate the MS-based protein identification strategy, a combination of western blot analysis as well as immunofluorescence and immunohistochemical analysis for selected candidate proteins was performed using tissue biopsy samples. This study provides the first comprehensive list of proteins expressed by RS cells of HL-derived cells.

Ma et al analyzed the secretome of the same two HL cell lines (L428 and KMH2), as well as an additional HL cell line (L1236).³¹ A subset of the identified proteins was validated using either ELISA or immunohistochemistry. Importantly, the expression of identified secreted proteins was demonstrated in HL patient tissue (i.e., CD44, CD71, MIF, RANTES, and TARC) by immunohistochemistry. Furthermore, ELISA studies showed increased serum levels of HL secretome proteins (i.e., ALCAM, cathepsin S, CD26, CD44, IL1R2, MIF, and TARC) in HL patient specimens, compared to controls. These studies demonstrate how secreted proteins identified in the culture medium may represent potential biomarkers for the detection of HL in patient serum samples. Future studies will determine the significance of these proteins, particularly those involved in key signaling pathways, in the diagnosis, prognosis, and pathogenesis of HL.

Proteomic Studies for Identification of Potential Lymphoma Biomarkers

Patients with lymphoma who are diagnosed earlier and at lower clinical stage have a better prognosis, yet biomarkers for the early detection of lymphomas are not available. Zhang et al utilized SELDI-TOF-MS proteomic patterns of serum to distinguish DLBCL patients from controls patients and identify DLBCL patients with a worse prognosis.³³ They evenly divided 132 DLBCL samples and 75 control samples into training groups and test groups. The training groups were used to identify discriminating peaks and develop decision trees that were then applied to the test groups. They were able to distinguish DLBCL patients from controls in the test set with a sensitivity of 94% and a specificity of 94%. Importantly, stage I patients were effectively distinguished from controls (15/15, 100% sensitivity).

In a similar manner, training groups were established to distinguish patients with a good prognosis (alive at 36 months) from patients with a bad prognosis and to distinguish patients who relapsed at 36 months. In the test set, they identified poor prognosis patients with a sensitivity of 94% and a specificity of 92%. Interestingly, the International Prognostic Index was only able to achieve 61% sensitivity and 80% specificity for poor prognosis in the same cohort of patients. Finally, the sensitivity and specificity for the detection of patients who relapsed were 92 and 90%, respectively.

The diagnosis of primary central nervous system (CNS) lymphomas is complicated by the difficulty and risk associated with direct biopsy of brain tissue. A recent study has employed a liquid chromatography/mass spectrometry (LC/

MS)-based approach to identify potential protein biomarkers of CNS lymphoma in the cerebrospinal fluid (CSF).³⁴ The investigators performed a large-scale identification of proteins in order to identify CSF proteins that could distinguish B-cell lymphomas from benign conditions. CSF from patients with lymphoma or from control patients with nonneoplastic conditions were subjected to LC/MS for differential quantification followed by LC-MS/MS protein identification. One hundred ninety-six proteins were identified that were expressed at either higher or lower levels in lymphoma patients compared to controls.

CSF from lymphoma patients showed higher levels of proteins implicated in tumor invasion and extracellular matrix production (i.e., matrix metalloproteinase-2, vitronectin, and fibulin 3) and lower levels of several regulatory proteins involved in normal brain function (i.e., neuronal cell adhesion molecules, cadherin 13, contactin-1, and chromogranin A).³⁴ Further validation studies were performed to evaluate the novel potential biomarker antithrombin III (ATIII). Increased expression was demonstrated in CNS lymphoma tissues by RT-PCR and immunohistochemistry, and increased CSF levels were confirmed by immunoblotting and ELISA. CSF ATIII identified CNS lymphoma patients with a sensitivity of 75% and a specificity of 98.7%. Furthermore, elevated CSF ATIII levels (measured by ELISA) correlated with relapse and decreased survival in CNS lymphoma patients. These results demonstrate the power of MS-based proteomics for the nonbiased identification of candidate biomarkers in the CSF of lymphoma patients.

There is an active search for biomarkers that may be utilized for the diagnosis and monitoring of human malignancies. Biomarkers could have a role in the early detection of a malignancy, allowing for earlier therapeutic intervention with the possibility of improving outcomes. In addition, a reliable biomarker may be used to monitor response to therapy and allow for the early detection of relapsed disease. In particular, a secreted biomarker would allow screening of routine peripheral blood or serum samples, rather than a biopsy procedure. MS-based methods for the identification of proteins in biological systems are advantageous because of their ability to identify large numbers of proteins present in complex mixtures. In addition, these methods may be adapted to high throughput systems for screening large numbers of samples.

Recently, an MS-based approach to identify proteins released by follicular lymphoma (FL)-derived cells was utilized to identify candidate biomarkers in FL.³⁵ A cell line derived from FL cells was utilized to provide a pure tumor cell population, and serum-free media was used to avoid the interference of high levels of serum proteins (i.e., albumin). Multiple replicates of the culture media were collected, and proteins were enzymatically digested and analyzed by LC-MS/MS to detect released proteins. Two hundred nine proteins were detected by MS/MS analysis and database searching at a maximum error rate of 5.6%. Identified proteins included known B cell-associated proteins, proteins

not previously identified in FL cells, and proteins previously only associated with other types of cancer. We also identified proteins that have previously been associated with other types of cancer, including activated leukocyte cell adhesion molecule (ALCAM; CD166). ALCAM has been shown to be over expressed in melanoma,³⁶ prostate cancer,³⁷ and colorectal carcinoma.³⁸ ALCAM expression has been shown to correlate with invasiveness, prognosis, and/or survival in these cancers, but these studies have not yet been done in FL patients.

Future studies will be required to determine the utility of these potential biomarkers in patient specimens. Practical issues include the depletion of the high-abundance serum proteins and determining the effect of the numerous proteins released by normal cells in patient samples. Beyond these issues, extensive validation will be required for any proposed biomarker to correlate detection and/or expression levels with disease status. The identification of proteins secreted by FL cells in cultures lays the groundwork for these future studies.

The presence of autoantibodies against L-plastin in plasma of patients with non-Hodgkin lymphoma has also been successfully detected using MALDI/TOF MS and tandem MS. The presence of autoantibody against L-plastin was significantly higher in patients with non-Hodgkin lymphoma than in patients with HL or autoimmune disease or in healthy controls.³⁹

Protein Identification From FFPE Cells

The development of strategies to permit utilization of the universal FFPE specimens will be important in leveraging the application of powerful mass spectrometry-based proteomic approaches into the investigation of archival clinical specimens. One such strategy has demonstrated the ability to perform MS-based proteomic studies on archived paraffin embedded tissue.⁶ In this study, a paraffin block was derived from cultured lymphoma cells by collecting the cells into a clot preparation, which was then fixed and embedded in a paraffin block. The cell material was then collected in a microfuge tube and paraffin was removed using xylene. After hydration in graded ethanol washes, protein was collected in RIPA lysate buffer and quantitated. Finally, proteins were subjected to trypsin digestion and repeated analysis by tandem MS. Protein analysis from fixed, paraffin embedded cells was compared in parallel to protein from fresh cell lysates. Trypsin digestions yielded 133 protein identifications in the FFPE sample and 231 from the fresh lymphoma lysate, while the gluC-digest resulted in 71 proteins identified from the FFPE cells, and 86 from the fresh lysate. Using peptides from both digests (trypsin and gluC), 120 proteins were identified from the FFPE material and 197 proteins identified from fresh cell lysate. A total of 324 unique proteins were identified from the FFPE cells and 514 from the fresh lysate, with 263 in common to both approaches. Thus, more than half (52%) of the proteins identified in the fresh

cell lysate were also identified from the 3-year-old FFPE material. The deparaffinizing step was the only additional labor required beyond that of preparing traditional samples for analysis by LC-MS/MS. The overall yield of extracted protein, peptide chromatography, and quality of MS/MS spectra was comparable to that of fresh cell lysate.

Protein kinase C ϵ (PKC ϵ) was among the proteins identified from the FFPE sample. The PKC isoenzyme family members are signal transduction regulators that exhibit cell and tissue-specific expression, and PKC ϵ has been implicated in the mediation of proliferative signals important in the pathogenesis and progression of some human cancers. In cultured lymphoma cells, a PKC inhibitor showed a dose- and time-dependent inhibition of cell growth. These results further validate the utility of FFPE-based proteomics approaches for the identification of biologically relevant proteins which may serve as therapeutic targets. Future studies examining FFPE specimens will be required to determine the effects of varying lengths of material storage prior to analysis.

These studies demonstrate the value of application of MS proteomics to patient tissue specimens. The ability to perform mass spectrometry based proteomic analysis on FFPE samples provides significant opportunities for biomarker and therapeutic target discovery in archival samples with well-documented clinical follow-up.

Proteomic Studies of Anaplastic Large Cell Lymphoma

Anaplastic large cell lymphoma (ALCL) is an aggressive T-lineage lymphoma harboring chromosomal translocations involving the ALK tyrosine kinase. The most common translocation in ALCL is the t(2;5)(p23;q35). This results in the formation of a chimeric fusion kinase NPM/ALK. Anaplastic lymphoma kinase (CD236) is a receptor tyrosine kinase of the insulin receptor subfamily^{40,41} with homology to leukocyte tyrosine kinase. Its expression is highly tissue-specific and is normally restricted to cells of the nervous system^{42,43} via its ligands pleiotrophin⁴⁴ and midkine.⁴⁵ An N-terminal oligomerization domain within NPM facilitates the dimerization of NPM/ALK, leading to autophosphorylation and constitutive activation of the ALK tyrosine kinase,⁴⁶ which is the causative oncogene in t(2;5) positive ALCLs.^{41,47}

Activated ALK induces multiple downstream signaling molecules, including phospholipase C γ (PLC γ),⁴⁸ phosphatidylinositol-3-kinase (PI3K),⁴⁹ RAC-serine/threonine-protein kinase (AKT),⁵⁰ Janus kinase 3 (JAK3),⁵¹ signal transducer and activator of transcription 3 (STAT3),⁵² and the nonreceptor protein kinase (SRC),⁵³ which leads to enhanced cell proliferation, survival, and apoptosis inhibition.⁵⁴ Several aspects of the ALCL proteome have been elucidated by recent MS-driven proteomic studies, which expand the current understanding of the molecular pathogenesis of ALCL and provides the basis for the identification of biomarkers and targets for novel therapeutic agents.

Global Profiling of ALCL Proteome

The signaling pathways and biological mechanisms involved in the pathogenesis of NPM/ALK-positive ALCL are not completely understood and a comprehensive profile of its proteome has yet to be performed. Large-scale profiling of the proteome of ALCL will provide the proteomic building blocks and will be of utility in the discovery of pathogenic mechanisms, diagnostic biomarkers, and treatment strategies.

To identify and catalog a comprehensive list of proteins expressed in the NPM/ALK-positive ALCL, lysates from the SUDHL-1 cell line were used to obtain 3 sub-cellular fractions: nuclear, cytoplasmic, and membrane. The sub-cellular fractions were separated by size using 13% SDS-PAGE, after which each of the lanes were cut into 32 slices, subjected to in gel digestion, and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS).⁵⁵ This approach was simple, fast, and effective in simplification of sample complexity, and allowed for the identification of proteins with molecular weights ranging from 7 to 620 kDa.

A total of 623 proteins consisting of 210 membrane, 229 cytoplasm, and 184 nuclear proteins were identified with a $\leq 5\%$ error rate. A thorough literature-based annotation of a subset of 209 proteins indicated that 19.9% were previously reported to be expressed in T-cells and 44.7% were reported to have important function in cancers. Notably, only 2.4% of the proteins have been previously reported in the literature to be implicated in ALCL pathogenesis. GoMinerTM⁵⁶ protein function analysis on the ALCL proteome revealed a highly complex system and identified proteins involved in diverse cellular functions, such as catalytic, enzyme, signaling, translation, transcription, structural, and motor.

Cussac et al⁵⁷ utilized 2-DE followed by MALDI-TOF MS to compare the proteome of the cytoplasmic soluble proteins of an NPM/ALK-positive cell line (SUDHL-1) with that of NPM/ALK-negative cell line (FE-PD). The spot patterns of the 2DE gels were analyzed with an image analysis software, which detected 82 proteins that were differentially expressed between the two cell lines. Identification of the proteins demonstrated that they belonged to five broad functional categories, including signaling, cytoskeleton and motility, chaperones, control of protein expression and degradation, and homeostasis and metabolism. A comparison of cell lines representative of two morphologic subtypes of NPM/ALK-positive ALCLs, namely the common type (SUDHL-1) and the small cell variant (COST), revealed distinct set of proteins which were selectively differentially expressed within each category. Furthermore the relative overexpression of one protein, carbonic anhydrase II, was validated in tissue biopsy samples of the small cell variant of ALCL. The differential proteomic analysis was extended by performing 2-D liquid chromatography, which allowed for the separation of a larger panel of proteins and increased the number of differentially expressed proteins to 175.

Identification of ALK Interactome

Because ALK plays a critical role in the activation of multiple signaling pathways, determination of its interacting proteins and those that form the complex of proteins within its “interactome” may provide novel insights as to its function, as well as reveal potential targets for novel therapies. A mass spectrometry-based proteomic strategy was used to determine the identity of proteins that interact with NPM/ALK tyrosine kinase.⁵⁸ The ALK interactome was enriched using a monoclonal antibody against ALK and the components separated by 1D-SDS-PAGE. Distinct bands (which were present in the ALK-immunocomplex but not in the IgG control immunoprecipitation) were digested and subjected to liquid chromatography (LC) and ESI-MS/MS. Among the 40 odd proteins identified in the ALK interactome, 9 proteins had been previously reported to be important mediators of the ALK signal pathway and interacted with ALK. These include phospholipase C-gamma (PLC γ [gamma]1),⁴⁸ phosphoinositide-3-kinase (PI3-K),^{49,50} Jak2,⁵⁹ Jak3,⁵² STAT3,^{52,60} and IRS.⁴¹ More importantly, many proteins previously not recognized to be associated with NPM/ALK, but with potential NPM/ALK interacting protein domains were identified. These included adaptor molecules (i.e., SOCS, Rho-GTPase activating protein, and RAB35), kinases (i.e., MEK kinase 1 and 4, PKC, MLCK, cyclin G-associated kinase, EphA1, EphB, JNK kinase, and MAP kinase 1), phosphatases (i.e., meprin, PTPK, and protein phosphatase 2 subunit), and heat shock proteins (i.e., Hsp60 precursor). Importantly, a subset of the proteins that were identified by MS was confirmed by western blotting and reciprocal immunoprecipitation. Interestingly, two of the proteins that were identified by MS, but not reported in the final manuscript due to their low scores for identification (i.e., NIPA⁶¹ and SRC⁵³), were subsequently reported by two independent studies to interact with ALK and have important biologic functions in cellular transformation in ALK-positive lymphoma. The significant role of STAT3 in the pathogenesis of ALK-mediated lymphomagenesis has been demonstrated in mice, where tumor formation was inhibited by genetic ablation using antisense Stat3 oligonucleotides.⁶² Similar strategies have been utilized to implicate the role of the tyrosine phosphatase Shp2⁶³ and p130Cas⁶⁴ in cell growth and migratory properties of ALCL. These studies demonstrate the utility of mass spectrometry-based approaches for the identification of novel proteins within signaling complexes and potential functional definition of their role in lymphomagenesis.

Proteomic Changes Induced by HSP90

Recent studies have identified heat shock protein 90 (Hsp90) as the primary target of geldanamycin (GA),⁶⁵ a benzoquinone ansamycin, which elicits antitumor activity in ALCL.^{66,67} Hsp90 is a highly conserved, ubiquitous molecular chaperone, that is required for the stability and conformational maturation of a diverse group of client proteins, including

components of signaling pathways exploited by cancer cells for survival and proliferation.⁶⁸ Hsp90 client proteins include the following: receptor tyrosine kinases, such as human epidermal growth factor receptor (HER) family kinases, breakpoint cluster region-Abelson (BCR-ABL), and NPM-ALK; cytosolic signaling proteins, such as AKT, v-raf-1 murine leukemia viral oncogene homolog 1 (RAF-1), and inhibitor of nuclear factor kappa (IKK); and cell cycle regulators, including cyclin-dependent kinase 4 (cdk4), polo-like kinase 1 (PLK1), and survivin.^{65,68,69} Inhibition of Hsp90 by ansamycins in ALK-positive ALCL cells results in down-regulation of NPM/ALK protein kinase activity,⁶⁷ leading to cellular apoptosis.⁶⁶ Clearly, there are multiple cellular targets of Hsp90, yet the comprehensive effects of Hsp90 inhibition in ALK-positive ALCL cells are unknown. Comparative quantitative analysis of cells in response to selective small molecular inhibitors of deregulated signaling proteins/pathways may aid in understanding the biologic mechanisms of its action.

Differential quantitative proteomic profiling was performed to evaluate the proteomic changes that occur as a result of Hsp90 inhibition by GA in ALCL-derived lymphoma cells. Equal mounts of lysates from vehicle-treated and cells exposed to 10 μ M GA were subjected to ICAT labeling after 12 h of incubation, followed by LC-MS/MS analysis of proteins. A total of 314 unique proteins were differentially expressed as a result of GA exposure. Forty-nine proteins were upregulated 1.5-fold or greater and 70 proteins downregulated 1.5-fold or greater in GA-treated cells. Importantly, proteins involved in diverse cellular functions, including signal transduction, DNA metabolism, nucleic acid metabolism, protein metabolism, cell growth and maintenance, and energy pathways were identified. Some of the downregulated proteins are known to be involved in described signaling cascades, such as JAK/STAT and MAPK, as well as pathways previously unreported in ALK-positive ALCL, including WNT, NF- κ (kappa)B, and TGF β (beta). These studies demonstrate some of the molecular mechanisms by which Hsp90 inhibition reduced viability of ALK-positive ALCL cells and illustrate the diverse proteins whose expression is changed due to GA inhibition of Hsp90.

Conclusions and Future Directions

The most critical (but challenging) step in mass spectrometry-based proteomic studies for human hematolymphoid malignancies as for other disease models, remains the functional validation of proteins and signaling pathways using clinical samples. Recent developments in protein microarrays, which allow for high throughput analysis of nanoliter volume of serum against tens of thousands of peptides/proteins, provide an important approach for the identification of secreted biomarkers and potential secretomic signature of patients with hematolymphoid disorders. Tissue microarrays constructed

from biopsy samples obtained from well-characterized clinical cohorts/clinical trials are also a critical component in the translation of protein identification to validation of biomarkers for diagnosis and prognosis. Application of novel technologies, such as imaging mass spectrometry,⁷⁰ has been used for the in situ analyses of proteins in tissue sections, thereby allowing imaging and comparison of protein expression between normal and disease tissues. Future studies utilizing targeted MS-driven proteomic strategies using multiple reaction monitoring (MRM)⁷¹ will lead to high sensitivity biomarker detection in biofluids, such as serum and plasma.

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Mouse Models of Hematolymphoid Malignancies

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Introduction

Sequencing of the mouse genome has revealed numerous advantages of using the mouse model, compared to other model organisms, to study the molecular pathogenesis of human diseases:

- both the human and mouse genomes contain approximately 30,000 protein-coding genes
- synteny or conserved gene order on chromosomes in both species is over 90%
- 40% of human and mouse genes align directly
- 99% of human genes have homologous gene(s) in the mouse.¹

These features, combined with the tremendous advancements in our abilities to manipulate the mouse genome, have resulted in the generation of countless genetically engineered mice (GEM) carrying transgenes, targeted mutations, and chemically induced mutations.

GEM, together with xenograft models, have provided invaluable information concerning multiple aspects of hematolymphoid neoplasms, including characterization of various chromosomal aberrations, elucidation of the biological/clinical disease states, and preclinical evaluation of therapeutic efficacy. The utility of these mouse models is ultimately dependent on their phenotype, and as anticipated, not all models recapitulate all features of the human disease.

The numbers of spontaneous,^{2,3} genetically engineered³⁻⁶ and xenograft⁶⁻¹⁰ mouse models of hematolymphoid malignancies are far too vast to explore in detail here and have been extensively covered in other sources. The following pages will list the consensus classification and nomenclature for lymphoid (Table 15.1) and nonlymphoid (Table 15.2) hematolymphoid neoplasms in mice, as recommended by the United States National Cancer Institute Mouse Models of Human Cancer Consortium.^{11,12} These classifications were established in an attempt to standardize the characterization

of disease in GEM and xenograft models while relating the murine disease entities to human disorders. Pertinent examples of mouse models of select neoplasms will be described. The subsequent discussion will be enhanced by an initial overview of normal murine hematopoiesis and the approaches to phenotypic characterization of new mouse models.

Hematopoiesis

Hematopoiesis, or the series of interrelated processes resulting in blood cell production, occurs in two phases in utero, primitive and definitive, and at different locations within the embryo.¹³ Murine primitive hematopoiesis is initiated in blood islands of the yolk sac between 7.5 and 9.0 days postcoitum (dpc). Blood islands consist primarily of large, nucleated erythroid cells that express embryonic globins but can also give rise to granulocytes and macrophages. Around 9.5 dpc, definitive hematopoiesis begins within the aorta-gonad-mesonephros (AGM), which derives from the para-aortic splanchnopleura. Hematopoiesis within the fetal liver is well underway by 11.0 dpc with colonization of stem cells from both the yolk sac and the AGM that then differentiate, and continues until after birth. Thymic colonization with lymphoid cells begins around 15 dpc. Seeding of the bone marrow and spleen, which remain sites of hematopoiesis throughout adult life, initiates around 15.5 dpc.

Hematopoiesis in humans similarly begins within blood islands of the secondary yolk sac by week 2 of gestation with pluripotent colony-forming activity detected at 25.0–30.0 dpc.¹³ By 42.0 dpc, the liver has completely replaced the yolk sac as the major hematopoietic organ and seeding of the bone marrow is completed by week 16 of gestation.

Significant differences between the hematopoietic tissues of mice and humans exist. Lymphocytes are the predominant circulating white blood cell in mice in contrast to neutrophils in humans. The splenic red pulp is an ongoing and active site

TABLE 15.1. Classification of murine lymphoid neoplasms [12].

B cell neoplasms

- Precursor B cell neoplasm
 - o Precursor B cell lymphoblastic lymphoma/leukemia
- Mature B cell neoplasms
 - o Small B cell lymphoma
 - o Splenic marginal zone B cell lymphoma
 - o Follicular B cell lymphoma
 - o Diffuse large B cell lymphoma
 - Morphologic variants
 - Centroblastic
 - Immunoblastic
 - Histiocyte associated
 - Subtypes
 - Primary mediastinal (thymic) diffuse large B cell
 - o Classic Burkitt lymphoma
 - o Burkitt-like lymphoma
 - o Plasma cell neoplasms
 - Plasmacytoma
 - Extrasosseous plasmacytoma
 - Anaplastic plasmacytoma
 - o B-natural killer cell lymphoma

T cell neoplasms

- Precursor T cell neoplasm
 - o Precursor T cell lymphoblastic lymphoma/leukemia
- Mature T cell neoplasms
 - o Small T cell lymphoma
 - o T-natural killer cell lymphoma
- T cell neoplasm, character undefined
 - o Large cell anaplastic lymphoma

TABLE 15.2. Classification of murine nonlymphoid neoplasms [11].

Myeloid leukemias

- Myeloid leukemia without maturation
 - o Myeloid leukemia with maturation
 - o Myeloproliferative disease-like myeloid leukemia
 - o Myelomonocytic leukemia
 - o Monocytic leukemia
- Erythroid leukemia
- Megakaryocytic leukemia
- Biphenotypic leukemia
- Nonlymphoid hematopoietic sarcomas
 - o Granulocytic sarcoma
 - o Histiocytic sarcoma
 - o Mast cell sarcoma
- Myeloid dysplasias
 - o Myelodysplastic syndrome
 - o Cytopenia with increased blasts
- Myeloid proliferations (nonreactive)
 - o Myeloproliferation (genetic)
 - o Myeloproliferative disease

of physiologic extramedullary hematopoiesis in mice whereas splenic extramedullary hematopoiesis is pathologic in humans. While the thymus persists well into adulthood in mice, it begins to involute after puberty in humans. In addition, bone marrow cavities in virtually all murine bones are hypercellular throughout adult life in contrast to cellular marrow in humans,

which is progressively replaced by adipose tissue with age. Although these fundamental differences ultimately translate into different manifestations of hematolymphoid disease between the two species, such as primary splenic and rare bone marrow involvement in mice, modeling in the mouse has proved invaluable.

Characterization of Mouse Models

Phenotypic characterization requires knowledge of normal mouse anatomy, histology, and physiology, as well as age-^{14–16} and strain-related^{17–19} background lesions and potential environmental influences. Phenotypic protocols often involve multiple tiers that combine antemortem clinical assessments with postmortem pathological examinations and more specific ancillary testing.^{17,20} Details regarding experimental manipulation are required for accurate interpretation of the phenotype since endogenous retroviruses (murine leukemia viruses, MuLVs),^{21,22} radiation,^{23–25} and chemicals²⁶ are known to induce leukemias in various mouse strains. Once the phenotype associated with a single genotype has been determined, mice of various genotypes are often crossed in order to investigate the full impact of overexpressing, mutating or knocking out a gene in the milieu of other linked genes and pathways. This mandates subsequent phenotypic characterization of each new line or cross with inclusion of appropriate age-, sex- and genotype-matched controls.

Clinical Pathology

Complete Blood Count

The most common clinical pathology test performed on mouse models of hematolymphoid disease, often sequentially during the course of disease progression, is the complete blood count, which provides absolute numbers of erythrocytes, leukocytes, and platelets. It is important to obtain automatic counts utilizing hematology machines equipped with veterinary software. Values from age-, sex-, genotype- and treatment-control mice on the same background strain should be used as “normal” reference ranges. It is also necessary to critically analyze the values and scattergrams obtained from automatic hematology machines in conjunction with microscopic evaluation of a peripheral blood smear stained with Romanowsky-type stains in the absence of formalin exposure. Large, blastoid, neoplastic cells and nucleated erythrocytes may be incorrectly sorted as monocytes and lymphocytes, respectively. Furthermore, observations including agglutination of erythrocytes, structures in or on erythrocytes such as Heinz bodies, specific erythrocyte shape abnormalities such as echinocytosis, platelet clumps, and mitotic figures can only be made by evaluating the blood smear.

Biochemical Parameters

Various biochemical parameters may also supplement the clinical pathologic characterization of hematolymphoid diseases in mouse models. Elevated levels of calcium and parathyroid hormone-related protein support a diagnosis of humoral hypercalcemia of malignancy. Monitoring levels of calcium and the bone isoenzyme of alkaline phosphatase may correlate with the presence of neoplastic cells in the bone marrow. Serum protein electrophoresis may be performed in instances of hyperglobulinemia to differentiate monoclonal from polyclonal gammopathies, especially in multiple myeloma models. Lastly, elevated levels of potassium, phosphate, and uric acid provide biochemical evidence of acute tumor lysis syndrome.²⁷

Anatomic Pathology

Macroscopic Evaluation

Assessment of physical appearance includes observations on size, body weight, posture and gait, pelage, eyes, nostrils, teeth, coloration of pinnae and foot pads, urination, defecation, food and water intake, home cage and reproductive activities, and any abnormal spontaneous behaviors. Body weights and weights of hematopoietic tissues such as thymus, liver, spleen, and the various lymph nodes should be recorded. Color of the pathologic hematopoietic tissue should be noted; bone marrow is normally red, and lymphoid infiltrates are typically pale tan while myeloid infiltrates frequently have a green hue. Tissue imprints and bone marrow smears should be prepared and immediately stained with Romanowsky-type stains in the absence of formalin exposure.

Microscopic Evaluation

Representative sections of all tissues, including those without overt evidence of disease, should be preserved in neutral-buffered formalin, processed by routine methods, embedded in paraffin, sectioned at 5 μ m, stained with hematoxylin and eosin, and evaluated by light microscopy. Tissues that appear macroscopically normal may also have histologic evidence of disease. Furthermore, such a thorough evaluation will aid in the characterization of localized versus disseminated disease. Microscopic evaluation of tissue imprints and bone marrow smears will provide enhanced cellular and nuclear detail of neoplastic cells. Various cytochemical and immunohistochemical stains are also commonly used to phenotypically characterize neoplastic cells.²⁸ Examples of stains include: CD3 and CD19 for the identification of T and B lymphocytes, respectively; CD71 and Ter119 for erythroid cells; myeloperoxidase, Sudan black B, and Ly-6G (Gr-1) for myeloid cells; nonspecific esterase, Mac-2, Ly-71 (F4/80) and CD11b for monocytic cells; and, von Willebrand factor, CD41, and glycoprotein V for megakaryocytes/platelets. Certain antibodies require

the use of frozen sections rather than paraffin sections, so portions of fresh hematopoietic tissues should also be stored at -80°C for this purpose.

Ancillary Testing

Specialized imaging modalities routinely used in human medicine, such as radiography, dual-energy X-ray absorptiometry, ultrasonography, fluoroscopy, magnetic resonance imaging, X-ray computed tomography, positron emission tomography, and single photon emission computed tomography have been specifically adapted for the mouse.²⁹ With these modalities, investigative staff may effectively and non-invasively monitor the progression of disease over time in an individual mouse, thereby reducing the number of different time points for which mice must be evaluated.

Fresh and/or frozen tissue samples should also be taken at the time of necropsy for various ancillary diagnostics. Many of the same antibodies noted above for immunohistochemistry are used for flow cytometry of blood and tissues. Cytogenetic techniques, such as fluorescent in situ hybridization³⁰ and comparative genomic hybridization,³¹ aid in the identification of chromosomal rearrangements, loss of heterozygosity, and retroviral integration.

Lymphoid Neoplasms

The most common form of leukemia in the Western world, with a median age at diagnosis of 70 years, is chronic lymphocytic leukemia (CLL), which is characterized by an accumulation of CD5+ B cells in peripheral blood, bone marrow, and lymphoid tissues.³² Expression of the antiapoptotic BCL-2 protein and proapoptotic BCL-X protein are increased and decreased, respectively, in neoplastic B cells. One of the most frequent chromosomal abnormalities associated with CLL is a deletion in 13q14.3.³³ Two critical microRNAs, *miR-15a* and *miR-16-1*, which have been shown to negatively regulate expression of the BCL-2 antiapoptotic protein, are located in this region.^{34,35}

New Zealand Black (NZB) mice are typically crossed with New Zealand White (NZW) mice, generating the (NZB $\times$ NZW) F1 hybrid model of an autoimmune disease resembling human systemic lupus erythematosus. With advancing age, NZB mice develop a clonal expansion of CD5+ B cells, as well as autoimmune hemolytic anemia, similar to a subset of human CLL patients. Lymphoproliferative disease in these mice has been linked to a locus on murine chromosome 14 with synteny to human 13q14. Additionally, affected mice were shown to have reduced levels of miR-16-1. Furthermore, when exogenous miR-16 was delivered to a malignant B cell line derived from an affected NZB mouse, apoptosis was significantly increased. Phase I/IIa studies of BCL-2 inhibitors are ongoing in human patients with lymphoid malignancies.^{36,37}

Nonlymphoid Neoplasms

Myeloid Leukemia

Chromosomal translocations involving the retinoic acid receptor α (RAR α) gene located on chromosome 17 and various genes (X genes) are characteristic of acute promyelocytic leukemia (APL) in humans.³⁸ The resultant reciprocal X-RAR α and RAR α -X fusion oncoproteins have a dominant negative effect on both RAR α and X-gene pathways critical for development and differentiation, the consequence of which is the accumulation of promyelocytes in the blood and bone marrow. In 98% of cases, RAR α fuses to the promyelocytic leukemia (PML) gene [t(15;17) (q22; q11.2–12)]. Fusion to additional genes including promyelocytic leukemia zinc finger (PLZF) [t(11;17) (q23; q11.2–12)], nucleophosmin (NPM) [t(5;17) (5q32; q11.2–12)], nuclear matrix protein (NuMA) [t(11;17) (q13; q11.2–12)], and signal transducer and activator of transcription protein 5b (STAT5b) [t(17;17) (q11; q11.2–12)] have also been reported.^{38,39} In APL cases involving all but the RAR α -PLZF fusion, treatment with all trans retinoic acid (AtRA) and/or arsenic trioxide (As₂O₃) targets the fusion oncoproteins resulting in promyelocytic terminal differentiation with subsequent apoptosis.³⁸

Independent transgenic mouse constructs have been generated, in which expression of PML-RAR α , PLZF-RAR α , or NPM-RAR α is driven by the human cathepsin G (hCG) promoter, which restricts transgenic expression at the transition from myeloblasts to myelocytes.^{40,41} All three models develop leukemia. Disease latency and frequency are similar between hCG-PML/RAR α and hCG-NPM/RAR α mice (12–16 months and 10–12%, respectively) but different from hCG-PLZF/RAR α mice (6–18 months and 95%, respectively). Hepatosplenomegaly is accompanied by leukocytosis, anemia, and thrombocytopenia in all three models. Striking differences between the models include large numbers of promyelocytes in the peripheral blood and bone marrow of hCG-PML/RAR α mice resembling human APL, in contrast to large numbers of mature granulocytes in the bone marrow of hCG-PLZF/RAR α mice and bone marrow leukemic blasts with monocytoid features in hCG-NPM/RAR α mice, reminiscent of human chronic myelogenous leukemia and acute myelomonocytic leukemia, respectively. Interestingly, treatment of transgenic mice and nude mice transplanted with leukemic cells from transgenic mice with AtRA and/or As₂O₃ recapitulates the therapeutic efficacy in humans according to the respective fusion oncoprotein. Disease remission characterized by clearance of leukemic cells, increased numbers of mature granulocytic cells in peripheral blood and bone marrow, and prolonged survival is achieved by treatment of hCG-PML/RAR α and hCG-NPM/RAR α

mice with AtRA and As₂O₃, alone or in combination.^{41,42} In contrast, hCG-PLZF/RAR α mice are nonresponsive to AtRA/As₂O₃ therapy, similar to humans with RAR α -PLZF translocations. However, treatment with a histone deacetylase inhibitor inhibits proliferation, induces apoptosis, and increases AtRA-induced growth inhibition in these mice.⁴³ As a result of the research using the above mouse models, histone deacetylase inhibitors are now applied to therapeutic protocols of human APL patients with RAR α -PLZF translocations.⁴⁴

Erythroid Leukemia

Erythroleukemia, either de novo or secondary to chemotherapy or immunosuppressive therapy, is rare in humans with an incidence of 3–8%.⁴⁵ In mice, spontaneous disease is similarly rare but erythroleukemia consistently develops following infection with Friend retrovirus, a complex comprised of a pathogenic, replication-defective spleen focus forming virus (SFFV) and a replication competent Friend murine leukemia virus.⁴⁶ Elucidation of the pathogenesis of Friend-induced disease has led to the generation of spi-1 transgenic mice, which together exemplify the multi-stage model of leukemogenesis. gp55, a glycoprotein derived from a deleted and mutated *env* gene that remains within the infected cell, initially binds to the erythropoietin (Epo) receptor and the short form of the stem-cell kinase (sf-Stk) receptor promoting Epo-independent proliferation and terminal differentiation of erythroid precursors, establishing a preleukemic stage. SFFV transforms Epo-responsive erythroid progenitors via insertional mutagenesis at the *SFFV proviral integration-1* (*spi-1*) locus resulting in aberrant overexpression of the encoded transcription factor, PU.1 and subsequent clonal expansion of individual cells progressing to leukemia. Mutations in p53 are also consistently found in Friend leukemia and appear to promote the acquisition of various other mutations thereby ensuring growth and survival of the leukemic cells independent of Epo.

Transgenic mice overexpressing spi-1 under control of the SFFV long terminal repeat promoter develop preleukemia characterized by hepatosplenomegaly, anemia, and large numbers of nucleated proerythroblasts in the peripheral blood.⁴⁷ Short-lived regression of the splenomegaly and disappearance of the circulating erythroblasts occur following erythrocyte transfusions thereby indirectly demonstrating Epo-dependent proliferation in vivo. Late in the course of disease, spi-1 transgenic mice acquire mutations in the *kit* gene, which has ligand-independent tyrosine kinase activity resulting in autonomous growth and leukemia.⁴⁸ Furthermore, activating mutations in *kit* have been shown to result in phosphorylation of the membrane cytoskeletal cross-linker ezrin, which also plays a role in the growth and survival of leukemic cells.⁴⁹

Conclusion

Xenograft and genetically engineered mouse models are valuable translational tools employed to elucidate the molecular genetics of hematologic malignancies and evaluate the therapeutic efficacy of new chemotherapeutics. Their increasing use necessitates systematic phenotypic evaluation by a comparative pathologist knowledgeable in the classification of mouse lymphoid and nonlymphoid neoplasms, normal mouse anatomy and histology, age- and strain-related background lesions, and potentiating effects by endogenous retroviruses, irradiation, and chemicals.

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Section III

Molecular Pathology of Hematolymphoid Neoplasms: Specific Subtypes

Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma

Patricia Aoun

Introduction

Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) is a clonal lymphoproliferative disorder of morphologically and immunophenotypically mature small B-cells that accumulate in the peripheral blood (PB), bone marrow, lymph nodes, spleen, and other extramedullary sites.¹ In the majority of cases, the neoplastic B-cells exhibit a low mitotic rate and a high resistance to apoptosis. The criteria for diagnosis of chronic lymphocytic leukemia (CLL) have been updated recently by the International Workshop on Chronic Lymphocytic Leukemia.² For a diagnosis of CLL, the revised recommendations require the presence of an absolute B-cell lymphocytosis of at least $5.0 \times 10^9/L$ persisting for greater than 3 months and expressing the characteristic immunophenotype (i.e., CD5+/CD19+/CD20^{dim}/CD23+/monotypic light chain^{dim}). The clonal B-cells are typically negative (or only weakly positive) for CD79b and FMC7. Cases in which the number of clonal B-cells in the PB is less than $5.0 \times 10^9/L$ may be diagnosed as CLL if cytopenias or disease-related symptoms are present, or as SLL if lymphadenopathy and/or splenomegaly are present and the diagnosis is confirmed by tissue biopsy.²

The use of increasingly sophisticated multicolor flow cytometric analysis has resulted in the identification of small monoclonal B-cell populations that are immunophenotypically indistinguishable from CLL in up to 12% of otherwise healthy adults.^{3–8} These clonal populations, now referred to as monoclonal B-cell lymphocytosis (MBL), are present in the PB of asymptomatic patients who have absolute B-lymphocyte counts of less than $5.0 \times 10^9/L$, and who do not otherwise meet criteria for the diagnosis of CLL or SLL.^{2,9,10} In the general population, the prevalence of MBL increases after the age of 60 years, and recent studies suggest that progression to CLL may occur at a rate of about 1% per year.^{3,8,10–12}

CLL/SLL occurs predominantly in older adults, with a mean age at diagnosis of 65 years.¹³ The majority of patients are asymptomatic at the time of presentation, and the disease course is typically indolent. The Rai et al¹⁴ and Binet et al¹⁵

clinical staging systems have proven the most useful for predicting prognosis in patients with B-CLL and are widely used, because of the ease with which they may be implemented in routine clinical practice.² Because there is no curative therapy, palliative treatment is delayed until patients demonstrate disease-related symptoms or evidence of progression. In most cases, the disease progresses slowly. However, the clinical course of patients with CLL may be variable, driving the search for additional prognostic features that will help develop and refine risk-adapted treatment strategies. This search, in turn, has resulted in a greater understanding of the molecular biology driving leukemogenesis.

Familial Predisposition

First degree relatives of CLL patients are at high risk of developing CLL, and familial clusters of CLL that are morphologically and immunophenotypically indistinguishable from sporadic cases have been reported, indicating a role for hereditary predisposition in a subset of cases.^{16–30} A limited number of studies have compared the biological features of sporadic and familial cases, demonstrating similarities in terms of recurrent cytogenetic abnormalities,^{30–34} CD38 and ZAP-70 expression,^{27,35,36} and preferential usage of the VH1, VH3, and VH4 gene families.^{22,37–39} MBL has been identified in up to 18% of unaffected members of families with B-CLL,^{9,27,29,35} a rate that is significantly higher than in the general population. Significantly, MBL has been detected in unaffected family members under the age of 40 years.^{27,30,35,40}

Cytogenetic Features

Conventional cytogenetic studies of CLL/SLL cases are often negative due to the low mitotic rate of the neoplastic cells. However, karyotyping is useful for the detection of complex chromosomal aberrations. Most cases (i.e., 65%) with

an abnormal karyotype have a single chromosome abnormality, with the remainder having two or more abnormalities.^{41–43} Patients with normal karyotypes have been found to have a better overall survival, when compared to those with clonal chromosomal abnormalities.^{41,42} Like most lymphoid malignancies, complex karyotypes and a high percentage of abnormal metaphases are associated a worse prognosis than karyotypes with only a single or dual chromosomal abnormalities.^{41,42}

In contrast to conventional karyotyping, molecular cytogenetic studies using interphase fluorescence in situ hybridization (FISH) have demonstrated that chromosomal abnormalities occur in over 80% of cases of CLL, with the majority consisting of chromosomal deletions. A summary of the cytogenetic abnormalities in CLL with their prognostic impact is provided in Table 16.1. The finding of chromosomal deletions suggests that the loss of tumor suppressor genes plays a critical role in the pathogenesis of the disease. The most common isolated chromosomal abnormality associated with CLL/SLL is deletion 13q14 (del 13q14), which is present in more than 60% of cases studied by FISH.^{43–46} This deletion is believed to be a primary event in B-CLL, as it is present in a majority of the tumor cells and is frequently the sole abnormality.^{43,45} When present as an isolated abnormality, del 13q14 is associated with the typical morphology and immunophenotype of CLL. The specific pathogenetic gene(s) affected by this deletion have not yet been conclusively identified. However, recent studies have suggested several candidates, including microRNA clusters miR-15a and miR-16-1, to be discussed subsequently,^{47–51} and the tumor suppressor gene ARLTS1, a member of the ADP-ribosylation factor family.⁵² The prognosis of patients with isolated del 13q14 appears to be significantly better, and is similar to that of patients with a normal karyotype.^{41–43,53}

The second most common abnormality is deletion 11q22–23 (del 11q22–23), which is detected in as many as 20% of CLL. This deletion is associated with distinct clinical features, including a younger age at presentation, male sex, bulky adenopathy, and poor prognosis.^{43,45,54–56} The deleted region contains the ataxia telangiectasia mutated (ATM) gene, strongly suggesting a pathogenetic role for the ATM gene in CLL. In addition to deletions, ATM mutations may also be seen at diagnosis, and have been demonstrated to

result in impaired responses to DNA in vitro, and to be an independent predictor of poor prognosis.⁵⁷

Deletions of 17p13 are rare at diagnosis, occurring in less than 10% of patients, and arise more commonly later in the course of disease as a secondary abnormality.^{43,45,46,58–61} The deletions involve the TP53 locus and are associated with rapid disease progression, poor response to therapy, and shorter survivals. Mutations of the TP53 gene in the absence of deletions are also rare (less than 5% of cases), and are also associated with a poor prognosis.^{61,62}

Deletions of chromosome 6q (del 6q) have been reported to occur at a low frequency (i.e., 3–6%) of CLL,^{63–65} but in more (i.e., up to 26%) of SLL cases.⁶⁶ The specific gene(s) with pathogenetic significance has(have) not yet been identified. Del 6q is typically detected as a secondary abnormality (or as part of a complex karyotype), and is associated with high white blood cell counts, atypical morphology, significant adenopathy, and splenomegaly. The prognostic significance of the del 6q is controversial, with some studies suggesting decreased progression free survival and others showing no effect on progression free or overall survivals.

The most common chromosomal gain in CLL is trisomy 12, which occurs in up to 20% of cases and is associated with atypical morphology and immunophenotype, high proliferation rate, and advanced clinical stage at presentation. Trisomy 12 is most likely a secondary genetic abnormality in CLL, as it is usually identified in a minority of the tumor cells. Although the molecular mechanism by which this abnormality contributes to leukemogenesis is unknown, the presence of this abnormality appears to confer a worse prognosis.^{41,42,44,54,59,67–70}

In contrast to many other lymphoid malignancies, chromosomal translocations are rare in CLL. The presence of chromosomal translocations is associated with a poor prognosis.^{41,43,54,67,71–73} Chromosomal translocations typically involve the immunoglobulin heavy chain gene at 14q32 and have been detected in 4–9% of cases in most series.^{43,46,73–75} Due to the rarity of the finding, the clinical and biological features of CLL cases associated with 14q32 translocations are not well understood. In most cases, the translocation partners for the 14q32 are unknown. Those characterized include BCL2 (18q21), CCND3 (6p21), CDK6 (7q21), and BCL11A (2p12).^{43,45,46,72,73} The morphological, immunophenotypic, and clinical features of these cases vary from typical to atypical, and additional cytogenetic abnormalities may be present, although these do not appear to affect prognosis.⁷³

The t(14;19)(q32;q13) involving the BCL3 and IGH genes is a rare cytogenetic abnormality that has been described in a variety of B-cell lymphoproliferative disorders, the majority of which are CLL.^{76–81} This translocation juxtaposes the BCL3 gene at chromosome 19q13 next to the immunoglobulin heavy chain gene at 14q32, resulting in over-expression of BCL3 protein, which may be detected by immunohistochemistry. The translocation is typically present in association with other chromosomal abnormalities, and often as

TABLE 16.1. Cytogenetic abnormalities and prognostic impact.

Cytogenetic abnormality	Frequency	Prognostic impact
Del 13q14	>60%	+
Del 11q22–23	20%	– ^a
Del 17p13	<10%	– ^a
Del 6q	3–6%	Controversial
Trisomy 12	20%	–
Translocations involving 14q32	4–9%	–

+ = better/good prognosis; – = worst/poor prognosis.

^aOnly these two cytogenetic abnormalities correlate with a worst prognosis in clinical chemotherapeutic trials.

part of a complex karyotype.⁸¹ Presence of this translocation is associated with younger age, advanced stage, adenopathy, and an aggressive clinical course.^{78,80,81} Additional atypical features include cytological heterogeneity, irregular nuclear contours, and an atypical immunophenotype with strong expression of CD20, CD38, surface immunoglobulin, and FMC-7. The phenotype may suggest mantle cell lymphoma; however, the cells are negative for the t(11;14;)(q13;q32) and cyclin D1.

The t(2;14)(p13;q32) juxtaposing the BCL11A gene with the immunoglobulin heavy chain gene occurs rarely in CLL and is usually an isolated abnormality.^{73,82–84} Presence of the translocation has also been associated with young age, advanced stage, and an aggressive clinical course. Although the phenotype is usually typical, the morphological features are atypical with plasmacytoid differentiation, irregular nuclei, and increased prolymphocytes. Expression of ZAP-70 and an association with unmutated IGHV genes have also been described.^{83,84}

Most studies have examined the frequency and clinical relevance of single chromosomal aberrations in patients with CLL. In many cases, however, various chromosomal abnormalities may be present in the same tumor, complicating the interpretation of prognosis. The seminal study by Dohner et al⁴³ examined the prognostic importance of various abnormalities, when ranked according to a hierarchical model developed using regression analysis. In this and in subsequent studies that have used the hierarchical model, deletions of 17p and 11q are independent indicators of poor prognosis, whereas isolated deletions of 13q14 confer a better prognosis.

Immunoglobulin Genes

Numerous studies have now demonstrated that the mutation status of the immunoglobulin heavy chain variable (IGHV) region genes and VH gene usage are also important independent prognostic factors in CLL (also see Chap. 13). Somatic hypermutation of immunoglobulin genes normally occurs in germinal centers following B-cell exposure to antigens. Approximately 50% of CLL cases demonstrate somatic hypermutation in the variable regions of the immunoglobulin heavy chain genes, and the presence of somatic hypermutation is associated with a more indolent clinical course and longer survival.^{70,85–87} It is now clear that the mutational status of the immunoglobulin heavy chain variable region gene is among the most important independent prognostic markers in CLL. Unfortunately, determination of mutational status remains too complex and too expensive for most laboratories to implement as a clinical test.⁸⁷ As a result, investigators have searched for surrogate markers that correlate with mutation status and that may be used in routine clinical practice. The first surrogate marker of mutational status described was flow cytometric detection of CD38 expression. CD38

expression in more than 30% of CLL cells appeared to be a good surrogate marker for unmutated heavy chain genes.⁸⁶ Subsequent studies, however, have found that CD38 expression may vary during the course of a patient's disease and may be discrepant with the mutation status in up to 30% of cases.⁸⁸

The identification of subsets CLL that showed either mutated or unmutated immunoglobulin variable region sequences initially suggested the possibility of two diseases; however, mutated and unmutated CLL show a common distinctive signature by gene expression profiling that appears most closely related to memory B-cells and that is distinct from normal B-cells and from other B-cell lymphomas.^{89,90} Despite a common signature, a small number of genes are able to distinguish between mutated and unmutated cases of CLL.^{89,90} In 2001, Rosenwald and colleagues used GEP to demonstrate that mutated and unmutated cases of CLL may be distinguished from each other by the expression of a few hundred genes, including ZAP-70, which was the most differentially expressed gene.⁹⁰ Subsequent studies suggested that ZAP-70 expression is a good predictor of mutation status, and that it identifies patients with a more aggressive clinical course even at low clinical stage.^{90–97} ZAP-70 is encoded by a gene on chromosome 2, and is a member of a family of intracellular tyrosine kinases that play important roles in lymphocyte activation. ZAP-70 is normally expressed at high levels in T and NK cells, and plays a critical role in signal transduction following binding of the T-cell receptor. Recent investigations have demonstrated that ZAP-70 is normally present in a subset of normal splenic and tonsillar B-cells and in hematogones, and may be induced in activated peripheral blood B-cells.^{98–101} Following activation of the B-cell receptor, ZAP-70 is phosphorylated, although its specific role in signal transduction is not completely understood. Despite its initial promise as a surrogate marker of mutational status, studies have demonstrated that ZAP-70 expression and mutational status are discordant in 8–25% of cases,⁹⁶ a finding that may be related to the presence of high risk genetic abnormalities, such as deletions of 11q and 17p, and/or expression of VH3-21.^{102–104} Routine assessment of ZAP-70 in clinical practice has been limited by technical considerations, with numerous methodologies proposed and compared.^{91,96,105–113} The lack of standardization of assays for ZAP-70 has greatly limited its implementation in clinical practice.

Activation-induced cytidine deaminase (AID) is normally expressed by germinal center B-cells and is critical for somatic hypermutation and class switch recombination. AID levels are normally downregulated during B-cell maturation. Several studies have described high expression of AID in CLL cases,^{114–117} usually associated with and predictive of unmutated IGHV status and poor prognosis.^{114,116} An association with high-risk cytogenetics (i.e., del 17p13, del 11q and trisomy 12) has also been reported.¹¹⁷ Despite these associations, high levels of AID have not been considered surrogate markers of IGHV mutation status, in part because

the high expression is limited to a subset of the leukemic cells and because AID expression may also be detected in nearly 50% of mutated cases.¹¹⁷

Additional relationships between IGHV mutation status and cytogenetic abnormalities have also been noted. For example, somatic IGHV mutations are frequently observed in cases with del 13q14,^{118,119} but are rare in cases with trisomy 12, and a mixed pattern in rare cases with both chromosomal abnormalities.¹¹⁸

Both mutated and unmutated cases of CLL show preferential use of the VH1, VH3, and VH4 gene families, suggesting that chronic antigen stimulation may play a pathogenetic role.^{70,85,104,120–123} In CLL, the most frequently expressed genes are VH1-69, VH3-7, VH3-21, VH4-34, and VH4-39. A biological relationship between Ig gene segments and somatic mutation appears to exist, with mutations more commonly found in cases that utilize the VH3-7, VH3-23, VH3-48, and VH4-34 gene segments, and lack of mutations in cases that utilize VH1-69 and VH4-39 genes.^{70,85,120,123} The use of VH3-21 is associated with a poor prognosis, regardless of mutation status.^{103,104,121,123–125}

In addition to preferential use of selected immunoglobulin heavy chain variable genes, CLL cells have also been shown to have “stereotyped” B-cell receptors with very similar antigen binding sites coded by both heavy and light chain genes, suggesting that recognition of antigens that are structurally similar may play a role in the selection of leukemic clones during disease development^{123,126–128} and may have prognostic significance.^{125,127} The “stereotyped” receptors are the result of highly similar complementarity-determining region 3 sequences, and have been described in unmutated as well as mutated cases of CLL.^{122,123,129,130} The nature of the environmental or auto-antigens that the stereotyped BCRs might be recognizing and the importance of the stereotyped BCRs in the pathogenesis of CLL remain unknown. However, sustained BCR signaling has been shown to result in decreased apoptosis and increased expression of the antiapoptotic proteins (i.e., Mcl-1, Bcl-XL, and XIAP) through activation of the Akt and ERK kinases.^{131,132}

Epigenetic Factors

Epigenetic alterations of genes that do not change the DNA sequences but are carried to progeny cells are now recognized as important contributors to the development of malignancy. In CLL, the major epigenetic alterations described to date are in DNA methylation and altered expression of microRNAs.

DNA methylation is the addition of a methyl group to position 5 of a cytosine ring in a C-G dinucleotide. Promoter sequences rich in C-G sequences (i.e., CpG islands) that undergo methylation may be bound by protein domains of large repressor complexes, resulting in decreased gene

expression. Increased methylation of promoter sequences effectively silences genes; whereas, decreased methylation may be expected to result in over-expression. Both hypo- and hyper-methylation have been described in CLL. Global hypomethylation of genomic DNA has been demonstrated in CLL cells, and recent evidence suggests that the losses gradually increase over time.^{133–135} Genes affected by hypomethylation in CLL include the antiapoptotic gene BCL2 and the pro-survival gene TCL1A.^{136,137} Hypermethylation also occurs frequently in CLL and results in silencing of genes involved in apoptosis (i.e., DAPK1, WIF1, ID4, and SFRPs), regulation of the cell cycle (i.e., CDKN2A and CDKN2B), mismatch repair (MLH1), regulation of the p53 pathway (TWIST2), and expression of tyrosine kinases (i.e., ZAP-70 and PTPRO).^{138–147} The BCL11A gene also has a large 5' CpG island.⁸²

MicroRNAs (miRNAs) are small noncoding single stranded RNAs of approximately 20 nucleotides that regulate genes involved in cell cycle, differentiation, and apoptosis^{148,149}). MiRNAs are incorporated into a complex known as RNA-induced silencing complex (RISC). As part of the RISC, miRNAs then bind to messenger RNA (mRNA) targets at the 3' untranslated region (3'UTR) with incomplete complementarity, preventing translation or allowing degradation of the mRNA. The result of this interaction is reduced or absent levels of the encoded protein. A single miRNA may bind as many as 200 genes with diverse functions, and target genes may bind several miRNAs, suggesting a complex regulatory system of gene expression.¹⁴⁸ Dysregulation of miRNA expression may lead to tumor formation through alterations in expression of tumor suppressor genes and/or oncogenes.

The initial report linking miRNAs to cancer was the demonstration of deletions of MIR-15a and MIR-16-1 genes in CLL patients with deletion 13q14.⁴⁷ Both genes are clustered within a 30 kb region at 13q14.3 (miR-15a and miR-16-1), are highly expressed in normal tissues, and normally function to downregulate Bcl-2 protein expression by interfering with transcription of the BCL2 mRNA.¹⁵⁰ Loss of miR-15 and miR-16 by the hemi- or homozygous deletions of 13q14 occurs in a majority of patients with CLL and may result in increased levels of Bcl-2 protein expression by the tumor cells.^{47,150} A pathogenetic mutation in the miR-15a–miR-16-1 cluster has also been identified and found to be associated with low levels of miRNA expression.⁴⁹

Genome-wide microarray GEP studies of miRNAs have demonstrated signatures in CLL cells distinct from those found in normal lymphoid cells, including normal CD5+ B-cells.^{48,151} In addition to miR-15 and miR-16 described above, deregulated expression of miR-21, miR-92, miR101, miR-150, miR-155, and miR-181a has been described in CLL.^{48,49,151,152} recent studies have suggested that miRNA expression patterns in lymph nodes and PB of patients with CLL are similar.¹⁵³ Furthermore, miRNA signatures which distinguish between cases with or without del 13q14,

IGHV mutations, or ZAP-70 expression have also been identified.^{48,49} Calin et al described a signature containing 13 miRNAs able to correctly classify ZAP-70 status, and nine of these miRNAs are able to distinguish between patients with long or short time to treatment.⁴⁹ More recent studies have suggested that the expression levels of as few as four miRNAs (i.e., miR-29b, miR-29c, miR-150, and miR-223) correlate with IGVH mutation status.¹⁵²

Down-regulation of miR-29c and miR-223 has been associated with progression to a higher stage, need for treatment, and death.¹⁵⁴ Cases of CLL with poor prognosis also have been shown to have low levels of miR-29b and miR-181a.¹⁵¹ Expression of miR-29b, miR-29c, and miR-181a is inversely correlated with expression of the T-cell leukemia/lymphoma 1 (TCL1) gene.^{50,154} Tc11 is a coactivator of the Akt oncoprotein, an important factor in the transduction of antiapoptotic signals. High levels of Tc11 protein in CLL are associated with unmutated IGVH status and an aggressive clinical course. The expression of the antiapoptotic protein Mcl-1 is also regulated by miR-29, with decreased Mcl-1 protein levels observed when there is an increased miR-29b expression.¹⁵⁵

Downregulation of miR-29c, miR-34a, and miR-17-5p has been demonstrated in cases of CLL harboring deletions or mutations of the TP53 gene.¹⁵⁶ Low levels of miR-34a have also been correlated with impaired response to DNA damage that is independent of TP53 abnormalities, resistance to apoptosis, and resistance to chemotherapy.¹⁵⁷ The evidence to date strongly suggests that deregulation of multiple miRNAs, and the resultant changes in the expression of important components of cell cycle and survival pathways, plays an important role in the pathogenesis and progression of CLL.

Clinical Implications

The clinical course of patients with CLL is highly variable. Many patients who are diagnosed at an early stage may remain asymptomatic and never require treatment; whereas, others may develop highly aggressive disease over a variable amount time. Multiple studies of patients with early stage disease, who have been studied in trials comparing immediate with deferred chemotherapy using alkylating agents, have not demonstrated a survival benefit to early therapy.¹⁵⁸ Therefore, current treatment guidelines recommend therapy only in those patients with symptomatic, advanced, or progressive disease, since treatment is generally considered to be palliative.² Although the Rai and Binet staging systems remain powerful tools for risk stratification, the molecular features of CLL that have been recently elucidated demonstrate that factors other than stage may predict time to progression and treatment, as well as overall survival. As previously mentioned, molecular features associated with poor prognosis include high-risk cytogenetics (i.e., del 11q

or del 17p), unmutated IGVH status, usage of the IGHV3-21 gene segment, as well as CD38 and ZAP-70 expression.

A recent prospective randomized clinical trial evaluated the prognostic effect of IGVH mutational status, ZAP-70 and CD38 expression, cytogenetic abnormalities detected by FISH, TP53 mutations, and expression of apoptosis-related proteins (i.e., Bcl-2, Bax, Mcl-1, Bag-1, XIAP, Caspase-3, and TRAF-1) in previously untreated patients who received fludarabine-based chemotherapy regimens.¹⁵⁹ In this study, only the high-risk cytogenetic abnormalities – del 17p13 and del 11q22 – correlated with shortened progression-free survival and were significant risk factors for early relapse. Other prospective clinical trials have also demonstrated that these high-risk cytogenetic abnormalities and IGVH mutational status are associated with poor responses to therapy.¹⁶⁰ Significant improvement in progression-free survivals has been seen with the use of chemo-immunotherapy that includes rituximab.¹⁶¹ However, CLL patients with unmutated IGVH or high-risk cytogenetics have poorer responses to this therapy with shorter progression free and overall survivals.¹⁶² The value of incorporating these factors into risk assessment for the purposes of determining treatment in routine clinical practice is not yet clear. Clinical trials designed to answer these specific questions are ongoing.

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Marginal Zone B-Cell Lymphoma

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Introduction

Marginal zone lymphoma (MZL) of extranodal mucosa-associated lymphoid tissue (MALT lymphoma), nodal MZL, and splenic B-cell MZL share similar morphologic and immunophenotypic features.¹ All are proliferations of small B lymphoid cells that colonize the marginal zone of reactive germinal centers (GC). These cells were previously described as “centrocyte-like” because their nuclei resemble those of small cleaved follicle center B cells, with slightly irregular nuclear contours, condensed chromatin, and inconspicuous nucleoli, but with more abundant pale cytoplasm.² Although the different subtypes of MZL share histologic and immunophenotypic features, they have different clinical, cytogenetic, and molecular genetic features.

The MALT lymphoma concept emerged from the work of Isaacson and Wright, first published in 1983.³ They showed that cases previously diagnosed as “pseudolymphoma” in stomach and bowel were actually extranodal low-grade B-cell lymphomas that recapitulated the morphology of Peyer patch marginal zone B cells, and typically arose in sites with no organized lymphoid tissue. They hypothesized that persistent antigen stimulation and chronic inflammation induced by infection with *Helicobacter pylori* organisms leads to the accumulation of extranodal lymphoid tissue, which forms the substrate for the development of extranodal MALT lymphoma. Since then, the identification of specific chromosomal translocations that involve a common signaling pathway in extranodal MALT lymphomas arising in the gastrointestinal tract and in other sites has further defined this subtype.^{2,4} In contrast to MALT lymphoma, *nodal marginal zone lymphoma* (NMZL) and *splenic B-cell marginal zone lymphoma* (SMZL) are predominantly defined based on their anatomic site of involvement because their etiologies are less well understood.

Extranodal Marginal Zone Lymphoma of Mucosa-Associated Lymphoid Tissue (MALT Lymphoma)

Definition

In the WHO classification, MALT lymphoma is defined as an extranodal lymphoma composed of a cytologically heterogeneous proliferation of small B cells including marginal zone (centrocyte-like) cells, cells resembling monocytoid cells, small lymphocytes, and scattered immunoblasts and centroblast-like cells.¹ Some cases show plasmacytic differentiation. The neoplastic cells infiltrate the marginal zones of reactive lymphoid follicles and extend into interfollicular region. In epithelial tissues, the neoplastic cells invade the epithelium to form lymphoepithelial lesions. The postulated normal counterpart is a postgerminal center B cell.

Clinical Features

MALT lymphoma, which comprises about 8% of all B-cell lymphomas, is the most common type of primary extranodal lymphoma.⁵ It affects predominantly adults with a median age of 61 years, and women are affected slightly more often than men (male:female=1:1.2).⁵ About half of all MALT lymphomas present in the gastrointestinal tract, the most common site of involvement. Within the gastrointestinal tract, the stomach is most frequently affected, accounting for 85% of all cases.⁶ Other common sites of origin are salivary gland, lung, head and neck, ocular adnexae, skin, thyroid gland, and breast.⁷

Many patients with extranodal MALT lymphoma have a history of chronic inflammation. In the stomach, the majority of cases (up to 90% in some studies) are associated with *H. pylori* infection.¹ In this setting, the development of MALT lymphoma is initiated by malignant transformation and subsequent expansion of postgerminal center B cells in response

to antigen-stimulated tumor-infiltrating T cells. In these patients, the proliferation of the lymphoma cells depends on the presence of *H. pylori*-antigen-specific activated T cells.⁸ Of the cases that are associated with *H. pylori* infection, about 75% of cases are cured following treatment to eradicate the organisms.⁹ Antibiotic therapy is ineffective in the minority of cases, in which the organisms are not identified. Infectious agents also play a role in the development of MALT lymphoma at other sites. MALT lymphoma is associated with infection by *Chlamydia psittaci* in the ocular adnexae,^{10,11} by *Borrelia burgdorferi* in skin,¹² and by *Campylobacter jejuni* in the small intestine.¹³ An infectious agent has not been identified in pulmonary MALT lymphoma.¹⁴ MALT lymphomas may also arise in the setting of autoimmune disease. Patients with Sjögren's syndrome or lymphoepithelial sialadenitis have a significantly increased risk of developing MALT lymphoma of the salivary gland.^{1,2} Similarly, patients with Hashimoto thyroiditis have a significantly increased risk of developing lymphoma of the thyroid gland.

Most patients with MALT lymphoma present with early stage (I or II) disease. Bone marrow involvement is uncommon, but is more likely to occur with MALT lymphomas that arise in lung or ocular adnexae than in stomach.^{7,15,16} At presentation, multiple extranodal sites may be involved in up to 25% of gastric cases and in almost half of extra-gastric cases.¹⁷ About one-third of patients, particularly those with MALT lymphomas that have arisen in the setting of Hashimoto thyroiditis or Sjögren's syndrome, have lymph node involvement at the time of presentation, which must be distinguished from primary nodal MZL.^{15,17} About one-third of patients have a small serum paraprotein.¹⁸ However, immunoproliferative small intestinal disease (IPSID), a subtype of MALT lymphoma, is characterized by an aberrant immunoglobulin alpha heavy chain proteinemia, as described below.

Morphology and Immunophenotype

Extranodal MALT lymphoma is composed of a cytologically heterogeneous population of small lymphoid cells that include marginal zone (centrocyte-like cells) cells, cells resembling monocytoid cells which have abundant pale cytoplasm, small lymphocytes, and scattered occasional large transformed-appearing cells. The neoplastic cells may also show plasmacytic differentiation, which is seen in about one-third of gastric MALT lymphoma cases, is usually conspicuous in cutaneous and thyroid cases, and is characteristic of IPSID.¹

In extranodal sites, the neoplastic cells surround and invade the mantle zones of reactive follicles, and form sheets that replace the reactive follicles and normal structures. In sites with epithelium, the neoplastic cells invade and destroy the epithelium to form lymphoepithelial lesions. In lymph nodes, the neoplastic cells invade the marginal zones and expand the interfollicular region. They may form aggregates of monocytoid cells, most conspicuous in the parafollicular regions or adjacent to sinusoids. The neoplastic cells also invade and may colonize germinal centers (follicular colonization),

which may be difficult to distinguish from follicular lymphoma without ancillary studies.¹

The neoplastic cells express pan-B-cell markers (i.e., CD19, CD20, CD22, and CD79a) and monotypic surface immunoglobulin, usually IgM, but occasionally IgA or IgG. The neoplastic cells express bc12, and may also express CD43 and CD11c. They are negative for CD10, CD23, and cyclin D1.¹⁹ Rare cases are positive for CD5.²⁰ Antibodies to the marginal zone cell-associated antigens CD21 and CD35 stain the neoplastic cells and also accentuate the disrupted follicular dendritic cell network of colonized lymphoid follicles.¹

Prognosis and Predictive Factors

The disease is indolent and slow to disseminate. About two-thirds of patients present with localized disease (stage I or IIE) and about one-third present with stage III or stage IV disease.¹⁵ Regardless of stage at presentation, about 80% of patients survive 10 years or more. Involvement of multiple extranodal sites at diagnosis, including bone marrow, does not confer worse prognosis. Thus, the staging systems used for nodal lymphomas may not be appropriate for MALT lymphomas.¹ Recurrences, which are more common in extra-gastric MALT lymphomas, tend to occur after many years and to involve other extranodal sites.^{15,17} Progression to large B-cell lymphoma, characterized by sheets of large cells, occurs in fewer than 10% of cases, and confers a worse prognosis.¹⁵ The WHO classification indicates that cases which meet the criteria for the diagnosis as DLBCL should be classified as such, with mention of the presence of the accompanying MALT lymphoma.¹ Further, the authors state that the term "high-grade MALT lymphoma" should not be used, even if the DLBCL has arisen in a MALT site or contains lymphoepithelial lesions.

Cytogenetic and Molecular Genetic Features

To date, four different chromosomal translocations have been shown to be associated with extranodal MALT lymphoma: t(11;18)(q21;q21), t(14;18)(q32;q21), t(1;14)(p22;q32), and t(3;14)(p14;q32), described below. Their incidence varies depending upon the site of origin of the MALT lymphoma. The first three are specific for MALT lymphoma. Although they involve different genes, their oncogenic activities are believed to be linked through activation of a common NF-κB pathway. The t(3;14) occurs in MALT lymphoma, but also in other B-cell lymphomas, and its pathogenetic mechanism is unknown.

t(11;18)(q21;q21)/API2-MALT1

The t(11;18) is the most common chromosomal abnormality in MALT lymphoma. It is found in about one-half of pulmonary MALT lymphomas and up to one-quarter of gastric MALT lymphomas; it is uncommon in MALT lymphomas of salivary gland, ocular adnexae, thyroid, and skin.^{4,21,22} The t(11;18) is

specific for MALT lymphoma; it is not found in nodal MZL, splenic MZL, or other types of lymphomas. In cases with the t(11;18), it is the sole cytogenetic abnormality.

The t(11;18) generates a fusion transcript between *API2* (apoptosis inhibitor 2) on chromosome 11 and *MALT1* on chromosome 18.^{23–25} *API2* is normally expressed strongly in lymphoid cells. It is a member of the inhibitors of apoptosis (IAP) gene family.^{26,27} Members of this gene family have three copies of a BIR motif, followed by a caspase recruitment domain (CARD), and a C-terminal really interesting new gene (RING) finger domain. Several proteins encoded by members of this gene family are known to inhibit apoptosis by inhibiting activated caspases through interaction with TNF-receptor-associated factor (TRAF) proteins. The protein encoded by *API2*, cIAP2, suppresses apoptosis by inhibiting the activity of caspase 3 and caspase 7, and also by inhibiting the activation of procaspase 9 by cytochrome C.²⁸ *MALT1* is normally involved in antigen-receptor-mediated activation of NF- κ B. The protein contains an N-terminal death domain followed by two immunoglobulin-like domains and a caspase-like domain.

There are four known breakpoints in both *API2* and *MALT1*, which generate eight variant fusion transcripts.^{4,23,29,30} In *API2*, the breakpoints are always downstream of the third BIR domain, and upstream of the RING domain, within intron 7 or exon 8. In *MALT1*, the breakpoints are upstream of the C-terminal caspase-like domain, which is essential for NF- κ B activation, in introns 4, 6, 7, or 8. Thus, the fusion protein always contains the N-terminus of *API2* with three BIR domains, and the C-terminus of *MALT1* with the caspase-like domain. The fusion gene products form homodimers through the *API2*-encoded portion of the fusion protein. These homodimers activate NF- κ B and inhibit apoptosis, both directly and indirectly. The *MALT1/API2* fusion transcript is not detected.

In gastric MALT lymphomas, the *API2*–*MALT1* fusion protein is found in 10–20% of cases and has important prognostic implications.^{4,21,22} These cases tend to present at an advanced clinical stage, spread deeply into the gastric wall, and often involve lymph nodes.³¹ However, they show low-grade histology, without an increase in large cells, and rarely undergo histologic progression, even at an advanced stage. There is a significant association between the t(11;18) and infection with strains of *H. pylori* that incite inflammation with neutrophils.³² It is hypothesized that reactive oxygen species generated by the neutrophils damage DNA and induce double-stranded breaks that increase the probability of translocation. Patients with the translocation may initially respond to antibiotic therapy, but later relapse even in the absence of *H. pylori* re-infection. In lung, the t(11;18) is also associated with low-grade histology. In MALT lymphomas with the t(11;18), the translocation is usually the sole cytogenetic abnormality.⁴

Of the approximately 80% of cases that lack the *API2*–*MALT1* fusion protein, more than half respond to *H. pylori* eradication therapy.^{2,4,31} These cases tend to present at a low clinical stage, show superficial involvement of the gastric

wall, and show no increase in large cells. They also show a bias toward VH3-23 or VH3-30 usage, which suggests that they are derived from *H. pylori*-associated B cell subset and may not progress in the absence of antigenic stimulation. The remaining fusion-negative cases do not respond to eradication therapy.^{2,29,31} These cases often present with clinically advanced-stage disease and may show an increase in large cells, which suggests that they may be more likely to undergo histologic progression. They show no bias in VH gene usage and are more likely to have genomic imbalances than the eradication sensitive cases.

t(14;18)(q32;q21)/IGH-MALT1

The t(14;18) is found in about 5–10% of extranodal MALT lymphomas, predominantly in those that arise in nongastrointestinal sites, such as liver, lung, ocular adnexae, skin, and salivary gland.^{21,33–35} This translocation brings the coding region of *MALT1* on chromosome 18 under the control of IGH enhancer elements on chromosome 14, and results in overexpression of *MALT1* protein.³⁵ The breakpoint on 18q21 is only 5 Mb centromeric to *BCL2*. Thus, this abnormality and the t(14;18) in follicular lymphoma, which involves *IGH* and *BCL2*, are indistinguishable on conventional karyotypic studies, but can be distinguished using specific FISH probes.³⁵ Unlike cases with the t(11;18), cases with the t(14;18) often show additional genomic abnormalities, in particular, trisomy 3, 12, and 18.^{21,22,34,35} Its clinical significance is unknown.

t(1;14)(p22;q32)/BCL10-IgH

The t(1;14) is found about 2% of all MALT lymphomas, predominantly those that arise in lung and stomach, and infrequently in those that arise in intestines, salivary gland, and skin.^{21,22,36} It is seen in up to 10% of cases in lung. This translocation brings *BCL10* on chromosome 1 under the control of *IGH* enhancer elements on chromosome 14, and results in overexpression of *BCL10* protein.^{36–38} *BCL10* is required for the normal development and function of B cells and T cells. It also links antigen-receptor signaling to the NF- κ B pathway.³⁹ The t(1;2) is an uncommon variant of t(1;14), in which *BCL10* is brought under the control of the kappa light chain gene.⁴⁰ Like the t(14;18), but unlike the t(11;18), cases with t(1;14), often show additional genomic abnormalities.^{21,22} Similar to patients with the t(11;18), patients with the t(1;14) may be more likely to present with advanced stage disease and less likely to respond to eradication of *H. pylori*.³⁶

The t(11;18), t(14;18), and t(1;14) Activate the NF- κ B Pathway

The oncogenic activities of t(11;18), t(1;14), and t(14;18) are believed to be linked through the NF- κ B pathway, which is activated from signaling through the antigen receptor.^{2,4,39} NF- κ B is a family of proteins that form many different homodimers and heterodimers which differentially regulate target gene transcription in normal B-cell and T-cell development.

In resting cells, most NF- κ B dimers are retained in cytoplasm and are bound by specific inhibitors, the inhibitors of NF- κ Bs (I κ Bs). Infection with *H. pylori* in the stomach leads to T-cell-dependent activation of the B-cell receptor. Although the precise mechanisms are not completely known, activation of antigen-receptor associated tyrosine kinases result in the formation of a complex that includes CARMA1, oligomerized BCL10, and oligomerized MALT1. These proteins are critical for the subsequent activation of the inhibitor of the NF- κ B (IKK) complex. Activated IKK phosphorylates the I κ B proteins that are bound to the NF- κ B proteins and targets them for polyubiquitination and degradation. The NF- κ B proteins are then free to enter nucleus, where they increase the transcription of genes that encode cytokines and chemokines, enzymes that modulate immune responses, and inhibitors of apoptosis.

In MALT lymphomas with the t(1;14), it is believed that overexpressed BCL10 interacts with MALT1 and CARMA1, which leads to ubiquitination and degradation of NF- κ B essential modulator (NEMO), a component of the IKK complex and I κ B.^{2,4,39} In those with the t(14;18), overexpressed MALT1 is believed to stabilize BCL10 protein and CARMA1.^{2,4,39} Finally, in MALT lymphomas with the t(11;18), the cIAP2 portion of the fusion protein induces oligomerization of the of the caspase-like domain of MALT1 portion of the fusion protein.^{2,4,39} Thus, each of these translocations results in constitutive activation of the NF- κ B pathway, which enhances cell proliferation and survival, and contributes to lymphomagenesis.

t(3;14)(p14;q32)/Forkhead Box P1-IGH

The t(3;14) is a recently described translocation found in extranodal MALT lymphomas of thyroid (50%), ocular adnexae (20%), and skin (10%).³³ However, unlike the translocations described above, the t(3;14) is not specific for MALT lymphomas; it is also found in DLBCL.^{41–43} This translocation brings *FOXP1* on chromosome 3 under the control of *IGH* enhancer elements on chromosome 14, and results in overexpression of a structurally normal FOXP1 protein.^{33,44} *FOXP1* is a member of the *FOXP* subfamily of forkhead transcription factors, which are characterized by a DNA binding winged helix, a leucine zipper, a zinc finger, and a

polyglutamine tract.⁴⁵ The FOXP1 protein is ubiquitously expressed in normal adult and fetal tissues, with its highest expression in lymphoid and gastrointestinal tissues, localized predominantly to the nucleus.⁴⁶ Little is known about how these proteins regulate transcription. However, FOXP1 is involved in RAG1 and RAG2 expression and is, therefore, critical in B-cell development.⁴⁷ Expression of FOXP1 has been detected by immunohistochemical stain in a subset of DLBCL, where it is associated with a poor prognosis.⁴² The mechanisms by which this translocation contributes to lymphomagenesis are unknown.

The anatomical distribution, inciting agent, frequency of chromosomal aberrations, and involved genes in MALT lymphomas are summarized in Table 17.1.

Translocation-Negative Cases

The molecular pathogenesis of translocation-negative MALT lymphomas remains poorly understood. Although trisomies 3 and 18 are frequently identified in translocation-negative cases, these abnormalities may be seen in other lymphoid malignancies, and their prognostic significance is unknown. Recent array comparative genomic hybridization (aCGH) studies performed on translocation-negative cases of gastric, salivary and ocular MALT lymphoma have identified partial or complete gains of chromosomes 3, 12, 18 and 22, as well as gains at 9q34 in translocation-negative cases of gastric MALT lymphoma.⁴⁸ A similar study performed on translocation-negative salivary gland MALT lymphomas identified recurrent chromosomal gains at 1p32, 9q33–34, 11q11–13, 17 and 18q21–22, as well as frequent concurrent chromosomal gains at 9q34, 11q13 and 18q21–22.⁴⁹ Array CGH studies performed on translocation-negative cases of ocular MALT lymphoma have identified recurrent losses at 6q23.3, 7q36.3, and 13q34, and recurrent gains of chromosomes 3, 15, 18q and 6p.⁵⁰ It has been suggested that extra copies of *TRAF2* or *CARD9* at 9q34, *RelA* at 11q13, or *MALT1* at 18q21–22 in gastric and salivary MALT lymphomas activate the NF- κ B pathway, similar to cases with the characteristic MALT lymphoma translocation. In addition, the novel deletion 6q23.3 may have a crucial genetic role in the pathogenesis of ocular MALT lymphoma.

TABLE 17.1. Anatomical distribution, inciting agent, frequency of chromosomal aberrations, and involved genes in MALT lymphomas.

Anatomic site	Inciting agent	Chromosomal aberrations ^a	Most frequent translocation	Involved genes
Stomach	<i>Helicobacter pylori</i>	t(11;18)(q21;q21), +3, +18, t(14;18)(q32;q21)	t(11;18)(q21;q21)	API2-MALT1
Salivary glands	Sjögren syndrome	+3, +18, t(14;18)(q32;q21), t(11;18)(q21;q21), t(1;14)(p22;q32)	t(14;18)(q32;q21)	IGH-MALT1
Lung		t(11;18)(q21;q21), +3, +18, t(14;18)(q32;q21), t(1;14)(p22;q32)	t(11;18)(q21;q21)	API2-MALT1
Ocular adnexae	<i>Chlamydia psittaci</i>	Trisomy 3, t(14;18)(q32;q21), t(3;14)(p14;q32), trisomy 18, t(11;18)(q21;q21)	t(14;18)(q32;q21) ^b	IGH-MALT1
Skin	<i>Borrelia burgdorferi</i>	+3, t(14;18)(q32;q21), t(3;14)(p14;q32), t(11;18)(q21;q21), +18	t(3;14)(p14;q32)	FOXP1-IGH
Thyroid	Hashimoto thyroiditis	t(3;14)(p14;q32), +3, t(11;18)(q21;q21)	t(3;14)(p14;q32)	FOXP1-IGH

^aAberrations are listed in decreasing order of frequency.

^bOccurs at a frequency similar to t(3;14)(p14;q32).

Differential Diagnosis

The differential diagnosis includes extranodal involvement by other B-cell lymphomas composed of predominantly small lymphoid cells, such as chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), follicular lymphoma (FL), and mantle cell lymphoma (MCL). Features that aid in the distinction of extranodal marginal zone lymphoma from these other entities include the heterogeneity of the cellular infiltrate, the presence of reactive germinal centers and lymphoepithelial lesions, the lack of expression of CD10 and cyclin D1, and the lack of expression of CD5 in the vast majority of cases. In addition, the differential diagnosis should include lymphoplasmacytic lymphoma. This differential diagnosis is further discussed in Chap. 18.

The differential diagnosis also includes reactive lesions, particularly those that precede extranodal MALT lymphoma, which can be difficult to distinguish because of the cellular heterogeneity of extranodal MALT lymphoma and the generally small size of endoscopic biopsies. However, extranodal MALT lymphomas are typically diffuse and destructive. The neoplastic cells demonstrate monotypic surface immunoglobulin expression by flow cytometry, and by immunohistochemical stains for immunoglobulin light chains in some cases. Molecular diagnostic studies demonstrate clonal immunoglobulin heavy chain gene rearrangements. Conventional cytogenetic and fluorescence in situ hybridization studies may demonstrate characteristic translocations and/or trisomies.

Immunoproliferative Small Intestinal Disease

Definition

In the WHO classification, immunoproliferative small intestinal disease (IPSID), previously referred to as alpha heavy chain disease, is considered a variant of MALT lymphoma.⁵¹

Clinical Features

Immunoproliferative small intestinal disease is the most common immunoglobulin heavy chain disease. Unlike most patients with MALT lymphoma, patients with IPSID are typically young, with a peak incidence in the second and third decades. There is no sex predilection. The geographical distribution of IPSID is characteristic.^{52–54} It is most common in countries that surround the Mediterranean, in particular, Israel, Egypt, Saudi Arabia, and North Africa. In these countries, IPSID is associated with low socioeconomic status, poor hygiene and nutrition, and frequent gastrointestinal infections. In some cases, it has been associated *Campylobacter jejuni* infection.⁵⁵ Patients typically present with signs and symptoms of malabsorption, such as abdominal pain, vomiting, diarrhea, steatorrhea, and hypocalcemia.⁵¹

Because progression to DLBCL is common, the disease is often fatal. However, if IPSID is detected before it progresses to DLBCL, it may respond to therapy with broad spectrum antibiotics.^{54,56}

Morphology and Immunophenotype

The neoplastic infiltrate typically involves the small intestine and mesenteric lymph nodes, but may also involve stomach and colon.⁵¹ Bone marrow and other organs are usually spared. The lamina propria contains a pronounced infiltrate of plasma cells with admixed small lymphocytes, which may invade the epithelium to form lymphoepithelial lesions. The villi may appear atrophic. Progression to DLBCL is characterized by destructive sheets of large plasmacytoid cells and immunoblasts that ulcerate and destroy the bowel wall.⁵⁷ The small lymphoid cells have the characteristic immunophenotype of marginal zone B cells, as described above. The plasma cells express CD138 and are negative for CD20.⁵⁸ Both components express aberrant monotypic cytoplasmic alpha heavy chain that lacks light chain.

Cytogenetic and Molecular Genetic Features

IPSID demonstrates clonally rearranged immunoglobulin heavy and light chains, with a high degree of somatic mutation.⁵⁷ Deletions in the *VH* and *CH1* regions of the alpha heavy chain result in a defective heavy chain protein that cannot bind light chain.⁵⁷ Thus, the serum protein electrophoresis is normal or may show hypogammaglobulinemia, but immunofixation demonstrates an abnormal IgA molecule.⁵⁴ There are no reported consistent cytogenetic abnormalities.

Nodal Marginal Zone Lymphoma

Definition

In the WHO classification, nodal marginal zone lymphoma is defined as a primary nodal B-cell neoplasm that morphologically resembles lymph nodes involved by MZL of MALT or splenic types, but without evidence of extranodal or splenic disease.⁵⁹ Because the neoplastic lymphocytes may have abundant pale cytoplasm, nodal MZL has also been called monocytoid B-cell lymphoma. The postulated normal counterpart is a postgerminal center B cell.

Clinical Features

Nodal marginal zone lymphoma is an uncommon neoplasm, comprising 1–2% of all lymphoid neoplasms.^{60,61} It affects predominantly adults, with a median age of approximately 60 years and no sex predilection.⁶² Most patients present with localized or generalized lymphadenopathy, usually without peripheral blood or bone marrow involvement.⁶² An association with Hepatitis C virus (HCV) infection has been found

in up to 50% of patients in some studies, but not in others, possibly reflecting geographic variation in the incidence of HCV infection.^{62–66} The disease course is generally indolent, with 60–80% of patients surviving for more than 5 years.^{62,67} However, progression to large B-cell lymphoma may occur in a minority of cases.^{67,68} In children, nodal marginal zone lymphoma occurs infrequently and has distinctive clinical and morphologic features (see below).

Morphology and Immunophenotype

The lymph node architecture is usually subtotally effaced; the neoplastic cells tend to surround reactive lymphoid follicles, infiltrate the mantle zones, and expand the interfollicular region, making it appear pale and vaguely nodular.⁵⁹ Follicular colonization is commonly seen. Many cases show plasmacytic differentiation. The immunophenotype is similar to that of MALT lymphoma.⁶⁹ The neoplastic cells express pan-B-cell markers and monotypic surface immunoglobulin. Most cases are also positive for BCL2, and about half express CD43. The neoplastic cells are negative for CD23, CD10, BCL6, and cyclin D1, and the vast majority of cases are negative for CD5. A minority of cases progress to DLBCL, characterized by sheets of large transformed cells.^{67,68}

Differential Diagnosis

Approximately one third of adult patients who present with lymph nodes involved by MZL actually have disseminated MALT lymphoma. Thus, presentation with nodal MZL must be distinguished from MALT lymphoma that has spread to lymph node. This is especially true of patients with a history of Hashimoto thyroiditis or Sjögren syndrome, who may present with disseminated disease.^{69,70} Like MALT lymphoma, the differential diagnosis includes other lymphoid malignancies composed predominantly of small B cells. Cases that show extensive follicular colonization may be difficult to distinguish from follicular lymphoma based solely on morphology. However, immunophenotypic studies should allow a distinction between MZL and FL, as well as other small B-cell neoplasms. Patients with splenic MZL, in contrast to those with nodal MZL, typically present with marked splenomegaly without peripheral lymphadenopathy.⁶⁰ The distinction between nodal MZL and nodal lymphoplasmacytic lymphoma is controversial; both have a similar immunophenotype and may show plasmacytic differentiation.⁵⁹

Cytogenetic and Molecular Genetic Features

Nodal marginal zone lymphoma demonstrates clonally rearranged immunoglobulin genes. Most cases show mutated immunoglobulin heavy chain genes with a bias toward VH3 and VH4 families, although occasional cases are mutated.^{65,68,71–73} Cases that arise in the setting of HCV infection preferentially

use the VH1-69 gene segment, whereas HCV-negative cases have a bias toward VH3-34 segment usage.⁶³ Nodal MZL cases lack the translocations that are associated with MALT lymphomas. Very little is known about the molecular events that lead to histologic progression. Because nodal MZL is rare, there are relatively few cytogenetic or genetic studies. Conventional cytogenetic studies show no characteristic cytogenetic abnormalities, although trisomies of chromosomes 3, 7, 12, and 18, deletions 6q, and rearrangements in chromosome 1 have been reported.^{66,74} Studies using array comparative genomic hybridization (array CGH) have identified additional abnormalities, including deletions in 1p36, 6q23, and 19q13.2, and gains in 6p.⁷⁵ In general, the translocations identified in extranodal marginal zone lymphomas are not found.

Pediatric Nodal Marginal Zone Lymphoma

The vast majority of children with nodal marginal zone lymphoma are boys (male:female=20:1) who present with asymptomatic lymphadenopathy confined to the head and neck.^{59,76} The morphologic features are similar to those seen in adults, except that the neoplastic proliferation is usually accompanied by marked progressive transformation of germinal centers. The immunophenotype is also similar to that seen in adults. Because of the pronounced progressive transformation of germinal centers, the differential diagnosis includes reactive processes. However, molecular diagnostic studies for immunoglobulin heavy chain gene rearrangement demonstrate that the B-cell proliferation is clonal. The prognosis is excellent, with a low relapse rate and long survival with conservative therapy.

Splenic B-Cell Marginal Zone Lymphoma

Definition

In the WHO classification, splenic B-cell marginal zone lymphoma is composed of small B lymphocytes that surround and replace the splenic white pulp germinal centers, efface the follicle mantle, and merge with a peripheral (marginal) zone of larger cells that includes scattered transformed blasts.⁷⁷ Both the both small and larger cell components infiltrate the red pulp. In the past, splenic marginal zone lymphoma has been referred to as splenic lymphoma with villous lymphocytes. The postulated normal counterpart is unknown.

Clinical Features

Splenic marginal zone lymphoma is an uncommon neoplasm, comprising less than 2% of all lymphoid neoplasms.¹⁶ It affects predominantly adults over the age of 50, with no sex predilection. Patients typically present with splenomegaly.⁶⁰ The splenic hilar lymph nodes and bone marrow are often involved, but peripheral lymph node involvement and

extranodal tissue involvement are uncommon. When the peripheral blood is involved, the neoplastic cells have the appearance of villous lymphocytes. About one-third of patients have a small paraprotein, and they may also have autoimmune thrombocytopenia or anemia.⁷⁸ In southern Europe, this lymphoma has been associated with HCV virus infection, similar to nodal MZL.⁶⁴

Prognosis and Predictive Factors

The clinical course in indolent, even in patients with bone marrow involvement, with an overall median survival of about 10 years.⁷⁸ Although patients generally respond poorly to chemotherapeutic regimens used to treat other chronic B-cell lymphoproliferative processes, many patients achieve hematologic responses and survive for many years following splenectomy. Patients who are HCV-positive may respond to antiviral therapy.⁷⁹ The presence of a large tumor mass, poor performance status, unmutated somatic mutation status of the immunoglobulin heavy chain variable region genes, the presence of deletions in the long arm of chromosome 7, and mutations in *TP53* have been associated with a poor prognosis.^{62,68,78} High serum $\beta 2$ microglobulin concentrations, development of lymphadenopathy, involvement of nonhematopoietic sites, peripheral blood leukocytosis, and peripheral blood lymphocytosis have also been associated with shorter overall survival and progression free survival.^{68,78} Gene expression profiling studies using cDNA microarrays have demonstrated shorter survival in cases that express a subset of NF- κ B pathway genes, including TRAF5, REL and PKC- α .⁶⁸ In aCGH studies, increased chromosome loss was associated with shorter survival.

Despite its indolent clinical course and response to splenectomy, the incidence of progression of splenic MZL to DLBCL is around 10–15%.^{67,68} This incidence is similar to that seen in CLL/SLL, but lower than that seen with mantle cell lymphoma or follicular lymphoma.⁶⁸ Features associated with progression to DLBCL include peripheral lymph node involvement at diagnosis, a high tumor growth fraction, and the presence of 7q deletion.⁶⁸ Further, the site of progression influences the response to treatment and overall survival.⁸⁰ Progression within the bone marrow is often refractory to therapy and associated with poor outcome; whereas, progression within lymph node tends to respond well to chemotherapy, with durable progression-free and overall survival.

Morphology and Immunophenotype

In the spleen, the neoplastic cells infiltrate both the red and white pulp.^{77,78} In the white pulp, the small neoplastic lymphoid cells surround and replace reactive germinal centers, and efface the normal mantle zones. The mantle zone merges with a peripheral zone composed predominantly of small to medium-sized cells with more dispersed chromatin and abundant

pale cytoplasm, which resemble marginal zone cells, with scattered occasional transformed blasts. The neoplastic cells may show plasmacytic differentiation. The neoplastic cells infiltrate the red pulp in a nodular to diffuse pattern and may invade the sinusoids. The sinuses of the splenic hilar lymph nodes are typically dilated. The neoplastic cells surround and replace germinal centers, but a marginal zone pattern is usually not apparent. The neoplastic cells infiltrate the bone marrow in a nodular and interstitial pattern, and may occasionally surround reactive germinal centers. Immunohistochemical staining with antibody to markers of B-cell differentiation accentuates the presence of the lymphoma cells within sinusoids. When the peripheral blood is involved, the neoplastic cells have the appearance of villous lymphocytes. These cells are small to medium-sized with condensed chromatin, moderately abundant pale cytoplasm, and short polar villi. They may also appear plasmacytoid.

The neoplastic cells express pan-B-cell markers, surface IgM, and usually surface IgD; they are negative for CD5, CD10, CD23, CD43, annexin A1, and cyclin D1, and are usually negative for CD103.^{77,78} Staining for Ki67 shows a distinctive targetoid pattern because of an increased growth fraction in the residual germinal centers and marginal zones.⁷⁷

Differential Diagnosis

The differential diagnosis includes splenic involvement by other B-cell lymphomas composed of predominantly small lymphoid cells, such as CLL/SLL, FL, and MCL. Features that help to distinguish splenic MZL from these other entities include the clinical presentation of splenomegaly in the absence of peripheral lymphadenopathy, expression of monotypic surface IgD, and lack of expression of CD5, CD10, CD43, and cyclinD1. Because they share the clinical features of splenomegaly with bone marrow and peripheral blood involvement, the differential diagnosis of splenic MZL also includes hairy cell leukemia (HCL) and hairy cell leukemia variant (HCL-v).⁷⁸ However, patients with HCL generally have peripheral cytopenias and a characteristic monocytopenia. Further, each entity has characteristic morphologic and immunophenotypic features that allow them to be distinguished.

Cytogenetic and Molecular Genetic Features

Splenic MZL demonstrates clonally rearranged immunoglobulin genes. The heavy chain genes may be either mutated or unmutated, and show a bias toward VH1-2 family usage. This bias suggests that cases may arise in the setting of chronic antigen stimulation and B-cell proliferation.⁸¹ However, other than the approximately 20% of cases that are associated with HCV infection, an inciting agent has not been identified in most cases.

About 70–80% of splenic MZL cases have an abnormal, usually complex, karyotype on conventional cytogenetic

TABLE 17.2. Anatomic distribution, immunophenotypic features, cytogenetic, and molecular genetic features of MZL lymphomas.

Lymphoma sub-type	Sites of involvement	Bone marrow involvement	Immunophenotype	Cytogenetic features	Molecular features
MALT	Gastrointestinal tract, salivary glands, lung, head and neck, ocular adnexae, skin, thyroid, breast	2–20%, more common in lung/ocular adnexal MALT; lowest incidence in gastric MALT	CD19+, CD79a+, CD20+, CD22+, CD5–, CD10–, CD23, CD43+/-, CD11c+/-, IgM+, light chain class restriction	t(11;18)(q21;q21), t(1;14)(p22;q32), t(14;18)(q32;q21), t(3;14)(p14;q32), +3, +18	Clonal IGH and IGL rearrangements, SHM
Nodal MZL	Lymph nodes	Rare	As above	+3, +18, +7, 6q21–25 deletion	Clonal IGH rearrangements, SHM with VH3 and VH4 family predominance
Splenic MZL	Spleen, splenic hilar lymph nodes, peripheral blood	Typically present	Similar to MALT and NMZL except CD43– and IgD+	7q21–36 deletion, +3	Clonal IGH and IGL rearrangements, SHM with VH1-2 family predominance in 50% of cases

IGH immunoglobulin heavy chain; IGL immunoglobulin light chain; SHM somatic hypermutation.

analysis, most often involving chromosomes 1, 3, 6, 7, 8, 12, and 14.^{68,78,82} The most frequent abnormality, detected by both conventional and molecular cytogenetic analyses, is deletion in the long arm of chromosome 7 at bands q21–36, which is seen in up to 50% of cases. The consensus deleted region is 7q32–7q36, with the highest incidence of loss found in band 7q32. Deletion 7q, which is relatively specific for splenic MZL, has been associated with a more aggressive clinical course. Other common abnormalities are gains of 12q (15–20%) and 3q (20–30%), and trisomies of chromosomes 7 and 18. Trisomy 3 is less common in splenic MZL than other subtypes of MZL. A recent analysis of 23 cases of splenic MZL with complex karyotypes using spectral karyotyping (SKY) demonstrated a much higher incidence of translocations involving the immunoglobulin heavy chain locus on chromosome 14q32 (21%) than previously reported, as well as translocations involving chromosomes 3, 6, 8, 9, and 12.⁸³ Recent aCGH experiments have identified frequent chromosomal imbalances including gains of 3q, 4q, 5q, 9q, 12q, 20q, and loss of 6q, 7q, 14q and 17p.^{68,75,84–86} Potential genes within the deleted regions that have been implicated in the pathogenesis of splenic MZL include *CAVI*, *CAV2*, *GNG11*, and *POT1* located in the 7q31–7q32 region and *SHH* located in 7q36 region.^{68,82} Genes within the 3q23–q29 region have also been identified that may play a role in its pathogenesis.⁷⁸ The translocations associated with extranodal MALT lymphoma are not seen.

The anatomic distribution, immunophenotypic features, cytogenetic, and molecular genetic features of the MZL lymphomas are summarized in Table 17.2.

Gene expression profiling studies have also identified potential diagnostic markers and pathogenic pathways in splenic MZL.⁸⁶ The molecular signature of SMZL includes upregulation of genes involved in apoptosis, B-cell receptor activation and NF- κ B activation, such as *SYK*, *BTk*, *BIRC3*, *TRAF3*, *TRAF5*, *CD40*, and *LTB*. Other genes of interest include *TCL1*, whose increased expression has been linked

with the upregulation of genes associated with intracellular signaling through the AKT1 pathway.

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Lymphoplasmacytic Lymphoma

Pei Lin

Introduction

Lymphoplasmacytic lymphoma (LPL), by definition, is a low-grade B cell lymphoma, which displays variable degrees of plasmacytic differentiation and is associated with a serum monoclonal protein, usually of IgM type, but may occasionally be of IgA or IgG type. The term LPL has been used interchangeably with Waldenstrom macroglobulinemia (WM), and yet a variety of low-grade B cell lymphomas, such as small lymphocytic lymphoma/chronic lymphocytic leukemia (CLL) and marginal zone B cell lymphoma (MZL), may also present with IgM macroglobulinemia. The current recommendation is that when the term WM is used clinically to designate a disease entity, it should be applied exclusively to patients with an underlying LPL.¹

Unlike other low-grade B cell lymphomas, such as follicular lymphoma or CLL, in which a distinct cytogenetic aberration or a characteristic immunophenotypic profile allows for precise classification, LPL/WM lacks a defining molecular genetic hallmark that readily distinguishes itself from other lymphomas, in particular MZL. The classical description of WM patients by Jan Waldenström in 1944² is that of hyperviscosity and associated complications related to high levels of IgM macroglobulinemia. Many LPL/WM patients nowadays are diagnosed early during their disease course with serum IgM levels below 3 g/dL and lacking a full spectrum of the classical syndrome. No specific serum IgM cutoff reliably separates LPL/WM from other B-cell lymphomas.³ The confirmation of LPL/WM primarily remains by excluding alternative diagnoses with all available data.

The major challenge lies in distinguishing LPL and MZL, as they share overlapping morphologic and immunophenotypic features. Even among experts,⁴ a diagnosis of LPL may be made on histological ground alone in slightly over 50% of cases. Additional immunophenotypic and clinical data only improves diagnostic accuracy to 66%. The limited diagnostic power by conventional tools in LPL is recapitulated in the diagnosis of nodal MZL, underscoring the lack of clear-cut distinction between the two (Table 18.1). Other confounding

factors are multiple terms applied historically to designate LPL in different classification schemes (Table 18.2). Synonyms listed for LPL include small lymphocytic lymphoma, plasmacytoid (Working Formulation), immunocytoma (Kiel classification), and lymphoplasmacytoid lymphoma (REAL classification). The category of small lymphocytic lymphoma, plasmacytoid, is a rather broad terminology, which could be conceivably applied to tumors otherwise considered MZL or other low-grade lymphomas with plasmacytoid differentiation.

Given these dilemmas, we choose to adopt the concept proposed by the International WM Workshop for the purpose of this chapter.¹ LPL is defined as a low-grade lymphoma that primarily involves bone marrow and is invariably associated with serum monoclonal IgM of any levels. Extramedullary spread to lymph node, spleen, and skin occurs as the disease progresses.⁵ The characteristic immunophenotype of LPL/WM is CD5– CD10– CD19+ CD20+ CD22+ CD23– CD25+ CD27+ FMC7+ CD103– and CD138–, although CD5 and/or CD23 expression may be observed.⁶ LPL with monoclonal IgA or IgG protein, LPL without a serum monoclonal protein, and LPL involving primarily the lymph nodes, as described in the newly revised World Health Organization (WHO) classification,⁷ are all possible variants of LPL; however, their distinction from MZL requires further studies. We believe that the best approach to defining the molecular signature of LPL should start with the prototype of LPL/WM, followed by comparing it with variants.

Another issue encountered in LPL studies is that many of the published data in the literature are based on cases classified as WM, as this term has been much more widely used in the clinical settings than LPL. We have included data that are compiled on such cases in this chapter. Although this approach could potentially include cases of IgM-producing splenic MZL, a disease that is sometimes indistinguishable from LPL clinically when a serum IgM protein is also present, we believe that this inclusion is inevitable before we can more precisely dissect LPL from MZL.

TABLE 18.1. Reproducibility of LPL and nodal MZL classified according to availability of histologic, immunophenotypic, and clinical data.

Consensus diagnosis	H	H+I	H+I+C
Nodal MZL	55%	63%	63%
LPL/WM	53%	56%	56%

H histologic findings, I immunophenotype, C clinical data.

TABLE 18.2. The terms used to designate lymphomas associated with WM in different classification systems.

Classifications	Terminology
Rappaport	Well-differentiated lymphocytic, plasmacytoid
Lukes-Collins	Plasmacytic-lymphocytic
Kiel	Immunocytoma, lymphoplasmacytic type
Working Formulation	Small lymphocytic, plasmacytoid
REAL	Lymphoplasmacytoid lymphoma (immunocytoma)
WHO	Lymphoplasmacytic lymphoma/Waldenstrom macroglobulinemia

Epidemiology

LPL/WM has an overall incidence of approximately three per million persons per year and approximately 1,500 new cases are diagnosed per year in the USA. It is more common in males than females (2:1) and Caucasians, and the median age at onset is 65 years old. Though a predominantly sporadic disease, WM shows familial clustering, suggesting a genetic predisposition. Patients with a familial history of WM have an earlier onset of disease with more tumor burden compared to the sporadic cases. The first degree relatives of 18.7% of LPL/WM patients have a B-cell neoplasm, including LPL/WM, monoclonal gammopathy of undetermined significance (MGUS), CLL, to other non-Hodgkin lymphoma (NHL).^{8–10} The risk for PCM or classical Hodgkin lymphoma is not similarly increased.

MGUS is considered the major risk factor for the development of WM, conferring a 46× higher relative risk than the general population.¹¹ A possible association with hepatitis C virus or autoimmune disorders have been proposed.⁹

Cell of Origin

LPL/WM is believed to arise from postgerminal center B-cells (IgM⁺ or IgM⁺IgD⁺) with hypermutated IGH variable (V_H) region genes.^{12–17} Mutations of switch regions (essential for isotype switching) are also reported to be usually absent. Isotype switching does not occur even when the tumor cells are stimulated in vitro, suggesting that the neoplastic cells are constitutively unable to or being prevented from carrying out isotype switching.^{15,16} The neoplastic clone preferentially uses the V_H3/J_H4 family in >90% of cases.¹⁸ Although this finding suggests antigenic selection, one study analyzing the mutation pattern

of the framework/complementary determine region (FR/CDR) suggests the contrary in a subset of cases.¹⁷ The clonal B-cells retain the ability to differentiate to plasma cells but secrete IgM.

Another phenomenon described in LPL/WM is that the tumor may harbor more than one clone, with one germline and the other somatically mutated, or both mutated – but with different patterns of somatic mutations, despite originating from a common IgM progenitor.¹⁹ Rare cases with sequential development of IgM and IgG monoclonal protein (that are derived from a common precursor clone based on analysis of CDR3 and somatic mutation pattern) have been reported and seem to dispute the conventional concept that isotype switching is consistently absent in LPL/WM.²⁰

Only a small subset of the clonal B-cells in LPL/WM expresses activation-induced cytosine deaminase (AID), an enzyme that is expressed by germinal center B-cells and essential for somatic hypermutation and isotype switching. ZAP-70, a marker indicative of unmutated status of the neoplastic cells in SLL/CLL, is usually not expressed in LPL/WM.²¹ Data regarding CD27 expression, a marker of memory B-cells, in LPL/WM are controversial.^{15,22}

Cytogenetics

Deletion of 6q is the most common finding in LPL/WM,²³ with 6q deletion being observed in 42% of patients by interphase fluorescence in situ hybridization (FISH).²⁴ However, deletion of 6q is a nonspecific finding, also seen in other types of lymphomas. Patients with deletion of 6q, deletion of 17p13, deletion of 13q14, or a complex karyotype have been shown to have a more aggressive disease and shorter survival.^{23,25,26} High resolution array-based comparative genomic hybridization (aCGH) has demonstrated chromosomal aberrations in 83% of cases of WM and each case usually carries a median of 3 aberrations. Gain of 6p, which usually is concurrent with 6q deletion, is the second most common aberrations after 6q deletion, in 17% of cases. Other aberrations such as whole or partial gain of chromosome 3 and 18, reported previously in MZL, are also observed in WM. On the other hand, gains of 4 and 8q are more specific for WM. Trisomy 12, common in CLL is not observed usually in WM. However, interstitial deletion of 13q14, spanning a region that overlaps with that in CLL, is reported in 10% of WM cases.²⁷

Most LPL/WM cases do not carry translocations of immunoglobulin heavy chain (IGH) gene.^{28,29} The t(9;14)(p13;q32), originally considered to be present in nearly half of cases of LPL, is neither common nor specific for LPL/WM. This translocation juxtaposes the PAX5 gene encoding for B-cell-specific activator protein (BSAP), an essential B-cell transcription factor, with the joining region of IGH resulting in deregulated expression of BSAP. Studies by karyotyping and FISH, using probes specific for the candidate genes, have failed to confirm the prevalence of t(9;14)(p13;q32) in LPL/WM.^{23,30} B-cell neoplasms that were found to harbor t(9;14) are tumors that were classified under a variety of categories, including diffuse large B cell lymphoma, MZL, FL, and PCM.^{31–34}

Four minimal deleted regions (MDR-1 to MDR-4) on 6q have been identified and MDR2 and MDR3 are most common. Candidate genes in these regions include ATM1, PRDM1 and TNFAIP3. TNFAIP3 is a negative regulators of nuclear factor-KB (NF-KB) signalling pathway.³⁵

Studies of high risk families by genome-wide linkage screening have found the evidence of linkage on chromosomes 1q and 4q.³⁶

Molecular Genetics

The advent of genomic technologies has allowed the global investigation of genes altered in LPL/WM. DNA oligonucleotide microarrays have revealed that LPL/WM is more similar to CLL than PCM. For example, *CYCLIN D3* is overexpressed in both CLL and LPL/WM. Only a small set of genes are uniquely overexpressed in LPL/WM, and these include *IL-6* and its related mitogen-activated protein kinase (MAPK) pathway,^{37,38} as well as *CD1c*. In another study, the investigators found that *IL4R* and *BACH2* – a gene regulating class switching – are down regulated in B-cells of the LPL samples. A set of four genes is differentially expressed between the neoplastic B-cells of LPL/WM and CLL: *LEF1* (WNT/ β (beta)-catenin pathway), *MARCKS*, *ATXN1*, and *FMOD*. Compared to the PCM cells, the plasma cells from WM overexpress *PAX5* and its three target genes (i.e., *CD79*, *BLNK*, and *SYK*); whereas, *IRF4* and *BLIMP1*, which are usually upregulated in PCM, remain downregulated, indicating that the B-cells and plasma cells from WM are different from their counterparts in CLL and PCM.^{38,39}

Other cytokines and chemokines upregulated in WM include B-lymphocyte stimulator (BLyS), IL-6, CD40 ligand, BAFF, APRIL, and stromal derived factor (SDF-1).^{38–40} These molecules enhance survival and proliferation of the tumor cells.

The mechanism for LPL/WM to be preferentially localized to bone marrow (BM) vs. lymph node is not entirely clear; however, the tumor cells express adhesion receptors, CXCR4 and VLA-4. Interaction between CXCR4/SDF-1 axis and VLA-4 may regulate migration and adhesion of tumor cells to BM stromal cells.⁴¹ Inhibition of CXCR4 or VLA-4 has been shown to decrease adhesion and increase apoptosis of tumor cells. The role of mast cells in promoting WM proliferation has also been investigated. The mast cells in WM samples express CD40 ligand (CD154), a potent inducer of B-cell expansion, while the tumor cells express CD154. The CD154–CD40 signaling induces pERK phosphorylation of tumor cells and sustains tumor survival.⁴²

Proteinomics

Antibody-based protein microarrays have been used to compare the patterns of protein expression between neoplastic cells in LPL/WM and normal BM controls.⁴³

The Ras family proteins (Rab-4 and p62DOK) and Rho family proteins (CDC42GAP and ROK α) have been shown to be upregulated. Rab4 is a Ras-like small GTPase that is associated with prolonged activation of MAP kinase in some malignancies.⁴⁴ Other upregulated proteins include cyclin-dependent kinases, apoptosis regulators, and histone deacetylases (HDAC). HDACs have been shown to be involved in large B cell lymphoma and PCM.⁴⁵ Similar patterns of expression have been observed between symptomatic and asymptomatic LPL/WM patient samples suggesting that the dysregulation of signaling pathways is an early event. Three proteins that are differentially expressed in symptomatic vs. asymptomatic cases include the heat shock protein HSP90, the Ras family protein CDC25C, and the chemotaxis protein p43/EMAPII.⁴³

Activated Signaling Pathways

The NF-KB pathway, Akt/PI3K/mTOR and extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathways are activated in LPL/WM.^{35,46} Akt downregulation by Akt knockdown leads to significant inhibition of proliferation and induction of apoptosis in LPL/WM cells in vitro and in vivo. Akt pathway downregulation also inhibits migration and adhesion, as well as homing of tumor cells to the BM.

Studies on pathways activated in LPL/WM provide a framework for targeted therapy. Novel agents, including the proteasome inhibitor (bortezomib), Akt/mTor inhibitors (perifosine and Rad001) and immunomodulatory agents (thalidomide and lenalidomide) have been used to treat refractory disease.⁴⁷ These agents target pathways key to the tumor survival.^{43,46,48} For example, the proteasome inhibitors act through inhibition of the canonical and non-canonical NF- κ (kappa)B pathways by inhibiting nuclear translocation of p65NF- κ (kappa)B. This effect appears to be mediated through a combined reduction of the PI3K/Akt and ERK signaling pathways.⁴⁹ The inhibition leads to increased apoptosis and decreased drug resistance conferred by the mesenchymal cells or IL-6.⁴⁹ Similarly, the cytotoxic effect of simvastatin in LPL/WM is mediated through inhibition of Akt and extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK), as well as an increased activity of stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), leading to increased tumor cell apoptosis.⁵⁰ Enzastaurin, a Protein Kinase C β (beta) inhibitor, blocks PKC β (beta) activity and induces a significant decrease of proliferation. Enzastaurin also inhibits Akt phosphorylation and Akt kinase activity.⁵¹

Differential Diagnosis

Lymphadenopathy occurs in 15–20% of LPL/WM patients at presentation, and usually is not as prominent as is seen in patients with other types of non-Hodgkin lymphoma.

Splenomegaly occurs in 10–20% of patients. When extramedullary disease occurs, other B-cell lymphomas are considered in the differential diagnosis.

Distinguishing LPL from SLL/CLL, mantle cell lymphoma, FL, and PCM is usually based on a combination of morphological, immunophenotypic, and cytogenetic studies. These are usually not difficult to achieve. For example, in CLL, del(13)(q14) is detectable in approximately 50% of cases with del(11q) and trisomy 12 in a smaller subset of cases. The t(11;14)(q13;q32) and cyclin D1 overexpression support the diagnosis of MCL. Most cases of FL are positive for CD10 and BCL-6; CD10 expression is rare in LPL/WM. Detection of the t(14;18)(q32;q21) (or *BCL-2* gene rearrangement) supports a diagnosis of FL.

IgM-secreting PCM or the small cell variant of PCM (that frequently expresses CD20) may resemble LPL/WM. Lytic bone lesions and renal failure are common in PCM and rare in LPL/WM. The small lymphocytes in LPL are typically CD19+CD138–; the plasmacytoid lymphocytes in LPL are usually CD19+CD138+; the clonal plasma cells in LPL are typically CD19–CD138+; and PCM cells are usually uniformly CD19–CD138+. Deletion of 13q and translocations involving the IGH locus 14q32 are common in PCM, but are distinctly rare in LPL/WM.^{52,53} Cyclin D1 is overexpressed in 30–40% of PCM (particularly in the small cell variant of PCM) and may aid in the differential diagnosis.

LPL/WM disseminated to skin, lung, and gastrointestinal tract may resemble extranodal MZL (MALT lymphoma).⁵ A small subset of patients with MALT lymphoma may also have a serum IgM paraprotein, and the serum level may be >3 g/dL in rare patients.⁵⁴ At the molecular level, translocations characteristic of MALT-lymphoma include the t(11;18)(q21;q21) involving *API2* and *MALT1*, the t(14;18)(q32;q21) involving *IGH-MALT1*, and t(1;14)(p22;32) involving *BCL10-IGH*. These translocations have been detected in 13.5%, 10.8%, and 1.6% of MALT-lymphomas, respectively, and have not yet been reported in cases of LPL/WM. The t(3;14)(p14.1;q32) which involves *FOXP1* and *IGH* and occurs in a subset of orbital and thyroid MALT lymphoma has not been described in LPL/WM either.^{55,56}

Ectopic nuclear BCL-10 expression is described to be present in most cases of MALT-lymphoma with the t(11;18)(q21;q21). However, MALT-lymphoma without recurrent translocations may also demonstrate a nuclear staining pattern.⁵⁷ BCL-10 expression in cases of MALT-lymphoma with the t(14;18)(q32;q21) and t(1;14)(p22;32) are less well studied. While some studies show that the pattern of BCL-10 expression in LPL/WM is usually negative or weak with nuclear or cytoplasmic staining,^{55,58} a study of a large series of LPL/WM found that BCL-10 nuclear staining correlates with advanced diseases in LPL/WM.⁵⁹

The distinction between LPL/WM and IgM-secreting splenic MZL may be particularly difficult.⁶⁰ Bone marrow involvement may occur in SMZL, and monoclonal serum IgM is present in up to 45% of patients.⁶¹ In most patients with splenic MZL,

fortunately, the M protein levels generally do not exceed 3 g/dL. Complex chromosomal aberrations are common (80% of cases) in splenic MZL and usually involve chromosomes 1, 3, 6, 7, 8, 12, and 14. The most frequent cytogenetic aberrations are deletion of 7q22–36, mostly at band 7q32–7q35 (30–40%), followed by gains of 3q29–q32 (20–30% of cases) and 12q (15–20% of cases).^{62–64} Prevalence of chromosome 3 has not yet similarly demonstrated in LPL/WM, and other aberrations have not been extensively tested. SMZL have been divided into two subsets based on the mutation status of *IgVH* gene: one mutated and the other unmutated, presumably simulating the normal cellular composition of the splenic marginal zone.^{65,66} It has been proposed that those with a mutated IGH and associated IgM comprise a subset of LPL/WM recognized clinically.⁶¹ A more recent study comparing the *IGVH* somatic mutation pattern and *CDR3* length between SMZL and LPL/WM appear to support that most SMZL have a pattern consistent with selection by autoantigens outside GC, while LPL tumor cells are GC-experienced memory B-cells.⁶⁷

Although nodal MZL is (by definition) primarily a lymph node-based disease, it may spread to BM at a frequency reported to be 45%.⁶¹ The presence of monocytoid B-cells is an unreliable marker for MZL, and may also be seen in LPL/WM.⁶⁸ Berger et al.⁶⁴ reported that 8% of patients with nodal MZL carry a serum IgM M protein. A study of nodal-based LPL using fluorescence immunophenotypic and interphase cytogenetics (FICTION) has failed to demonstrate those abnormalities prevalent in BM-based LPL/WM. In this study, CD79a antibody was used to identify B-cells along with multiple probes designed to detect trisomies of chromosome 3, 12, and, 18; rearrangements of IGH, BCL6, PAX5, and MALT1; and deletion of 6q21.⁶⁹ One of the major hurdles in studying nodal-based LPL is how to select indisputable cases of LPL, given that many LPL and MZL are indistinguishable and how to deal with cases associated with IgA or IgG monoclonal protein.

Some investigators have proposed that MZL, nodal or splenic, and LPL should all be considered part of a spectrum of one disease.^{52,70} This approach may be practical and convenient for the clinical management of patients given that gain of chromosome 3 and 18 have been demonstrated in both LPL and MZL, but more studies are needed to further elucidate their relationship at the molecular level.

Large cell transformation occurs in LPL/WM, similar to Richter transformation in CLL/SLL, and the high-grade tumor may or may not arise from the same clone.^{35,71,72} Transformation may occur subsequent to (or concurrent with) the low-grade LPL and as a result of new genetic events acquired by the existing clone [or de novo with unrelated B-cells infected with Epstein–Barr virus (EBV)].⁷³ The onset of large cell transformation is usually accompanied by new onset of (or increasing) lymphadenopathy, organomegaly, cytopenias, and rarely hypercalcemia. Serum IgM levels may paradoxically decrease at the time of transformation, as a result of dedifferentiation of the neoplastic cells.³⁵ Classical Hodgkin lymphoma may also occur in LPL/WM patients, probably related to EBV as well.⁷⁴

Conclusions

LPL is a low grade B-cell lymphoma typically associated with WM. The lymphoma cells arise from antigen exposed B cells. The most common cytogenetic aberration found in LPL is 6q deletion, a nonspecific finding notable in many other lymphomas. The t(9;14) is uncommon in LPL. More studies are needed to better define LPL and its distinction from MZL at the molecular genetic level. Clearly, the relationship between LPL and nodal MZL or a subset of splenic MZL needs to be further explored.

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Molecular Pathology of Plasma Cell Neoplasms

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Introduction

The plasma cell neoplasms are a heterogeneous category of disorders that are defined by a combination of clinical, pathologic, and radiologic criteria and range from the very indolent (monoclonal gammopathy of undetermined significance (MGUS)) to clinically aggressive, overt malignancies (such as plasma cell leukemia).¹ The majority of the molecular pathology literature in plasma cell neoplasms has focused on plasma cell myeloma (PCM).^{2–6} However, the molecular abnormalities identified in PCM are not unique to this disorder, and may also be found in other plasma cell neoplasms, such as plasma cell leukemia or solitary plasmacytomas. For this reason, molecular studies do not assist in the classification of plasma cell neoplasms. Once a diagnosis of PCM is ascertained, however, molecular studies may be very helpful in assessing a patient's prognosis. Several molecular abnormalities have been shown to be of prognostic significance in patients treated with standard chemotherapy, or with high dose chemotherapy and single or tandem bone marrow (BM) transplants. Through an assessment for the molecular abnormalities described in this chapter, patients with PCM may be divided into those with “high-risk” or “standard-risk” disease, and risk-stratified treatment regimens may therefore be possible.^{7,8} It must be kept in mind, however, that therapeutic regimens for patients with PCM continue to evolve, with the introduction of immunomodulatory agents, such as thalidomide and its derivatives and other novel agents such as bortezomib. Whether the molecular abnormalities described below maintain their prognostic significance in the face of these new therapeutic options remains to be determined.

Techniques for the Assessment of Molecular Abnormalities in Plasma Cell Neoplasms

Several techniques are commonly employed to detect molecular abnormalities in PCM (Figure 19.1). Each of these techniques is associated with specific advantages

and disadvantages, and an optimal approach to the routine evaluation of PCM requires a combination of these methods.

Metaphase Cytogenetic Studies

Metaphase cytogenetic studies are routinely performed on BM biopsies at many institutions, including cases performed for evaluation of plasma cell neoplasms. Although chromosomal abnormalities are present in nearly all cases of PCM, standard metaphase cytogenetic analysis identifies abnormal karyotypes in only 30–40% of cases.^{4,9} The relatively low yield of standard cytogenetic studies is thought to reflect the poor in vitro growth of the malignant plasma cells. Despite this low yield, metaphase cytogenetic studies may provide useful prognostic information in PCM. Overall, the presence of any abnormal karyotype is associated with an adverse prognosis compared to cases that yield apparently normal karyotypes. The identification of an abnormal karyotype may thus serve as a surrogate marker for the proliferative rate of the neoplastic plasma cells, another known adverse prognostic indicator. The major advantage of metaphase cytogenetic studies is that they allow for an assessment of the global karyotype, rather than simply the presence or absence of specific abnormalities [as is the case with fluorescent in situ hybridization (FISH) studies]. The presence of a hyperdiploid karyotype is generally associated with a favorable prognosis, while a hypodiploid karyotype is associated with an adverse prognosis.^{4,10,11}

Fluorescence In Situ Hybridization Analysis

In contrast to the low yield of metaphase cytogenetic analysis, FISH studies have shown that cytogenetic abnormalities are essentially universal in PCM.^{4,12} Therefore, FISH studies have therefore become the gold standard for molecular cytogenetic analysis of plasma cell neoplasms. FISH studies in PCM, however, are also associated with several practical limitations. Firstly, FISH studies provide information only regarding the specific abnormality being studied. FISH studies provide no information in regards to the overall global

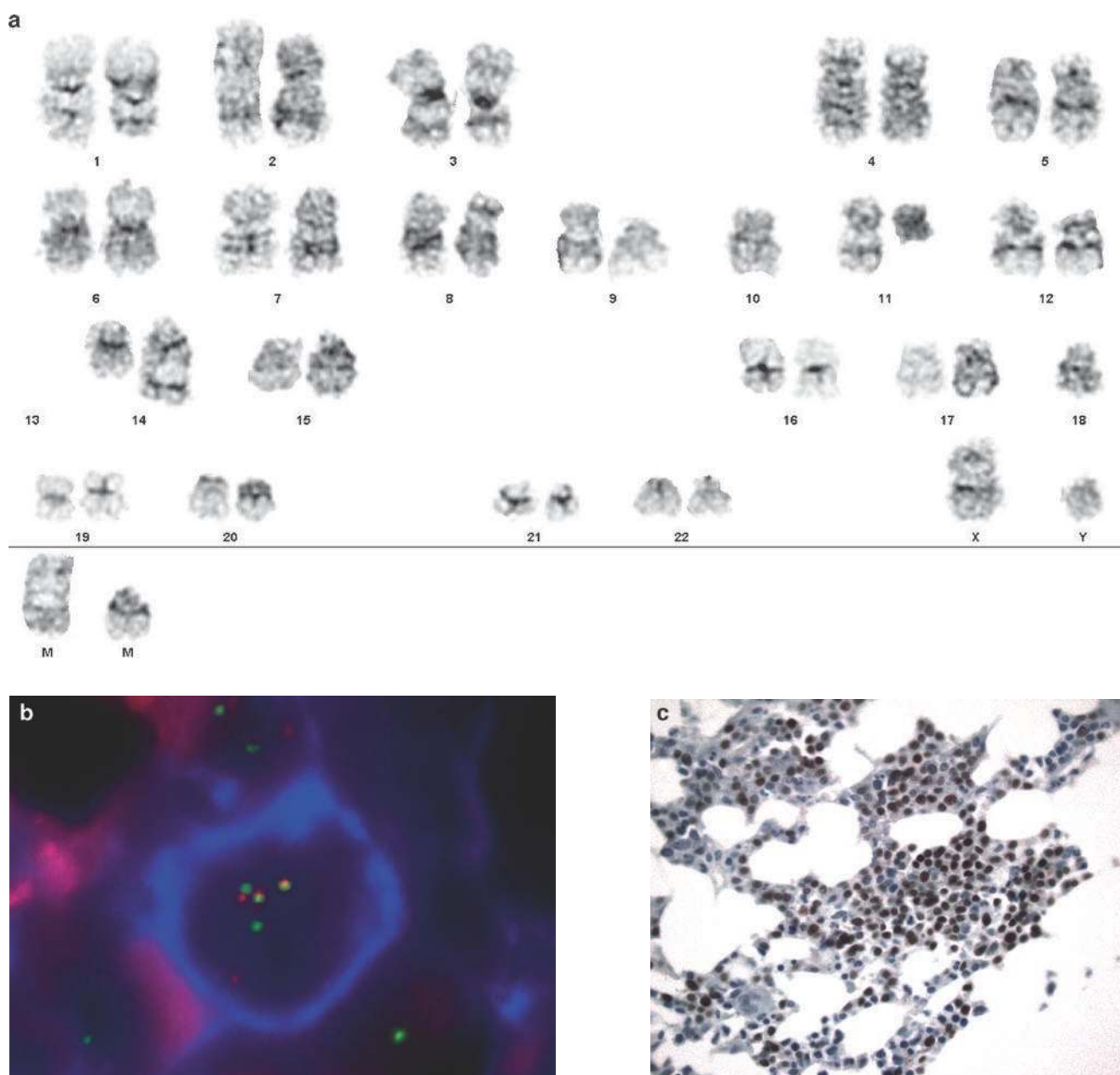


FIG. 19.1. Methods of detection of clinically significant molecular abnormalities in plasma cell myeloma. (a) Metaphase cytogenetics – this metaphase preparation displays t(11;14)(q13;q32) and loss of both chromosomes 13. The complete karyotype for this case was as follows: 45,XY,-10,t(11;14)(q13;q32)[3]/43,idem,del(1)(q32),der(3)add(3)(p25)inv(3)(p21q21),add(8)(p21),del(9)(p13),-13,-13,-18,-22,+mar1,+mar2[6]/46,XY[11]. (b) FISH – Plasma cells are

identified by blue cytoplasmic fluorescent staining for CD138. FISH using a probe for *IGH* and *MMSET* identifies separate red and green signals on the uninvolved chromosomes 4 and 14, respectively. 3 sets of red/green fusions are present, consistent with an *IGH/MMSET* translocation and gain of one of the derivative chromosomes. (c) Immunohistochemistry – plasma cells show positive nuclear staining for p53 protein.

karyotype, or for other secondary abnormalities not covered by the specific probes employed. For this reason, FISH studies should be employed as a supplement to, and not a replacement for, metaphase cytogenetics. Secondly, FISH studies of plasma cells may be challenging due to the variable numbers of plasma cells that may be found in a BM aspirate. When plasma cells are found in relatively low numbers, FISH analysis of an unsorted BM aspirate preparation may be impeded by

the large number of benign admixed BM elements that may mask the presence of a neoplastic clone. Many groups have therefore developed special procedures for FISH analysis of plasma cells, including purification of plasma cells using anti-CD138 antibodies prior to analysis,^{13–15} or FISH performed using simultaneous immunofluorescence for CD138¹⁶ or cytoplasmic immunoglobulin light chains^{3,17} to allow for specific scoring of only plasma cell nuclei.

Immunohistochemistry

Several cytogenetic abnormalities in PCM, such as the t(4;14) or p53 abnormalities discussed below, are associated with phenotypic abnormalities that may be detected by immunohistochemistry (IHC). Although immunostaining serves only as a surrogate marker for the presence of specific cytogenetic abnormalities, IHC has the important advantage of being available in most routine pathology laboratories.

Array-Based Genotyping

Several groups of investigators have performed gene expression profiling microarray studies of PCM.^{6,18,19} These studies have confirmed the presence of biologically distinct subtypes of PCM associated with specific molecular abnormalities, such as the immunoglobulin heavy chain translocations described below. These studies have also demonstrated that abnormalities of either Cyclin D1, Cyclin D2, or Cyclin D3 are nearly universal in PCM, providing important clues to the molecular pathogenesis of this malignancy. While this approach represents an important research tool, array-based studies are not currently employed for routine evaluation of PCM, and these studies will therefore not be further discussed.

Clinically Significant Molecular Abnormalities in Plasma Cell Myeloma

Table 19.1 shows some of the clinically significant abnormalities in PCM.

Chromosome 13 Abnormalities

Monosomy of chromosome 13 or deletions of chromosome 13q represent one of the most frequent abnormalities in PCM, being detectable by FISH in approximately 50% of cases.^{4,12,20} In cases with interstitial 13q deletions, the deleted segment appears to be centered around 13q14. However, in the great majority of cases, chromosome 13 abnormalities consist of monosomy 13.²¹ In cases with chromosome 13 changes detected by FISH, the percent of plasma cell nuclei carrying the deletion is variable from approximately 25%

to >95%, suggesting this abnormality represents a secondary change in the pathogenesis of PCM. Nevertheless, since chromosome 13 abnormalities are also detectable in 25–50% of cases of MGUS, this abnormality appears to occur relatively early in the evolution of plasma cell neoplasms.^{4,12}

In patients treated with either standard chemotherapy or high dose chemotherapy and autologous BM transplantation (ABMT), the presence of a chromosome 13 abnormality is associated with an adverse prognosis.^{22–26} Chromosome 13 abnormalities are highly associated with other molecular abnormalities of adverse significance, such as t(4;14), as discussed further below. The impact of chromosome 13 abnormalities on prognosis is larger when the abnormality is detected by metaphase cytogenetics rather than by FISH, likely reflecting the combined influence of chromosome 13 changes and plasma cell proliferative rate.^{27,28}

Del(17p) and p53 Abnormalities

Somatic point mutations or hemizygous deletions of the tumor suppressor gene p53 (*TP53*), located at locus 17p13, are observed in many malignant neoplasms.²⁹ As detected by FISH analysis, deletions of the 17p13 locus are found in 5–10% of cases of PCM. 17p deletions have been shown to be more common in patients with advanced PCM and in more than 60% of human myeloma cell lines, suggesting that *TP53* mutations and deletions develop as secondary abnormalities during the course of disease progression.^{4,12} In patients treated with conventional chemotherapy or high dose chemotherapy with ABMT, the presence of 17p13 deletions as detected by FISH is associated with short survival.^{3,30–32}

Although most of the data in the literature regarding the clinical and prognostic significance of *TP53* abnormalities comes from studies employing FISH, other techniques may also be of clinical utility. For example, 17p deletions may be detected by metaphase cytogenetics in some patients. Furthermore, deletions of the 17p13 locus may represent a marker for functional abnormalities of the p53 tumor suppressor pathway, rather than being of intrinsic biologic significance. In general, the hemizygous loss of one *TP53* locus is thought to be associated with acquired mutations in the remaining locus that lead to abnormal function. Sequencing based studies have suggested that point mutations in *TP53* are less common than 17p deletions.³³ Furthermore, because *TP53* mutations lead to an abnormally long half-life of the protein, the presence of *TP53* mutations is associated with increased nuclear p53 expression as detected by IHC. The presence of nuclear p53 staining by IHC has been shown to be associated with deletions of 17p13 by FISH, and with poor survival.³⁴ Immunohistochemical studies, which are much more widely available in routine pathology practice than FISH studies, may therefore be a useful technique to examine for p53 abnormalities in PCM. It is currently unclear which of these methodologies (FISH, sequencing, or IHC) provides the most clinically relevant assessment of the p53 pathway in PCM.

TABLE 19.1. Prognostically significant molecular abnormalities in plasma cell myeloma.

Abnormality	Genes involved	Frequency ^a	Prognosis
–13/–13q	Unknown	~50%	Unfavorable
–17p	<i>TP53</i>	10%	Unfavorable
t(11;14)(q13;q32)	<i>CCND1/IGH</i>	15–20%	Neutral to favorable
t(4;14)(p16;q32)	<i>MMSET/IGH</i>	15–20%	Unfavorable
t(14;16)(q32;q23)	<i>CMAF/IGH</i>	5–10%	Unfavorable

^aFrequency defined by FISH analysis.

Immunoglobulin Heavy Chain (14q32) Translocations

Translocations involving the immunoglobulin heavy chain locus (*IGH*) at chromosome 14q32 are identified in approximately 50–60% of cases of PCM, and are associated with non-hyperdiploid karyotypes.^{4,17} The incidence of *IGH* translocations has also been shown to be inversely correlated with patient age.³⁵ Unlike several B-cell non-Hodgkin lymphomas that are associated with specific translocations involving the *IGH* locus and one partner gene (i.e., *IGH/BCL2* in follicular lymphoma), numerous *IGH* translocation partner genes have been described in PCM. The major recurring partner genes include *CCND1* (11q13), *MMSET* (4p16), and *CMAF* (14q62). These recurring translocations may be detected in cases of MGUS, and are therefore thought to arise early in the pathogenesis of PCM.^{4,36} Other *IGH* translocations, however, such as the *IGH/CMYC* translocation, are thought to represent secondary abnormalities acquired during disease progression.³⁷ The major recurring translocations, as discussed further below, are associated with specific pathologic and clinical features.

t(11;14)(q13;q32) *IGH/CCND1*

The t(11;14)(q13;q32) is found in approximately 15–20% of cases of PCM using FISH techniques.^{4,12,38} This translocation is also found in essentially all cases of mantle cell lymphoma (MCL). The precise translocation breakpoints, however, differ between PCM and MCL, consistent with differing molecular pathogenesis (i.e., translocations arising during VDJ recombination for MCL versus during class switching for PCM).^{4,39,40}

Cases of PCM containing the *IGH/CCND1* translocation are associated with distinct clinicopathologic features, including a small, lymphoid morphology and CD20 expression, as also discussed in Chap. 18.^{41–43} In some cases, these findings create a differential diagnosis with B-cell lymphomas with plasmacytic differentiation. Oligosecretory/nonsecretory PCM and IgM PCM are also associated with the presence of the *IGH/CCND1*.^{15,44,45} The influence of *IGH/CCND1* on prognosis has been controversial. In general, studies using conventional chemotherapy or high dose chemotherapy with autologous stem cell transplantation have shown either no effect or a slightly favorable effect on prognosis.^{15,17,32}

Several techniques have been used for the detection of *IGH/CCND1* in routine practice. When informative karyotypes are available, the translocation is typically readily identified by metaphase cytogenetics. Many *IGH/CCND1* positive cases, however, may yield non-informative results by standard karyotyping. Using modern, sensitive antibodies to Cyclin D1 protein, the vast majority of cases containing the *IGH/CCND1* show strong, uniform positivity for Cyclin D1 protein by IHC. However, approximately 30% of PCM also show weak, partial expression of Cyclin D1 in the absence of the t(11;14) through other mechanisms of dysregulation of the *CCND1* locus.⁴³ The detection of Cyclin D1 mRNA by RT-PCR has also been shown to be of prognostic significance; however,

this technique did not discriminate between *IGH/CCND1* and other forms of *CCND1* dysregulation.⁴⁶ In general, FISH studies offer the most sensitive and specific method for detection of this translocation, especially when using simultaneous phenotyping (such as cIg-FISH or CD138 gating) or purification of plasma cells prior to FISH analysis.

t(4;14)(p16;q32) *MMSET/IGH*

The t(4;14)(p16;q32) is found by FISH studies in approximately 15–20% of cases of PCM.^{15,47–49} The translocation leads to the production of an *IGH/MMSET* fusion gene. The normal function of the *MMSET* gene, and the role of the dysregulated *IGH/MMSET* fusion gene in PCM pathogenesis, is largely unknown. In addition, the t(4;14) also leads to dysregulation of the *FGFR3* gene at chromosome 4p16 in about 80% of cases containing this translocation. Dysregulation of the FGFR3 protein is also thought to contribute to the molecular pathogenesis of t(4;14)-positive PCM.^{4,12,47}

In PCM treated with either conventional chemotherapy or high dose chemotherapy with ABMT, the presence of the t(4;14) has been associated with an adverse prognosis.^{3,15,47} The prognosis appears to be poor in cases with the t(4;14), regardless of whether or not *FGFR3* expression is detectable.⁵⁰ This latter observation suggests that the *IGH/MMSET* fusion gene contributes directly to the adverse prognosis in these patients. Cases with the t(4;14) also show a strong association with chromosome 13q abnormalities, that may also contribute to the adverse prognosis.^{4,12,49} PCM cases containing the t(4;14) are also associated with specific clinicopathologic features, including a blastoid morphology, IgA paraproteins, and preferential use of lambda light chains.^{4,12,51} Novel small molecule inhibitors targeting the function of FGFR3 and cytotoxic anti-FGFR3 antibodies are currently in development for the treatment of t(4;14)-positive PCM.^{52–54}

The t(4;14)(p16;q32) is cryptic by routine banded karyotyping. The *IGH/MMSET* fusion transcript can be detected by RT-PCR studies, or interphase FISH studies may be employed.^{4,12} More recently, studies have shown that IHC for FGFR3 protein can also be used to screen for the presence of t(4;14).^{55,56} Because FGFR3 protein expression is found in only 75–80% of cases containing the t(4;14); however, FISH studies remain the gold standard for detection of this abnormality.

t(14;16)(q32;q23) *IGH/CMAF*

A t(14;16)(q32;q23) involving the *IGH* locus and the *CMAF* oncogene is identified in 2–10% of PCM cases by using FISH.^{4,12} Due to the rarity of this translocation, there is limited data regarding the prognosis of cases carrying this abnormality. However, the available findings suggest that the abnormality is associated with an adverse prognosis.³ The *IGH/CMAF* translocation is generally cryptic by metaphase cytogenetics, and is best detected by interphase FISH studies, and dual fusion FISH probes specific for this abnormality are

commercially available. Rare PCM cases contain a variant t(14;20)(q32;q12), involving *IGH* and another member of the *maf* family, *MAFB*.⁵⁷ Although only a small number of cases with this abnormality have been described, it also appears to be associated with short survival.

Other *IGH* Translocations

Several other *IGH* translocation partner genes have also been identified in PCM. Translocations involving *MYC* are frequent in PCM cell lines, but are found in only a small percentage of PCM patient samples.^{58,59} The *IGH/CMYC* translocations are generally found in patients with advanced disease, and are thought to generally represent secondary, acquired abnormalities. A t(6;14)(p21;q32) involving *IGH* (and the cyclin D3 gene (*CCND3*)) is found in <5% of cases. A t(6;14)(p25;q32) involving the *MUM1/IRF4* locus has also been described in several patients.^{4,12,60,61} The clinical significance of *CCND3* and *MUM1/IRF4* translocations has not been well characterized to date.

Other Chromosomal Abnormalities

Abnormalities of chromosome 1 are frequently found in PCM. Duplication or amplification of the *CKS1B* gene on chromosome 1q21 is found in approximately 30% of PCM cases by using FISH.^{62,63} The *CKS1B* protein regulates the degradation of p27, thereby promoting cell cycle progression. Amplification of the *CKS1B* locus is associated with the presence of other prognostically significant abnormalities, including 17p13 deletions and chromosome 13 abnormalities; the presence of *CKS1B* amplification conveys poor survival. Another chromosome 1 abnormality, deletion of chromosome 1p21, has been reported in 20% of PCM.⁶⁴ The deleted locus contains the cyclin dependent kinase phosphatase, *CDC14A*. Deletion of 1p21 is also associated with other chromosomal abnormalities, including chromosome 13 abnormalities and the t(4;14)(p16;q32). Cases with the 1p21 deletion display an adverse prognosis in cases treated with high dose chemotherapy and ABMT.

A Practical Approach to Molecular Evaluation of Plasma Cell Myeloma

Until recently, the demonstration of molecular abnormalities in PCM identified patients at risk of poor outcome, but these studies did not generally alter the choice of treatment. A recent consensus publication, however, has identified a set of prognostically significant abnormalities as defining patients with “high-risk” PCM.^{7,65} So-called “high-risk” abnormalities include chromosome 13 abnormalities detected by metaphase cytogenetics, t(4;14)(p16;q32) or t(14;16)(q32;q23) identified by FISH, or abnormalities of the p53 locus. Treatment algorithms have been proposed, using such abnormalities to allow for risk stratification, analogous to the risk-stratified approach to treating childhood acute lymphoblastic leukemia.^{7,20}

To allow for identification of these abnormalities in routine practice, metaphase cytogenetic studies and FISH studies for *IGH* translocations are suggested in all cases at initial diagnosis, and the p53 pathway should be assessed using FISH or IHC. Although other recurring abnormalities may also be of prognostic significance, it is currently unclear how such information should be incorporated into routine practice.

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The Roles of Molecular Techniques in the Diagnosis and Management of Follicular Lymphoma

W. Richard Burack

Introduction

The first section of this chapter is an overview focused on the practical applications intended for trainees and nonspecialist practitioners. The second section reviews how the parameters assessed in molecular genetic assays reflect the parthenogenesis of follicular lymphoma (FL).

Practical Aspects of Molecular Genetic Testing in the Diagnosis and Management of Follicular Lymphoma

Of all the non-Hodgkin lymphomas, FL is perhaps the one for which molecular genetic analyses are least often required for diagnosis. Still, there are a number of reasons that molecular tests for suspected FL are needed. First, there are a lot of FL specimens: given the long clinical course of this generally indolent disease, it is the most prevalent NHL, and so this histology is probably the single histology most frequently seen in biopsies. Second, FL may be difficult to recognize in small biopsies or aspirates, in which it is not possible to see the nodular pattern. Third, there are several histologic and immunophenotypic variants that may call for additional molecular genetic studies to discern these from other entities.

Molecular genetic studies are only rarely needed when an excisional biopsy is in hand. For example, while karyotypes may help recognize FL and distinguish it from other NHLs, cytogenetic analyses performed on 279 consecutive excisional biopsies with adequate histology confirmed the impression that routine G-banding has limited value in the diagnosis of FL, or NHL in general.¹ Given an ample excisional biopsy with flow cytometric data, the scenarios in which molecular genetic tests might be necessary to resolve a differential diagnosis that includes FL are relatively uncommon but examples may include:

- A morphologic differential that includes reactive hyperplasia and a BCL2-negative FL or “FL in situ.”
- A morphologic differential that includes FL with marginal zone differentiation and a nodal marginal zone lymphoma.
- A differential diagnosis of primary cutaneous follicle center cell lymphoma (PCFCL) or cutaneous involvement by systemic FL.
- Synchronous or metachronous lymphomas with dissimilar histologies.

In practice, the most common scenario prompting the consideration of molecular studies of a possible FL involves a poor or small specimen. Unfortunately, the results obtainable by molecular assays are usually not sufficient to overcome these limitations. Therefore, whenever molecular genetic studies are contemplated, it is useful to first consider if the patient would be better served by obtaining another specimen. This is especially true when confronted with atypical lymphoid proliferations in a bone marrow (BM) biopsy in a patient not previously diagnosed with lymphoma. If these represent FL, the patient will almost certainly have significant adenopathy, and the patient is most often better served by an excisional node biopsy for definitive diagnosis and grading, rather than by pursuing molecular analysis of the BM.

Minimal residual disease (MRD) detection is often suggested as a role for molecular genetic testing. There is currently no accepted role for MRD detection in the case of a patient with any mature B cell neoplasm, and there are reasons that MRD detection specifically by molecular genetic techniques would be technically difficult in FL even were it ever thought to be useful. Grade 1/2 FL is generally an indolent disease and may be incurable with all current therapies. Therefore, the disease is generally not treated with intent to cure. When the disease progresses or recurs to an extent that warrants exposing a patient to the risks of current chemotherapies, the disease burden is anything but minimal and residual. Even if sufficiently nontoxic therapies were available to warrant a study of retreatment in

patients positive for MRD, other factors suggest that MRD assays would have to be much more complex than those that are currently in use. Highly sensitive PCR assays indicate that all healthy humans probably have several “non-malignant” B cell clones, including clones that carry the t(14;18)(q21;q32) lesion that is considered the hallmark of FL.² Less sensitive flow cytometric assays also detect B cell clones in about 5% of healthy adults.³ While it may be possible to distinguish these “benign clones” from a patient’s previously diagnosed malignant FL, the assays would have to be more complex than those currently in use. In summary, the use of molecular genetic testing in MRD detection remains strictly in the research realm.

There is, however, a limited role for molecular genetic tests in the workup of BM biopsies obtained for staging of FL. If BM biopsies do not show overt involvement by morphologic criteria, the meaning of any molecular genetic results is unclear since no prospective clinical trials to date have used these data. For the rare patient with a single node (or nodal group) who has Grade 1/2 FL, a diagnosis of BM involvement may profoundly alter treatment planning by shifting the stage from 1 to 4. There are not sufficient data available on any molecular genetic techniques to warrant using these alone to upstage patients. If the BM contains a few lymphoid aggregates, classification of these is best first approached by immunohistochemical techniques. If these fail to yield unequivocal evidence of involvement then consideration could be given to molecular genetic analyses. Probably, the most common reason for equivocal results on a staging BM is small sample size; the National Comprehensive Cancer Network (NCCN) guidelines suggest at least 2 cm of BM and encourage bilateral specimens.⁴ Again, the perceived need for additional molecular genetic testing is often the result of a failure to obtain a high quality specimen for routine morphologic evaluation.

Molecular Genetic Tests Available for FL

Karyotypes may be occasionally useful in the classification of FL. However, performing karyotypes on potential NHL specimens is not routine outside of a subset of academic laboratories and a large retrospective analysis suggests that these results will only very rarely materially affect the diagnosis.¹ Given a specimen that is good enough to spare fresh material for a karyotype, there is invariably adequate material for more routine immunophenotypic methods. Obtaining sufficient numbers of metaphases from FL is technically difficult. Finally, the t(14;18)(q21;q32) is not specific to FL: the t(14;18)(q21;q32) lesion has been reported in specimens with essentially every lymphoma histology.⁵

The single most reliable molecular genetic test for the confirmation of FL is probably FISH for the IGH:BCL2 translocation.⁶⁻⁹ The probes for at least one version of this assay are commercially available and, assuming that the level of expertise of the laboratory is appropriate, the test has much higher sensitivity on formalin-fixed, paraffin-embedded

(FFPE) specimens than PCR-based methods. This assay has the advantage of both detecting clonality and indicating the presence specifically of the IGH:BCL2 translocations. It may also suggest the presence of other lesions that disrupt the IGH locus, such as the t(3;14)(q27;q32) (IGH:BCL6), which occurs in a substantial fraction of BCL2-negative FL. This assay also has the advantage of being amenable to small specimens, such as core and endoscopic biopsies, which are frequently only available as FFPE specimens. Some laboratories perform this assay only on air-dried touch preparations, a restriction that makes this test very rarely useful. In general, if there is sufficient material for additional air-dried touch preparations, then there is sufficient material for other sorts of ancillary studies that prove diagnostic, particularly flow cytometry.

PCR-based methods for the IGH:BCL2 translocation are also not widely available in clinical laboratories. This reflects the relative insensitivity of these assays. Many assays varying in the details of primers have been published (reviewed in ref. ¹⁰). Although estimates vary, it appears that only 50–70% of specimens positive for t(14;18)(q32;q21) by karyotype will be positive by these PCR methods. (The probable molecular basis for this insensitivity is discussed below.) In short, if acceptable sections of FFPE material are available, the FISH assay is a better choice.

Rearrangements of the V-(D)-J elements of the IG loci can be used to detect clonality. The reliability of PCR methods for clonality detection varies greatly with the specimen type. Most laboratories use the BIOMED-2 or similar primer systems.¹¹ These systems are reliable when performed on optimal fresh/frozen material. In contrast, specimens for which molecular tests are needed are usually far from optimal. The current BIOMED-2 IGH PCR system fails on about 50% of FFPE FL specimens, even when the DNA is ample and the specimen is largely tumor.¹² This high failure rate appears to be specific to coupling of FL histology and FFPE source. In contrast, the IGH assays are far more reliable, detecting clonality in at least 95% of these same specimens. (The probable molecular basis for this distinction is discussed below.)

In summary, molecular genetic testing are currently suited for helping to confirm a diagnosis or resolve a limited differential diagnosis. While these tests will evolve and improve, in their current state they are the last resort in a diagnostic dilemma.

The Molecular Pathogenesis of FL and Its Impact on Molecular Diagnostics

A number of excellent reviews discuss how the “mutagenic environment” of the normal germinal center (GC) gives rise to many types of lymphomas. Although GC B cells comprise a small fraction of the total B cells in the adult human, the vast preponderance of B cell lymphomas appear to originate from B cells in the GC.¹³⁻¹⁵ In lymphomas as varied as classical Hodgkin lymphoma, Burkitt lymphoma, and follicular lymphoma, the “cell of origin” shows evidence

of “follicular differentiation” or genetic features indicating that the neoplastic clone originates within a cell that has “experienced” the GC. B cells in the GC carry out two processes that put their own genomes at risk: somatic hypermutation (SHM) and class switch recombination (CSR). Both these processes physiologically target the IG loci, but may also aberrantly target other sites in the genome. These physiologic processes have led to the concept that the GC process puts B cells at risk for neoplastic transformation. It is currently unclear if the canonical “first hit” (i.e., the translocation of BCL2) is acquired in the BM or in the GC. But there is overwhelming evidence that the GC provides the greatest opportunity to acquire the “second hit,” and even the “third hit” that drives large cell transformation.

GC B cells are poised to die. Lack of expression of BCL2, the potent and archetypal “anti-apoptosis” molecule, is probably critical in allowing the elimination of B cells, which have been rendered “less fit” through SHM. The mechanism of SHM and CSR suggest another mystery: these processes require double-stranded breaks to form, and then be repaired in the B cells’ genome. In most cell types, these breaks would be detected by p53, which should drive apoptosis, especially given that no BCL2 is expressed in GC B cells. How do B cells in the GC tolerate the DNA damage required for CSR and SHM occur? GC B cells have no discernable expression of p53. In fact, p53 expression is directly repressed by BCL6, a nuclear factor that is characteristic of GC B cells.^{16,17} Translocations that result in BCL6 overexpression are observed in about 5% of FLs, and may augment the survival of genetically damaged B cells by ensuring the repression of p53 expression.

What Goes Wrong to Make Follicular Lymphoma? The “First” Hit

The translocation of the BCL2 gene into the IGH locus appears to rely in many cases on recombination activation gene (RAG)-mediated recombination: in essence, RAG mistakes the BCL2 gene for a V region.¹⁸ The overexpressed BCL2 gene is physically located on the derivative chromosome 14 in FL. The remnants of the IG genes and the intact BCL2 gene are combined in opposite orientations with respect to that used in their native sites for transcription. In this way, expression of the BCL2 is driven by its own native promoter, and the “over-expression” of BCL2 is apparently due to the now “downstream” immunoglobulin enhancers. Because RAGs are expressed in early B cells residing in the BM (and not in GC B cells), it was for many years assumed that the BCL2 translocation was acquired in the BM. Characterization of B cells with a t(14;18) in normal healthy individuals has transformed this understanding.

In healthy individuals, about 1 in 10⁵–10⁶ circulating cells contain the t(14;18) lesion, suggesting that all humans have multiple B cells or multiple small clones of B cells, each with a t(14;18)(q32;q21) (reviewed in ref. ²). It is not entirely clear that these cells are the precursors of FL, and no case has

yet been described in which FL evolves from this presumed precursor. Even though the t(14;18) could have occurred in the BM, the cells carrying this lesion in healthy individuals are more similar to cells which have experienced the GC, such as memory B cells. These cells from healthy individuals share a number of features with FL cells in addition to the t(14;18), which suggest that they are the true precursors of FL.^{19,20} Features in these cells suggesting a prolonged “exposure” to the GC environment include:

- Most express CD27, a marker associated with transit through the GC.
- As in FL, these presumed precursors also show “class switching” on the IGH allele translocated to the expressed BCL2 locus. The most telomeric of the IGH constant regions (particularly mu and delta) are deleted. Class switching is not a feature of “naïve” B cells; rather class switching is a process that is thought to be strictly limited to the GC.
- As in FL, a substantial number of these presumed precursors have an apparently unique “class switch” on the productively rearranged IGH allele, in which the gamma and alpha switch regions recombine to delete intervening sequences, but the Mu switch region is not involved. This recombination is distinct from that which occurs in normal B cell development (in which the Mu switch region recombines with either the alpha or gamma).

Whether or not these cells represent the precursor to FL, their existence indicates that the t(14;18) is not sufficient for lymphomagenesis. The features identified by Roulland et al suggest that these cells are suspended in the midst of the GC reaction, and are therefore subjected to repeated and prolonged aberrant SHM, a process that drives lymphomagenesis.¹⁹

The molecular data for FL studies appear to reflect what is obvious from the histology: the FL clone is “trapped” in a GC. Sequencing of IG genes from FL specimens suggests that there is a high degree of intra-clonal variation (although this has also been disputed).¹³ Trapped in a GC, the FL clone continues to mutate both its IG genes and mistargets other genes as well. Among the several reports that demonstrate this intra-clonal variation is a strikingly elegant single-cell PCR study; individual tumor cells were plucked from adjacent malignant follicles, and sequencing of IG loci showed that there is ongoing SHM and that the daughter cells migrate between follicles.^{21,22}

The “Second” Hit

The “second” hit(s) in FL is(are) more obscure. There is a plausible mechanism to explain the origin of these mutations: aberrant somatic hypermutation (aSHM). In essence, the B cells “misfire” the mutagenesis that is physiologically directed toward the rearranged IG genes. The result is the accumulation of point mutations in many other expressed genes, including proto-oncogenes. The amount of aSHM in FL is controversial. Early descriptions of aSHM indicated that aSHM is less frequent in FL, when compared to diffuse large B-cell lymphoma (DLBCL).²³ While further studies have corroborated

this impression, these studies have also indicated that aSHM occurs in FL, and may result in mutations in the same regions of the same proto-oncogenes, that have been considered plausibly related to the pathogenesis of DLBCL.^{24,25} However, no mutations with definitively causal roles have been identified. SHM requires expression of activation-induced cytidine deaminase (AID), an enzyme which initiates DNA damage. Therefore, AID activity is a possible cause of the mutations that drive oncogenesis. One study has reported a linear relationship between activation-induced cytidine deaminase (AID) levels and intra-clonal heterogeneity, as measured by variations in IGH sequences.²² The FL cell's "permanent resident" status in the GC environment with its accompanying AID-mediated genotoxic risks may give rise to the genetic variability that allows evolution of the disease.

Other mechanisms possibly providing the "second hit" include additional chromosomal lesions, such as translocations and acquired copy number changes (deletions and amplifications or copy number aberrations (CNA)), and the loss of heterozygosity by uniparental disomy (UPD). The Mittleman collections of 1208 FL karyotypes does not strongly suggest any recurrent translocations that might constitute a common "second hit" occurring after a BCL2 translocation. However, both karyotypes and more recent comparative genomic hybridization data have suggested a number of recurrent copy number changes that may provide the "second hit." A sophisticated analysis of a large collection of classic karyotypes suggested a limited number of recurrent abnormalities accrued in the development of FL, particularly 6q-, +7, or +der18, with del117p and +12 associated with adverse outcome.²⁶ It is possible that many or even all these represent "passenger" mutations rather than "driver" mutations. The genes involved are not clear. Higher resolution studies for CNA and UPD using array-based technologies have been applied in an effort to pinpoint involved genes, and therefore provide better evidence of a pathogenetic role for these lesions.^{27,28} While the bulk of the recurrent CNAs identified by these high resolution platforms were actually sufficiently large to have been identified by standard karyotype, some important insights were gleaned: the del117p in most cases did not affect the p53 locus, and a minimal region of 6q23.3–24.1 is a common deletion.²⁸ Furthermore, a region containing this locus on 6q and another region on 1q were defined as having prognostic relevance.²⁷

The abundance of genetic changes induced by this "permanent GC" state makes it difficult to discern pathogenetic (i.e., "driver") mutations from incidentally occurring (i.e., "passenger") mutations. Whole genome discovery methods, beginning with karyotypes, have been used to search for recurrent lesions which presumably have some pathogenetic relevance. But even if a lesion is recurrent, this is not proof that it is pathogenetically relevant, or if it merely represents a site or form of genetic damage to which FL cells are particularly susceptible. This is particularly problematic when considering chromosomal lesions.

Proven secondary changes with plausible roles in the oncogenesis of FL include deletions of genes for the cyclin dependent kinase inhibitors 2A and 2B (CDKN2A/B, also known as p15/16, and INK4A/B) and lesions of p53. For example, minimal deletions in CDKN2A/B coding regions, each less than 1 MB, were detected in three of ten cases of FL (Halldorsdottir and Burack, unpublished data). Furthermore, a larger region containing these genes has been reported to undergo UPD, suggesting that homozygous deletion may result from the deletion of one allele followed by replacement of the wild-type allele. Loss of the tp53 gene has been described in transformed FL.²⁹ While point mutations of p53 have been reported in low grade FL, CNA for the tp53 gene has thus far only been reported in transformed FL. Mutations in tp53 have recently been reported to portend a poor prognosis.³⁰ A role of tp53 mutations in permitting genetic instability, and hence disease progression, is easily understood. However, it seems unlikely that tp53 mutations could comprise the "second" hit for transformation of t(14;18) cells because expression of this protein is effectively repressed in GC B cells by BCL6 expression. Therefore, it seems likely that tp53 mutations are likely to be late events in FL evolution, only exerting any effect on cellular function, after the BCL6-mediated repression of the locus is rendered inoperable.

Immunoglobulin Genes in FL

FL characteristically express high levels of surface IG, although surface IG negative cases do occur. The isotype is most often IGM (or IGM/D), but about a third of cases express IGG. Several aspects of the IG locus are consistent with the GC origin of FL, and suggest that the cell of origin is subjected to selective pressures of the GC.

- The IG genes have undergone SHM, with levels of SHM up to 30% reported, but averaging about 10%.
- The framework regions are generally conserved (contain mutations classified as "silent"), and indicating that preservation of IG function is likely required for FL.
- The "replacement" mutations in the IGH genes have a high frequency of creating N-glycosylation sites. These sites are proposed to enhance antigen binding, further suggesting that functional IG is necessary to support the development of FL.³¹
- In IGM-expressing cases, the productively rearranged heavy chain allele has often undergone a unique downstream rearrangement that appears to involve the gamma and alpha switch regions. This peculiar rearrangement, which does not affect the IG coding region, has also recently been reported in activated B-cell (ABC)-type of DLBCLs and has not been reported in any other normal or malignant B cell populations.^{32,33} Because class switching is an activity only supported by the GC environment, it has been suggested that this "hallmark" aberrant class switch reflects the "permanent residency" of FL cells in a GC state.

Molecular Genetic Analysis by Gene Expression Profiling Largely Reflects the Microenvironment (Also See Chap. 13)

The T cell and macrophage components may in some way determine the clinical course of the disease. Low grade FL has a remarkably high rate of spontaneous regression, which has been reported as high as 25% in untreated disease.³⁴ This feature suggests that the patient's immune response or another intrinsic feature may affect the outcome more than acquired genetic lesions in the FL cells. Gene expression studies performed on whole tumor specimens appeared to reflect features that may be evident by examining the cellular composition of the tumors: that increased T cells is associated with a good prognosis, while increased macrophages is associated with a poor prognosis.³⁵ The association of increased macrophages with poor prognosis may be as trivial as the association of apoptosis that typically accompanies increased proliferation. However, other studies have not clearly corroborated these results.³⁶ Regardless, with the advent of immunotherapy, particularly those targeting CD20, the relevance of the immune infiltrate appears all that much greater. In addition, several other parameters of the microenvironment have been suggested as prognostic factors, such as sclerosis, vascularity, and mast cell number, which might be reflected in gene expression profiling (GEP).

The “Third Hit”: Mechanisms of Large Cell Transformation

About 25% of patients with low grade FL will transform to large cell lymphoma, most often with a diffuse histology. The rate of transformation is greatest at the time of diagnosis, and then falls so that if a patient survives 10 years with low grade FL, they are unlikely to subsequently transform to large cell lymphoma. This time course implies that an intrinsic feature of the lymphoma (or the patient) present at diagnosis could determine the risk of transformation.

The mutations that drive transformation may be distinct or overlapping with the genetic lesions which comprise the “second hit” required to cause FL.³⁷ Most of the lesions described in transformed FL (i.e., mutations in p53, loss of p53, loss of CDKN2A/B, karyotypic abnormalities, accumulation of mutations attributable to aSHM, etc.) have also been described in low grade FL, although usually at a lower frequency. For example, mutations in the 5' noncoding regions of BCL6 were initially described as exclusively occurring in transformed FL,³⁸ but additional studies have shown that these mutations also occur in low grade FL. Mutations in multiple proto-oncogenes (i.e., MUM1, PIM1, MYC, and RHO), attributable to aSHM, are as frequent in low grade FL as in transformed FL, and similar numbers accumulate in stable, low grade FL as accumulate with transformation of FL.²⁴

An interaction between “microenvironment” and intrinsic propensity of the FL to transform to large cell FL has also

been suggested.³⁹ The nature of the T cell infiltrate and the function of the follicular dendritic cells may vary with the intrinsic properties of the tumor cells.

Molecular Variants of FL

BCL2-negative FL is fairly common, reported to comprise 10–15% of all cases. Virtually all cases positive for t(14;18) are positive for BCL2 protein expression by IHC. On the contrary, about one-third of cases that are negative for t(14;18) (or any other detectable abnormality of the BCL2 locus) are still positive for BCL2 protein by IHC. The remaining two-thirds of cases that are negative for any chromosomal lesions of the BCL2 locus are also negative for expression of BCL2 protein by standard IHC methods.

A large series of t(14;18)-negative FL have indicated that about 10% of these cases have a lesion of the 18q21 band, likely affecting BCL2.⁴⁰ This includes a handful of cases with a t(2;18)(p11;q21), which likely represents a translocation of the BCL2 gene into the IGH locus. Rare translocations involving the IGL locus have also been reported. The majority of 18q21-negative cases had an abnormality of 3q27, the location of BCL6; many of these cases have a t(3;14)(q27;q32), which places BCL6 under the control of the IGH locus. Distinct translocations of BCL6 have been observed in DLBCL and FL, and may have mechanistic implications.

Implications of Pathogenesis for Molecular Assays

Clonality Detection by IG PCR

The high level of SHM in FL has implications for the use of PCR methods to detect clonality. The BIOMED-2 primer systems for B cell neoplasms are, in general, highly sensitive for FL. However, the mutations in the primer binding sites caused by SHM may decrease primer binding and result in false negative results. Using DNA prepared from fresh or frozen tissue, the mismatch caused by SHM is not sufficient to decrease the sensitivity of the BIOMED-2 IGH assays. In contrast, the BIOMED-2 IGH assay detects clonality in only 50% of FFPE FL specimens. We suggest that this loss of sensitivity for FFPE FL is because the process of formalin fixation/paraffin-embedding and the SHM synergistically damage the template for the Jh consensus primers. Because the BIOMED-2 IGH primer system works well with FFPE CLL/SLL specimens (which have on average considerably less SHM) and on fresh/frozen FL specimens, neither effect is alone sufficient to degrade sensitivity. The IG kappa locus is much less affected by SHM for unknown reasons. Consistent with the hypothesis that FFPE preparation and SHM synergistically damage the DNA template, >95% of FFPE FL specimens show clonality with the BIOMED-2 IGK primers. Therefore, the IGK primer system is the current method of choice for the detection of clonality in FFPE FL specimens.

Detection of BCL2 Translocations

FISH for detection of the t(14;18) is more reliable than PCR in all comparative studies. While the breakpoint within the IGH locus is restricted to a small area just telomeric to the Jh genes, the breakpoints within the BCL2 locus are scattered over at least 30 KB on chr18. Within the BCL2 locus, there are several loosely defined “clusters,” which together comprise ~70% of the breakpoints seen in FL: these are termed major breakpoint region (MBR), a more 3' region (3' MBR), and a more distal region, termed the minor cluster region (mcr). Many primer systems have been assessed; the current BIOMED-2 system has reported sensitivity not exceeding 70% when performed on fresh specimens. There is a technical reason that this method may be more reliable for the detection of clonality in FFPE FL specimens than by IGH PCR methods: it is likely that the Jh consensus sequences in the productively rearranged IGH allele will be altered by SHM, and that this effect will hinder binding of the Jh consensus primers. In contrast, the Jh consensus sequences in the derivative chromosome 14 are less likely to be affected by aSHM. Therefore, for any FFPE FL specimen, the BIOMED-2 BCL2 assay is likely to be more sensitive than the BIOMED-2 IGH assay. Of course, a positive result with the BCL2 assay will both demonstrate clonality and suggest FL. However, if the major concern is simply to demonstrate clonality, the BIOMED-2 IGK assay is more sensitive than either BCL2 or the IGH assays for this specimen type. For fresh/frozen specimens, the IGK and IGH assay systems are likely to have similar sensitivities, while the sensitivity of the BCL2 system will be considerably lower.

This relative unreliability of the BCL2-PCR assays is due to the biologic variability in the location of the chromosomal breakpoint on chromosome 18, with breakpoints “clustered” within a large (30 KBp) region. For clinical assays, this region is too large to sample with a limited number of PCR primers. Furthermore, any breakpoints falling outside of this region will not be detected by most clinical PCR methods. Long Distance PCR methods have been described, but these have not been widely adopted in the clinical setting. Although a substantial number of studies have addressed the possible relationship between prognosis or other clinical features and the precise site of breakpoint, no correlations have been validated.^{41,42}

See Chap. 14 regarding the proteomics of FL.

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21

Mantle Cell Lymphoma

Kai Fu and Qinglong Hu

Introduction

Mantle cell lymphoma (MCL) is a distinct subtype of mature B-cell neoplasm with characteristic histologic, immunophenotypic, genetic, and clinical features. The neoplastic cells of MCL appear to correspond to naïve B cells that normally home to, and reside in, primary lymphoid follicles and mantle zones of the secondary follicles. MCL brings together the worst characteristics of high-grade and low-grade lymphomas; the course of the disease is not indolent, and the disease is rarely curable. Novel and better therapies are definitely needed for this group of lymphomas.

Epidemiology

MCL comprises 5–10% of human B-cell malignancies.^{1–3} Patients who have MCL have a median age of approximately 60 years, with males predominance.^{4,5} A recent epidemiological study based on the data from the Surveillance, Epidemiology, and End Results (SEER) Tumor registries showed that of the 87,166 patients diagnosed with non-Hodgkin's lymphoma during the 13-year period between 1992 and 2004 in the United States, 2,459 (2.8%) had confirmed MCL. The overall incidence of MCL (per 100,000) was 0.55, which increased with age: 0.07 in patients aged <50 years, 2.97 in patients aged 70–79 years, and 2.78 in those aged ≥80 years. The median age at diagnosis was 68 years. The incidence of MCL was higher in men (0.84 of 100,000) than in women (0.34 of 100,000) ($P < 0.05$), and was higher in Caucasians (0.61 of 100,000) than in African-Americans (0.32 of 100,000).⁶

Clinical Presentation

The majority of the patients (74.6%) presented with advanced disease (stage III or IV), usually with generalized lymphadenopathy and bone marrow involvement. Spleen and peripheral blood involvement is also common⁷. Lymphomatous papulosis represents the initial or secondary involvement

of the intestinal tract with numerous confluent polyps.⁸ The median survival of patients with MCL has ranged between 3 and 4 years in most series.^{4,5} This survival is significantly shorter than the survival of patients who have other forms of lymphocytic lymphomas. The disease is considered incurable using the current standard therapy.¹

Histopathology

Morphologically, the involved lymph nodes show partial or complete enfacement of the nodal architecture by a monomorphic lymphoid infiltrate with a vaguely nodular, diffuse, mantle zone (or rarely follicular) growth pattern.^{9–11} The nodular pattern resembles enlarged primary lymphoid follicles with no definitive germinal centers (GCs); while mantle zone pattern shows small naked GCs and large expanding mantle zones. Rarely, the so-called “in situ MCL” has been described, in which the neoplastic lymphoid infiltrate almost exclusively restricts to the inner mantle zones or to the narrow mantle.¹² The lymphoma cells are monotonous with medium-sized nuclei and variably irregular nuclear contours, dense chromatin, and small to moderate amounts of cytoplasm. Scattered histiocytes may be present in the diffuse or interfollicular areas. Histological transformation to typical large cell lymphoma does not occur; however, loss of a mantle zone or nodular growth pattern, increase in cell size and nuclear polymorphism, and an increase in proliferative activity may occur during the course of the disease or at relapse.^{4,10,13} Polymorphic and blastoid variants have also been described and may be associated with a worst prognosis.^{4,10}

Immunophenotype

MCL is a distinct type of mature B cell lymphoma, expressing CD19, CD20, CD22, FMC7, PAX5, surface IgM and/or IgD, and the T-cell-associated antigen, CD5. They are usually negative for surface CD23, CD10, and BCL6, distinguishing it with chronic lymphocytic leukemia/small lymphocytic

lymphoma and follicular lymphoma, respectively. More importantly, almost all cases express nuclear cyclin D1, including rare cases that are CD5-negative.^{14–16} Cyclin D1-negative MCLs have been identified and display the morphologic and immunophenotypic features of conventional cyclin D1-positive MCL, but show no evidence of a t(11;14) translocation or cyclin D1 overexpression.¹⁷

Pathogenesis

Initial Oncogenic Event

Cytogenetically, MCL is characterized by the chromosomal translocation t(11;14)(q13;q32), which results in deregulated aberrant expression of cyclin D1.^{18–22} The translocation occurs in the early stage of B cell development and juxtaposes proto-oncogene *CCND1*, which encodes cyclin D1, on chromosome 11q13 to the proximity of the immunoglobulin heavy chain gene enhancer on chromosome 14q32, resulting in the constitutively overexpression of cyclin D1.^{19,23,24} This genetic alteration is thought to be the primary event in the pathogenesis of MCL, probably through the deregulation of the cell cycle at the G1-S phase transition. Cyclin D1 forms a complex with cyclin-dependent kinase 4 (CDK4)/CDK6, which phosphorylates retinoblastoma 1 (RB1) protein, resulting in the release of E2F transcription factors from RB1 and subsequent progression of the cell cycle from G1 to S phase.^{25–27} However, recent discovery of intragenic deletions of RB1, leading to a total lack of protein expression in some cases of MCL, suggests that cyclin D1 may have an oncogenic role independent of RB1 in MCL.²⁸

Occasional cyclin D1-negative or t(11;14)-negative MCLs have been described, and share the same morphologic, immunophenotypic, and clinical features with conventional MCL.^{17,29} Interestingly, all six cases described in the original series expressed either cyclin D2 or D3.¹⁷ Although none of these cases showed a chromosomal alteration affecting cyclin D2 or D3 locus based on interphase FISH study, subsequent studies have shown that some cyclin D1-negative MCL cases may carry a t(2;12)(p12;p13) (fusing cyclin D2 to the kappa light chain gene locus³⁰), some may carry a t(12;14)(p13;q32) (fusing cyclin D2 to IgH gene locus^{29,31}), and other cases may carry a t(6;14)(q21;q32) (fusing cyclin D3 to IgH gene locus).²⁹ Chromosome translocations involving cyclin D2 or cyclin D3 gene locus may be other mechanisms for the upregulation of these D-type cyclin genes, which may functionally substitute for cyclin D1 and play important roles in the pathogenesis of cyclin D1-negative MCL.^{17,29} Moreover, a comparative chromosome hybridization (CGH) study has shown that cyclin D1-negative MCL cases share similar cytogenetics alterations with their cyclin D1-positive counterpart.³²

Secondary Genetic Alterations

Despite the apparent essential role of the t(11;14) translocation and constitutive overexpression of cyclin D1 in MCL, several lines of evidence suggest that the t(11;14) itself may not be sufficient for the full transformation of the cells. Transgenic mice (in which *CCND1* was linked to Ig gene regulatory elements) did not develop spontaneous lymphomas, and lymphogenesis in these animals occurs only in cooperation with the overexpression of other oncogenes, such as *MYC*.^{33–35} More recently, mature B-cell lymphomas developed in a mouse model expressing a cyclin D1 mutant, that is refractory to nuclear exportation and therefore the degradation of cyclin D1 protein. The tumor cells in this model showed alterations in the p53-MDM2-ARF pathway and *BCL2* overexpression. These studies suggest that the t(11;14)(q13;q32) alone is not sufficient to result in lymphoma, and that additional genetic alterations are necessary.

Advances in gene expression microarray and high resolution array-based CGH has improved our understanding of the secondary genetic events involved in MCL lymphomagenesis. Cytogenetics studies have revealed frequent recurrent genetic alterations in MCL and suggest that MCL is one of the malignant lymphoid neoplasms with the highest level of genomic instability, probably secondary to alteration in the ataxia-telangiectasia mutated (*ATM*) gene.³²

Chromosome-based CGH is a useful technique, that is performed with genomic DNAs from lymphoma and normal control tissues, which are differentially labeled with fluorochromes and then hybridized to the metaphase chromosome of normal donor cells. Comparative analysis is then performed to identify the variation in DNA copy number chromosome-wide, such as amplification, duplication, and deletion. Since the hybridization was performed on the metaphase chromosome, its resolution is similar to conventional cytogenetics, ranging from 5 to 10 Mb. Secondary chromosomal imbalances previously detected by conventional CGH are gains of 3q and 8q, and losses of 1q, 6q, 11q, and 13q.^{36–38} Array-based CGH (aCGH) technique is a revolutionary advance in medicine during the last several years.³⁹ Using BAC/PAC CGH microarray, Rubio-Moscardo and colleagues studied 68 MCL patients and nine MCL-derived cell lines. Multiple recurrent genetic abnormalities were identified, some of which are distinct for MCL, such as deletions of 1p21 and 11q22.3 with coincident 10p12 gene amplification. The most common secondary chromosomal abnormalities are gains of 3q25-qter, 4p12–13, 7p21–22, and 8q21-qter, and losses of 1p13–p31, 6q23–q27, 9p13–p24, 9q13–q31, 11q22–q23, 13q11–q13, 13q14–q34, and 17p13-qter.^{40–42} While some of these alterations, such as losses of 11q and 13q, occur at similar frequencies in all histological variants of MCL, the highly proliferative and clinically aggressive variants of MCL tend to have more complex karyotypes with frequent gains of 3q and 12q, and losses of 9p, 9q, and 17p. Loss of

9q22–24 was predictive of poor overall survival, while deletion of 1q21 correlated with a favorable prognosis. Loss of 8p21.3 was associated with leukemic MCL, which showed preferential use of VH4-39 with high frequency of somatic mutation.⁴³ High-resolution genome-wide tiling aCGH has reached 32 kb in resolution and may identify genetic imbalance with an interval of less than 50 kb. Recent studies of MCL with high-resolution aCGH have led to the identification of cryptic genetic gains and losses. However, more studies are required to determine the association between the genetic imbalances and the concomitant effects on the gene expression and function.^{40,41}

Pathway Dysregulations in MCL

Gene expression profiling (GEP) is another powerful tool for the study of molecular pathogenesis, classification, and gene-targeted therapy for MCL. The mRNA prepared from purified tumor cells of fresh or frozen tissue is labeled and hybridized to commercially available gene array chips, such as Affymetrix arrays. Differentially expressed genes are identified by comparing with the normal B cell counterpart after data processing and analysis. The results obtained from these studies may be further validated by RT-PCR, Western blot, and immunohistochemical staining. Functional validation has been developed recently by small interfering RNA (siRNA) transfection in MCL cell lines.⁴⁴

Correlatively, analysis of the gene expression and genomic profiling data has generated insights for the pathogenesis of MCL. These studies suggest that most dysregulated target genes in MCL development are involved in two common pathogenetic pathways, the cell cycle regulation, and the DNA damage response. Recent studies also suggest that genes involved in cell proliferation, cell survival, and apoptosis may also be targeted by oncogenic events in MCL, and dysregulations of these genes may affect the tumor response to chemotherapy.

Dysregulations in Cell Cycle Control

Rosenward and his coworkers profiled 92 MCL tumor biopsy samples using the Lymphochip cDNA array.⁴⁵ They found that the proliferation signature (i.e., an average expression level of 20 genes related to cellular proliferation) closely correlates with the clinical outcome in patients with MCL. Many highly proliferative MCL have genetic alterations affecting two closely related regulatory pathways, INK4a-CDK4-RB1 and ARF-MDM2-p53, which are responsible for controlling the cell cycle and senescence.

The *CDKN2A* locus on chromosome 9p21 encodes two key regulatory factors, the CDK4 inhibitor (INK4a) and the p53 regulator (ARF). Homozygous deletions of this locus have been detected in up to 30% of the highly proliferative MCL cases, but in less than 5% of the classical cases.^{46,47} INK4a inhibits CDK4/6, thus, INK4a deletion cooperates

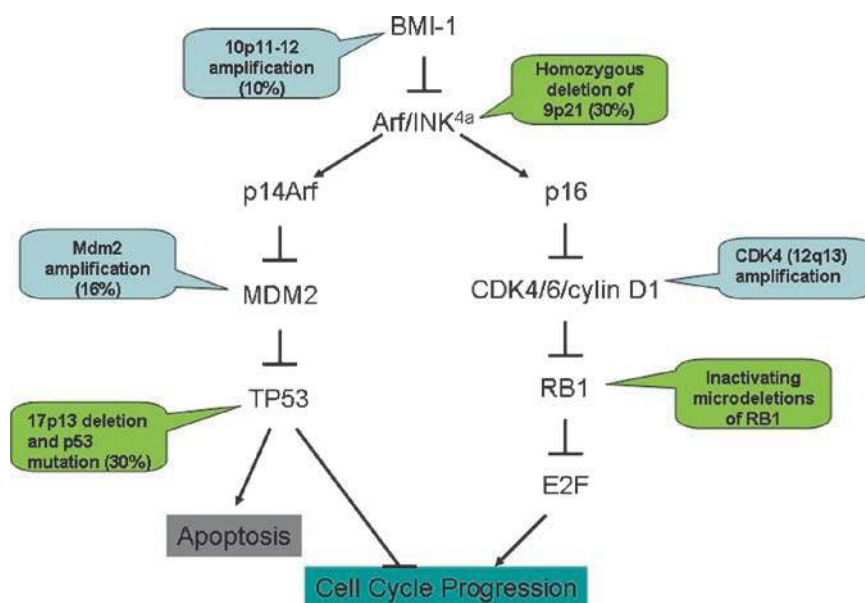
with the increased levels of cyclin D1 in accelerating the G1/S-phase transition in MCL cells. Conversely, amplification of CDK4 locus (12q13) and overexpression of CDK4 has been detected in some highly proliferative MCL cases. Interestingly, the amplification occurs almost exclusively in MCL cases with a wild-type *CDKN2A* locus.⁴⁸ More recently, studies have shown that the presence of inactivating microdeletions of RB1 gene, in some cases of highly proliferative MCL, results in truncated mRNAs and a total lack of RB1 protein.²⁸ Similarly, these microdeletions occur almost exclusively in tumors with a wild-type *CDKN2A* locus. Moreover, gain/amplification of 10p11–12, which targets a gene of the Polycomb group (*BMI1*) has been detected in some MCL cases with a wild-type *CDKN2A* locus.^{49–51} Overexpression of *BMI1*, a transcriptional repressor of the *CDKN2A* locus, suppresses the expression of INK4a and ARF. It is therefore apparent that the tumor cells in MCL may have found different approaches, including amplification of 10p11–12 and overexpression of *BMI1*, homozygous deletions of 9p21/*CDKN2A* locus, amplification of 12q13 and overexpression of CDK4, and inactivating microdeletions of RB1 gene, in order to induce the dysregulation of INK4a-CDK4-RB1 pathway (Figure 21.1).⁵² The presence of mutual exclusiveness supports the notion that oncogenic activation of more than one member of certain pathways does not provide a biological advantage to the tumor cells.

Deletion of the *CDKN2A* locus also affects the expression of ARF. ARF binds to MDM2 and prevents the degradation of p53; thus, inactivating this locus leads to deregulation of the ARF-MDM2-p53 pathway as well. Mutations of the TP53 gene, usually associated with 17p13 deletions, are observed in approximately 30% of the MCL cases with a high proliferative activity and a poor prognosis. Deletion of 17p is more common in the blastoid variant of MCL, indicating its role in high grade transformation of the tumor.^{51,53,54} Conversely, overexpression of MDM2 may be detected in up to 16% of MCL cases, although the mechanism of this upregulation is still elusive.^{48,55} While inactivation of *CDKN2A* leads to simultaneous dysregulation of not only the cell cycle, but also the p53 pathway, inactivating mutations of p53 occur in tumors with a wild type *CDKN2A* locus and are commonly associated with CDK4 amplification, or RB1 inactivations. The findings suggest that tumor cells acquire a selective advantage by inactivating both ARF-MDM2-p53 and INK4a-CDK4-RB1 pathways. The double inactivation may be achieved through homozygous deletions of the *CDKN2A* locus, *BMI1* amplification, or simultaneous mutations of TP53 combined with CDK4 amplification or RB1 inactivation⁵² (Figure 21.1).

Dysregulations in DNA Damage Response Pathway

Among the most frequent secondary cytogenetic alterations in MCL are deletions of 11q22–23, which target the ataxia-telangiectasia mutated (ATM) gene. The ATM gene

FIG. 21.1. Dysregulation of pathways in MCL.



encodes a phosphoprotein kinase that belongs to the PI3K-related superfamily and has a central role in the cellular response to DNA damage, such as DNA double strand break repair.^{56,57} ATM is normally expressed in naïve B-cells within the mantle zone of the lymphoid follicles, but it is negative in immature B-cell precursors and follicular center B-cells. Deletion of 11q22 region involves ATM and occurs in 55% of the MCL cases; high ATM mutation has been demonstrated recently in MCL by oligonucleotide microarray (43%, 12/28).⁵⁸ Inactivation of ATM in naïve B-cells may enhance the genomic instability and increased tumorigenesis in these cells. ATM mutations, usually associated with the loss of the other allele, have been detected in up to 75% of MCL cases.^{54,58,59} These mutations mainly affect the PI3K domain, or lead to truncated and unstable ATM protein.

Dysregulations in Cell Survival Pathway

The PI3K/Akt pathway is involved in various fundamental cellular processes, including cell cycle progression, cell proliferation, apoptosis, and survival. Constitutive activation of the pathway contributes to the pathogenesis of many types of cancer. Recent GEP and proteomic studies have identified several genes in this pathway, including *PIK3CA*, *AKT-1*, *PDK-1*, and *PPP1R2*, overexpressed in primary MCL tumors and MCL cell lines.^{60,61} Additional studies have shown that the PI3K/AKT pathway is constitutively activated in a subset of primary MCL tumors, including all of the aggressive blastoid MCL variants and a small subset of classical MCL cases, suggesting its role in the pathogenesis of MCL.⁶² The AKT activation may be associated with the loss of phosphatase and tension homologue (PTEN) expression. More recently, our laboratory has discovered that overexpression of miRNA-17-92 cluster, which is present in cases with 13q31-32 amplification (a common secondary genetic alteration in MCL) may down-modulate

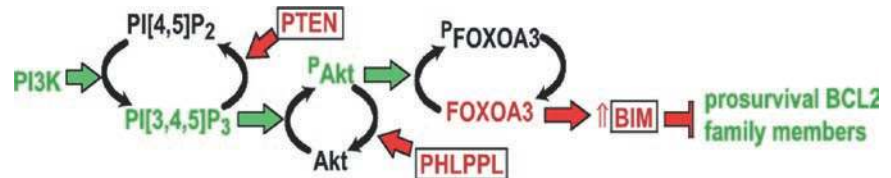
PTEN or PHLPP2, and thereby activate PI3K/AKT pathway (see below).⁶³ The presence of the active, phosphorylated form of this kinase, p-AKT, was accompanied by the increased phosphorylation of p27^{kip1}, the FOXO family, MDM2, BAD, mTOR, and p70S6K. Inhibition of the PI3K/Akt pathway in MCL cell lines reduced the phosphorylation of AKT and its targets, and led to cell-cycle arrest and apoptosis. These data suggest that inhibition of this pathway may be effective in the treatment of AKT-activated MCL.⁶²

BCL-2-interacting mediator of cell death (BIM) is one of the most potent pro-apoptotic BH3-only proteins; it binds to all pro-survival BCL2 family members with high affinity,^{64,65} thereby releasing BAX or BAK proteins, the critical downstream effectors of the BCL2-dependent pathway of apoptosis.⁶⁶ Thus, BIM is a tumor suppressor, as demonstrated by the accelerated Myc-induced lymphomagenesis in Eμ-myc mice lacking *BIM*⁶⁷ and increased tumorigenesis of *BIM*^{-/-} epithelial cells.³¹ B-lymphocytes lacking BIM become refractory to the apoptosis induced by B-cell receptor ligation in vitro. More recently, using aCGH, two groups have shown loss of 2q13 and homozygous deletion of *BIM* in primary MCL tumors or MCL cell lines.^{68,69} Furthermore, we showed that overexpression of miRNA-17-92 cluster may also target BIM, a proapoptotic protein, and prevent cellular apoptosis in MCL (see below).⁶³ These findings strongly suggest that BIM serves as a tumor suppressor in promoting cellular apoptosis and its downregulation may contribute to lymphomagenesis in MCL. Conversely, amplification of 18q11-q23 and overexpression of BCL2 protein has been observed in MCL cell lines and primary tumors.

Dysregulations in MicroRNA-17-92 Expression

Secondary genomic alterations are frequently detected in MCL, of which chromosome 13q31-q32 gain/amplification is one of the most frequent.^{32,70,71} Amplification at chromo-

Fig. 21.2. Interaction among putative miR-17-92 targets that promote apoptosis.



some 13q31-q32 targets a microRNA cluster, miR-17-92, which resides within intron 3 of *c13orf25*, a nonprotein-coding gene at 13q31.3.^{72,73} The miR-17-92 cluster consists of six miRNAs, and overexpression of the cluster accelerates the development of MYC-induced lymphoma in mice^{72,74} and contributes to carcinogenesis in the lung.⁷⁴ Nevertheless, the functional role of miR-17-92 in MCL has not been investigated. Based on a GEP study of 82 primary MCL biopsy specimens using whole genome Affymetrix U133 2.0 plus arrays, we found that overexpression of miR-17-92 was associated with a worse overall survival in patients with MCL ($P=0.021$).⁶³ We further demonstrated that enforced expression of miR-17-92 reduced chemotherapy-induced apoptosis in retrovirally transduced MCL cell lines, and that overexpression of miR-17-92 in MCL cells increased the phosphorylation of AKT and its downstream targets. Moreover, we demonstrated that overexpression of miR-17-92 in MCL cells down-modulates multiple proteins involved in PI3K/Akt signaling and apoptosis, including PTEN, PHLPP2, and BIM, and that downregulation of these targets collaboratively enhances cell growth and chemoresistance in tumor cells (Figure 21.2). As a consequence, overexpression of miR-17-92 may be associated with poorer survival in MCL patients.⁶³ Our findings disclose a novel oncogenic pathway in MCL and suggest that targeting the miR-17-92 cluster may provide a novel therapeutic approach for this disease, which is incurable using current chemotherapeutic regimens.

Molecular Diagnosis

The t(11;14)(q13;q32) is present in almost all cases. However, conventional cytogenetics demonstrate only a 70–75% positive rate in the detection of the t(11;14)(q13;q32) translocation.²¹ Interphase FISH analysis using a break-apart probe has been used to detect the cyclin D1 translocation with sensitivity and specificity close to 100%. The FISH analysis may be performed on peripheral blood and bone marrow smears, imprint slides, and formalin-fixed, paraffin-embedded tissue sections. Because the breakpoints in the cyclin D1 locus are not tightly clustered, molecular studies using DNA amplification may only detect the t(11;14) translocation in approximately 30% of cases. However, DNA amplification has proven useful in monitoring the minimal residual disease.

Clonality analysis of B cell receptor genes has also been used to aid in the diagnosis of MCL, in combination with the morphologic and immunophenotypic features. PCR with

fresh or formalin-fixed tissues for the detection of clonal rearrangement of IgH gene is the most commonly used technique and has shown nearly 100% sensitivity and up to 90% specificity in MCL.^{75,76}

Proteomics and Antibody-Based Protein Array

Protein expression is regulated by several important mechanisms, which include DNA modification, mRNA transcription, and pre- and posttranslational modifications. The information, therefore, obtained from aCGH and GEP may not exactly reflect the changes in cellular protein level of the tumor cells. Current proteomics has been established for the detection of global cellular protein profiles by 2-D polyacrylamide gel electrophoresis (PAGE) coupled with mass spectrometry.^{77,78} Using 2-D PAGE and MALDI-TOF mass spectrometry, Weinkauff et al recently studied the protein expression in three MCL cell lines and three follicular lymphoma cell lines. Among 38 proteins highly expressed in the cell lines were those involved in DNA repair (Rad50), cell cycle regulation (mad1L1), transcription (SAFB), and apoptosis (Luca 15 and bcl-2). Modest correlation was observed between protein and mRNA expression, as detected by parallel RNA array, emphasizing the relevance of posttranslational regulation in the lymphomagenesis.⁷⁹ However, analysis of human proteome by 2-D PAGE and MALDI-TOF mass spectrometry is considered time-consuming and is biased toward high abundance of proteins. When the entire tumor tissue is used, the protein expression profile reflects a combination of tumor cells and stromal tissue.

Antibody-based protein microarray technique has shown significant progress during the last several years. Ghobrial et al studied protein expression in seven MCLs, using a protein microarray chip containing 512 monoclonal antibodies that showed overexpression of more than 13 proteins involved in cell cycle regulation (RCC1), phosphatase inhibitor (inhibitor 2), p53 regulator (MDM2), Hsp90, and kinase KRIC.⁶¹ More recently, Ek et al used expression profiling to preselect dysregulated genes in MCLs. Then, they used protein expression signature tag (PrEST) to develop polyclonal antibodies against the protein products of the genes. Most of the antibodies (84%) showed reactivity with MCLs, and 25% of the antibodies had tumor specificity.⁸⁰ Further investigation is needed to prove that antibodies might have great clinical benefit in the diagnosis and therapy of MCL in the future.

Prognosis

MCL is a clinically aggressive B cell malignancy, whereas their clinical courses are quite variable with survival ranges from a few months to more than 10 years.^{81–83} Efforts have been made to identify risk factors associated with clinical outcome during the last several years. The prognostic significance of a different morphologic variant is controversial, even though blastoid/pleomorphic variants have shown unfavorable prognosis in the majority of studies.⁸⁴ Lack of reproducibility in morphologic observation among pathologists may be one of the factors that attributes to the inconsistency. Clinical features, such as International Performance Index (IPI) and staging, have demonstrated significant correlation with patient survival in diffuse large B cell lymphoma and other types of non-Hodgkin lymphoma. However, they have shown no prognostic significance in MCL.^{81,82}

Ki67 as a surrogate marker of proliferation index has been consistently proved to be a reliable prognostic indicator for MCL, and has been used as part of the diagnostic procedure in many centers. Increased expression of Ki67 in MCL is a risk factor for poor prognosis.^{81–83} Variability in scoring of Ki67 expression by immunohistochemical (IHC) staining of the tissue sections is a potential problem. However, a moderate agreement may be reached by category scores.⁸⁵ Some authors have suggested a three tier scoring system, namely less than 10%, 10–40%, and above 40%. Whereas most classical cases of MCL show Ki67 of 20–30%, the more aggressive MCL cases often show a higher proliferative rate and, rarely, some indolent MCL cases may have a proliferative index of less than 10%.

Using gene expression microarray, Rosenwald and et al showed that proliferation signature of genes is highly predictive for the survival of MCL patients.⁴⁵ Based on the results and previous reports, they performed quantitative real time PCR to detect the expression of 33 selected genes with both fresh frozen tissue and formalin-fixed tissue. A model of five genes, including MYC, RAN, SCL29A2, POLE2, and TNFRSF10B, was developed and demonstrated strong predictive value for the survival of MCL patients. High expression of MYC, RAN, SCL29A2, and POLE2 genes correlated to a worse prognosis; whereas, an increased expression of TNFRSF10B correlated with a more favorable clinical course.⁸⁶ MYC gene dysregulation is commonly involved in many hematologic malignancies. Ran gene encodes a guanosine triphosphatase binding protein associated with the control of RNA and protein transportation, DNA synthesis, cell cycle progression, and cell proliferation. POLE2 is a component of DNA polymerase and participates in the regulation of DNA replication and cell cycle control. SCL29A2 is a nucleoside carrier, and transports many nucleosides, such as purines and pyrimidines. TNFRSF10B is a member of TNF receptor superfamily and triggers apoptosis upon activation by cytokine TRAIL (TNF-related apoptosis inducing ligand). If this observation is proven, a risk-based practical model may be used at initial diagnosis to stratify the patients before making a decision if an aggressive treatment should be given to these patients.

Therapy

MCL is clinically aggressive and considered incurable using the current chemotherapy regimen. The median overall survival of MCL patients is about 3–5 years. The benchmark chemotherapy regimen is CHOP plus rituximab. Although more intensive fractionated chemotherapy (i.e., Hyper-CVAD) plus rituximab followed by bone marrow stem cell transplantation achieves a higher therapeutic response with up to 70–90% complete remission, its use is discouraged because of severe toxicity and limited improvement in overall survival. More recently, novel agents, such as bortezomib, mammalian target of rapamycin (mTOR) inhibitors, cladribine, and thalidomide have provided new hope for patients with MCL.^{87–89}

Summary

MCL is an aggressive malignant lymphoma with distinct pathologic and genetic features. Chromosome t(11;14) translocation with cyclin D1 overexpression in MCL is considered an initial event of pathogenesis in majority of the cases. Multiple secondary genetic events, which may play important roles in lymphomagenesis, have been identified and provide new information regarding the pathogenesis of this tumor. MCL lymphoma appears to be a heterogeneous disease, both morphologically and clinically. Further studies to determine the correlation of these secondary genetic events with clinical features will aid in our understanding of the pathogenesis and progression of the disease. Combined targeted molecular therapy may then become possible, and provide new hope for the cure of MCL patients.

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Diffuse Large B-Cell Lymphomas

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Introduction

De novo diffuse large B-cell lymphoma (DLBCL) is the most common lymphoma worldwide, accounting for approximately 30–40% of adult non-Hodgkin lymphomas. It is defined as a diffuse proliferation of large neoplastic B lymphoid cells with a nuclear size equal to or exceeding that of a normal macrophage or more than twice the size of a normal lymphocyte. As such, de novo DLBCL is heterogeneous in morphology, immunophenotypic features, and biologic behavior, including response to chemotherapy and ultimate outcome. In this chapter, we discuss the applications of gene expression profiling (GEP) to this large, heterogeneous group of lymphoma, in terms of diagnosis, differentiation from other B-cell lymphomas, and biologic behavior.

Distinction of De Novo DLBCL from DLBCL of Follicular Origin

Follicular lymphomas (FLs) may progress/transform to DLBCL and most DLBCLs are of follicle center cell origin; however, DLBCLs may also arise from de novo. In a study by Lossos et al, DLBCLs derived from FLs were clearly distinguishable from de novo DLBCLs by GEP.¹ The most prominent differences were in proliferation and cell cycle genes, c-MYC target genes, MHC genes, and a miscellaneous group of other genes, including CD20, CD52w, WAS, LYN, and SYK. When compared with de novo FL, the GEP of transformed DLBCL was closer to FL than to de novo DLBCL. In the transformed group, proliferation, basic metabolism, and invasion related genes were all upregulated, while antiapoptotic genes and accessory and T-cell related genes were downregulated.² In another study by Huang et al, all DLBCLs carrying a translocation (14;18) belonged to the germinal center B (GCB)-like group.³ As will be discussed, the distinction between these entities is important for prognostic implications.

Subgrouping of DLBCL and Prediction of Prognosis

The sentinel study by Alizadeh et al revealed that the GEPs of DLBCLs were largely distinct from those of CLL and FL and showed additional biological complexity.⁴ The genes that defined germinal center B-cell (GCB)-like DLBCL were highly expressed in normal GC B-cells. In contrast, most of the genes that defined activated B-like (ABC) DLBCL were not expressed in normal GC B cells. Known markers of GC differentiation included CD10, CD38, the nuclear factor A-myb, the DNA repair protein 8-oxoguanine DNA glycosylase (OGG1), bcl-6, and a host of new genes (bcl-7A and LMO2). The ABC-DLBCL signature also included a gene translocated in lymphoid malignancies, IRF4 (MUM1/LSIRF). Another notable feature of the GEP of ABC-DLBCLs was the expression of two genes whose products inhibit programmed cell death: FLIP (FLICE-like inhibitory protein, which can block apoptosis) and the key antiapoptotic gene bcl-2 (which is fourfold higher than in germinal center B-cells). Of note, this overexpression did not correlate with the bcl-2 translocation.

Importantly, GCB-like and ABC-DLBCLs were associated with statistically significant differences in OS ($p < 0.01$) and event-free survival (EFS). 76% of GCB-like DLBCL patients were still alive after 5 years, as compared with only 16% of ABC-DLBCL patients. This difference held up when compared within the International Prognostic Index (IPI) score. Thus, the GEP of DLBCL and the IPI apparently identify different features of these patients that influence survival.

Rosenwald et al later used DNA microarrays (MAs) and identified three gene-expression subgroups of DLBCL based on hierarchical clustering (HC): GCB-like; ABC; and type 3 DLBCL (not expressing either set of genes at a high level and associated with an intermediate outcome) (Figure 22.1).⁵ There were four GE signatures that correlated with survival: GC B-cells, proliferating cells (“proliferation signature”), reactive stromal and immune cells in the lymph node (“LN signature”),

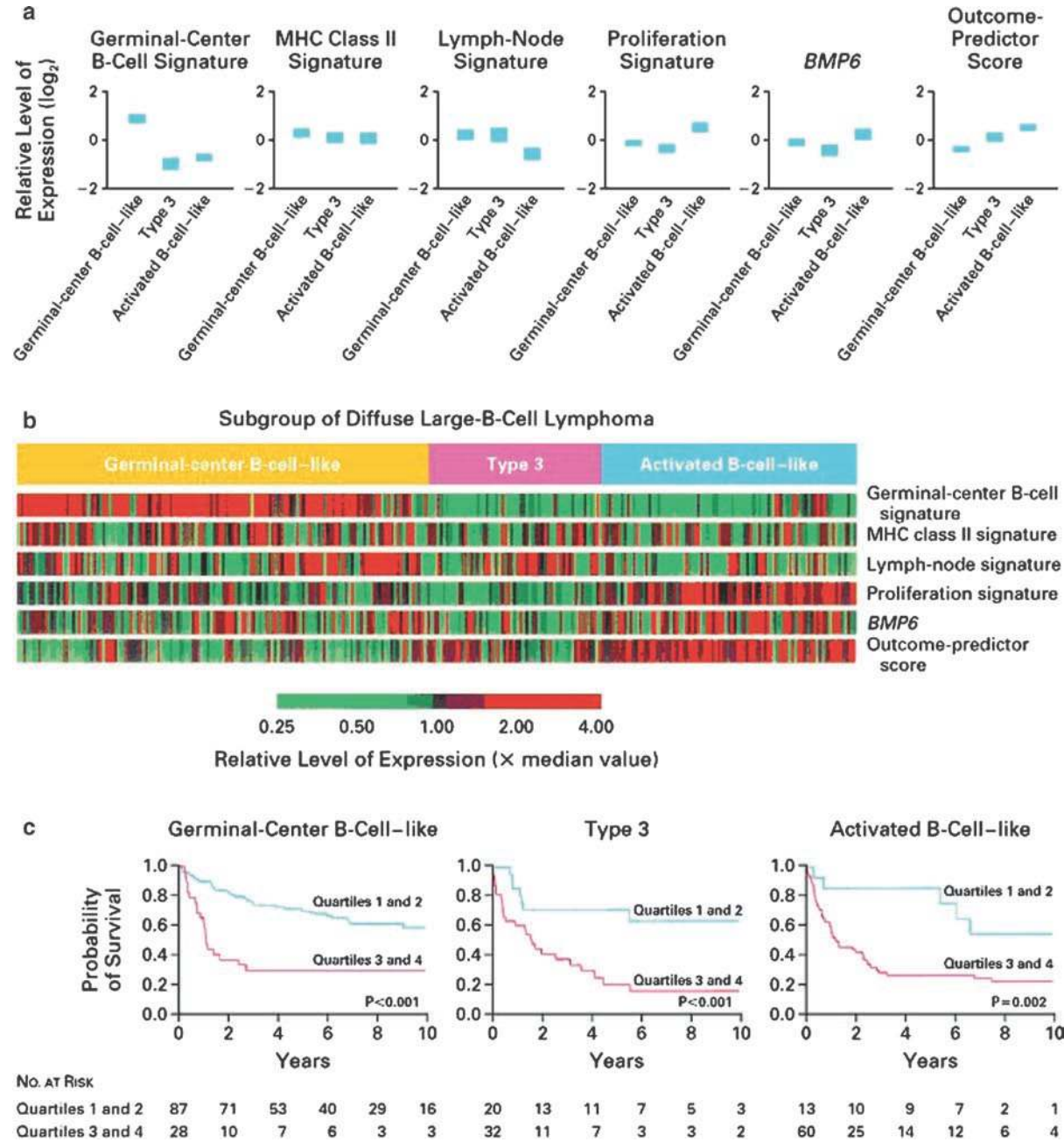


FIG. 22.1. Subgroups of diffuse large-B-cell lymphoma according to gene-expression profiles. Hierarchical clustering of diffuse large-B-cell lymphomas from 240 patients with untreated disease and 34 patients who had previously been treated or who had a preexisting low-grade lymphoma, according to the level of expression of 100 genes is shown. *Red areas* indicate increased expression, and *green areas* decreased expression. Each column represents a single diffuse large-B-cell lymphoma, and each row represents a single gene. Genes that are characteristically expressed in germinal-center-B-cell-like diffuse large-B-cell lymphomas or activated B-cell-like diffuse large-B-cell lymphomas are indicated. The dendrogram at the top shows the number of samples with amplification of the *c-rel* locus and *bcl-2* trans-

locations in subgroups of diffuse large-B-cell lymphoma. The ratio of genomic copy number for the *c-rel* and β (β)₂-microglobulin loci was determined by a quantitative polymerase-chain-reaction assay, and ratios greater than 2 were considered to indicate *c-rel* amplification. The *bcl-2* translocations were detected with the use of a PCR assay for the main break-point cluster region that is frequently involved in the t(14;18) translocation. Data are from patients who had untreated diffuse large-B-cell lymphomas without preexisting cancer. Panel C shows Kaplan-Meier estimates of overall survival after chemotherapy among the 240 previously untreated patients, according to the gene-expression subgroup. (Reprinted from Rosenwald et al,⁵ with permission.)

or major-histocompatibility-complex class II complex. Seventeen genes, including genes from these four signatures and BMP6 (associated with poor outcome), were used to construct a predictor of OS after chemotherapy. The GCB-like subgroup had the highest 5-year survival rate (60%), compared to 35% for the ABC group and 39% for the type 3 DLBCL group. This gene-based predictor and the IPI were independent prognostic indicators. Of interest, two common oncogenic events in DLBCL (i.e., *bcl-2* translocation and *c-rel* amplification) were detected only in the GCB-like subgroup; the ABC subgroup had the highest level of expression of the “proliferation signature” and BMP6 and the lowest level of expression of the “LN signature.” Wright et al further described that the ABC subgroup expressed a subset of genes characteristic of plasma cells, particularly those encoding endoplasmic reticulum and golgi proteins involved in secretion; they also confirmed that GC and ABC subgroups had significantly different 5-year survival rates after multiagent chemotherapy (62 vs. 26%; $p=0.0051$).⁶

Of further interest and significance, Wang et al used first self-organizing map (SOM) with subsequent HC and K-means clustering and identified four prominent GEP patterns in DLBCL, distinguished by gene clusters 10, 11, 1, and the large group of clusters 7 and 9.⁷ Cluster 10 contained genes expressed in GC B cells (FAK, WIP, CD10, CD27, CD38, FMR2, *bcl-6*, and *bcl-7A*). Cluster 11 contained genes specifically expressed by T-cells (CD3, CD2, TCR), NK cells (NK4), macrophages (CD14, CD63, CD64, CD115), lymph node dendritic cells (S100), and genes coding for chemokines and chemokine receptors (RANTES, BLC, IP10, SLC, FPR, STRL33.1, and MIP1), which play a role in the chemo-attraction of inflammatory cells. DLBCL variably expressed genes in the adjacent clusters 1, 7, and 9. Cluster 1 included genes associated with proliferation (Ki-67, cyclin A, BUB1, cyclin B1, thymidine kinase); whereas, clusters 7 and 9 include genes associated with cell survival (*bcl-XL*, defender against cell death 1, Bfl-1, BAK, Bag-1, MCL1) and plasma cell differentiation (XBP-1, STAT3, IRF-4, ribosomal proteins). Similar to the previous results, they confirmed the better survival for cases expressing GC-related genes (gene cluster 10). In addition, they showed a significant improved survival of cases expressing inflammation-related genes (gene cluster 11) and a significantly reduced survival of cases expressing genes related to cell proliferation, antiapoptosis, and plasma cell differentiation (clusters 1,7,9).

In contrast to the sentinel study by Alizadeh, Shipp et al subsequently reported successful outcome prediction in a series of 58 DLBCL patients using GE data from oligonucleotide MAs with supervised learning methods and identified molecular correlates of outcome that were *independent* of the “cell of origin” distinction previously described, suggesting that additional factors may be important in determining therapeutic response of DLBCL.⁸ Genes implicated in DLBCL outcome included ones that regulate responses

to B-cell-receptor signaling (PKC- β (beta)), critical serine/threonine phosphorylation pathways (PDE4B), and apoptosis (NOR1). All three of these outcome genes regulate apoptotic responses to antigen-receptor engagement and, potentially, cytotoxic chemotherapy. PKC- β (beta) was overexpressed in fatal/refractory DLBCL. The consequences of B-cell-receptor signaling were dependent upon associated activation of PKC- β (beta). In the presence of an intact PKC- β (beta) pathway, B-cell-receptor engagement resulted in B-cell proliferation. These studies suggest that PKC- β (beta) activity enhances B-cell proliferation and survival. The cyclic AMP (cAMP)-specific phosphodiesterase (PDE4B) was also overexpressed in fatal/refractory DLBCL. It may also be an attractive therapeutic target in fatal/refractory DLBCLs. The mitogen-inducible nuclear orphan receptor (MINOR), or NOR1, was overexpressed in cured DLBCL. It is possible that NOR1 increases the apoptotic response to chemotherapy in curable DLBCL.

When analyzed as single markers in Alizadeh’s original dataset, NOR1 ($p=0.05$) and PDE4B ($p=0.07$) were clearly correlated with outcome. In addition, two clones specific for the PKC- β (beta) isoform also correlated with outcome in this dataset ($p=0.04$). Thus, these results from an independent dataset supported the previous results and highlighted the value of publicly accessible GE databases for rapid, computational validation of hypotheses.

The potential extension of MA-based outcome prediction was further explored using immunohistochemistry (IHC). A tissue array of the study DLBCLs was analyzed by IHC for PKC- β (beta). Its protein expression was highly correlated with MA-determined transcript abundance in the DLBCLs and was closely associated with worse clinical outcome in these patients ($p=0.03$).

Interestingly, as seen in the studies by Rosenwald et al, Wang et al, Glas et al, and Dave et al, the presence of a prominent T-cell and follicular dendritic-cell signature in the FLs also indicated that MA GEP can be used to capture additional nonmalignant components of the tumor microenvironment. This study also highlighted the important difference between unsupervised (clustering) and supervised machine learning analytical approaches.

Lossos et al subsequently applied significance analysis of MAs (a supervised method for the identification of genes with a statistically significant association with survival) to the data set of Alizadeh et al in order to identify genes that may have been missed in the unsupervised analyses.⁹ Expression of 36 genes whose expression had been reported to predict survival in DLBCL was measured in 66 independent lymphoma samples by quantitative real-time polymerase chain reaction (qRT-PCR) analyses and the results were related to OS. Then, in a univariate analysis, genes were ranked on the basis of their ability to predict survival and a multivariate model was developed (validated in two independent MA data sets) based on the expression of the six “strongest predictor” genes (LMO2, *bcl-6*, FN1, CCND2, SCYA3, and *bcl-2*).

These genes occur in the GCB-like signature (LMO2 and *bcl-6*), the ABC signature (*bcl-2*, *CCND2*, *SCYA3*), and the LN signature (FN1). *Bcl-6* is known to downregulate the expression of *CCND2* and *SCYA3*. The expression of LMO2, *bcl-6*, and FN1 correlated with prolonged survival. The expression of *Bcl-2*, *CCND2*, and *SCYA3* correlated with shorter survival. None of these genes alone independently predicted OS at a statistically significant level. The model was independent of the IPI and added to its predictive power. However, it should be noted that serum LDH was the only independent predictor of OS ($p=0.004$) and it was not included in the model. This study concluded that measurement of the expression of these six genes is sufficient to predict OS in DLBCL.

A subsequent review, also by Lossos et al in 2006,¹⁰ pointed out that in contrast to *bcl-6* gene rearrangements, which have not been associated with an improved survival, *bcl-6* mRNA or protein expression has been shown to portend an overall longer survival.¹¹ Although *bcl-2* translocations do not correlate with survival, most studies have shown *bcl-2* expression to be an indicator of poor prognosis. HGAL expression is essentially restricted to lymphomas of germinal center origin, and its presence has been shown to be an independent predictor of better outcome in DLBCL. FOXP1 expression is associated with an inferior survival.

Lossos et al also pointed out that the uniqueness of prognostic biomarkers is dependent on the effectiveness of therapy. When several of these markers have been more recently reevaluated in the era of immunochemotherapy [i.e., rituximab plus CHOP (R-CHOP)] for DLBCL, the prognostic advantage or disadvantage appears to be mitigated. For example, the negative impact of *bcl-2* expression is alleviated, as is the unfavorable survival outcome in the non-GC subtype of DLBCL.^{12,13} Mounier et al found that the therapeutic addition of rituximab eliminated the effect of chemotherapeutic resistance in elderly patients with *bcl-2*+ DLBCL, thus resulting in similar outcomes in both groups. Winter et al reported that by administering R-CHOP, the difference between *bcl-6*+ and *bcl-6*− cases was essentially eliminated.¹⁴

Correlation of Immunohistochemical Analyses with GEP Data in DLBCL

Interestingly, a study analyzing the expression and prognostic significance of CD44s (CD44v4, CD44v6, and CD44v9) in DLBCLs: 22 GCB-like (*bcl-6*+/*CD10*+/*bcl-2*−), 25 ABC (*bcl-6*−/*CD10*−/*bcl-2*+), and 35 unclassifiable DLBCLs found that CD44v6 was coexpressed with *bcl-2*, appeared predominantly on *bcl-6*− cases, and correlated with higher disease stage.¹⁵ CD44v6− cases had an OS of 82% at 70 months and CD44v6+, 58%. Expression of CD44v6 correlated with higher disease stage and has been suggested to contribute to lymphoma dissemination. CD44v6 was expressed

predominantly in ABC–DLBCL, and in CD44-negative cases, CD44v6 expression was associated with worse OS.

Linderroth et al analyzed the application of IHC markers (*Bcl-6*, CD10, *bcl-2*, *bax*, CD138, CD40, and CD23) to 125 DLBCL samples to differentiate histogenetic origin and prognosis, as have been described by GEP.¹⁶ *Bcl-6*, CD10, and CD40 were considered markers of GC phenotype, CD23 as pre/early GC origin, and CD138 as postgerminal center origin. *Bcl-2* and *bax* were considered apoptotic regulators. There was no prognostic significance of CD10, *bcl-6*, or CD138 IHC results. CD40 was expressed in 76% of cases, and this group was associated with superior time to treatment failure (FFS) ($p=0.027$) and OS ($p=0.0068$), independent of IPI. CD23 was expressed in 16% of cases (all CD5-negative and all CD40-positive). This group showed a strong tendency for better OS ($p=0.033$). CD40 expression correlated with *bax* but not with *bcl-2* expression.

However, an additional IHC study evaluated if using a panel of GC B-cell (CD10 and *Bcl-6*) and activation (MUM1/IRF4 and CD138) markers defined prognosis in 42 de novo DLBCL patients.¹⁷ They were classified into three expression patterns: (a) GC B-cell pattern expressing CD10 and/or *bcl-6* but not activation markers, (b) activated GC B-cell pattern expressing at least one of the GC B-cell markers and one of the activation markers, and (c) activated non-GC B-cell pattern, expressing MUM1/IRF4 and/or CD138 but not GC B-cell markers. Patients with pattern A had much better OS than those with the other two patterns ($p<0.008$). The IPI scores and the expression pattern of these markers were independent prognostic markers; and, thus, these IHC markers have been practically applied (Table 22.1). Although with the era of immunochemotherapy (i.e., R-CHOP), the unfavorable survival outcome in the non-GC subtype of DLBCL seems to be mitigated, it is the hope that therapeutic regimens will become more customized and tailored to each subtype. Although currently there is no consensus opinion regarding the best IHC model to describe the heterogeneity of DLBCL, identified candidate proteins with known contribution to survival should be examined further in effort to define subtypes with different biologic behaviors, which may benefit from tailored, customized therapy, in an attempt to achieve longer remissions and potential cures.

Correlation of GEP Data and Immunohistochemical Patterns with Cytogenetic Abnormalities in DLBCL

Correlation of GEPs of de novo primary DLBCL with cytogenetic abnormalities, particularly, the occurrence of the t(14;18) (q32;q21) in the GCB-like and the ABC subgroups of DLBCL has shown the t(14;18) being detected in 7/35 (20%) of cases, all with a GCB-like GEP, representing 35% of the cases in this subgroup. Six of these seven cases had very similar GEPs.³ The expression of *bcl-2* (75% in GCB-like vs. 67% in ABC)

TABLE 22.1. Immunohistochemical markers associated with prediction of outcome in DLBCL.

Expression pattern of DLBCL	ICH markers expressed	Association with prognosis
(A) GCB pattern	CD10+ and/or bcl-6+	Best OS
(B) "Activated" GCB pattern	At least 1 GCB marker (CD10, bcl-6) and 1	Worse OS than pattern A
(C) Activated non-GCB pattern	mum1 and/or CD138	Worse OS than pattern A

Based on GEP data.¹⁷

GCB germinal center B-cell.

and bcl-6 proteins was not significantly different between the t(14;18)+ and -negative cases. However, CD10 was detected only in the GCB-like GEP group (55% of these cases), and CD10 was most frequently expressed in the t(14;18)+ cases. This study supported the validity of subdividing DLBCL into two major subgroups by GEP, with the t(14;18) being an important event in the pathogenesis of a subset of GCB-like DLBCL. This study also supported CD10 protein expression as useful in identifying cases of GCB-like DLBCL and as often expressed in cases with t(14;18).

Similarly, a study evaluating for the 14;18 translocation by fluorescent in situ hybridization (FISH) in 141 DLBCLs, previously analyzed by GEP, detected this translocation in 17% of DLBCLs and in 34% of the GCB-like group, which contained the vast majority of positive cases.¹⁸ In addition, 12 t(14;18)+ cases detected by PCR on additional samples were added to the FISH+ cases. In contrast to the previous study, IHC indicated bcl-2, bcl-6, and CD10 protein were preferentially expressed in the 14;18+ cases as compared to the 14;18- cases. Within the GCB-like subgroup, the expression of bcl-2 and CD10, but not bcl-6, differed significantly between cases with or without the 14;18 translocation: 88 vs. 24% for bcl-2 and 72 vs. 32% for CD10, respectively. In the GCB-like subgroup, a heterogeneous group of genes was overexpressed in the t(14;18)+ subset, among which bcl-2 was a significant discriminator. The t(14;18)- subset within the GCB-like subgroup was dominated by overexpression of cell cycle-associated genes, indicating that these tumors are significantly more proliferative, suggesting distinctive pathogenetic mechanisms. However, despite this higher proliferative activity, there was no significant difference in survival (OS or FFS) between the t(14;18)+ and t(14;18)- subsets within the GCB-like subgroup.

Future Directions

Interestingly, several known downstream targets of the NF- κ (kappa)B transcription factors were expressed highly in many cases of ABC-DLBCL and not GCB-like DLBCL.¹⁹ The NF- κ (kappa)B family is a group of homo- and heterodimeric transcription factors that play critical roles in the development, lymphocyte activation, and the prevention of apoptosis.²⁰ NF- κ (kappa)B transcription factors are latent in the cytoplasm of cells in a complex with a

member of the I κ B family of inhibitory proteins. These findings establish the NF- κ (kappa)B pathway as a new therapeutic target for those DLBCL cases that are refractory to current therapies.²¹ Also, as mentioned previously, the extrapolation of the GEP data and application of immunohistochemical markers have helped define subtypes with different biologic behaviors, which may benefit from tailored, customized therapy, in an attempt to achieve longer remissions and potential cures.

Differentiation from Burkitt Lymphoma

DLBCL may usually be differentiated from Burkitt lymphoma (BL) by the defined characteristics for BL. Characteristics set for BL include a typical morphology, a mature B-cell phenotype of CD10+, Bcl-6+ and BCL2- tumor cells, a proliferation rate of >95%, and the presence of C-MYC rearrangements in the absence of t(14;18)(q32;q21).²²

Of interest, comparison of the GEPs of cell lines from transformed FL (tFL), EBV- BL, EBV+ BL, and de novo DLBCL with HC, based upon the levels of 43 genes, has highlighted characteristic expression patterns of the first three lymphoma subtypes.²³ Genes expressed at higher levels in tFL than in BL included calcium/calmodulin-dependent protein kinase (CAMK1) and mitogen-activated protein kinase 10 (MAPK10). EBV-negative BL was characterized by high-level expression of amyloid β (beta) precursor protein (APP), heat shock 27 kD protein 1 (HSPB1), and mothers against decapentaplegic homolog (MADH1). Gardner-Rasheed feline sarcoma viral oncogene homolog (FGR) was the most significant gene to delineate EBV+ BL. A subtype prediction algorithm using 34 genes correctly classified 92% of the first three types of lymphoma. By comparison with normal B-cells, the expression patterns of the selected genes were characteristic of lymphomas. The study extended the HC analysis to cell lines from de novo DLBCL. The de novo DLBCL cell lines either separated from the first three types of lymphoma or segregated with the EBV+ BL, possibly reflecting variable genetic abnormalities. The associations of CAMK1 with tFL, APP and MADH1 with EBV- BL, FGR with EBV+ BL, and bcl-2 with tFL and DLBCL were confirmed by RT-PCR. This study provided new molecular markers, expressions of which were closely associated with BCL subtypes.

Differentiation of De Novo DLBCL, De Novo CD5+ DLBCL, and Mantle Cell Lymphoma

Obviously, de novo DLBCL is usually differentiated from de novo CD5+ DLBCL by the expression of CD5, which may be detected by flow cytometry or IHC. More challenging is the differentiation of CD5+ DLBCL from a “large cell,” or aggressive blastoid, variant of mantle cell lymphoma (MCL).

Mantle cell lymphoma is known to be associated with the t(11;14)(q13;q32), resulting in deregulated cyclin D1 expression.²⁴ High levels of cyclin D1 are associated with higher proliferation and poorer survival.²⁵ Determination of these abnormalities are important for a diagnosis of MCL

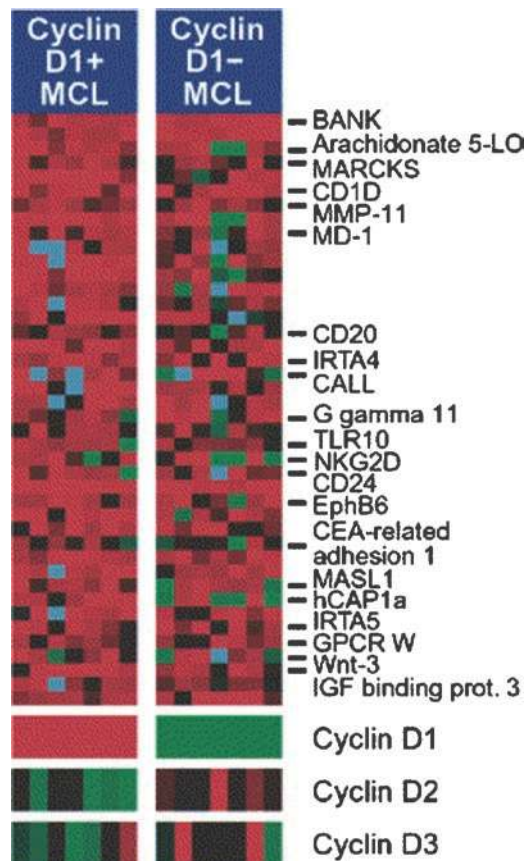


FIG. 22.2. Expression of MCL signature genes in seven cyclin D1-positive and seven cyclin D1-negative lymphoma cases. Cyclin D1-negative cases had MCL morphology and immunophenotype and were classified as MCL based on their gene expression profile. Shown is the relative gene expression of cyclin D1 (as measured by quantitative RT-PCR) and cyclins D2 and D3 (as measured by DNA microarray analysis). (Reprinted from Rosenwald et al,²⁶ with permission.)

and have conventionally been detected by routine cytogenetics and FISH techniques for the t(11;14) and IHC for the cyclin D1 protein over-expression, respectively.

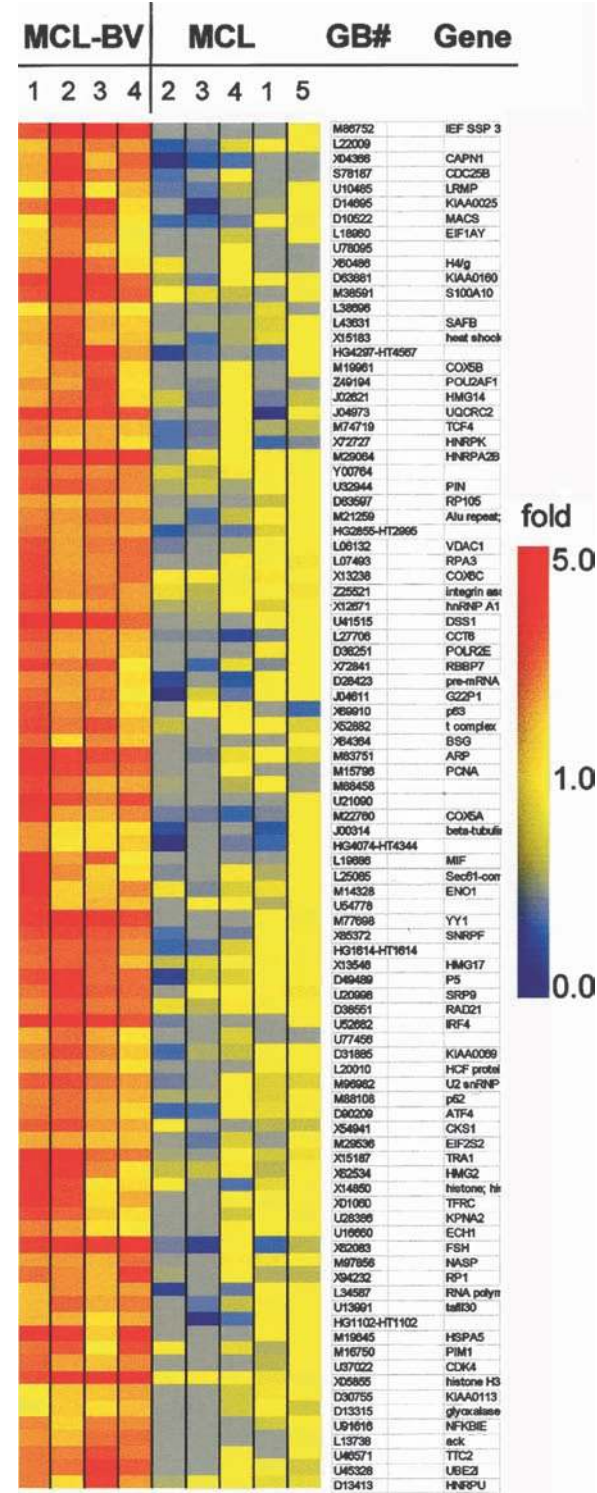


FIG. 22.3. Hierarchical clustering of differentially expressed genes in MCL and MCL-BV. The genes displayed were identified by two independent search strategies. Five samples of MCL and four samples of MCL-BV are shown. Red increased expression, blue decreased expression, yellow unchanged. (Reprinted from de Vos S et al,²⁷ with permission.)

More recently, a GEP study was performed on 101 lymphoma cases morphologically consistent with MCL.²⁶ Of these, 92 cases showed high expression of cyclin D1 mRNA by quantitative RT-PCR. More than 1,000 genes were found to be differentially expressed between cyclin D1+ MCLs and other lymphoma subtypes with high statistical significance. A GE-based predictor of MCL was fashioned from 42 of the most discriminatory genes, yielding a “MCL signature.” Of note, cyclin D1 was excluded to test whether cyclin D1– MCLs could be identified by the predictor. The predictor correctly classified 98% of cyclin D1+ MCLs. More interestingly, of the nine cyclin D1– MCLs, seven were classified as MCL by their expression of the “MCL signature” genes. Of interest, three of these tumors expressed high levels of cyclin D3 or cyclin D2, suggesting that these proteins may functionally substitute for cyclin D1 in these MCLs (Figure 22.2). Existence of cyclin D1– MCL with an “MCL signature” GEP, some with overexpression of cyclin D2 or D3, has been confirmed in an additional study, indicating overexpression of cyclin D1-related cyclins may have a pathogenetic role in these cases.²⁵

In the aggressive blastoid variant of MCL (MCL-BV), overexpression of genes involved in cell cycle control at the G1/S and G2/M checkpoints and in apoptosis inhibition has been identified. Studies using gene array and comparing the GEPs of micro-dissected normal mantle cells (NMCs), MCL, and MCL-BV by oligonucleotide MAs and qRT-PCR have identified 118 genes with significant differential expression between MCL and MCL-BV, including tumor suppressors, transcription factors, proto-oncogenes, and genes associated with cell cycle, proliferation, chromatin assembly, and mitosis/spindle assembly. The highly expressed cyclin dependent kinase 4 (CDK4) is a cell cycle kinase that associates with cyclin D1 for the progression through the G1/S checkpoint; whereas, overexpression of cdc28 protein kinase 1 (CKS1) blocks the inhibition of the cyclinD1/CDK4 complex by the CDK inhibitor p27/Kip1. Other highly expressed genes in

MCL-BV that promote cells through the G1/S checkpoint include the oncogenes (B-Myb, PIM1, and PIM2), and passage through the G2/M checkpoint is enhanced by high levels of cdc25B. In addition, two highly expressed genes that inhibit apoptosis are defender against cell death (DAD1) and RSK1. In addition, the transcription factor YY1, which is involved in cyclin D1 overexpression has been shown to be increased in MCL–BV (Figure 22.3).^{27,28} These findings suggest a potential pathogenetic role of these genes in the evolution of MCL–BV.

Furthermore, analysis of GEP, using cDNA MA technology, in 9 CD5– DLBCLs, 11 de novo CD5+ DLBCLs, and 10 MCLs has identified a series of genes distinguishing these three lymphoma types.²⁹ Integrin β (beta)1 (also confirmed by IHC) and/or CD36 adhesion molecules were overexpressed in most cases of CD5+ DLBCL. Integrin β (beta)1 over-expression may account for the high extranodal involvement and poor prognosis of CD5+ DLBCLs. Of interest, CD36 was overexpressed on vascular endothelia in CD5+ DLBCLs, although there was no difference in vascularity detected by von Willebrand factor antibody between CD5+ and CD5– DLBCLs. These results suggested that CD5+ and CD5– DLBCLs have different gene expression signatures in both tumor cells and their vascular systems.

AIDS-Related DLBCL

Patrone et al extensively analyzed 18 candidate genes and showed distinct patterns of expression in both AIDS–DLBCL and DLBCL samples.³⁰ However, none of these genes were preferentially associated with either AIDS–DLBCL or DLBCL. The study data suggested that the increased incidence and severity of AIDS–DLBCL compared to DLBCL is likely due to crippled immune surveillance rather than to markedly different GEPs.

TABLE 22.2. Characteristic features of PCFCL and PCLBCL, leg type.

	PCFCL	PCLBCL-leg
Morphology	Predominance of centrocytes that are often large, especially in diffuse lesions. Centroblasts may be present, but not in confluent sheets. Growth pattern may be follicular, follicular and diffuse, or diffuse (a continuum without distinct categories or grades).	Predominance or confluent sheets of medium-sized to large B cells with round nuclei, prominent nucleoli, and coarse chromatin resembling centroblasts and/or immunoblasts. Diffuse growth pattern.
Phenotype	Bcl-2: –/+ ^a Bcl-6: + CD10: –/+ ^c FOXP1–	Bcl-2: ++ ^b Bcl-6: +/- CD10: – FOXP1+
Clinical features	Middle-aged adults. Localized lesions on head or trunk (90%). Multifocal lesions in rare cases.	Elderly, especially females. Lesions localized on leg(s), most often below the knee. Rare cases with lesions at other sites than the leg (10%).

^aFaint staining, when present; minority of cells.

^bStrong staining; in most neoplastic cells.

^cDiffuse lesions, mostly CD10–.

Cutaneous Large B-Cell Lymphoma

According to the WHO-EORTC classification, primary cutaneous DLBCLs are divided into those of follicle center cell origin (PCFCL), those of “leg type” (PCLBCL-leg), and those designated “other,” representing rare cases of LBCL arising in the skin, which do not belong to the first two types.³¹ This latter subtype includes morphologic variants of DLBCL, such as anaplastic or plasmablastic subtypes or T-cell/histiocyte rich large B-cell lymphomas. Such cases are generally a skin manifestation of a systemic lymphoma. The characteristic features of these first two subtypes are outlined in Table 22.2. In addition, FISH analysis for t(14;18) (IGH;BCL2) has revealed a substantial proportion (41%) of PCFCLs with this abnormality.³²

Hoefnagel et al further investigated the GEPs of 21 primary cutaneous large B-cell lymphomas (PCLBCLs) by oligonucleotide MA analysis to establish a molecular basis for their subdivision into PCFCL and PCLBCL-leg.³³ HC, based on a B-cell signature of 7,450 genes, classified PCLBCL into two distinct groups consisting of 8 PCFCL and 13 PCLBCL-leg. PCLBCL-leg showed increased expression of genes associated with cell proliferation, the proto-oncogenes Pim-1, Pim-2, and c-myc, and the transcription factors MUM1/IRF4 and Oct-2. Pim-kinases are known to cooperate with c-myc and N-Myc to generate T- and B-cell lymphomas. In the group of PCFCL, high expression of SPINK2 was observed. The SPINK2 gene encodes a Kazal type serine threonine kinase with ill-defined functions in physiological and pathological cellular processes. Further analysis suggested that PCFCL and PCLBCL leg have expression profiles similar to that of GCB-like and ABC–DLBCL, respectively. These results suggested different pathogenetic mechanisms are involved in the development of these lymphomas, and their GEPs may predict prognosis as in the distinction of their noncutaneous DLBCL counterparts

Primary Mediastinal Large B-cell Lymphoma

Primary mediastinal large B-cell lymphoma (PMBL) is a subtype of DLBCL, arising in the mediastinum and of putative thymic B-cell origin, with distinctive clinical, immunophenotypic, and genotypic features. It is characterized by the following immunophenotype: CD45+, CD19+, CD20+, CD30+ (weak), CD10–, CD5–, and CD15–.³⁴

Of interest, PMBL demonstrates a unique GEP that bears similarity to that of Hodgkin lymphoma (HL)-derived cell lines. In a study by Rosenwald et al, over one third of the genes that were more highly expressed in PMBL than in other DLBCLs were also characteristically expressed in HL cells (Figure 22.4).³⁵ In this study, PDL2, which encodes a regulator of T-cell activation, was the gene that best discriminated

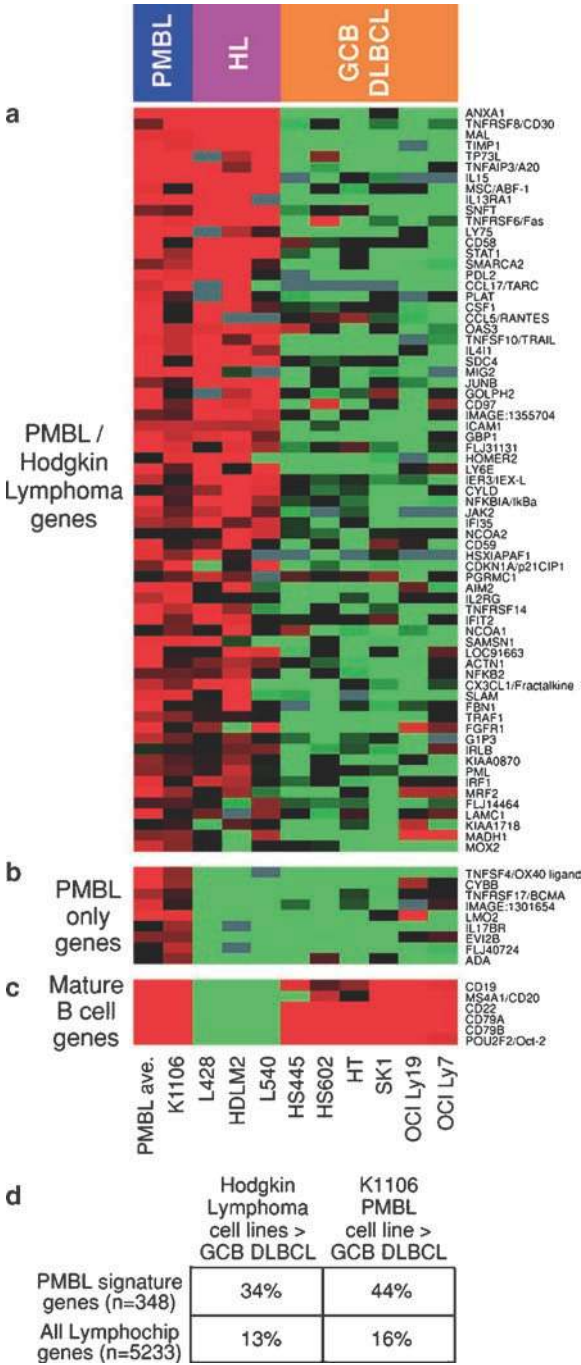


FIG. 22.4. Relationship of PMBL to Hodgkin lymphoma. Relative gene expression is shown in primary PMBLs (average of all biopsy samples), the PMBL cell line K1106, three Hodgkin lymphoma (HL) cell lines, and six GCB DLBCL cell lines. (a) PMBL signature genes that are also expressed at high levels in Hodgkin lymphoma cell lines compared with GCB DLBCL cell lines. (b) PMBL signature genes not expressed in Hodgkin lymphoma cell lines. (c) Mature B cell markers expressed in PMBL and GCB DLBCL but not in Hodgkin lymphoma. (d) Enrichment within the set of PMBL signature genes of genes highly expressed in Hodgkin lymphoma cell lines or in the K1106 PMBL cell line relative to GCB DLBCL cell lines. (Reprinted from Rosenwald A et al,³⁵ with permission.)

PMBL from other DLBCLs and was also highly expressed in HL cells. Savage et al reported that mediastinal LBCLs had low levels of expression of multiple components of the B-cell receptor (BCR) signaling cascade, a profile resembling that of classical HL (cHL) cells.³⁶ Like cHL, PMBL also had high levels of expression of interleukin 13 receptor and downstream effectors of IL-13 signaling [(Janus kinase-2 and signal transducer and activator of transcription (STAT1) (also confirmed by IHC)], tumor necrosis factor (TNF) family members, and TNF receptor-associated factor-1 (TRAF1) (also confirmed by IHC). In addition, in almost all PMBLs, c-REL was localized to the nucleus, consistent with activation of the NF- κ (kappa)B pathway. These studies identified a molecular link between PMBL and cHL and a shared survival pathway. The findings of low levels of expression of BCR signaling pathway components, a distinctive cytokine pathway signature, and activation of NF- κ (kappa)B are strikingly similar to cHL. The distinction between DLBCL and PMBL is important, since interestingly, PMBL patients had a better 5-year survival rate (64%) than all DLBCL patients after therapy (46%).³⁶

Summary

Gene expression profiling has contributed significantly to defining the differences in biologic behavior of de novo DLBCL and in distinguishing this group of lymphoma from other types of DLBCL and Burkitt lymphoma. Continued extrapolation of the data to immunohistochemical markers should help in applying the GEP data to clinical trials for better therapeutic customization in this large, heterogeneous group of lymphoma.

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The Molecular Pathology of Burkitt Lymphoma

Claudio Mosse and Karen Weck

History and Clinical Features of Burkitt Lymphoma

In 1958, Dr. Denis P. Burkitt reported on a series of 32 children presenting with large malignant tumors of the jaw at Mulago Hospital in Uganda and six other district hospitals.¹ The syndrome was notable for starting in the mandible and often spreading to other jaw quadrants, as well as to the adrenals, kidneys, and liver. No involvement of spleen or lymph nodes was detected in these initial 38 patients. Of note, in that initial report, the histopathology was described as “strongly resembling lymphocytes... [and] in some cases the tumor [resembled] a lymphosarcoma.” Definitive classification of this as a lymphoma would await O’Conor and Davies’ description of these and other cases in 1960.²

Of interest, the peculiar distribution of this tumor in the malaria belt of Africa was noted at the outset, with a few cases being reported in North or South Africa, and the bulk of cases coming from tropical areas. To better delineate the regions of endemic tumor, Burkitt and two companions, Drs. Ted Williams and Cliff Nelson, traveled 10,000 miles through ten countries in order to map the tumor endemic areas of Africa.³ They also took this opportunity to measure the extent of other diseases in different areas. The end result of this “geographical biopsy” was a map that showed tumor prevalence only in areas with mean temperatures consistently over 15°C and rainfall over 20 in. per annum. This map very accurately predicted the areas of endemic “Burkitt lymphoma” (BL), as well as intense endemic malaria. This extensive epidemiologic work linking malaria and BL was confirmed in more detailed examinations of differentially affected areas of Africa,⁴ as well as comparing malaria-protected sickle-cell trait children with controls,⁵ and in mouse studies of lymphomagenesis.⁶

Since Burkitt’s original description in 1958, BL has been described outside of the malaria belt of Africa. Interestingly, there are notable clinicopathologic differences between African (or endemic) BL and the sporadic BL described in

the developed world.⁷ Sporadic Burkitt lymphoma represents approximately 1–2% of lymphomas in Western Europe and the USA, although in pediatrics it represents 40% of all lymphomas. Sporadic BL typically involves lymphoid tissue in the terminal third of the ileum or Waldeyer’s ring. Although massive abdominal involvement is not rare in sporadic BL, jaw involvement is distinctly rare. Furthermore, advanced cases of the sporadic type present with bone marrow involvement or circulating lymphoma cells. Previously, these were diagnosed as mature B cell acute lymphoblastic leukemia (ALL) or L3-morphology ALL. The FAB classification of L3 ALL is now considered leukemic presentation of BL in the most recent WHO classification.

More recently, a third clinical subset of BL has been described in patients with immunodeficiency and is most commonly seen in patients with HIV infection.^{8–12} Immunodeficiency-associated BL is interesting in that unlike other HIV-associated B cell lymphomas it typically occurs in patients with CD4 counts greater than 200 cells per microliter. In fact, because HIV symptoms are often absent at this CD4 count, lymphoma can be the AIDS-defining criterion. Compared to other HIV-associated B cell lymphomas, BL patients are typically younger with higher CD4 counts and with a shorter history of HIV infection. Induction of HAART therapy augments chemotherapy in these cases.¹³ Solid organ transplant patients are another population of patients with immunodeficiency-associated BL. These patients typically present with BL four to 5 years after organ transplantation. Again, restoration of immune function by relieving immunosuppression may augment response to therapy. Stem cell transplant patients are less likely to present with immunodeficiency-associated BL, likely reflecting their lower levels of immunosuppression. Immunodeficiency-associated BL is the subtype to most commonly involve lymph nodes. Of interest, these three clinical subtypes – endemic, sporadic and immunodeficiency-associated – all have similar histologic, immunophenotypic, and molecular presentations, although there are unique features to each.

EBV and Burkitt Lymphoma (Also See Chap. 7)

In 1961, soon after Burkitt described this lymphoma, he began collaboration with Dr. M.A. Epstein, a young experimental pathologist. Three years later, Epstein discovered that the endemic BL specimens were all infected by a novel virus that would later be called Epstein–Barr virus (EBV).¹⁴ This was the first virus shown to be associated with a human malignancy. The role of EBV in lymphomagenesis would continue to be unraveled over the next 45 years with active basic research still underway.

Interestingly, although EBV is found in over 95% of endemic BLs, it plays a less significant role in sporadic and immunodeficiency-associated BL. Most series in the literature from Western Europe and North America have consistently shown EBV infection in 5–30% of sporadic BLs, depending on the population in the study. Of note, some developing countries (such as Brazil and Egypt) have shown an intermediate rate of EBV association, with 60–80% of “sporadic” BLs showing EBV infection.^{15–17} Immunodeficiency-associated BL is associated with EBV infection in approximately one out of three cases.¹⁸ These varying rates of infection with EBV in the different clinical subtypes of BL clearly demonstrate that EBV infection is not necessary for transformation to BL. In that case, what is the role for EBV when it is associated with BL?

EBV is a ubiquitous gamma-herpes virus that infects approximately 95% of people in most populations studied and establishes life-long latency in B cells. EBV has evolved a complicated pattern of latency programs, which allows the virus to manipulate B cell differentiation and establish long-term latency in the memory B cell reservoir.¹⁹ There are three patterns of EBV latent infection.²⁰ Latency III is characterized by expression of all EBV latency genes, including Epstein–Barr nuclear antigens (i.e., EBNA1, 2, 3a, 3b, 3c and LP), latent membrane proteins (i.e., LMP1 and 2), and small noncoding RNAs (called EBERs). This pattern of infection occurs during primary infection of B cells and in EBV+ posttransplant lymphoproliferative disorders (PTLDs). EBV Latency III is associated with B cell activation and proliferation, primarily because of the roles of LMP1 and EBNA2 in B cell transformation. In germinal center B cells, EBV switches to Latency II, expressing EBNA1 and the LMPs. Latency II expression may drive B cell differentiation into memory B cells, the long-term latent reservoir of EBV, in which all latent protein expression may be downregulated.¹⁹ In BL cells of EBV+ BL, only the EBERs and EBNA1 are consistently seen, regardless of clinical subtype. This pattern of expression is considered the Latency I expression pattern of EBV. BLs with LMP2A expression²¹ or with EBNA1, 3a, 3b, and 3c expression²² have been reported, but likely represent a minority of cases.

The precise role of EBV in lymphomagenesis of BL is still debated. The Latency I program of EBV is not associated with immortalization of B cells, and the bulk of evidence indicates that EBNA1 is not transforming.²³ EBNA1 is expressed in all replicating EBV-infected cells and is required for the maintenance and replication of the viral genome.²⁴ EBV Latency III is associated with immune recognition of infected B cells by cytotoxic T lymphocytes specific for EBV latent proteins and, conversely, immunosuppression may result in the risk of expansion of activated B cells with Latency III EBV expression, as is seen in EBV+ PTLD. On the other hand, Latency I BL cells are immunologically silent and are not recognized by virus-specific cytotoxic T lymphocytes.²⁰ A Gly-Ala repeat domain within EBNA1 has been shown to inhibit antigen processing by preventing proteasome-mediated degradation.²⁵ Decreased expression of HLA class I, TAP, and proteasome subunit LMP7 have all been associated with Latency I expression patterns. These factors may play a role in protecting EBV-infected BL cells from immune surveillance and clearance of infected cells by cytotoxic T cells.

EBV infection may also promote cell survival and protect BL cells from apoptosis. Work with the Akata Burkitt cell line has demonstrated a cell survival role for both EBNA1 and the EBERs via virus-induced upregulation of the *TCL1* oncogene.^{23,26,27} EBV-negative subclones of this cell line are more sensitive to apoptosis induction. EBER expression in EBV-negative subclones has been shown to enhance tumorigenicity and resistance of apoptosis.^{28,29} There is evidence that this may occur through inhibition of the IFN α (alpha)-inducible dsRNA-activated protein kinase PKR.^{30,31} EBERs have also been shown to increase production of IL-10, a known B cell growth factor.³²

While these EBV-associated factors may all contribute to lymphomagenesis, none are absolutely required, since most sporadic and immunodeficiency-associated BLs are EBV-negative. In fact, the only required molecular abnormality in BL is abnormal expression of the *MYC* oncogene. EBV infection, which induces growth and activation of B cells, may produce an environment that is prone to acquiring *MYC* translocations, which may occur as an error of the somatic hypermutation process during B cell differentiation.^{20,23} Subsequent *Myc* upregulation may drive the switch from EBV Latency III to Latency I, as well as other events required for evolution to BL. Interestingly, this latter process has been successfully modeled in vitro.³³ Recent experimental evidence supports the hypothesis that a switch from the growth-promoting, immunogenic EBV Latency III to Latency I may be a key event in progression to BL.^{22,34,35}

The role of malaria infection in the pathogenesis of endemic BL is also not well understood, although it is associated with a 100-fold increased risk of BL. It has been proposed that malarial infection may act as a chronic stimulus of the B-cell system, recruit EBV-infected B cells into germinal centers, and increase the likelihood of *MYC* translocation.^{15,23}

The malarial parasite *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) contains a cysteine-rich interdomain region 1 alpha (C1DR1alpha), which binds to surface immunoglobulin (IG) and may directly activate B cells.^{36,37} HIV-infection may also serve as a chronic stimulator of B cells, through induction of IL-6 and IL-10.³⁸⁻⁴⁰ Alternatively, the immunodepressant effects of malaria and HIV infection may allow for the expansion of EBV and the outgrowth of BL cells. None of these hypotheses precisely explains why >95% of endemic BLs are EBV-positive. After over 40 years, it remains a mystery what the precise interplay is between EBV and malaria in the pathogenesis of endemic BL.

Myc and Burkitt Lymphoma

In 1972, Manolov and Monolova reported that cases of endemic BL were associated with an additional band on the long arm of chromosome 14.⁴¹ Several years later, George Klein's group identified that this material was from chromosome 8.⁴² In 1982, two separate groups reported that the t(8;14) translocated the *MYC* (c-myc) oncogene to the IG heavy chain gene locus.^{43,44} Since that time, it has been well established that the key molecular event associated with Burkitt lymphoma is translocation of the *MYC* gene on chromosome 8q24 to one of the immunoglobulin gene loci (i.e., *IGH*, *IGK* or *IGL*), resulting in the upregulation of *MYC*.^{33,45,46} The most common translocation associated with BL is t(8;14), resulting in translocation of *MYC* to the IG μ (mu) heavy chain gene (*IGH*) locus on chromosome 14q32. The resulting *IGH-MYC* gene rearrangement is seen in ~80% of BL and has been associated with all forms of BL. Translocation of *MYC* to one of the IG light chain loci may also occur in BL, with ~15% associated with t(2;8) and ~5% with t(8;22), resulting in fusion of the *MYC* gene with the kappa (*IGK*) or light chain (*IGL*) genes, respectively.^{47,48} Although *MYC* translocation is a consistent feature of BL, it is not specific for Burkitt lymphoma and may also be seen in some cases of diffuse large B-cell lymphoma (DLBCL).

Interestingly, although *MYC* gene translocation is associated with all forms of BL, the translocation breakpoints differ in endemic, sporadic, and immunodeficiency-associated BL (see Figure 23.1).⁴⁷⁻⁵¹ In endemic EBV+ BL, the *IGH* breakpoint is within the JH domain and the *MYC* breakpoint is over 100 kb upstream of *MYC*, resulting in fusion of *IGH* control elements upstream of the *MYC* promoter region. In sporadic and immunodeficiency-associated BL, the *IGH* breakpoint is usually within the S μ (mu) switch domain (occasionally S γ [gamma] or S α [alpha]) and the *MYC* breakpoint is within exon 1 or intron 1, translocating the coding region of *MYC* into the *IGH* locus. In sporadic BL with the variants t(2;8) or t(8;22), the breakpoint is downstream of the *MYC* gene, resulting in the translocation of *IGK* or *IGL* control elements downstream of *MYC*. The use of

alternative breakpoints may indicate that *MYC* translocation occurs during different stages of B cell differentiation, and that there may be differential regulation of *MYC* in the different forms of BL. The *IGH* JH breakpoints are not at canonical recombination signal sequences, but are located within J region introns or rearranged VDJ sequences, and are thought to occur during the somatic hypermutation process.^{20,52,53} Thus, *MYC* translocation likely occurs as an error of IG somatic hypermutation or class switching, both of which occur in mature germinal center B cells to produce antibody diversity.^{54,55}

The consistent translocation of the *MYC* proto-oncogene to a locus that is upregulated in B lymphocytes suggested soon after its discovery that *MYC* dysregulation is important to the pathogenesis of BL.^{43,44} *MYC* is normally downregulated during cellular differentiation and its upregulation in BL is likely an early event in lymphomagenesis. Myc is a transcriptional regulator that affects multiple downstream targets, including genes involved in signal transduction, cell cycle regulation, metabolism, cell differentiation, and apoptosis. The Myc protein is over 430 amino acids and includes several functional domains. The amino terminal transactivation domain contains two conserved Myc family domains, boxes I and II. The carboxy-terminal region includes a helix-loop-helix DNA binding domain and a dimerization domain that binds Max, which is required for Myc transactivation and transforming activity. Myc triggers apoptosis by inducing Bim, which inhibits Bcl-2, and p53 or ARF.⁵⁶ Recent studies have identified several microRNA targets of Myc regulation that are dysregulated in BL and may play a role in lymphomagenesis.^{57,58} Although the key downstream genes critical for Myc-induced transformation in BL are not well understood, pathways involved in cell proliferation, downregulation of the immune response, and antiapoptosis are key features of BL, that are likely to play important pathogenic roles.^{25,59,60}

Interestingly, while wild-type *MYC* is upregulated in many human cancers, in ~20% of BLs, the *MYC* gene is mutated at one of several hotspots in the Box I transactivation domain, including threonine-58 (which is a target for GSK3 β phosphorylation).^{56,61} The juxtaposition with IG enhancers may predispose *MYC* to somatic hypermutation. Mutations in the Box I transactivation domain may increase the tumorigenic potential of Myc by abrogating the inhibitory effect of p107, preventing proteasomal degradation, or by preventing activation of Bim and thus inhibiting Myc-mediated apoptosis.⁶²⁻⁶⁴ Other reported mechanisms of inhibition of Myc-induced apoptosis include mutations in the p53 gene *TP53* (which are seen in approximately one third of BLs), alterations in the p53/ARF/MDM2 pathway, and induction of the antiapoptotic kinase PIM-1.⁶⁵⁻⁶⁸ Interestingly, BL cells with inactivating mutations in *TP53* have been shown to lack *MYC* Box I domain mutations,⁶³ suggesting that only one antiapoptotic mechanism may be necessary for the survival of Burkitt lymphoma cells.

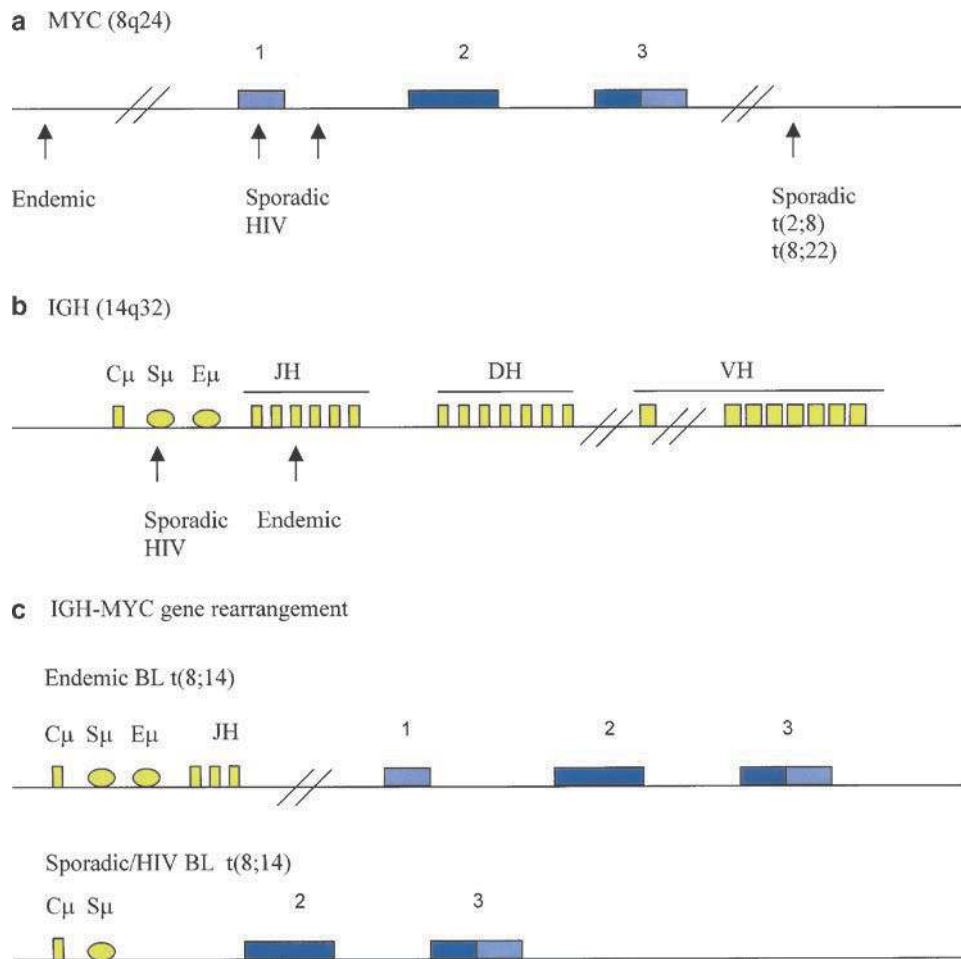


FIG. 23.1. *MYC* and *IGH* gene rearrangement in Burkitt lymphoma. (a) Genomic organization of *MYC*. Arrows denote the translocation breakpoints seen in endemic, sporadic and immunodeficiency-associated (HIV) BL. The three *MYC* exons are shown in blue: the dark boxes represent coding exons, the light hatched boxes denote untranslated regions. (b) Genomic organization of the *IGH* locus

showing the variant translocation breakpoints seen in endemic, sporadic and immunodeficiency-associated (HIV) BL. C μ constant region, S μ switch region, E μ enhancer element, JH joining region, DH diversity region, VH variable region. (c) Genomic structure of the *IGH-MYC* gene rearrangement seen in endemic versus sporadic BL.

Diagnosis of Burkitt Lymphoma

BL has a distinctive histological presentation. At low magnification, the characteristic “starry sky” pattern may be appreciated on hematoxylin and eosin staining. This pattern is composed of a “blue” background of tightly packed, medium-sized, round basophilic nuclei forming the sky on which the “stars” of interspersed tingible-body macrophages are scattered (see Figure 23.2a). At higher magnification, the lymphoma cells are intermediate-sized, monomorphic lymphocytes with scant blue cytoplasm. The nuclei are round with lacy chromatin and multiple small nucleoli (see Figure 23.2b). On touch imprints or aspirate smears, the cells are notable for cytoplasmic lipid vacuoles (see Figure 23.3). There is a rare plasmacytoid variant, that is more common in children and immunodeficiency-associated subtypes. This variant is notable for increased

polymorphism with a slightly eccentric nucleus containing a single, central nucleolus.⁶⁹ Atypical Burkitt or Burkitt-like lymphoma is a second variant. It is characterized by more pleomorphism in size and shape of the lymphoma nuclei with more prominent nucleoli.

BLs typically express monotypic surface IgM, CD20, CD10, and bcl-6, and have an MIB1/Ki-67 proliferative fraction >95%. They typically do not express TdT, bcl-2, or CD5. The expressions of CD10 and bcl-6 favor a germinal center (GC) origin for BL, which has been confirmed in gene expression analysis of BL cells.^{59,60} The GC origin of BL is consistent with the finding of somatic hypermutation of the *IGH* variable region in tumor cells and with evidence that *MYC* translocation occurs as an error of somatic hypermutation or class switching. This immunophenotype is distinguished from precursor B-cell lymphoblastic neoplasms, in

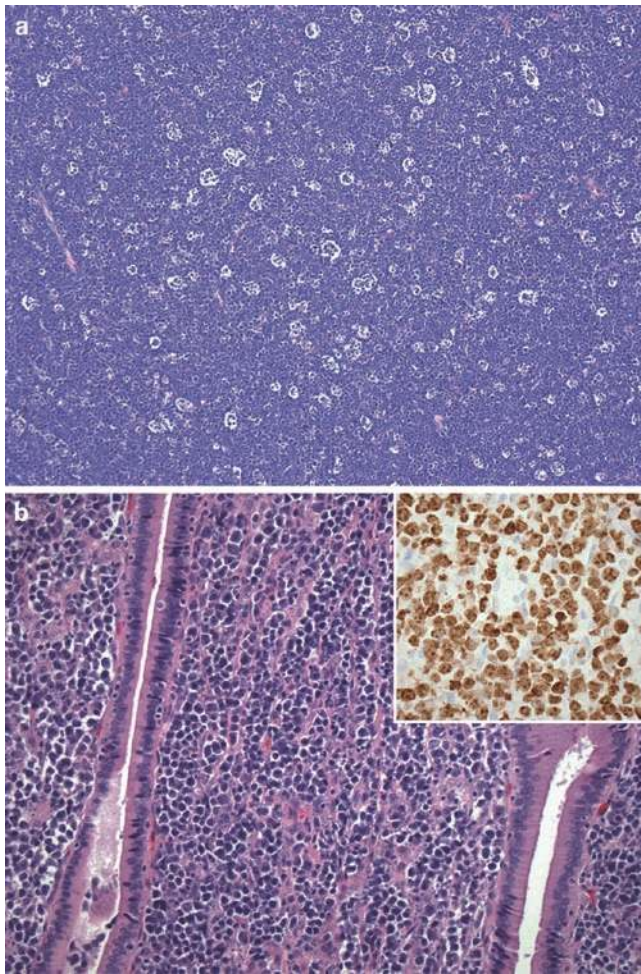


FIG. 23.2. Histology and immunohistochemical profile of Burkitt lymphoma. (a) The characteristic low power ($\times 100$) view of BL shows effacement of normal lymph node architecture by an infiltrate of intermediate sized cells with scant cytoplasm. There are numerous tingible-body macrophages interspersed throughout the lesion. (b) Sporadic BL often involves the GI tract filling the submucosal space ($\times 200$). All BLs show $>95\%$ MIB1 proliferative rate (see inset, $\times 400$).

that BL does not express TdT and shows surface light chain restriction by flow cytometry. While follicular lymphomas are similar to BLs in their expressions of CD10, CD20, and light chain restriction with TdT-negativity, they typically express bcl-2; whereas, BLs do not. Furthermore, the morphology of BL cells is quite distinct from the cleaved cells of follicular lymphoma. Diffuse large B cell lymphomas may be similar to BL, both morphologically and immunophenotypically; however, DLBCLs rarely have an MIB1 proliferative rate $>95\%$.

Occasionally, the sole definitive method to distinguish DLBCL from BL is with molecular techniques (also see Chap. 22). As noted previously, BLs are characterized by

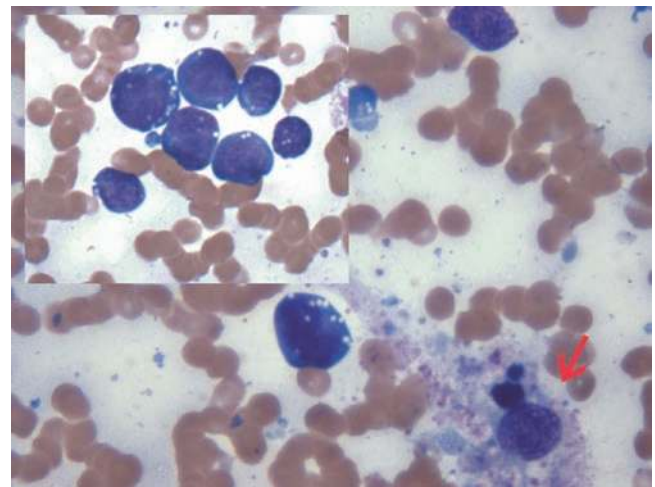


FIG. 23.3. Wright Stained Aspirate of Burkitt Lymphoma. The characteristic cytologic features of BL include the deep blue cytoplasm and round nucleus with multiple small nucleoli, that are often peripherally located. The cytoplasmic vacuoles are lipid-filled and easily recognized on cytology specimens (see inset). Frequently, aspirate and touch preparation will show tingible-body macrophages (red arrow).

MYC translocations – most commonly t(8;14), but also t(2;8) and t(8;22). In lymphomas with morphologic and immunophenotypic features of BL, these translocations are definitive when occurring in isolation. The mainstay of molecular diagnosis of BL is the use of FISH probes which span the *MYC* locus, allowing for the detection of all breakpoints.^{70,71} The wide range of breakpoints seen precludes efficient detection of *IGH-MYC* by conventional PCR, although long-range PCR has been used.^{72–76} Southern blot hybridization may be used to detect *MYC* gene rearrangement, but requires high-quality DNA and multiple probes to detect the various genomic breakpoints.^{49–51}

There have been numerous reports of lymphomas with Burkitt morphology or Burkitt-like morphology that contain a *MYC* translocation in addition to complex cytogenetics or other translocations, such as the t(14;18) associated with *IGH-BCL2* gene fusion that is seen in follicular lymphomas.¹² The new 2008 WHO classification of these lymphomas is “large B cell lymphoma with features intermediate between diffuse large B cell lymphoma and Burkitt lymphoma.” These cases may represent a transformation of a low-grade lymphoma, and the *MYC*-associated translocation generally portends a dismal prognosis.^{60,77} Likewise, it should also be recognized that there is a group of DLBCLs with *MYC* gene rearrangement (without a coexistent 14;18 translocation). Whether these lymphomas (i.e., “double-hit” lymphomas and DLBCL with *MYC* rearrangement) benefit from the Burkitt chemotherapy regimens is still unclear.

Microarray Profiling

As previously discussed, the distinction between BL and DLBCL is not always possible with routine diagnostic techniques and treatment for these two entities is different. Whereas *MYC* translocation is characteristic of Burkitt lymphoma, it may also be seen in DLBCL and some unusual Burkitt lymphomas lack *MYC* translocation. Several groups have recently employed microarray analysis to identify genomic or gene expression profiles that may distinguish Burkitt lymphoma from other mature, aggressive B cell lymphomas, including DLBCL (also see Chap. 22).

Hummel et al identified a molecular signature for Burkitt lymphoma (mBL) that was associated with classic and atypical BLs, as well as with several cases that had morphologic features of DLBCL or unclassifiable mature aggressive B-cell lymphomas.⁶⁰ Most cases with the mBL signature were *IGH-MYC* positive and lacked other chromosomal abnormalities, including *BCL2* or *BCL6* translocations. However, a few mBL cases were *MYC* negative or had *MYC* translocations to non-IG gene loci. Cases without the mBL signature were more likely to be *MYC* negative or involve *MYC* translocation to non-IG loci and to have rearrangement of *BLC2*, *BLC6*, and/or other complex chromosomal abnormalities. However, several intermediate cases that lacked the mBL were *IGH-MYC* positive and lacked other chromosomal changes. Cases with the mBL signature were associated with a better survival rate, regardless of *MYC* status or the presence of other chromosomal abnormalities, although in multivariate analysis survival could be attributable to young age or early stage.^{60,78}

Dave et al identified a molecular genetic signature for BL that was associated with high level expression of *Myc* target genes, expression of genes associated with GC B cells, and low level expression of HLA class I genes and NFκ(kappa)B target genes.⁵⁹ Patients with a Burkitt's signature had higher survival rates when treated with the intensive chemotherapy typically used for BL, rather than with lower dose regimens. Both groups found a significant percentage (17–34%) of cases with the BL genomic profile had been previously diagnosed as DLBCL or unclassifiable high-grade lymphoma.

In addition to providing a better understanding of the molecular pathways involved in pathogenesis of BL, these molecular studies have been useful to identify potential new immunophenotypic or molecular markers (i.e., over-expression of *TCL1* or downregulation of *HLAI* and *CD44*), that may better distinguish BL from other mature B cell lymphomas.⁷⁸ In the future, molecular profiling may be useful as diagnostic testing or to identify novel molecular targets of therapy.

Therapy of Burkitt Lymphoma/Leukemia

New chemotherapeutic regimens have greatly improved the previously grim prognosis of BL patients with 2-year disease free surviving fractions approaching 90% in some series.

Key to the improvement in therapy has been the development of shorter intensive courses of chemotherapy with higher doses of alkylating agents combined with intrathecal therapy and careful preventive management of tumor lysis syndrome. The German Multicenter Study Group for the treatment of adult ALL (GMALL) reported results of two protocols for the therapy of adult Burkitt leukemia, that showed 50% (BNHL83) and 71% (BNHL86) disease free survival at 8 and 4 years, respectively. These protocols both implemented a cytoreductive phase to prevent tumor lysis syndrome, followed by six cycles of fractionated cyclophosphamide, methotrexate, and low-dose cytarabine in alternating cycles. CODOX-M/IVAC (cyclophosphamide, vincristine, doxorubicin, high-dose methotrexate/ifosfamide, etoposide, high-dose cytarabine) developed by Magrath is notable for its higher doses of cytarabine and methotrexate, when compared to the BNHL trials. Magrath et al showed that the CODOX-M/IVAC regimen may lead to 2-year event-free survival (EFS) of 75% in children and 100% in adults.⁷⁹ Unfortunately, the original Magrath regimen was associated with high toxicities, particularly neurotoxicity, mucositis, and severe myelosuppression. Modified regimens with decreased methotrexate and reduced intrathecal cytarabine have decreased the associated toxicities without severely compromising effectiveness (i.e., 2-year EFS of 60% in high-risk patients and 100% in low risk patients, respectively).⁸⁰

Two other therapy regimens (not based on the BNHL studies) have been used. MD Andersen has reported that hyper-CVAD (high dose cyclophosphamide, doxorubicin, vincristine and dexamethasone alternating with methotrexate and cytarabine) led to a 3-year overall survival (OS) of 49% in an older patient population.⁸¹ Subpopulation analysis of patients younger than 60 years old showed a 3-year OS of 77%, which is comparable to the results in the Magrath and modified Magrath regimens. The CALGB regimen (i.e., cytoreduction with cyclophosphamide and prednisone followed by three cycles of ifosfamide, vincristine, etoposide, cytarabine, methotrexate, and dexamethasone alternating with cyclophosphamide, doxorubicin, vincristine, methotrexate and dexamethasone) was reported to have a 4-year disease-free survival of 50%; however, few patients were able to complete all cycles of therapy, due to severe toxicities, particularly neurologic toxicity. As noted previously, immunodeficiency-associated BL may be treated with high dose intensive chemotherapy, yet it benefits from concomitant HAART therapy in the case of HIV or removal of immunosuppression in solid organ transplant patients.

Autologous bone marrow transplantation (BMT) as consolidation therapy after high-dose chemotherapy has been used with mixed success. One phase II study showed comparable 5-year EFS and OS between standard chemotherapy-only patients and those who received autologous BMT after short intensive chemotherapy (that avoided high-dose methotrexate and cytarabine).⁸² Nevertheless, the lack of a clear benefit for most patients combined with the additional morbidity from

BMT has prevented the more widespread use of autologous BMT in first line therapy. A European group for Blood and Marrow Transplantation (EBMT) retrospective review of autoBMT, as salvage therapy for refractory or relapsed BL in second or greater remission, showed a 3-year OS of 72% in patients in first complete remission, 37% in chemo-sensitive relapsed patients, and 7% OS in chemo-resistant patients.⁸³ Allogeneic BMT has had even less critical investigation. Retrospective reports indicate that patients receiving allogeneic BMT did not have a longer OS than those receiving autologous BMT.⁸⁴ There have been other case reports, yet no large prospective trials have shown a clear benefit to allogeneic BMT.

New Therapeutic Agents

Rituximab, an anti-CD20 monoclonal antibody that induces apoptosis of B cells, has been added to hyper-CVAD-, CHOP-, and EPOCH-containing regimens with very promising preliminary results.^{85–87} Fayad et al⁸⁷ reported that the addition of rituximab to the MD Andersen hyper-CVAD protocol led to a 3-year OS of 89% in BL patients. Epratuzumab is an anti-CD22 monoclonal antibody that in vitro demonstrates a different and synergistic mechanism of inducing apoptosis in B cell lymphomas (than does rituximab).⁸⁸ Trials with these and other agents are still underway, but expectations are high for the synergistic actions of immunotherapy and chemotherapy in BL. Molecular-targeted therapies currently under investigation include histone deacetylase inhibitors, selective serotonin reuptake inhibitors, antisense oligonucleotides to Myc, proteasome inhibitors, and cyclin-dependent kinase inhibitors. These have all been used on Burkitt-derived cell lines in vitro, but have not yet made their way to clinical trials.

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Precursor B-Cell Acute Lymphoblastic Leukemia

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Introduction

Acute lymphoblastic leukemia (ALL) is a heterogeneous group of disorders caused by clonal expansion of immature lymphoid cells. The overall age-adjusted incidence is approximately 1.6 per 100,000 persons, with higher rates among children and adolescents than in adults.¹ Diagnosis is based on bone marrow (BM) morphology, immunophenotyping by flow cytometry and/or immunohistochemistry, and identification of chromosomal/genetic abnormalities by cytogenetic or molecular genetic analysis. Precursor-B ALL, characterized by a malignant proliferation of immature B-lineage lymphoid cells, comprises the majority of all leukemias in both adults and children.¹ Treatment of ALL involves multiple agents given in a complex regimen, typically lasting 2–3 years and involving numerous chemotherapeutic agents with different mechanisms of action.^{2–6} Patients who achieve clinical remission (<5% blasts in the BM) after an initial month-long induction phase receive intensified consolidation to eliminate residual leukemic blasts, and maintenance therapy to suppress re-emergence of therapy-resistant clones.

Nearly all children diagnosed with ALL are enrolled on large cooperative group pediatric trials,^{2,4–6} which use risk-based treatment stratification to balance the chance for cure with the risks of drug toxicity. The current risk assessment strategy of the Children's Oncology Group (COG)¹ uses multiple prognostic factors, including National Cancer Institute (NCI) risk groups based on age and white blood cell (WBC) count at diagnosis,⁷ presence of cytogenetic and/or molecular genetic abnormalities, BM blast percentage at day 8 or 15 of induction therapy, and minimal residual disease (MRD) status (see MRD section below) at the end of induction therapy.⁸ Event-free survival (EFS) rates for pediatric ALL have improved to approximately 80%, largely through the efforts of cooperative group trials.^{2,4–6} In contrast, the prognosis for adults with ALL has historically been poor,

approximating 10% overall survival (OS). More recently, OS rates for adult ALL patients have improved to an average of nearly 35% in trials that use either stem cell transplant or risk-based treatment stratification methods (based on those used for pediatric ALL).³

As shown in Table 24.1,^{9–51} the most frequent and/or prognostically important recurrent cytogenetic/molecular genetic abnormalities observed in children with precursor-B ALL include numerical abnormalities such as hyperdiploidy^{9,13–17} and hypodiploidy,^{9,13,14,16,21} as well as numerous structural rearrangements, including the *ETV6-RUNX1* gene fusion, resulting from a cryptic t(12;21)(p13;q22),^{21,28–34} rearrangements of the *MLL* gene at 11q23,^{9,13,14,16,17,37,38} the *TCF3-PBX1* gene fusion resulting from a t(1;19)(q23;p13),^{9,13,16,37,38} and the *BCR-ABL1* rearrangement (also called the Philadelphia (Ph) chromosome), resulting from t(9;22)(q34;q11.2).^{9,13,14,16,17,37–40} In addition, recent analyses suggest the presence of additional molecular defects, such as mutations in the *PAX5* gene on chromosome 9p⁴³ and gene amplification of *RUNX1*, in a significant percentage of children with precursor-B ALL.^{52,53} Additional 9p abnormalities also have been described.^{9,13,16,17,37,51} For reference, the official and common names of the genes discussed in this chapter are listed in Table 24.2 according to their Human Genome Organization (HUGO) nomenclature.⁵⁴

While similar translocations and gene rearrangements are apparent in both children and adults, the frequencies of specific abnormalities differ between the two age groups (Table 24.1; see reviews by Downing⁵⁵ and Faderl⁵⁶). In particular, adult ALL has a higher frequency of abnormalities with adverse prognostic significance, such as hypodiploidy and t(9;22)(q34;q11.2), the latter of which is the most frequently observed abnormality in adults with ALL, and a lower frequency of abnormalities that confer favorable outcome, such as *ETV6-RUNX1*, hyperdiploidy with >50 chromosomes, or trisomies of chromosomes 4, 10, 17, or 18.^{13,14,16,17,37–39} Adolescent ALL patients have intermediate features, as they are less likely than younger children to have high hyperdiploidy or an *ETV6-RUNX1*,^{10,23} and more

¹The COG was formed in 2000 from a merger of the Children's Cancer Group and the Pediatric Oncology Group.

TABLE 24.1. Estimated frequencies of recurrent and prognostically significant chromosomal abnormalities in ALL.

	Children ^a <19 years		Adults ^b	
Abnormality	References		References	
High hyperdiploidy (MN = 51–68)	24–39% ^c	9–12	5–10% ^c	9,13–17
Hypodiploidy MN < 46	5–9.4%	9,10,18–20	4–8%	9,13,14, 16,21
MN < 45	1.1–1.2%	8,22	4.1%	17
t(12;21)(p13;q22) <i>ETV6-RUNX1</i>	19–32%	21,23–27	0–4%	21,28–34
t(9;22)(q34;q11.2) <i>BCR-ABL1</i>	2.0–3.4%	9,10,35,36	11–29	9,13,14,16, 17,37–40
11q23 <i>MLL</i>				
Any 11q23 or 11q abnormality	6.2–8%	10,41,42	6–7%	13,16
<i>MLL</i> rearrangement	4.5–6%	21,43,44	NR	–
t(4;11)(q21;q23) <i>MLL-AFF1</i>	1.4–2.4%	9,10,42,45	3–7%	9,13,14,16, 17,37,38
t(1;19)(q23;p13) Pr1 <i>TCF3-PBX1</i>	2.5–6%	9,10,46–48	3–7% ^d	9,13,16, 37,38
Abnormal 9p	7.4–11%	9,10,49,50	6–15%	9,13,16,17, 37,51

MN modal number; NR not reported.

^aData reflect percentages based on all patients, including those with precursor-T ALL (typically 11–15% of children with ALL). Frequencies reported in some references do not include infants. Bloomfield et al¹⁴ cohort included six children with 8q24 abnormalities.

^bData reflect percentages based on all patients, including those with T-ALL (typically 25% of adults with ALL). Group Francais cohort¹³ included 21 adults with t(8;14)(q24;q32); Bloomfield et al¹⁴ cohort included ten adults with 8q24 abnormalities. In Moorman et al¹⁵ adult cohort was separated into Ph⁺ and Ph⁻, thus frequencies of abnormalities other than Ph chromosome reflect the Ph⁻ population.

^cBased on cohort of patients with >50 chromosomes and other structural chromosome abnormalities, except as noted for Moorman et al¹⁵ in footnote b.

^dFour of five references cited frequencies of 3%.

likely to have the *BCR-ABL1* fusion. Detailed descriptions of these cytogenetic and molecular genetic abnormalities are provided below.

Abnormalities of Chromosome Number

High Hyperdiploidy

As shown in Table 24.1, the frequency of high hyperdiploidy (>50 chromosomes), including cases that also had structural chromosome abnormalities, ranges from 24 to 39% in children with ALL.^{9–12} In adults, the frequency of high hyperdiploidy in the presence of other structural abnormalities is estimated to be 5–10%.^{9,13–16} The generation of the hyperdiploid cells is thought to occur prenatally^{57,58} and nonrandomly in one mitotic event, with gains of specific chromosomes at distinct modal numbers (MN).⁵⁹

High hyperdiploidy (>50 chromosomes) is associated with a good prognosis in children,^{10,14,22,60} which may be

TABLE 24.2. Official and common names for genes involved in ALL chromosomal abnormalities.

HGNC ^a official gene name	Chromosomal location	Aliases
<i>ETV6</i>	12p13	TEL
<i>RUNX1</i>	21q22.3	AML1, CBFA2, PEBP2A2, AMLCR1
<i>BCR</i>	22q11	D22S662, CML, PHL, ALL
<i>ABL1</i>	9q34.1	ABL, JTK7, c-ABL, p150
<i>MLL</i>	11q23	TRX1, HRX, ALL-1, HTRX1, CXXC7, MLL1A, KMT2A
<i>AFF1</i>	4q21	AF4
<i>MLLT1</i>	19p13.3	ENL, LTG19, YEATS1
<i>MLLT3</i>	9p22	AF9, YEATS3
<i>EP315</i>	1p32	AF-1P, MLLT5
<i>MLLT11</i>	1q21	AF1Q
<i>FOXO3</i>	6q21	AF6q21, FOXO2
<i>MLLT10</i>	10p12	AF10
<i>FOXO4</i>	Xq13.1	MLLT7, AFX1
<i>MLLT6</i>	17q21	AF17, FLJ23480
<i>TCF3</i>	19p13.3	E2A, ITF1, MGC129647, MGC129648, bHLHb21
<i>PBX1</i>	1q23.3	pr1
<i>PAX5</i>	9p13.2	BSAP

^aHUGO Gene Nomenclature Committee.⁵⁴

a result of altered drug sensitivity. When compared with nonhyperdiploid leukemic cells, those with hyperdiploidy have a higher *in vitro* sensitivity to several chemotherapeutic including methotrexate,^{61,62} mercaptopurine, thioguanine, cytarabine, and L-asparaginase,^{63,64} and are more likely to undergo apoptosis.⁶⁵ The prognostic significance of hyperdiploidy in adults is less clear, as some studies have reported an poor outcome,^{13,17} while others have reported improved outcome.^{9,16,66} However, in one study that found poor outcome, one-third of the high hyperdiploid patients were Ph⁺,¹³ and in one study the favorable effect was not maintained in multivariate analysis.⁹

Previous studies by the Children's Cancer Group (CCG) and the Pediatric Oncology Group (POG) found that trisomies of chromosomes 10, 17, or 18,¹¹ or trisomies 4 and 10,⁶⁷ respectively, rather than the presence of hyperdiploidy per se, predicted the best prognosis. Data from the United Kingdom showed that trisomy for either chromosomes 4 or 18 was associated with better event-free survival.¹² A recent combined analysis of CCG and POG data determined that combinations of trisomies for chromosomes 4, 10, and 17 conferred the best outcome.⁶⁸ According to the current COG risk stratification, in the absence of unfavorable features, patients with these three trisomies are assigned to the low-standard risk treatment group.⁸

Hypodiploidy

Hypodiploidy, defined as the presence of less than 46 chromosomes in a cell, is estimated to occur in approximately 5–9.4% of childhood ALL cases,^{9,10,18–20} and 4–8% of adult cases^{9,10,13,14,16} (Table 24.1). Careful diagnosis is critical, as

a frequent observation is chromosome doubling in a cell with only 24–39 chromosomes, which could be misinterpreted as hyperdiploidy. Patients with hypodiploidy have worse outcome than patients with normal or hyperdiploid leukemia.^{18–20,69,70} There is no apparent prognostic difference between patients in whom the chromosomes have doubled and those in whom the MN remains near-haploid.⁷¹ Patients in whom the leukemic clones have a modal number (MN) of 45 appear to have better event-free survival than those with an MN < 45.^{18,19} Indeed, progressively worse outcome has been reported with decreasing MN (i.e., 6-year EFS estimates were $65 \pm 8\%$ for MN of 45; $40 \pm 18\%$ for MN of 33–44; and $25 \pm 22\%$ for MN of 24–28).¹⁹ In a recent analysis of data combined from eight cooperative groups and two large cancer or children's hospitals in the United States and Europe, patients with an MN < 44 had an even worse outcome than those with an MN = 44 (8-year EFS = 30.1% vs. 52.2%). Although patients with an MN < 44 typically achieved remission at the end of induction therapy, they tended to relapse early, within 2 years of initiating therapy. In the current COG risk stratification system, patients with leukemic cells containing < 44 chromosomes or with a DNA index < 0.81 are considered very high risk, which ensures the allocation of more aggressive treatment than other ALL patients.⁸

Frequent Structural Chromosome Abnormalities

t(12;21)(p13;q22) *ETV6-RUNX1*

The cytogenetically cryptic t(12;21)(p13;q22) translocation, leading to the *ETV6-RUNX1* gene fusion, is the most frequent chromosomal abnormality in childhood ALL, occurring in 19 to 32% of pediatric ALL cases,^{21,23–27} but is present in $\leq 4\%$ of adult cases^{15,28–34} (Table 24.1). The finding of *ETV6-RUNX1*⁺ cells in archived neonatal blood spots of children with *ETV6-RUNX1*⁺ ALL has suggested a prenatal origin.⁷² Data from identical twins, one of whom developed ALL at age 5 and the other who developed ALL at age 14, has indicated that disease progression is variable, perhaps due to variation in the occurrence of additional transforming events.⁷³

Molecular Characterization of the *ETV6-RUNX1* Fusion (Figure 24.1a)⁷⁴

Leukemic blasts from patients with the *ETV6-RUNX1* gene fusion uniformly express *ETV6-RUNX1* mRNA and may also express a reverse transcript, *RUNX1-ETV6*.^{75,76} Usually, they also express wild type RUNX1 protein,⁷⁵ but typically do not express wild-type ETV6, which is most often deleted.^{77–79} The *ETV6-RUNX1* fusions encode the N-terminal sterile alpha motif/pointed (SAM/PNT) DNA binding domain of ETV6 and most of RUNX1, including the RUNT

DNA binding/homodimerization and RUNX domains. An alternative breakpoint leads to a transcript lacking exon 2 of *RUNX1*, but encodes a similar fusion protein.⁷⁵ Alternative splicing may also lead to the lack of exon 3 in the transcripts, leading to the expression of up to four different *ETV6-RUNX1* transcripts detected in a given patient. Duplication of the *ETV6-RUNX1* fusion may also occur.^{80,81}

The *ETV6-RUNX1* fusion gene may be detected in leukemic blasts using either reverse-transcriptase polymerase chain reaction (RT-PCR) or fluorescence in situ hybridization (FISH). RT-PCR detection typically utilizes primers in exon 5 of *ETV6* and exon 4 of *RUNX1*, allowing for the detection of all four of the common transcripts. Both FISH and RT-PCR methods detect the vast majority of positive cases, although rare cases positive by one or the other method exist (Gastier-Foster, JM, unpublished data).⁸² Rare cases that are positive by FISH and negative by RT-PCR have been described, where the resulting fusion does not include exon 5 of ETV6 (Gastier-Foster, JM, unpublished data).⁸²

Properties of ETV6, RUNX1, and the *ETV6-RUNX1* fusion protein have been reviewed by Hart and Foroni.⁸³ *ETV6*, a widely expressed member of the ETS family of transcription factor genes, contains an N-terminal homodimerization domain and a C-terminal DNA binding domain; *in vitro* evidence suggests that the ETV6 protein may act as a transcriptional repressor. *RUNX1* is one of several genes encoding an alpha subunit of the core DNA binding factor protein that is hypothesized to recruit and organize other transcription factors and is primarily expressed in hematopoietic tissues.⁸³ The fusion protein may form heterodimers with ETV6 and has been shown to block RUNX1 binding to DNA and to repress RUNX1-activated transcription.

Prognostic Significance of *ETV6-RUNX1*

Retrospective analyses show that children with ALL whose leukemic cells contain the *ETV6-RUNX1* fusion have a good prognosis on current risk-adjusted therapy, with estimated 4–5-year EFS between 89 and 100%.^{24,25,44,75,84,85} The favorable outcome on current risk-adjusted therapies for children whose leukemic blasts express *ETV6-RUNX1* appears to result from the high sensitivity of such cells to the commonly used chemotherapy agents (i.e., dexamethasone, vincristine, and asparaginase).^{86,87} A recent retrospective analysis of data from standard risk patients treated on legacy POG and CCG trials has demonstrated a 5-year EFS of 85 and 86%, respectively, for patients positive for *ETV6-RUNX1*.⁸ Currently, COG considers patients of 1–9 years of age, with precursor-B ALL and WBC counts < 50,000/ μ L at diagnosis who are positive for *ETV6-RUNX1* to be low-standard risk; patients ≥ 10 years, or with high WBC counts, are considered to be higher risk, regardless of *ETV6-RUNX1* status.⁸

The prognostic significance of *ETV6-RUNX1* in adult ALL patients is unclear, as most studies have too few *ETV6-RUNX1*⁺ patients to allow comparison of outcome with other patient subsets. However, Lee et al³² recently reported that

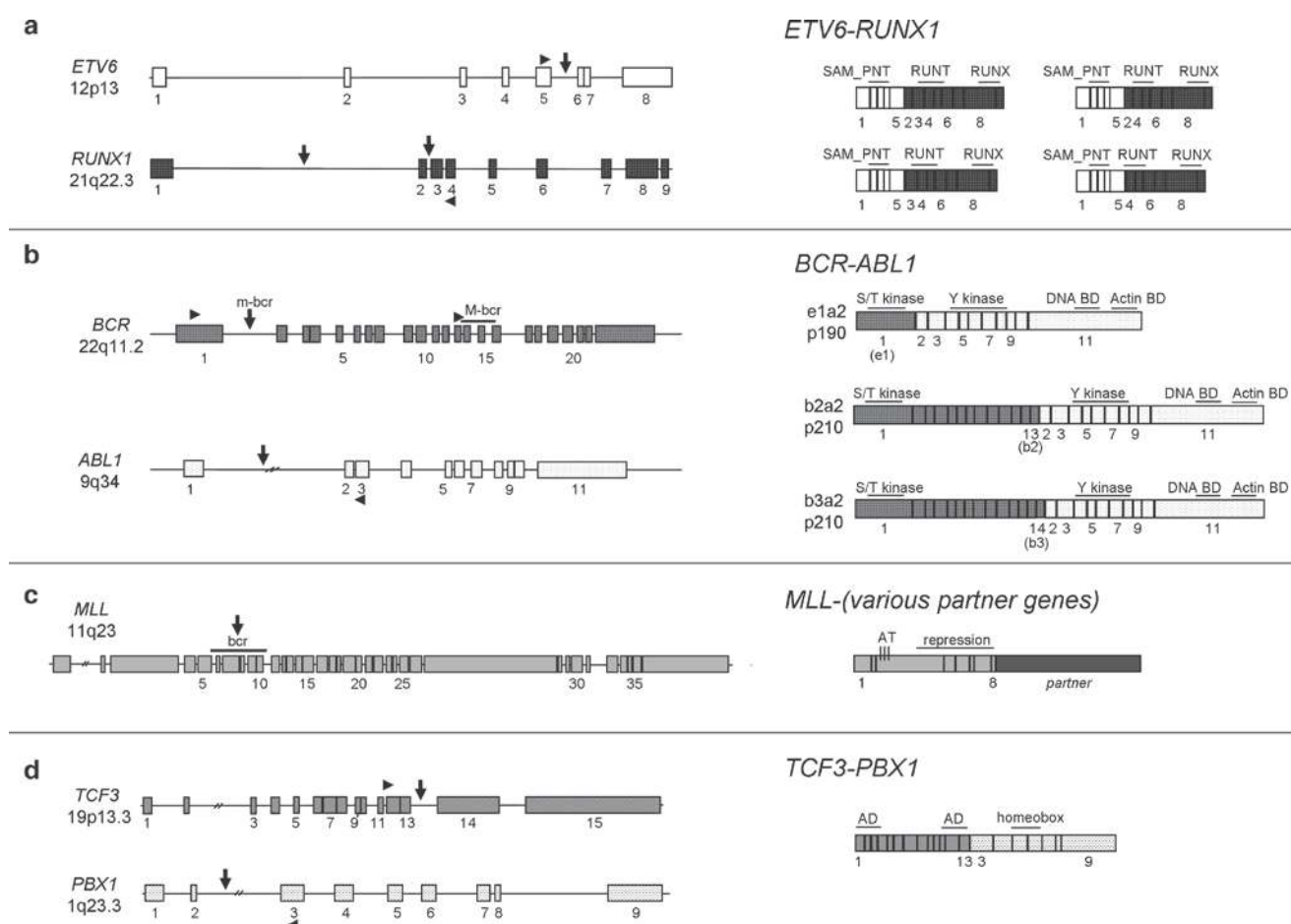


FIG. 24.1. Schematic representation of the common ALL chromosomal translocations. All genomic constructs are based on Ensembl⁶³ transcripts. The genomic structure, of each gene is shown to the left, with the most frequent gene fusions represented to the right. All of the genes have normal variant transcripts, so the transcript used to describe the intron/exon structure is indicated below. All breakpoints were marked in the appropriate intron by comparison of published fusion sequences to the exonic sequences in the chosen transcript (typically the HGNC-curated sequence). The genomic breakpoints are indicated with a *down arrow*, and the primers that have been used for RT-PCR amplification in ongoing COG studies are indicated by *arrow heads*. (a) Transcripts used for genomic structures: ENST00000266427 (*ETV6*) and ENST00000399245 (*RUNX1*). Fusion of the *ETV6* and *RUNX1* genes leads to two different fusion genes (with or without exon 2), and a total of 4 transcripts are possible, because exon 3 can be alternatively spliced out of either fusion. The resulting *ETV6-RUNX1* transcripts encode the sterile alpha motif/pointed (SAM/PNT) of the ETV6 protein and the RUNT and RUNX domains of the RUNX1 protein. (b) Transcripts used for genomic structures: ENST00000305877 (*BCR*) and ENST00000372348 (*ABL1*). In ALL, fusion of the *BCR* and *ABL1* genes leads to three possible fusions, depending on whether the

BCR breakpoint occurs in the minor breakpoint cluster region (m-bcr) or major breakpoint cluster region (M-bcr). The exons of *BCR* frequently have been called e1 (exon 1), b2 (exon 13), and b3 (exon 14), resulting in the common names of the fusions e1a2, b2a2, and b3a2 when combined with exon 2 of *ABL1* (a2). In ALL, the e1a2 fusion is observed most often, while the b2a2 and b3a2 fusions are observed more frequently, but not exclusively, in chronic myeloid leukemia. The domains retained in the fusions include the serine/threonine kinase domain (S/T kinase) of BCR and the tyrosine kinase (Y kinase), DNA binding (DNA BD), and F-actin binding (Actin BD) domains of ABL1. (c) Transcript used for genomic structure: ENST00000359313 (*MLL*). The *MLL* gene is involved in translocations with multiple fusion partners. *MLL* breakpoints are located within the breakpoint cluster region (bcr) between exons 5 and 11, and the resulting fusion proteins retain the AT-hooks (AT) and repression domain of the MLL protein. (d) Transcripts used for genomic structures: ENST00000395423 (*TCF3*) and ENST00000340699 (*PBX1*). Except for rare variants, all *TCF3-PBX1* fusions have common breakpoints in *TCF3* intron 13 and *PBX1* intron 2 which lead to fusion proteins containing the activation domains of TCF3 and the homeobox domain of PBX1.

two patients who were positive for *ETV6-RUNX1* and three patients with *ETV6* deletion had a median survival twice that of 69 patients without these abnormalities (33 months vs. 17 months).

ETV6-RUNX1 in Relapsed Pediatric ALL

The frequency of *ETV6-RUNX1* in relapsed pediatric ALL patients is unclear. In retrospective analyses, German investigators have reported frequencies similar to those observed at diagnosis⁸⁵; whereas, investigators at the Dana-Farber Cancer Institute have reported a very low frequency at relapse.⁸⁸ In a more recent prospective analysis, the frequency of *ETV6-RUNX1* at relapse was 7/44 (16%) of children with B-precursor ALL.²⁵ In both the retrospective and prospective analyses, patients positive for *ETV6-RUNX1* who relapsed had longer first remissions than other patients who relapsed (median of 43 months vs. 27–29 months).^{25,85}

Philadelphia Chromosome: t(9;22)(q34;q11.2) *BCR-ABL1*

The t(9;22)(q34;q11.2) translocation (also called the Philadelphia (Ph) chromosome), a hallmark of chronic myelogenous leukemia (CML), is also found in approximately 1–4% of pediatric ALL cases^{9,10,21,35,36} and 11–29% of adult ALL cases^{9,13,14,16,17,37–40} (Table 24.1). Detection of the t(9;22)(q34;q11.2) in previous large clinical studies used conventional cytogenetics; more recent studies have used FISH,⁸⁹ or various PCR methods, including those with sufficient sensitivity to allow quantification of minimal residual disease after induction therapy.^{90–92}

*Molecular Characterization of the BCR-ABL1 Fusion (Figure 24.1b)*⁷⁴

The Ph chromosome results from a fusion between the 5' sequences of the *BCR* gene at chromosome 22q11.2 and the 3' portion of the Abelson leukemia virus proto-oncogene (*ABL1*) at chromosome 9q34. In both CML and ALL, the breakpoints in *ABL1* cluster in intron 1.^{93–97} The *BCR* gene molecular breakpoints typically are different in the two forms of leukemia. In CML, the *BCR* breakpoints are most often found within the 5.8 kb major breakpoint cluster region (M-bcr), whereas in ALL, the breakpoints are either within the M-bcr or more commonly within intron 1, termed the minor BCR (m-bcr).^{94,97–103} These breakpoints result in the production of a 210 kD protein (p210) or a 180–190 kD protein (p190), respectively.^{95,104–107} Both children and adults may harbor either M-bcr or m-bcr breakpoints, with inconsistent reports on the percentages of each age group with either breakpoint.^{35,89,90,108–113} The fusion genes are detected in leukemic blasts using either RT-PCR or FISH. With RT-PCR, two sets of primers are required, a *BCR* primer specific to the m-bcr or M-bcr and an *ABL1* primer. Although wild-type *c-ABL1* contains sequences that encode the v-abl

tyrosine kinase activity, this activity is not expressed by c-abl *in vitro*.¹¹⁴ The mutant BCR-ABL1 fusion protein, however, presumably due to formation of tetramers,¹¹⁵ has constitutive tyrosine kinase activity in K562 Ph⁺ leukemia cells,¹¹⁴ which leads to leukemogenesis.^{116,117}

Prognostic Significance of BCR-ABL1

Presence of the t(9;22)(q34;q11.2) has been shown by numerous investigators to confer a poor outcome in both adults and children.^{14,66,118–122} Most studies have found no associations between the type of molecular breakpoint and outcome for either adults or children,^{35,108–112,123} although one study suggested a positive trend between boys and p210, and a higher relapse rate for m-bcr (4 of 8) when compared with M-bcr (2 of 11).⁹⁰ Although associated with older age and high WBC count at diagnosis in children,^{14,120} the presence of the t(9;22)(q34;q11.2) is an independent adverse risk factor for pediatric ALL.^{14,36} This adverse risk has persisted even with the advent of contemporary risk-adjusted therapy,^{2,4–6,36} including augmented postinduction therapy.^{36,124} However, UK based investigators have reported improvement to a 3-year EFS of 52%.¹²⁵ Similarly, the presence of the t(9;22)(q34;q11.2) in adults continues to be a major adverse risk factor despite intensive chemotherapy.¹⁰⁸

Current risk classification of the COG defines children with a Ph⁺ ALL as very high risk.⁸ Current treatment strategies for children with Ph⁺ ALL typically include intensive induction therapy and allogeneic bone marrow transplantation (BMT) in first remission.^{6,126–130} Similarly, adults with t(9;22)(q34;q11.2) who achieve remission after induction therapy also are assigned to related or unrelated donor allogeneic transplant.^{38,131–135} Nevertheless, outcome remains poor for both children and adults, with long-term EFS <50%, even among patients who undergo a matched-related donor BMT.^{38,126–130,133,136}

New Therapies for BCR-ABL1⁺ ALL

A novel targeted inhibitor therapy, imatinib mesylate (Gleevec®), which binds to the active site of the BCR-ABL fusion protein and inhibits its tyrosine kinase activity, has been shown to be effective for inducing remission in adults with CML and Ph⁺ ALL, but nearly all patients with ALL have demonstrated relapse within several months of remission.^{137–142} Molecular analysis of the *BCR-ABL1* gene in patients who relapsed after an initial response to imatinib revealed a high frequency of point mutations in the kinase domain, that resulted in amino acid changes, including threonine 315 to isoleucine (T315I).^{143–145} Initial data for children has shown similar results.^{146,147} A recent study, however, demonstrated highly promising results (≥95% EFS at 1 year) with a combination of high-dose chemotherapy and continuous dosing of imatinib during the postinduction phase, with or without BMT in children with Ph⁺ ALL.¹⁴⁸

An alternative agent, dasatinib, has been investigated for addressing imatinib resistance. Dasatinib inhibits both the ABL tyrosine kinase and intracellular downstream SRC kinases that are thought to be involved in leukemogenesis. Dasatinib is 325 times more potent at blocking the ABL tyrosine kinase than imatinib and is effective against almost all imatinib-resistant BCR-ABL proteins, with the exception of the T315I mutant.^{149,150} Talpaz et al¹⁵¹ recently reported a high response rate to dasatinib for ten patients (15–73 years of age) with Ph⁺ ALL or Ph⁺ CML in lymphoid blast crisis, who were resistant ($N=9$) or intolerant ($N=1$) to imatinib. None of the ten patients had the T315I BCR-ABL mutation. All but one patient relapsed within 1–8 months. Interim results of a Phase 2 study of dasatinib in 36 patients with Ph⁺ ALL showed a 42% hematologic response rate and a 58% cytogenetic response rate, although long-term event-free survival was not yet available.¹⁵² Thus, the efficacy of dual kinase inhibitor therapy is not yet proven for the treatment of Ph⁺ ALL.

Rearrangements of the *MLL* Gene at 11q23

Abnormalities in chromosome 11q, including t(4;11)(q21;q23), t(11;19)(q23;p13.3), and t(9;11)(p22;q23), all of which result in rearrangement of the *MLL* gene at 11q23, occur with estimated frequencies of 6–8% in children^{10,41,42,45} and 3–7% in adults^{13,16,39} (Table 24.1). These abnormalities are particularly prevalent in infants with ALL (by cytogenetic analysis, 11q23 abnormalities and t(4;11)(q21;q23) are observed in approximately 45 and 35%, respectively; and by molecular genetic analysis, *MLL* rearrangements are identified in 70–80% of infants).^{153–155} Evidence from newborn blood spots and studies of monozygotic twins with ALL and identical 11q23 rearrangements support an in utero origin of these leukemias in infants.^{156–158}

Molecular Description of *MLL*, Fusion Partners, and the *MLL* Fusion Genes (Figure 24.1c)⁷⁴

The wild-type *MLL* gene has high sequence homology, including six putative zinc fingers, with the gene for the *Drosophila* homeotic transcriptional regulator, trithorax; *MLL* also has cysteine-rich domains with homology to DNA methyltransferase, arginine-rich sequences found in the U1 small nucleoprotein particle (snRNP) 70 kD protein, and AT hook DNA binding motifs that are characteristic of high mobility group proteins, which bind to AT-rich domains in the DNA minor groove and act as transcriptional activators (see review by Cimino et al¹⁵⁹), and sequence homology with the genes for yeast SET proteins,¹⁶⁰ which are members of a family of highly conserved proteins that restructure chromatin to induce or repress transcription.¹⁶¹ Amino acids 2829–2883, containing the core sequence homology to DNA methyltransferase, are strong transcriptional activators *in vitro*.¹⁶² The amino terminus of *MLL* has been shown to confer nucleolar and nuclear matrix localization, cell cycle

arrest, and monocytic differentiation.^{163,164} Current data suggest that in normal cells, the *MLL* protein functions as a transcriptional regulator of several target genes, including the mammalian homeotic (HOX) gene family, whose properly coordinated expression is required during embryonic hematopoiesis (see review by Lawrence¹⁶⁵). Indeed, properly regulated expression of HOX genes by *MLL* has been shown to be a requirement for normal mammalian hematopoiesis.¹⁶⁶ *MLL* appears to regulate transcription by a combination of effects on histone acetylation and methylation, and by association with RNA polymerase II.^{160,167,168}

The three commonly observed 11q23 translocations in ALL, t(4;11)(q21;q23), t(11;19)(q23;p13.3), and t(9;11)(p22;q23) result in the fusion of the 5' portion of *MLL* with the 3' sequences of *AFF1*, *MLLT1*, and *MLLT3*, respectively.^{169–172} In addition, other less common translocations and their resulting fusion genes have been reported in patients with ALL (see review by Cimino et al¹⁵⁹), including *EP515* (1p32), *MLLT11* (1q21), *FOXO3* (6q21), *MLLT10* (10p12), and *FOXO4* (Xq13).

Nearly all B-precursor ALL patients with *MLL* rearrangements show breakpoints in a 5' *MLL* gene region of approximately 8.3 kb, termed the *MLL* breakpoint critical region (bcr), that maps roughly between exon 5 and exon 11, regardless of the fusion partner^{173–179} (Figure 24.1c).⁷⁴ These breakpoints have been further localized to two subregions, termed bcr1 and bcr2,^{174,176} that are upstream of a zinc finger domain that is deleted in approximately 25% of patients.¹⁸⁰ The resulting fusion mRNAs and proteins contain the 5' portion of *MLL*, including the AT-hooks and a transcription repression domain,¹⁸¹ and the 3' portion of the partner gene (see review by Cimino et al¹⁵⁹).

The fusion partners *AFF1*, *MLLT1*, and *MLLT3* appear to encode transcription factors.^{162,182–184} *AFF1* encodes a serine- and proline-rich putative transcription factor, with a glutamine-rich carboxy terminus that has homology with a region of the *Drosophila* trithorax gene, as well as a nuclear localization signal, and a transcription activation domain.¹⁶⁹ The *AFF1* protein also shows homology with the interleukin growth hormone gene cluster at chromosome 5q31.¹⁸⁵ Amino acids 480–560 of the *AFF1* protein, which are present in *MLL*-*AFF1*, have strong transcriptional activation activity *in vitro*.¹⁶² While the *AFF1* protein lacks direct sequence homology with *MLLT3* and *MLLT1* (see review by Cimino et al¹⁵⁹), it nevertheless shares structural motifs, including nuclear targeting sequences, serine-rich domains, proline-rich, or other basic amino acid-rich regions, serine/proline-rich or proline-rich transactivation domains, and positively-charged DNA binding domains, with the other fusion partners.¹⁷¹

The leukemogenic potential of various *MLL* fusions has been demonstrated *in vitro* and *in vivo*. The *MLLT1* protein has *in vitro* transactivation activity in lymphoid and myeloid cells.¹⁸³ Transactivation requires both the methyltransferase domain of *MLL* and the C-terminal 90 amino

acids of MLLT1, which are highly conserved with MLLT3 and present in both MLL-MLLT1 and MLL-MLLT3.^{183,184} *MLL-MLLT1* also transforms murine BM cells, which (when implanted into sub-lethally irradiated syngeneic mice or SCID mice) cause myeloid leukemia.¹⁸⁶ In addition, chimeric and heterozygous mice harboring an endogenous *MLL-MLLT3* “knock-in” mutation under control of the *MLL* promoter have been shown to develop myeloid and lymphoblastic leukemias.^{187,188} More recent data suggest that MLL-MLLT1 may recruit a transcriptional repressor that binds to MLLT3,¹⁸⁹ and that cellular transformation by *MLL-MLLT1* involves deregulation of HOX genes and the HOX coregulator MEIS1.¹⁹⁰ Consistent with these findings, other data have shown that the murine homolog of *MLLT3* is required for normal embryogenesis, controlling embryogenesis via regulation of HOX genes.¹⁹¹

Four of the other *MLL* fusion partners (some specific to AML) also appears to encode transcription factors or activators (i.e., *ELL*, *FOXO4*, *CREBBP*, and *FOXO3*); whereas, others appear to encode cytoplasmic proteins, some of which may be capable of dimerization (MLLT6) or protein–protein interaction (MLLT4) (see review by Cimino).¹⁵⁹ Like wild-type MLL, both types of fusion proteins may induce transcription of HOX genes, but unlike wild-type MLL, activation by the fusions results in overexpression of HOX A9 and MEIS1 protein,¹⁶⁷ which is presumed to be an initial leukemogenic event. The observed activation of transcription by MLL fusions with cytoplasmic proteins suggests that different mechanisms, involving protein–protein interaction or dimerization, may be involved for the different types of MLL fusions.¹⁹²

Prognostic Significance of MLL Rearrangements

Numerous early studies identified t(4;11)(q21;q23) as an adverse risk factor for childhood ALL, beginning with the findings of Arthur et al.¹⁹³ Large cooperative groups have continued to report that t(4;11) or other *MLL* gene rearrangements confer an adverse risk.^{41,44,153–155,194–201} Pui et al.¹⁵⁴ identified 11q23/*MLL* rearrangements as an independent adverse risk factor for a cohort of 30 infants with ALL.

Subsequent studies have examined the relationship between age and prognosis for 11q23 abnormalities in more detail. In a large dataset of children and infants compiled from numerous collaborative groups and institutions, all children with a t(4;11) had a poor outcome. Further analysis demonstrated that the 5-year EFS for infants with a t(4;11) was significantly worse than that of older children (19% for infants vs. 42% for age ≥1; log rank $P=0.0001$).²⁰⁰ Similarly, infants with t(11;19) had a significantly worse outcome (5-year EFS=26%) than older children with t(11;19) (5-year EFS=64%; log rank $P=0.003$). For t(9;11), outcome was similarly poor for both age groups (5-year EFS=38% for infants and 46% for age ≥1). Among infants, any 11q23 abnormality was associated with a poor outcome; whereas, in older children, t(4;11) and t(9;11) were associated with worse outcome than other 11q23 aberrations. These data

suggested that among children of 1–9 years of age, different 11q23 abnormalities were associated with different prognoses, although all were associated with poorer outcome than is observed in this age group in the absence of an 11q23 rearrangement.¹⁹⁸ Similar results were reported by Johansson et al.¹⁹⁷ and Rubnitz et al.²⁰²

Recent publications have added new insights regarding prognosis and 11q23 abnormalities. Moorman et al.²⁰³ demonstrated that other chromosomal abnormalities present in patients with an 11q23 rearrangement provided no additional prognostic significance. A side-by-side retrospective analysis of POG and CCG data demonstrated worse outcome for patients with a t(4;11) versus other 11q23 abnormalities among all patients and standard-risk patients from both POG and CCG and in high-risk POG patients, but not in CCG patients.⁸ Nevertheless, subsets with either t(4;11) or other 11q23 both had 5-year EFS outcomes >45%, which is the current cutoff for very high risk treatment assignment. However, children >1 year of age with a t(4;11) and a poor response at day 7 of induction had an EFS of <45%. Therefore, the current COG risk classification assigns patients with a t(4;11) or other 11q23 abnormality to the high risk treatment group, and those with a t(4;11) and a poor early induction response to the very high risk group.

Transplantation Therapy for ALL with MLL Rearrangements

Previous studies have suggested that transplantation does not improve outcome for patients with t(4;11).^{197,199,204} Recent reports, however, suggest that HSCT transplant in first remission may improve outcome for children with very high risk ALL, including those with a t(4;11). Balduzzi et al.²⁰⁵ reported that among children with very high risk features, including t(4;11), disease-free survival (DFS) at 5 years was 57% for those who received HSCT, when compared with 41% for those who received chemotherapy. Jacobsohn et al.²⁰⁶ reported that HSCT in first remission among 16 infants with ALL containing an *MLL* rearrangement resulted in 12 survivors, with a median survival of 4.7 years. Recently, intensive consolidation therapy, including alternating high-dose methotrexate and cytarabine, was reported to improve EFS outcome among children with t(4;11), or other very high risk features, to 61% at 9 years.²⁰⁷ These data provide promising trends for improving outcome among this high-risk subset of childhood ALL.

t(1;19)(q23;p13.3)TCF3-PBX1

The t(1;19)(q23;p13.3) abnormality occurs at estimated frequencies of 2.5–6% in children^{9,10,46–48} and 3–7% in adults^{9,13,16,37,38} (Table 24.1), and is found predominantly in patients with a precursor-B immunophenotype^{9,10,41,46,48,193} The chromosomal aberration in t(1;19)(q23;p13) may be balanced or unbalanced. The unbalanced translocation may arise by nondisjunction of the balanced translocation during

clonal evolution, based on the observation of mosaic karyotypes with both abnormalities.²⁰⁸ In the unbalanced translocation, the der(1) chromosome as well as the short arm of chromosome 19 distal to 19p13.3 are lost, and there is a duplication of the normal chromosome 1, resulting in trisomy for the long arm chromosome 1 distal to 1q23.

Molecular Description of the TCF3-PBX1 Fusion (Figure 24.1d)⁷⁴

The t(1;19)(q23;p13.3) leads to the fusion of *TCF3* and *PBX1* genes.^{209–213} *TCF3* encodes the E12 and E47 immunoglobulin gene enhancer binding factors.²¹⁴ *PBX1* encodes a DNA-binding homeoprotein with homology to the homeotic fly protein extradenticle (*exd*).^{215,216} PBX1 protein contains a 66-amino acid homeodomain motif that is related to the homeodomains in *exd* and other homeotic proteins in *Drosophila* and vertebrates, that regulate proper body/axis segmentation during embryogenesis (see reviews by Moens and Selleri²¹⁷ and Mann and Chan²¹⁸).

The TCF3-PBX1 fusion protein contains the N-terminal portion of TCF3, containing two transcription activation domains, and the C-terminal portion of PBX1, containing the 60-amino acid homeodomain.^{209,210,212,213} The breakpoints in *TCF3* are consistently observed within a single intron.^{210,213} Although several different forms of TCF3-PBX1 protein arise because of alternative splicing of the mRNA transcript, the two most abundant species are 77 and 85 kD proteins that differ in their carboxy termini.^{211,212} Initial studies of TCF3-PBX1 activity have shown that the fusion may constitutively activate transcription via the PBX1 recognition sequences (PRS),²¹⁹ transform fibroblasts,²²⁰ and cause acute myeloid leukemia or T-cell lymphoma in mice.^{221,222} Critical domains identified within TCF3 and PBX1 include two transcription activation domains in the N-terminus of TCF3 and the homeodomain and C-terminal 25 amino acids of PBX1 (see review by Hunger²²³).

PBX1, like *exd*, has been shown to interact with HOX proteins to regulate gene expression,^{217,218,223} and data from several studies suggest a model, in which PBX1 and HOX proteins bind cooperatively in heterocomplexes to their respective target sequences in a target gene, resulting in conformation changes that regulate transcriptional activation of the specific targets.^{224–229} The C-terminus of PBX1 is essential for interaction with HOX proteins.²³⁰ The TCF3-PBX1 fusion forms heterodimers with only a subset of the HOX proteins that normally heterodimerize with PBX1, thus altering the target genes affected.^{225,231,232} Transformation of mouse BM cells requires overexpression of both TCF3-PBX1 and HOXA9, consistent with the cooperative role of PBX1 and HOX proteins in regulating gene expression.²³³ Additionally, a repressor function of PBX1, localized to an internal domain of PBX1, has been identified and is independent of DNA binding via the homeodomain.²³⁴ Taken together, these data suggest that TCF3-PBX1 likely disrupts normal HOX-dependent gene expression. Other studies have identified

other potential target genes activated by TCF3-PBX1,^{235–241} but the contribution of these upregulated proteins to leukemogenesis is presently unknown.

Prognostic Significance of TCF3-PBX1

Numerous studies have identified t(1;19)(q23;p13) as an adverse risk factor in children with ALL.^{46,208,242–245} Other studies found better or similar outcome for the unbalanced t(1;19) when compared with the balanced t(1;19).^{205,241,242} The adverse risk of the t(1;19) appears to have been abrogated with more intensive therapy,^{8,48,246} and retrospective analysis of the large POG cohort of 273 t(1;19)⁺ patients enrolled between 1986 and 1999 showed no statistical difference in the outcome for balanced vs. unbalanced t(1;19).⁸ Thus, current COG risk stratification does not include t(1;19) for treatment allocation.⁸

Adults with ALL and a t(1;19)(q23;p13) have a poor outcome.^{40,244,247–250} Recently published results from a French study (LALA-94) have suggested that allogeneic SCT in first remission may improve outcome, as disease-free survival was 63% for the allogeneic transplant group versus 16% for those receiving autologous transplants, and 0% for those receiving chemotherapy, although the comparison did not reach statistical significance.²⁴⁸

Abnormalities of Chromosome Arm 9p

Abnormalities of the short arm of chromosome 9 are recurrent aberrations observed in both children and adults with ALL.^{9,10,49–51,251–253} By metaphase cytogenetics, the estimated frequency of 9p abnormalities is 7–11% in children,^{9,10,49,50} and 6–15% in adults.^{9,13,16,17,37,51} FISH may reveal a higher frequency. Although primarily found in patients with a precursor-B immunophenotype, 9p aberrations are also found in T-lineage ALL.^{49,50}

The most frequently observed 9p abnormalities in children with ALL are deletions, unbalanced translocations resulting in partial deletions, balanced translocations, and dicentric chromosomes, primarily involving 20q or 12p as partner chromosomes.^{49,50} The dic(9;12)(p11–p13;p11–p13) has been reported in infants, children, and young adults (ages 20–21), as well as one middle-aged adult with ALL.^{49,252,254–258} and has an estimated frequency of 0.65% in childhood ALL.^{49,258} The dic(9;12) appears to be rare in patients >21 years of age. The dic(9;20)(p11;q11) has been described in both children and adults with ALL, and appears to occur almost exclusively in patients with a precursor-B immunophenotype.^{49,259–263}

Molecular Characterization of Gene Mutations and Deletions Mapping to 9p

The high frequency of deletion of the 9p21–22 region suggests the presence of a tumor suppressor gene. Potential candidates for such a tumor suppressor include the interferon

(INF) gene cluster,^{264–266} methylthioadenosine phosphorylase gene (*MTAP*), which encodes an enzyme involved in purine metabolism,²⁶⁷ and the tandem genes *CDKN2* (also known as p16INK4a) and *CDKN2B* (also known as p15INK4b), which encode inhibitors of cyclin-dependent kinases.^{267–272} Deletions may be mono- or biallelic and involve one or more of these genes.^{264,265,267,268,270} In some studies, deletion of *CDKN2/CDKN2B* occurred at a higher frequency in patients with precursor-T ALL.^{268,270,272}

A unique fusion gene, *PAX5-ETV6*, has been identified in children with ALL and a dic(9;12)²⁷³ and in an adult patient with ALL harboring a t(9;12)(q11;p13).²⁷⁴ *PAX5*, which maps to 9p13,^{275,276} is a member of a gene family that encodes transcription factors containing paired DNA-binding domains and is thought to select for B-cell development over other hematopoietic fates by activating B-lineage specific genes and repressing genes specific to other lineages.^{43,277–279} Genome-wide single nucleotide polymorphism (SNP) array analysis has demonstrated that *PAX5* abnormalities occur at a frequency near 30%, and thus are one of the most frequent gene alterations in children with precursor-B ALL.⁴³ Investigators of the COG have identified 2 mutually exclusive rearrangements in ALL with dic(9;12).²⁸⁰ One subset had *ETV6-RUNX1* rearrangements suggesting a cryptic t(12;21)(p13;q22), and the other had *PAX5-ETV6* fusions, representing the dic(9;12). The dic(9;12) rearrangement remains to be elucidated in cases with a *ETV6-RUNX1* translocation.

Prognostic Significance of 9p Abnormalities

An initial large cohort study found no prognostic significance for 9p abnormalities,⁵⁰ whereas a later study of a larger series of children with ALL found significant adverse risk for patients with an abnormal 9p versus those with a normal 9p (6-year EFS: 61% vs. 76%; log rank $P < 0.0001$).⁴⁹ More recent data suggest that patients with deletion of the *CDKN2A* gene (or interferon loci at 9p) may have a poorer outcome than patients without these deletions.^{268,281,282} Significantly worse outcome also has been reported for children with ALL and homozygous vs. heterozygous interferon gene deletions.²⁸³

The prognostic significance of the dicentric chromosomes is unclear, as different investigators have reported a poor outcome among ALL patients with any 9p abnormality (including dic(9;12)),⁴⁹ good outcome (>88% EFS) for ALL patients with a dic(9;12),^{84,255,258} better outcome for dic(9;12) versus other 9p abnormalities,²⁵² or similar outcomes for patients with either a dic(9;12) or a dic(9;20), when compared with that of patients with other 9p abnormalities.⁴⁹ Limited data are available concerning dic(9;20), but a Swedish study of pediatric patients reported a 5-year EFS of 62% and 5-year OS of 82% for those with a dic(9;20).²⁵⁹ Current treatment allocation of the COG does not use the presence of 9p abnormalities in assigning risk-based therapy, as the subsets were too small to be analyzed in the cumulative CCG and POG data.⁸ Future studies may need to re-examine the prognostic

significance of some of these abnormalities, particularly with the discovery of the high frequency of *PAX5* mutations in ALL.

Intrachromosomal Amplification of Chromosome 21 and *RUNX1* Gene Amplification

Intrachromosomal amplification of chromosome 21, specifically 21q22, has been identified as a new recurrent abnormality in ALL, with an estimated frequency of 2%.^{51,53,81,284–286} Leukemic cells from such patients are characterized by the presence of multiple, tandemly repeated copies of *RUNX1*. In some patients, *RUNX1* amplification is associated in some patients with duplication of 21q; in others, the tandem repeats occur in the absence of an extra chromosome 21.^{52,53,81} The abnormality may have arisen via a breakage-fusion-bridge mechanism.²⁸⁷ Although *RUNX1* amplification has been reported to occur only in patients who do not have the *ETV6-RUNX1* fusion,^{53,81,285} one study has reported both abnormalities in the same patients.⁸⁰ In contrast to *ETV6-RUNX1*⁺ patients, those with *RUNX1* gene amplification appear to have a poor outcome, with reported 5- or 6-year EFSs <40%.^{288,289} Additional studies in large cohorts of patients are warranted to examine this recently identified recurrent abnormality that may have adverse prognostic significance in ALL.

Detection of Minimal Residual Disease by Molecular Methods in Patients with Chromosomal Abnormalities

Quantification of MRD (i.e., presence of leukemic cells undetectable by light microscopy, but detectable by flow cytometry or molecular methods) (see review by Moppett et al²⁹⁰) has been demonstrated using flow-cytometry for the detection of leukemia-specific surface antigens,^{291–294} and using molecular techniques, such as polymerase chain reaction (PCR) for the detection of clone-specific immunoglobulin (Ig) or T-cell receptor (TCR) rearrangements.^{295–297} See also Chapter 10 for additional description. Both methods may be used in >90% of patients with ALL and sensitivity varies from 10^{−3} to 10^{−4} by flow cytometry and 10^{−3} to 10^{−5} by Ig/TCR-based PCR.²⁹⁰ Both methods have shown that the presence of MRD (defined as ≥1% of BM cells by flow cytometry²⁹² and ≥10^{−2} residual blasts per 2 × 10⁵ mononuclear BM cells by Ig/TCR-based PCR²⁹⁵) at the end of induction is a significant adverse prognostic factor.^{291–298}

MRD may also be examined in specific subsets of patients, based on residual leukemic cell expression of the rearranged genetic loci described above. For example, numerous groups have developed PCR-based methods for quantification of the most common gene rearrangements in ALL, specifically *MLL* rearrangements in infants,^{173,299–302} *BCR-ABL1* rearrangements in adults and very high risk children,^{90–92,110,303–306}

and *ETV6-RUNX1* fusions in children.^{307–313} Methods also have been developed for the detection of *TCF3-PBX1* fusion transcripts.^{314–319} A consortium of European investigators has developed a standardized method for the various MRD detection methods, including those for specific fusion gene transcripts.³²⁰ Several studies have shown that the presence of postinduction MRD, based on detection of *BCR-ABL1* transcripts, has adverse prognostic significance in both children and adults.^{35,321–324} Additionally, detection of *BCR-ABL1* transcripts following allogeneic transplant has adverse prognostic significance.³²⁵ By contrast, the prognostic significance of low levels of residual *TEL-AML1*⁺ cells requires further study, as results are presently inconsistent.^{326–328}

B-Precursor Lymphoblastic Lymphoma

Lymphoblastic lymphoma (LYL) is a rare disorder with similarities to ALL. The key differences that distinguish LYL from ALL at diagnosis are the presence of bulky tumors at various sites^{329–331} and the lack of involvement of BM and peripheral blood.^{330–332} Whereas the majority (85%) of patients with ALL have a precursor-B immunophenotype, only 10% of LYL patients are of precursor-B cell origin.³³² The most common site of disease at diagnosis in precursor-B LYL is the skin^{329,331}; additional sites include head and neck, as well as bone or other internal organs; whereas, in T-cell LYL, the most frequent sites are the mediastinum and lymph nodes.³³⁰ Over 100 patients with precursor-B LYL have been described in the literature.^{329–331,333,334} Outcome appears relatively good for precursor-B LYL, based on a retrospective analysis of approximately 100 current and published cases by Maitra et al, which showed that 74% were alive with a median follow-up of 26 months.³³¹ Ten of 24 patients described by Lin et al were alive, either in complete clinical remission (nine patients) or with one relapse (one patient), with follow-up ranging from 12 to 144 months.³²⁹

Cytogenetic abnormalities in precursor-B LYL are not well described. Among the small group of patients with cytogenetic findings in various publications, the only recurrent observations were a gain of 21q material (which resulted from trisomy 21, tetrasomy 21, or add(21q22)) and IgH or TCR gamma gene rearrangements.^{329,330,334} Several observed abnormalities have not been reported in precursor-B ALL: t(12;15)(q24;q24), t(2;8)(p12;q12), del(8)(q11q13), del(9)(q12q32), add(13)(q32), and dup(10)(q22q24 or q24q26).^{330,334} Additional studies are warranted to more fully characterize cytogenetic and molecular genetic features in this uncommon form of lymphoma.

Conclusions and Future Directions

Molecular genetic analyses continue to identify new gene abnormalities in ALL. Thus, current and future studies are seeking to determine how these abnormal genes contribute to

leukemogenesis, and if targeting new or existing therapeutic agents toward these abnormalities may provide efficacious and safe treatment. Oligonucleotide microarray analysis has revealed distinct gene expression profiles for leukemic cells harboring *TCF3-PBX1*, *BCR-ABL1*, *ETV6-RUNX1* fusion genes, or a rearranged *MLL* gene.^{335,336} A recent study identified 14 genes, including *RUNX1*, that are involved in cell differentiation, cell proliferation, apoptosis, and cell motility or response to wounding and are upregulated in leukemic cells with *ETV6-RUNX1*.³³⁷ In another recent analysis, leukemic clones in patients with an early relapse (<36 months) were found to overexpress genes involved in biosynthesis and metabolism, DNA replication/repair, and inhibition of apoptosis, all of which favor cell proliferation, compared with clones from patients with a late relapse (>36 months).³³⁸ Additionally, while gene expression profiles were similar for early relapse vs. diagnosis, they were divergent for late relapse vs. diagnosis, suggesting the involvement of distinct mechanisms for the re-emergence of leukemia at early and late relapses. These and future novel molecular genetic findings should lead to new information regarding risk factors within each ALL subset, as well as to the discovery of novel therapeutic approaches for each subset.

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Molecular Genetics of Mature T/NK Neoplasms

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Introduction

The mature (postthymocyte; peripheral) T/natural killer (NK) lymphomas/leukemias represent 5–15% of all non-Hodgkin lymphoma (NHL) and vary according to geography.¹ Peripheral T cell lymphoma, not otherwise specified (PTCL, NOS), is the most common type worldwide. There are more nodal presentations in Europe and North America, where the second most common types in each region are angioimmunoblastic T cell lymphoma (AITL) and anaplastic large cell lymphoma (ALCL), respectively. There is more extranodal disease in Asia, due to Epstein–Barr virus related NK/T lymphoma and human T cell leukemia virus (HTLV)-1 associated adult T cell leukemia/lymphoma (ATLL). With the exceptions of the indolent mycosis fungoides (MF) and the chemo-sensitive anaplastic lymphoma kinase (ALK)-positive ALCL, the prognosis in most peripheral T/NK neoplasms is poor, with a 5-year survival less than 30%.² (Table 25.1)

Diagnosis of T/NK Neoplasms

Lennert in Europe and Lukes and Collins in the United States proposed an immunological classification of lymphomas in 1974.³ The description of these disorders and the subdivision into B and T/NK malignancies have evolved over time and were modified as the Revised European American Lymphoma (REAL) classification in 1994 and subsequently adopted by the World Health Organization (WHO).⁴ B cell lymphomas and leukemias have been better described because they are four times more common than T/NK neoplasms, usually have light-chain restriction to confirm clonality, and have had recurrent translocations involving the immunoglobulin (Ig) heavy-chain gene locus on chromosome 14q32. The goals of the WHO classification are to define pathologic entities by their clinical presentation, histology, immunophenotype, and cytogenetic/molecular data when available.

Cytogenetic and molecular analyses have contributed to the diagnosis and classification of peripheral T/NK lymphomas/leukemias.^{5,6} There are two subtypes of T cell

receptors (TCR): alpha-beta ($\alpha\beta$) and gamma-delta ($\gamma\delta$). Approximately 95% of normal, mature T lymphocytes express the $\alpha\beta$ heterodimer, while the remainder express the $\gamma\delta$ heterodimer. Alpha-beta and $\gamma\delta$ T cells develop from a common progenitor in the thymus. Gamma-delta T cells emigrate from the thymus to reside in the skin, intestinal epithelium, and splenic red pulp.^{7,8} T cell neoplasms may involve rearrangements at the site of TCR α and δ genes on chromosome 14q11, and the site of TCR β and γ genes on chromosome 7(7q34–36 and 7p15).^{9,10} Alpha-beta rearrangements are more common in PTCL, NOS, and ALCL and define subcutaneous panniculitis-like T-cell lymphoma (SCPTCL), while $\gamma\delta$ clonality is the more common type in hepatosplenic T cell lymphoma (HSTCL) and enteropathy-associated T cell lymphoma (EATL)⁵ and defines cutaneous $\gamma\delta$ T cell lymphoma.

Nonrandom recurrent chromosomal abnormalities in mature T/NK neoplasms include t(2;5) in ALK-positive ALCL, isochromosome 7q in HSTCL, trisomies 3,5, and 21 in AITL; complex karyotypes in PTCL, NOS, deletions of chromosomes 6q, 11q, 13q, and 17p in extranodal NK/T cell lymphoma, nasal-type; loss of heterozygosity of chromosome 9p21 in EATL, and chromosome 14 abnormalities in T-cell prolymphocytic leukemia (T-PLL).^{5,6} Frequent cytogenetic abnormalities in ALK-negative ALCL include gains of 1q and 3p and losses of 16pter, 6q13q21, 15, 16qter, and 17p13. In PTCL, NOS, frequent gains were identified at 7q22q31, 1q, 3p, 5p, 8q24qter, and losses occurred at 6q22q24 and 10p13pter.⁶ Although recurrent translocations are rarely observed in PTCL, a novel t(5;9)(q33;q22) involving *ITK* and *SYK* genes was identified in five (17%) of 30 PTCL, NOS cases.¹¹ Complex karyotypes (defined as >5 structurally aberrant chromosomes) are observed in approximately one-half of PTCL, NOS, and ALK-negative ALCL and have a worse survival than those without a complex karyotype.⁶

Many of the genes translocated to the breakpoints at the TCRs at chromosome 14, and more rarely at chromosome 7, encode transcription factors, and have been predominantly recognized in precursor T-lymphoblastic lymphoma (LL)/leukemias.¹² Examples of transcription factor genes occurring

TABLE 25.1 Characteristic clinical and genetic features of mature T/NK neoplasms.

Disease	Predominant TCR gene rearrangement	Chromosome abnormalities	Unique features	5-year survival (%)
<i>Cutaneous</i>				
Mycosis fungoides	$\alpha\beta$	Variable	Prognosis varies according to stage. Rearrangement of <i>TCR-γ</i> gene by PCR can identify clonality	10–100
Primary cutaneous anaplastic large cell lymphoma, ALK-negative	$\alpha\beta$	Variable	One-quarter of solitary lesions may spontaneously resolve; responsive to local therapy	80–90
<i>Nodal</i>				
Anaplastic large cell, ALK-positive	$\alpha\beta$	t(2;5) (p23;q35) and variants	Extranodal involvement (50–80%): skin (21–35%), bone (8–17%); chemosensitive	60–90
Anaplastic large cell, ALK-negative	$\alpha\beta$	Gains of 1q and 6p21	Distinguish from primary cutaneous anaplastic large cell lymphoma	10–45
Angioimmunoblastic	$\alpha\beta \approx \gamma\delta$	Trisomies 3, 5, and 21	Autoimmunity; follicular T (T_{FH}) cells express CD10 and CXCL13; B cell clones can be present	10–30
Peripheral T cell lymphoma, unspecified	$\alpha\beta$	Often complex, numerical and structural	Most common; survival dependent on IPI	15–35
<i>Extranodal</i>				
Nasal NK	Absent	Deletions of 6q; loss of heterozygosity of 13q; often complex	EBV association, central nervous system risk	50–70
Localized			Sites: skin, gastrointestinal tract, testis, orbit	5–10
Disseminated (nasal type)			Indolent, worse prognosis if hemophagocytosis	45–85
Subcutaneous panniculitis-like	$\alpha\beta$	Limited data	Aggressive, frequent hemophagocytosis	10–20
Cutaneous $\gamma\delta$ lymphoma	$\gamma\delta$	Isochromosome 7q has been reported	Can occur in organ transplants and Crohn disease	5–15
Hepatosplenic	$\gamma\delta > \alpha\beta$	Isochromosome 7q, trisomy 8	Celiac disease; small bowel obstruction	5–20
Enteropathy-associated	$\gamma\delta > \alpha\beta$	Loss of 9q21		
<i>Leukemia</i>				
T-prolymphocytic leukemia	$\alpha\beta$	Chromosome 14 abnormalities	One-fifth can have indolent phase	10–20
Adult T cell leukemia lymphoma	$\alpha\beta$	Breakpoints at 10p11, 14q11, and 14q32; complex	HTLV-1 association, hypercalcemia. Four types: acute (55–65%), chronic or smoldering leukemia and lymphoma (20–25%)	0–15
Large granular lymphocytic leukemia	$\alpha\beta$	Variable	Rheumatoid arthritis, neutropenia, red cell aplasia; high levels of Fas and Fas ligand	50–75
Aggressive NK leukemia	Absent	Deletions of 6q21–q25 and loss of 17p13	May represent leukemic phase of extranodal NK neoplasms	0–10

in T-acute lymphoblastic leukemia (T-ALL) include the *TAL1* in t(1;14)(p32;q11), *TAL2* in t(7;9)(q35;q34), and *HOX11* in t(10;14)(q24;q11). A rare translocation in T-ALL is t(7;9)(q34;q34.3), which involves *NOTCH1*, a transmembrane protein of the *NOTCH* gene family of membrane-receptor proteins that control cell differentiation. Activating mutations of *NOTCH1* signaling have been found in the majority of T-ALL/LL, and inhibitors of γ -secretase, a proteolytic enzyme that cleaves *NOTCH1* and leads to its activation, are in clinical trials.¹³

With the exception of T-PLL (*vide infra*), mature T/NK neoplasms rarely involve translocations with the TCR genes. Only two of 245 (0.8%) mature T cell lymphomas were found to have a chromosome breakpoint affecting the TCR

α/δ locus and none involved the TCR β and/or TCR γ loci.¹⁴ Two of 169 (1.2%) PTCLs had t(6;14)(p25;q11.2) involving the multiple myeloma oncogene -1/interferon regulatory factor -4 (*IRF4*) and the TCR α locus.^(14a) A t(14;19)(q11;q13) involving the TCR α/δ locus and *BCL3* has been identified in classical Hodgkin lymphoma (cHL) and PTCL.¹⁵

Inactivation of tumor suppressor genes plays a variable role in lymphomagenesis. Overexpression of p53 has been commonly observed in ALCL, PTCL, NOS, but is not observed in AITL or HSTCL; mutations of *p53* are rare in mature T/NK neoplasms.¹⁶ Co-expression of p53 and BCL-2 in T/NK neoplasms has been associated with advanced stage disease, high international prognostic index, and a worse survival.¹⁷ Cyclin-dependent kinase (cdk) inhibitors (p15, p16, p21, p27)

may also be involved in lymphomagenesis. Deletions of p15 and p16 are more common in the aggressive forms of ATLL, when compared to smoldering disease and are predictive of shorter survival.^{18,19} Partial or complete loss of PTEN, a tumor suppressor gene on chromosome 10q23, has been observed in 66.7% of ALCL, correlated with loss of p27, and was found in 12.5% of other mature T/NK lymphomas.²⁰

Gene expression profiles may distinguish subtypes of B cell lymphomas and are likely to be able to subdivide T/NK neoplasms. Genes involved in the NFκB pathway are present in PTCL and are absent in T-lymphoblastic lymphoma (T-LL).²¹ Preliminary data show that gene profiles may discriminate AILT from ALCL and ALK-positive ALCL from ALK-negative ALCL.^{22–24} AILT is represented by overexpression of B cell markers, such as immunoglobulin genes, whereas the ALCL signature displays genes active in the early immune response, as well as extracellular matrix and cell proliferation.²² Molecular profiles have identified three different overexpression patterns of genes in PTCL, NOS: (1) the cell cycle regulator *CCND2*; (2) genes involved in T cell activation and apoptosis, *NFKB1* and *BCL-2*; and (3) genes in the interferon/JAK/STAT pathway.²² Overexpression of genes in a proliferation signature includes genes associated with the cell cycle (i.e., *CCNA*, *CCNB*, *TOP2A*, and *PCNA*) and has been significantly associated with a shorter survival in patients with PTCL.²⁵

Epstein–Barr virus (EBV) and human T leukemia virus 1 (HTLV-1) may contribute to T/NK lymphomagenesis.²⁶ Also see Chap. 7. EBV is a ubiquitous herpes virus that is B cell tropic and associated with African Burkitt lymphomas in the immunocompromised host and cHL; however, it may also infect T and NK cells and is associated with extranodal NK/T cell lymphomas and aggressive NK cell leukemias in Asia and parts of South America. The tumor cells in nasal NK/T cell lymphoma express EBNA1 and LMP2; whereas, expression of LMP1 is more variable.²⁷ EBV positivity in the broad category of PTCL has had an inferior survival when compared to EBV-negative lymphoma.²⁸

HTLV-1 is a member of the deltaretrovirus genus of the retrovirus family and is endemic to southwestern Japan, the Caribbean basin, Central Africa, parts of South America, Melanesia, Papua New Guinea, and the Solomon Islands. The lifetime risk of developing ATLL among HTLV-1 carriers in Japan is 6.6% for men and 2.1% for women.²⁹ Similar to EBV promoting lymphomagenesis, HTLV1 contributes to a multistep process of worsening genetic instability by interfering with mitotic checkpoints and preventing DNA repair.³⁰ Also see Chap. 7.

Mycosis Fungoides

The genetics of MF are heterogeneous; cytogenetics, analysis of T cell receptor genes for clonality, and comparative genomic hybridization (CGH) correlate with prognosis. Numerical aberrations, particularly missing chromosomes,

are the most common cytogenetic abnormalities.³¹ The most common chromosomes lost include 10 (28% of cases), 9, 2, 17, 1, 13, and 16.³² The most common structural abnormalities include the deletion of 6q (18% of cases) and isochromosome 17q (17% of cases).³¹ Aberrations of chromosome 8 and 17 are associated with progressive disease.³³ Identifying identical clones of T cell populations by T-cell receptor gene rearrangements in dermatopathic lymph nodes and in skin predicts extent of disease and prognosis in MF.³⁴

The most common findings by CGH are losses in 10q25 → q26, 13q21 → q22, and 17p13 → p11, and amplifications in 8q24 → q24.3 and 17q21 → q25.³¹ Patients with more than five aberrations or loss in 6q, 10q, 13q, or gain in 7 or 8q have a shorter survival than those without these changes.³⁵

Gene expression signatures in MF have not been uniform, but may identify potential genes involved in pathogenesis. One study found that deletion of the *NAV3* gene encoded at 12q21 was present in the majority of patients with Sezary syndrome (SS).³⁶ Three potential molecular mechanisms have been suggested by CGH in another series of patients with SS: (1) gain of *cMYC* and loss of *cMYC* antagonists, (2) loss of *TP53* and genome maintenance genes (i.e., *RPA1/HIC1*), and (3) gain of genes (i.e., *STAT3/STAT5* and interleukin-2 (IL-2) receptor) involved in the IL-2 pathway.³⁷

Anaplastic Large Cell Lymphoma, ALK-Positive

ALK expression subdivides ALCL into three clinical subtypes: (1) ALK-positive systemic ALCL, (2) ALK-negative systemic ALCL, and (3) primary cutaneous ALCL (also ALK-negative). ALK-positive ALCL occurs at a younger median age (15–30 years) than ALK-negative systemic ALCL (45–65 years), has a male predominance, and usually has advanced-stage disease with B symptoms (40–75%) and extranodal involvement (50–80%).^{38–40} Skin (21–35%), soft tissue (17%) and bone (8–17%) are common extranodal sites; the gastrointestinal tract and central nervous system are rarely involved at diagnosis.^{38–40} Bone marrow involvement occurs in 10–15% by routine histology, but increases to 30% with immunohistochemistry, identifying isolated ALCL cells.^{38,41}

The morphology of ALCL is heterogeneous and may be misdiagnosed as metastatic carcinoma, malignant histiocytosis, and inflammatory processes. The common variant comprises ~70% of ALCL and usually is composed of large, pleomorphic, atypical tumor cells with abundant gray-blue cytoplasm.³⁸ The nuclei are large, horseshoe- or kidney shaped, and may be referred to as doughnut cells if there are pseudonuclear inclusions. Small cell and lymphohistiocytic variants each comprise ~10% of ALCL. Other rarer subtypes include a giant-cell-rich type and a sarcomatoid variant. The tumor cells are always CD30+, often express pan T cell

antigens CD2 and CD3, and express cytotoxic granule-associated proteins.³⁸

Few lymphomas have been recognized to contain translocations that produce a fusion protein, but the best described translocation in mature T cell neoplasms is the t(2;5)(p23;q35) associated with ALCL.⁴² Also see Chap. 14 for a discussion of the proteomics of ALCL. This translocation produces a fusion gene between the cytoplasmic part of ALK, a receptor tyrosine kinase of the insulin receptor subfamily on chromosome 2, and the amino-terminal portion of nucleophosmin (NPM) on chromosome 5⁴³ (Figure 25.1). Approximately 70–80% of ALK-positive ALCL express the NPM-ALK fusion protein. NPM encodes a 23 kDa multifunctional-protein that is involved in ribosomal transport, regulation of cell division, DNA repair, and transcription. The transcription of the 80 kDa chimeric fusion protein NPM-ALK results from the ALK gene coming under the control of the NPM promoter.

Approximately 20–25% of ALK-positive ALCL are negative for NPM-ALK, or the t(2;5), and involve other fusion partner genes⁴⁴(Figure 25.1). The second most common fusion gene partner in ALCL is nonmuscle tropomyosin (TPM3), forming t(1;2)(q21;p23) and the TPM3-ALK protein.⁴⁵ TPM4 is a homologue of TPM3 that is located on the short arm of chromosome 19, forming t(2;19)(p23, p13.1). Hernandez et al described three variant ALK rearrangements involving TRK-fused gene (TFG) on the long arm of chromosome 3.⁴⁶ TFG-ALK fusion proteins lack nuclear localization signals and are predominantly cytoplasmic. Three other nonnuclear ALK fusion gene partners and the cytogenetic abnormalities are as follows: (1) clathrin heavy polypeptide-like gene (*CLTC*) and t(2;17)(p23;q23), (2) 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/inosine-monophosphate-cyclohydrolase (*ATIC*) and inv (2)(p23;q35), and (3) moesin (*MSN*) and t(2;X)(p23;q11-12).^{44,47} CLTC-ALK and, to a lesser extent, NPM-ALK fusions have been rarely

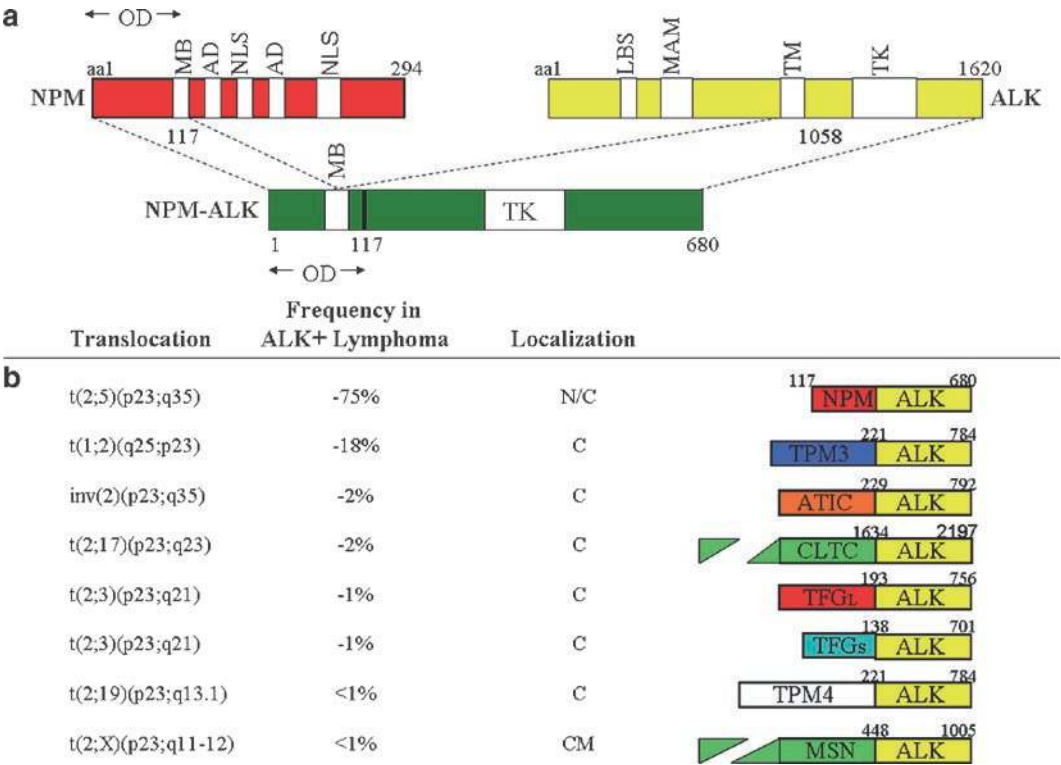


FIG. 25.1. Schematic (top) of the ALK receptor tyrosine kinase and the NPM-ALK fusion protein resulting from the t(2;5). Fusion of the chromosome 5 gene encoding nucleophosmin (NPM) to the chromosome 2 gene encoding anaplastic lymphoma kinase (ALK) generates the chimeric tyrosine kinase, NPM-ALK. NPM contains an oligomerization domain (OD; residues 1–117) a putative metal-binding domain (MB) (residues 104–115), two acidic amino acid clusters (AD) (Asp/Glu-rich acidic domain; residues 120–132 and 161–188) that function as acceptor regions for nucleolar targeting signals, and two nuclear localization signals (NLS) (residues 152–157 and 191–197). ALK contains a single MAM (Meprin/A5/protein tyrosine phosphatase Mu) domain, a

region of about 170 aa present in the extracellular portions of a number of functionally diverse proteins that may have an adhesive function (residues 480–635). The ligand-binding site (LBS) for pleiotrophin and midkine (ALK residues 391–401) is indicated. TM transmembrane domain; TK tyrosine kinase catalytic domain. ALK fusion proteins, the chromosomal rearrangements that generate them, their occurrence in ALK-positive lymphomas, and their subcellular localizations. ATIC 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase; C cytosolic; CM cell membrane; CLTC clathrin heavy chain; MSN moesin; N nuclear; TFG TRK-pomyosin-3; TPM4 non-muscle tropomyosin-4 (adapted from Morris et al¹²).

associated with a variant of large B cell lymphoma with plasmablastic/immunoblastic morphology and a poor prognosis.⁴⁸ At least 15 different ALK fusions have been described, and have been found in inflammatory myofibroblastic tumors, squamous cell cancer of the esophagus, and non-small-cell lung cancer.^{43,44}

NPM-ALK may be detected by fluorescent-in situ hybridization (FISH) analysis, or by reverse transcriptase-polymerase chain reaction (RT-PCR), but antibodies specific for ALK have been utilized to stain both the cytoplasm and nucleus in tissues containing ALK translocations (Figure 25.2). Immunoperoxidase staining for ALK is clinically important because its presence is not only associated with the t(2;5) or one of its variants, but predicts chemosensitivity. Complete remission (CR) rates following anthracycline therapy are over 75% for ALK-positive ALCL and 50–75% for ALK-negative patients.² Five-year overall survival is 60–93% for ALK-positive ALCL, when compared to 11–46% for ALK-negative ALCL.^{40,49} Factors associated with a worse prognosis in ALK-positive ALCL include B symptoms, a high International Prognostic Index (IPI), small cell variant histology, and expression of CD56 or survivin (a member of the inhibitor of apoptosis family).^{50,51}

ALK fusions have oncogenic potential, because of aberrant tyrosine kinase activity that promotes cell proliferation and survival through interconnected pathways: Ras-extracellular signal-regulated kinase (ERK), Janus kinase 3 (JAK3), signal transducer and activator of transcription 3 (STAT3), and phosphatidylinositol 3-kinase (PI3K)-AKT.^{52–54} The Ras-ERK pathway promotes ALCL proliferation; whereas, the JAK-STAT and PI3K-AKT pathways block apoptosis of ALK-positive cells. STAT3 is central to NPM-ALK

lymphomagenesis by mediating the transcription of both antiapoptotic factors and cell-cycle regulators, including BCL-X_L, survivin, cyclin D3, C/EBP β , and myeloid cell leukemia 1 (MCL1).^{52,55} CD30 expression is sustained through the phosphorylation of STAT3 and the ERK1 and ERK2-mediated upregulation of JUNB protein levels (Figure 25.3). CD30 engagement results in the degradation of tumor necrosis factor 2 (TRAF2) and phosphorylation of BCL3 and activates the canonical and alternative NF κ B pathways. Clinical trials are specifically targeting CD30 in ALCL and cHL and are also under development to target ALK, AKT, the mammalian target of rapamycin (mTOR), and NF κ B.⁵²

Angioimmunoblastic T Cell Lymphoma

AITL is difficult to diagnose and treat, because it may have both T and B cell clones, the presence of EBV usually in B cells, and a variable clinical course with autoimmune features.⁵⁶ AITL usually occurs in the elderly (median age, 57–68 years) as an advanced staged lymphoma with generalized lymphadenopathy, B symptoms (50–70%), a pruritic rash, pleural effusions, arthritis, eosinophilia, and a spectrum of immunologic abnormalities, including Coombs-positive hemolytic anemia, cold agglutinins, cryoglobulinemia, and hypergammaglobulinemia.^{2,5,56}

AITL is pathologically characterized by a polymorphous infiltrate with effacement of the normal nodal architecture with open or dilated peripheral sinuses. The neoplastic T cells are usually CD4+, are present in clusters in the paracortex, and have clear cytoplasm. There are usually prominent arborizing high endothelial venules (HEVs) and follicular dendritic cells (Figure 25.4). The lymph nodes contain polyclonal plasma cells and frequent large B immunoblasts, which are EBV-positive in the parafollicular areas. B cell follicles are usually regressed or absent, but follicular hyperplasia may be present early and obscure the diagnosis.

The neoplastic T cells express CD10 and sometimes BCL6 and are thought to have a germinal center origin (referred to as follicular T (T_{FH}) cells). The tumor cells usually express a unique marker, CXCL13, a chemokine that is produced by normal T_{FH} cells and recruits B cells to the lymph node. A hypothetical model of AITL proposes the EBV-positive B cells activate T_{FH} cells, which in turn produces CXCL13 and further B cell activation⁵⁶ (Figure 25.5). An antigen independent clone of T_{FH} cells might emerge to produce the neoplastic processes of AITL.

Gene expression profiles have identified genes characteristic of T_{FH} cells, including *CXCL13*, *BCL6*, *PDCD1*, *CD40L*, and *NFATC1*.⁵⁷ A strong microenvironment imprint has also been identified with overexpression of B cell and follicular dendritic cell-related genes, chemokines, and genes related to vascular biology. *PDGFRA*, *REL*, and *VEGF* are deregulated in AITL.⁵⁸ High expression of VEGF-A has been found

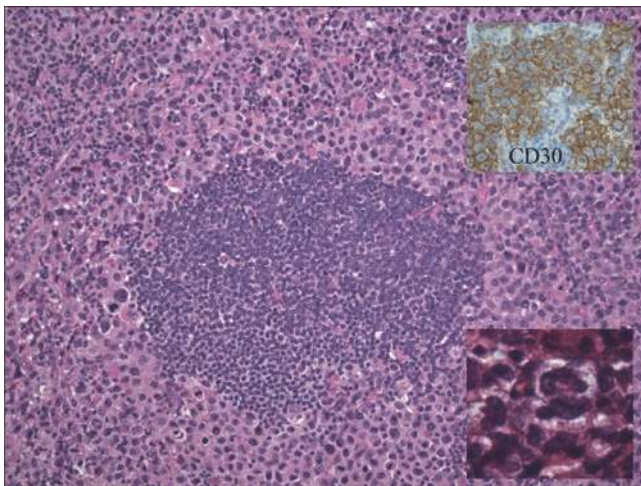


FIG. 25.2. Anaplastic large cell lymphoma showing an interfollicular pattern of large atypical cells with copious cytoplasm and pleomorphic nuclei surrounding a residual germinal center. These atypical cells show bright membranous staining for CD30 (upper right inset) and on higher magnification “hallmark” cells characterized by their distinctive horseshoe shaped nuclei are typically present.

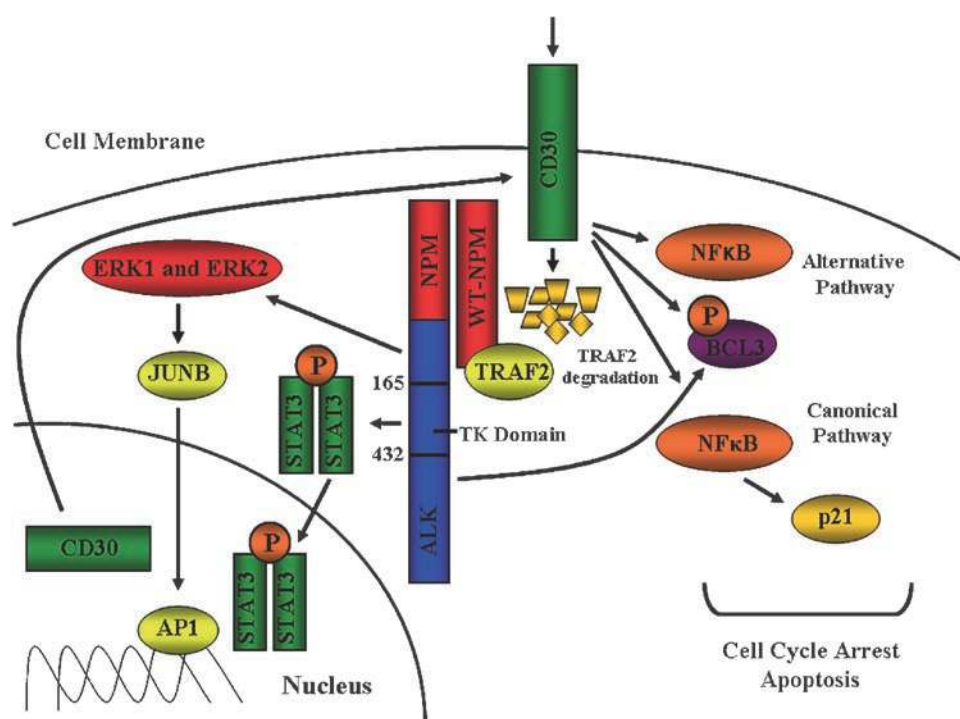


FIG. 25.3. ALK and CD30 signaling. CD30 expression is controlled by anaplastic lymphoma kinase (ALK) activity through the phosphorylation of signal transducer and activator of transcription 3 (STAT3) and the extracellular signal-regulated kinase 1 (ERK1)- and ERK2-mediated upregulation of JUNB protein levels. The nucleophosmin (NPM)-ALK fusion protein impedes full CD30 signaling and nuclear factor κ B (NF κ B) activation by titrating tumor

necrosis factor receptor-associated factor 2 (TRAF2) away from CD30 through dimerization with wild-type (WT) NPM. CD30 engagement results in TRAF2 degradation and BCL3 phosphorylation. The effect of CD30 engagement in ALCL cells is the activation of both the canonical and alternative NF κ B pathways, which result in apoptosis and p21-mediated cell-cycle arrest. *TK* tyrosine kinase (adapted from Chiarle et al⁵²).

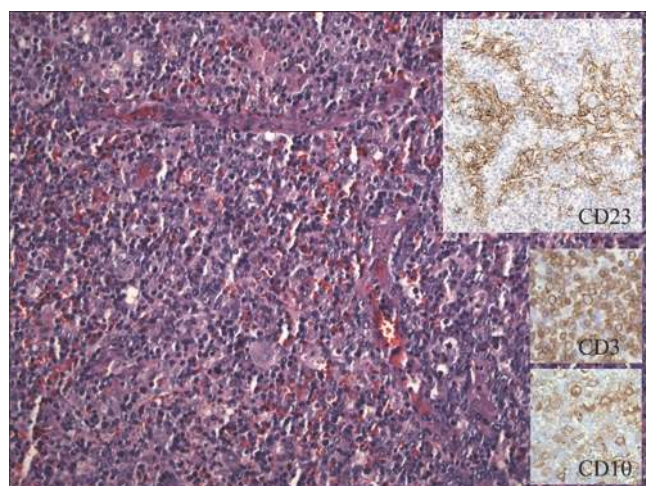


FIG. 25.4. Angioimmunoblastic T cell lymphoma. This lymph node is effaced by a heterogeneous infiltrate notable for predominantly medium-sized T cells some of which have a distinctive clear cytoplasm and interspersed large B cell immunoblasts which are often EBV infected. There is also a proliferation of arborizing high endothelial vessels. CD23+ (upper right inset) or CD21+ dendritic meshworks can be found extending from the vessels. The neoplastic T cells are derived from a follicular helper T cell that typically expresses CD3 (middle right inset), CD10 (lower right inset) and PD-1.

in AITL and has been shown to correlate with a poor prognosis.⁵⁹

Array-based CGH has identified unique cytogenetics findings in AITL, distinguishing it from PTCL, NOS.⁶⁰ The most recurrent changes in AITL include gains of 22q, 19, and 11p11–q14 and loss of 13q. Gains of 4q, 8q24 (*MYC* locus), and 17 are significantly more frequent in PTCL, NOS than in AITL. Trisomies 3 and 5, which have been described as common in AITL, have been identified in only a small number of PTCL, NOS cases.

Because histologic diagnosis may be difficult, TCR clonality may aid in the diagnosis. Specific patterns of T and B cell clonality may correlate with prognosis.⁶¹ Patients with both TCR β -chain gene and immunoglobulin gene rearrangements often have hemolytic anemia and may have spontaneous remissions, but do not respond as well to chemotherapy and have a worse survival than patients with only TCR clonality.⁶¹ Some cases of AITL will demonstrate oligo-clonality, and B cell clonality has been reported in up to 35% of cases.⁶²

AITL may respond to single agents, including steroids, cyclosporine, interferon, and nucleoside analogs. Combination chemotherapy is warranted once a diagnosis is made with a complete remission (CR) rate of 50–70%, following anthracycline-based therapy, but patients have frequent and

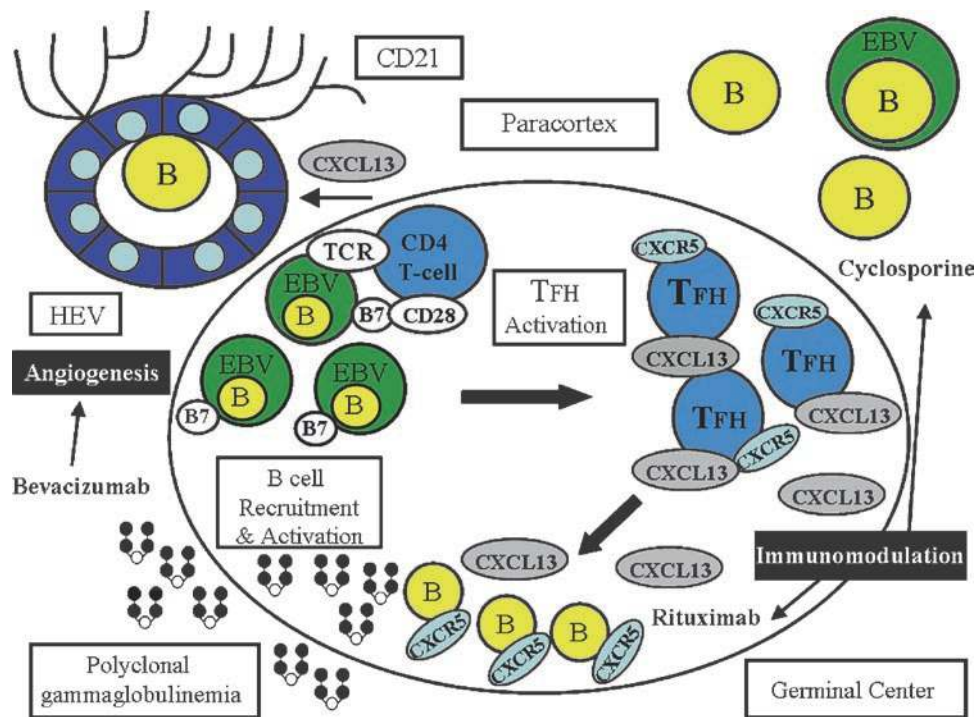


FIG. 25.5. Hypothetical model of angioimmunoblastic T cell lymphoma pathogenesis. Epstein–Barr virus (EBV)-positive B cells present EBV viral proteins (e.g., EBNA-1) in association with major histocompatibility complex (MHC) class II molecules, upregulate CD28 ligand (B7) and provide costimulatory signals for follicular helper T (T_{FH}) cell activation. T_{FH} cells upregulate CXCR5 and CXCL 13. CXCL 13 promotes B cell recruitment to

the lymph node through adherence of B cells on high endothelial venules (HEVs). Increased B cells expand in the paracortex and are activated. Some paracortical B cells become EBV transformed. CD21-positive dendritic cells expand from the HEVs. Unique types of therapy in AILT include immunomodulation by cyclosporine or rituximab and inhibition of angiogenesis by bevacizumab (adapted from Dunleavy et al⁵⁶).

early relapses or deaths because of infections with a median survival less than 36 months and 5 year overall survivals of 10–35%.^{2,63} Denileukin difitox, alemtuzumab (Campath), and even rituximab have had responses reported in AITL. Antiangiogenesis therapy has been proposed, due to the high expression VEGF-A, and case reports have shown responses with thalidomide and bevacizumab.⁵⁶

Natural Killer/T Cell Neoplasms

NK cells outnumber B cells in the circulation by a 3-to-1 ratio and contribute to innate immunity by recognition and lysis of tumor and virus-infected cells and by production of cytokines.⁶⁴ Interferon gamma ($IFN-\gamma$) is the prototypic NK-cell cytokine, which affects the Th1 immune response, activates antigen presenting cells which upregulate MHC class I expression, and activates macrophage killing of intracellular pathogens.⁶⁴ NK cells are differentiated from stem cells through myeloid-antigen positive NK/T bipotential progenitors, which lead to a relatively mature NK cell lineage progenitor (Figure 25.6). Mature NK cells are characterized by (1) large granular lymphocyte morphology (LGL), (2)

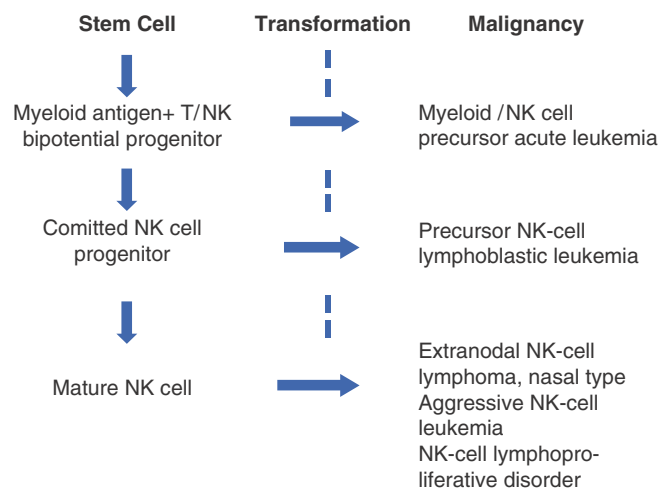


FIG. 25.6. NK-cells are differentiated from stem cells through myeloid-antigen positive NK/T bi-potential progenitors and lineage-committed progenitors. Myeloid/NK cell precursor acute leukemia is transformed from the myeloid antigen-positive progenitor. Blastic NK-cell lymphoma and precursor NK-cell lymphoblastic leukemia are derived from a relatively mature, NK-cell lineage committed progenitor. Two mature NK-cell neoplasms, aggressive NK-cell leukemia and extranodal NK-cell lymphoma, nasal type, are transformed from mature NK-cells (adapted from Suzuki et al⁷⁸).

surface CD3-negative, CD56-positive phenotype, and (3) germline configuration of the T cell receptor genes.

NK neoplasms (Table 25.2) are prevalent in Asia and Latin America. The WHO has recognized three types of NK neoplasms: (1) extranodal NK/T cell lymphoma, nasal type (ENKL), (2) aggressive NK-cell leukemia (now considered the leukemic form of no. 1), and (in the past) (3) blastic NK-cell lymphoma.⁶⁵ The latter diagnosis has now been termed CD4+, CD56+ hematodermic or blastic plasmacytoid dendritic cell neoplasm which is recognized as a tumor of plasmacytoid dendritic cell origin, and may have a myelomonocytic leukemia phase. Other neoplasms of possible NK cell lineage include myeloid /NK-cell precursor acute leukemia and precursor NK-cell acute lymphoblastic leukemia, which overlaps with blastic plasmacytoid dendritic cell neoplasm. Chronic NK-cell expansions, which may be clonal or (more often) reactive, may have features similar to T large granular lymphocyte leukemia.

EBV is consistently detected in tumor cells of ENKL and aggressive NK-cell leukemia, and its detection is supportive of a diagnosis. In situ hybridization for EBER may identify NK lymphoma cells in histopathologic sections; and the detection of EBV in sites where it is usually absent, such as the liver and bone marrow, is particularly helpful in diagnosing extranodal involvement. EBV-latent membrane protein (LMP-1) is expressed in most patients. Elevated levels of

EBV DNA in tumor tissue or serum has correlated with a poor prognosis in nasal NK/T cell lymphomas.^{66,67}

The pathologic diagnosis of NK/T neoplasms may be difficult because it can have a low-grade appearance mimicking a reactive process or have extensive necrosis with sparse tumor cells (Figure 25.7). The neoplastic infiltrate is polymorphic, often with angioinvasion and/or angiodestruction. The neoplastic cells have cytoplasmic azurophilic granules and a CD2+/CD3-/cCD3ε+/CD56+ immunophenotype. The nonneoplastic inflammatory cells may obscure the neoplastic cells and make the diagnosis problematic, particularly if the biopsy sample is small. Immunophenotypic variants, such as those expressing CD30, or lacking CD56 or EBV, may contribute to problems in diagnosis.⁶⁸

Conventional cytogenetic analysis of mature NK neoplasms is difficult, partly due to extensive necrosis and/or small sample size. Deletion of chromosome 6(q21–q25) is the most frequent cytogenetic abnormality.⁶⁹ Loss of heterozygosity at 13q has been observed in up to one-third of cases at diagnosis, but is uniformly present at relapse.⁶⁹ Abnormal karyotypes have been found in 23 of 30 (77%) of patients with NK neoplasms: pseudodiploidy (57%), hyperdiploidy (30%), and hypodiploidy (13%).

Genomic profiling (array CGH) has had variable results but shows genetic differences between ENKL and aggressive NK cell leukemia.⁷⁰ ENKL has shown gain at 2q and

TABLE 25.2. Clinicopathologic features of NK-cell lineage neoplasms.

	Myeloid/NK cell precursor acute leukemia	Precursor NK-cell lymphoblastic leukemia	Blastic NK-cell lymphoma/Plasmacytoid dendritic cell neoplasm (formerly blastic NK-cell lymphoma)	Aggressive NK-cell leukemia	Extranodal NK cell lymphoma, nasal type		NK cell lymphoproliferative disorder
					Limited stage	Advanced stage	
Morphology	Blastic	Blastic	Blastic	LGL	LGL		LGL
Azurophilic granule	–	–	–	+	+		+
Lymph node involvement	+	+	+/-	+	–	+/-	–
Extranodal involvement	Bone marrow, blood, mediastinum	Skin, bone marrow	Skin, bone marrow	Bone marrow, blood, liver, spleen	Nose, skin	Nose, skin, bone marrow, blood, GI tract, testes	Bone marrow
Surface marker	CD7+, CD33+, CD34+, CD56+	CD4+/-, CD7+, CD56+, TdT+	CD4+, CD56+, CD123+, TdT+/-	CD2+, CD16+, CD56+	CD2+, cyCD3+, CD56+		CD2+, cyCD3+, CD8+, CD16+, CD56+ (weak)
EBV	–	–	–	+/-	+		–
Clinical course	Aggressive	Aggressive	Usually aggressive	Aggressive	Sometimes indolent	Aggressive	Indolent
Therapy	AML chemotherapy	ALL chemotherapy	No standard therapy; consider AML chemotherapy in young patients	No standard therapy; consider ALL chemotherapy	Combined modality	No standard therapy	Immunosuppression/single agent chemotherapy
Prognosis	Poor	Poor	Poor	Very poor	Variable	Very poor	Good

LGL large granular lymphocyte; EBV Epstein–Barr virus; AML acute myeloid leukemia; ALL acute lymphoblastic leukemia.

Adapted from Suzuki et al.⁷⁸

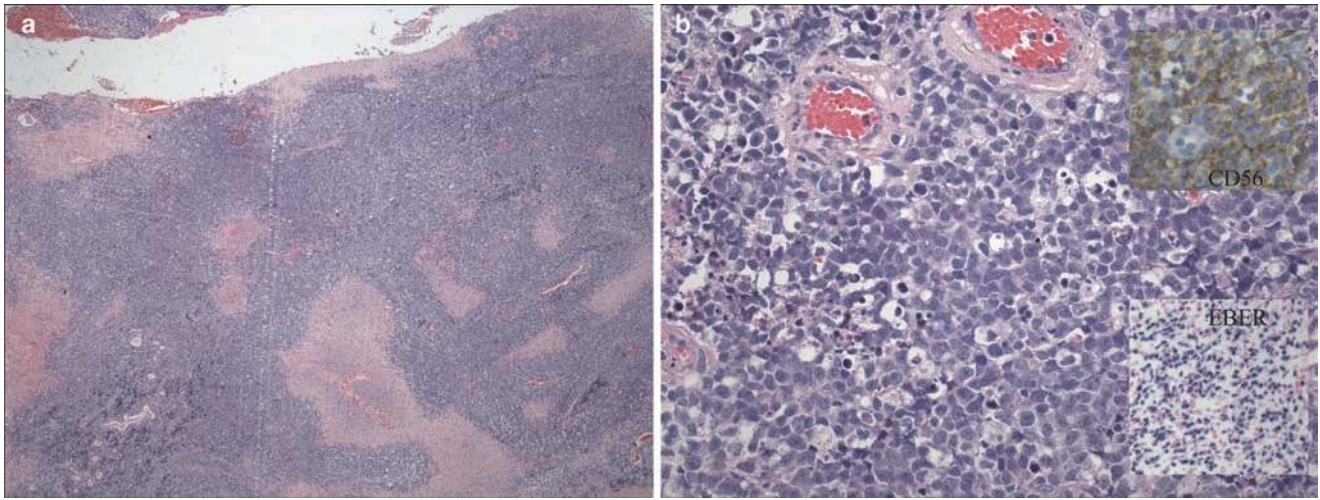


FIG. 25.7. Extranodal NK/T cell lymphoma, nasal type. (a) The characteristic low power findings of a diffuse lymphoid infiltrate showing angiocentrism and angiodestruction. These cells can range from small, angulated lymphocytes to larger more pleomorphic lymphocytes with variable amounts of background mitoses and apoptoses.

(b) The lymphocytes typically express membranous CD56 (upper right inset) with cytoplasmic CD3 and TIA-1. These NK/T cells are also commonly positive for EBV encoded RNAs (EBER) as seen in the lower right inset.

losses at 6q, 11q, 5p, 1p, 2p, and 4q; NK cell leukemia has shown gain at 1q and losses at 7p and 17p.⁷⁰

No consistent oncogenes or tumor suppressor genes have been identified in NK neoplasms. p53 is often overexpressed in patients with NK/T lymphoma nasal type and has been associated with a poor prognosis.^{16,71} Gene mutations in p53, C-KIT, and β -catenin have been described and have a geographic variation.^{72,73} Homozygous deletions of p15, p16, and p14 genes have also been described.⁷⁴ Methylation of p73 and p21 genes has been hypothesized to contribute to lymphomagenesis in NK cell disorders.⁷⁵ Mutations of FAS gene have been identified in 50–60% of nasal NK/T-cell lymphoma and may lead to resistance to apoptosis.^{72,76} Alterations in ATR, a gene responsive to DNA damage, have been detected in two of eight NK cell lines and four of ten clinical cases.⁷⁷

Patients with ENKL are middle aged (median age, 50–60 years) and may present with facial edema, nasal obstruction, or epistaxis.⁷⁸ Other sites of involvement include skin (nodule $\pm$ ulceration), orbit, gastrointestinal tract, and testes. CNS risk is estimated at 5–10%, and prophylaxis may be warranted. Lee et al developed a prognostic model for ENKL, which includes B symptoms, advanced stage, elevated lactate dehydrogenase level, and regional lymph nodes.⁷⁹ Five-year overall survival (OS) according to four risk groups was: 81%(0 risk factors), 64%(1), 32%(2), and 7%(3,4). Because of chemoresistance related to the presence of P-glycoprotein, concurrent chemoradiotherapy is recommended in ENKL.^{80,81} Agents that bypass P-glycoprotein in combination regimens for ENKL include methotrexate, etoposide, and L-asparaginase.^{80,82}

Aggressive NK cell leukemia develops in young patients (median age, 30–40 years) and has a progressive course with B symptoms, liver dysfunction, lymphadenopathy, hepatosplenomegaly, and hemophagocytosis.^{81,83,84} The most common cytogenetic abnormalities are deletion of 6q21–q25 and loss of 17p13. Median survival is 2 months. Because prognosis is so poor in the majority of NK neoplasms, limited data suggests an up-front role for both autologous and allogeneic stem cell transplantation. Other therapies under investigation include EBV-specific T lymphocytes, monoclonal antibodies, and phase I/II trials of chemotherapy agents.

Extranodal Peripheral T Cell Lymphomas

$\gamma\delta$ TCL comprise less than 10% of PTCL and occur most commonly at extranodal sites in subcutaneous, hepatosplenic, or intestinal forms. The TCR δ gene consists of six V δ gene segments. In one study, normal $\gamma\delta$ lymphocytes present in spleen, thymus, and intestine expressed the V δ 1 gene which is present in HSTCL, while the $\gamma\delta$ T cells in tonsils and skin expressed the V δ 2 gene which is present in the subcutaneous $\gamma\delta$ panniculitis-like T cell lymphoma (SPTCL).⁸⁵ This finding suggests that the $\gamma\delta$ T cell lymphomas are derived from local lymphoid tissue.

Within the group of PTCL that may present with subcutaneous involvement, distinction should be made between cases with the $\alpha\beta$ phenotype (cases of SPTCL) and the $\gamma\delta$ phenotype (cases of cutaneous $\gamma\delta$ T-cell lymphoma). SPTCL usually has a CD4–, CD8+, CD56– phenotype, less commonly is associated with hemophagocytosis (HPS), and a

favorable prognosis (5-year OS of 82%) On the other hand, primary cutaneous $\gamma\delta$ T-cell lymphoma usually has a CD4 $^{-}$, CD8 $^{-}$, CD56 $+/^{-}$ phenotype, frequent HPS, and a poor prognosis (5 year OS of 11%).⁸⁶ Both types have a predominantly subcutaneous infiltrate with rimming of the fat cells by neoplastic cells (Figure 25.8); however, the upper dermis and epidermis are more commonly involved in primary cutaneous $\gamma\delta$ T-cell lymphoma. EBV is usually negative in both types and isochromosome 7q has been reported in some cases of the $\gamma\delta$ type.⁸⁷

Hepatosplenic T-cell lymphoma (HSTCL) is mainly seen in young males (median age, 29–35 years.), presenting with B symptoms, hepatosplenomegaly, minimal to no lymphadenopathy, anemia, and severe thrombocytopenia.^{88–90}

The marrow is involved in three-quarters of patients, and HPS may occur.⁸⁹ HSTCL has been described in patients with solid organ transplants and with Crohn disease.⁹¹ Most patients have brief responses (or are refractory) to anthracycline therapy with median survivals less than 14 months.^{88–90} Patients may have a terminal leukemic phase.

HSTCL likely arises from $\gamma\delta$ T cells of the hepatic sinusoids and splenic red pulp (Figure 25.9). Most cases have clonal TCR γ -gene or δ -gene rearrangements with a characteristic T cell phenotype: CD2 $^{+}$, CD3 $^{+}$, CD4 $^{-}$, CD7 $^{+}$, and CD8 $^{-}$. NK-cell associated antigens, CD16 and CD56, and cytotoxic granule-associated proteins are often expressed.⁹¹ An $\alpha\beta$ variant has been described with a similar presentation and poor prognosis.⁹² HSTCL uniquely express

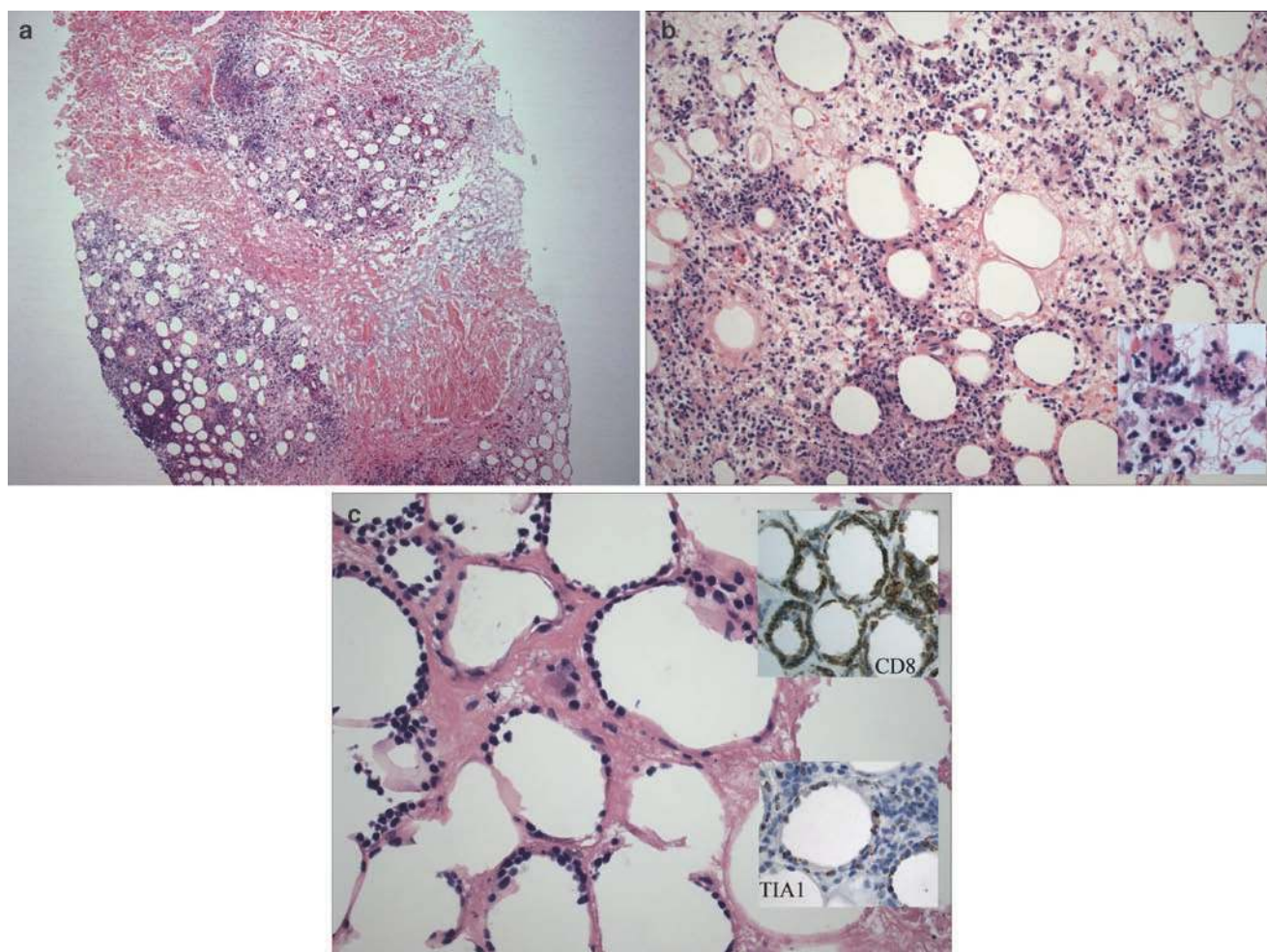


FIG. 25.8. (a) Cutaneous $\gamma\delta$ T cell lymphoma with a subcutaneous panniculitic-like T cell lymphoma pattern. Although $\gamma\delta$ T cell lymphomas can involve skin in a multitude of patterns, the panniculitic-like pattern seen here with lobules of subcutaneous fat showing involvement by a heterogeneous infiltrate comprised of small lymphocytes, plasma cells, eosinophils and macrophages is common. (b) Often there is significant apoptosis and necrosis in cutaneous $\gamma\delta$

T cell lymphoma (*lower right inset*). (c) At high power, examination of adipocytes often reveals them to be studded by CD3 $^{+}$ T cells that express the cytolytic protein TIA-1 (*lower right inset*). In cases of $\gamma\delta$ T cell lymphoma, the T cells are typically negative for both CD4 and CD8, whereas in most cases of subcutaneous panniculitic-like T cell lymphomas comprised of $\alpha\beta$ T cells there is expression of CD8 (*upper right inset*).

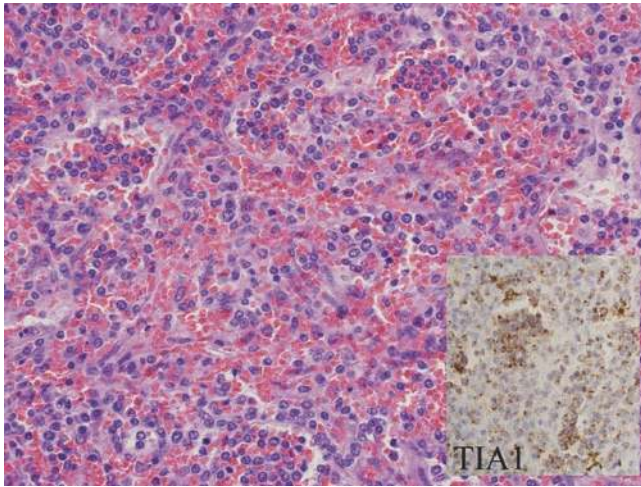


FIG. 25.9. Hepatosplenic T cell lymphoma. This section of an enlarged spleen shows clusters of atypical medium-sized lymphocytes in the splenic sinuses and with some *red pulp extension*. These lymphocytes are positive typically CD3+ T cells with expression of the cytolytic protein TIA-1 (*lower right inset*). There is often loss of CD5 or CD7 in these cases. Those hepatosplenic T cell lymphomas that are derived from $\gamma\delta$ T cells are often negative for both CD4 and CD8, whereas those that are $\alpha\beta$ T cell derived typically express CD8.

killer-cell Ig-like receptors (KIRs), indicating a derivation from memory T cells. EBV has been identified in a minority of HSTCL.⁵

The most common cytogenetic abnormality in HSTCL is isochromosome (i) 7(10q).^{89,90} Trisomy 8 is also frequently observed. Other cytogenetic abnormalities seen in HSTCL include deletion 11q, t(1;14) (q21;q13), der(21) t(7;21), and complex karyotype.⁸⁹

Enteropathy-associated T cell lymphoma (EATL) is a rare lymphoma of intraepithelial lymphocytes which are CD3+, CD4-, CD7+, CD8-/+, CD30+, and CD103+ (Figure 25.10). Patients often have had a prior history of gluten-sensitive enteropathy; and four-fifths of patients will have abdominal pain and weight loss, and one-third will have diarrhea or emesis.⁹³ Patients have multiple circumferential jejunal ulcers, and many will develop small bowel obstruction or perforation before a diagnosis is made at laparotomy. Prognosis is poor with median survival of 7.5 months and 1 year disease free-survival of less than 20%.^{93,94}

Serologic markers for celiac disease, such as positive antigliadin antibodies, and HLA types (DQ2 and DQ8) may be present at diagnosis.⁹⁵ The TCR genes are usually rearranged in patients with EATL (more commonly γ than β), and may be indicative of refractory celiac disease evolving into lymphoma.⁹⁶ Loss of heterozygosity of chromosome 9q21 has been found with EATL.⁹⁷ CGH revealed chromosome imbalances in 87% of cases with gains on chromosome 9q (58%), 7q (24%), 5q (18%), and 1q (16%) and losses on chromosome 8p (24%), 13q (24%), and 9p (18%).⁹⁸ Patients

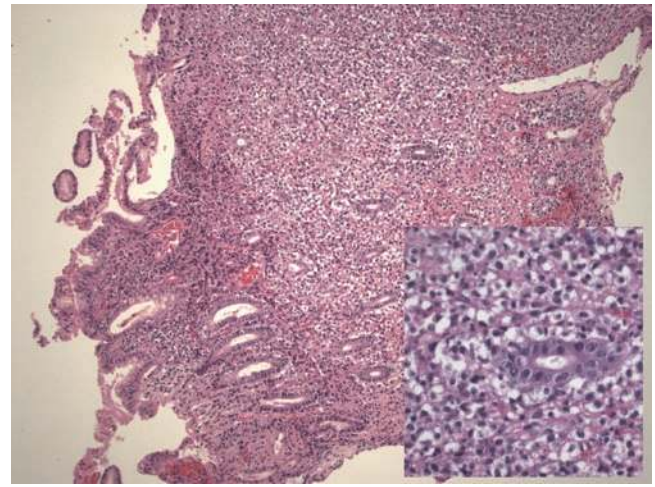


FIG. 25.10. Enteropathy associated T cell lymphoma. This T cell lymphoma is associated with celiac disease, and mucosal ulceration is a common finding. The infiltrative, neoplastic T cells are typically monomorphic, intermediate to large sized with round to angulated nuclei and clear cytoplasm. The T cells often invade the intestinal crypt epithelium (*inset*). In some cases, the neoplastic cells are nearly obscured by the associated inflammatory cell infiltrate of eosinophils and histiocytes. These lymphomas are comprised of CD3+ T cells that express CD103 without CD4 or CD5 and have variable CD8 expression.

with more than three chromosomal imbalances had a worse survival than those with less imbalances.⁹⁸ Analysis of microsatellite markers revealed two distinct subgroups of EATL: one with amplification of genomic material at 9q34 (loci for *c-ABL* and *NOTCH1*) and a smaller one showing allelic imbalances at 3q27.⁹⁹ EBV has varied according to geography with a prevalence in Mexico over Europe.¹⁰⁰

T-Prolymphocytic Leukemia

T-PLL represents approximately 2% of small lymphocytic leukemias in adults; median age is 57–69 years, and there is a slight male predominance. Patients usually have a lymphocytosis over $100 \times 10^9/L$ (75%), splenomegaly (73–79%), lymphadenopathy (46–53%); one-fifth of patients will have skin infiltration.^{101,102} Cell morphology is variable and nuclei are irregular with prominent nucleoli, and cytoplasmic protrusions are common. The majority of T-PLL are CD4+/CD8-; one-quarter coexpress CD4+/CD8+; and a small number will be CD4-/CD8+.¹⁰³

T-PLL usually has a complex karyotype, and the most common (70–80%) abnormality is an inversion of chromosome 14 (inv(14) (q11;q32)); 10% have a reciprocal translocation (t(14;14) (9q20;q1)) (Figure 25.11). These translocations juxtapose the locus of the TCR α/δ genes or, less commonly the TCR- β chain locus, with members of the *TCL-1* gene family. *TCL-1* interacts with the pleckstrin homology

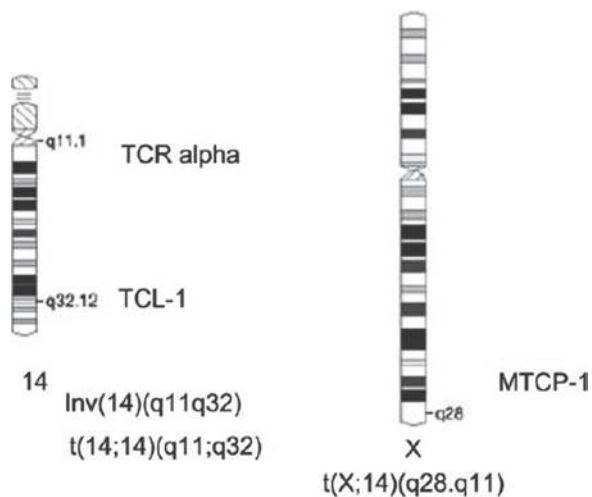


FIG. 25.11. Chromosomal translocations involving *TCL-1* and *MTCP-1* oncogenes with *TCR α* in T-prolymphocytic leukemia (with permission Dearden¹⁰¹).

domain of AKT and enhances AKT kinase activity, which affects pathways involved in controlling proliferation and survival of T cells.¹⁰⁴ Secondary chromosomal aberrations include gains of 8q (including isochromosome (8q)), deletions of 8p, and losses on 11 and 12p13 and (less frequently) on 6q and 22q).¹⁰⁵

Genome expression profiling in T-PLL identifies upregulation of genes involved in transcription, nucleosome assembly, and cell cycle control and downregulation of proapoptotic genes.¹⁰⁵ A gene dosage effect has been proposed as a pathogenetic mechanism in disease progression.¹⁰⁵ Mutations in the ataxia telangiectasia mutated (*ATM*) gene, a tumor suppressor located at 11q22–23, has been identified in T-PLL.¹⁰¹

Response rates to chemotherapy are 10–48% with few complete responses. Median survival is approximately 1 year, although 10–20% of patients can have an initial indolent phase. Alemtuzumab (Campath) is the most active single agent.¹⁰¹ Preliminary data suggest that the best survival occurs in patients who receive chemotherapy (that includes a nucleoside analog, alemtuzumab, and a transplant, preferably allogeneic).¹⁰¹

Adult T Cell Leukemia/Lymphoma

HTLV-1 is an RNA-containing retrovirus that infects a mature T cell (CD3+, CD4+, and HLA-DR+). Also see Chap. 7. The molecular pathogenesis revolves around Tax, a potent HTLV-1 transactivator protein, and HTLV-1 basic ZIP factor (HBZ)¹⁰⁶ (Figure 25.12). Tax is prominent during the initial infection by increasing the expression of viral genes through viral long terminal repeats (LTRs) and by stimulating the transcription of cellular genes through signaling

pathways of nuclear factor kappa B (NFκB), serum responsive factor (SRF), cyclic AMP response element binding protein (CREB), and activated protein 1 (AP-1). Cytotoxic T cells (CTLs) target Tax expression and control the infection; however, ATL cells lose Tax expression and escape the CTLs by three mechanisms: (1) the loss of 5'-LTR, which is a viral promoter for the transcription of the *TAX* gene; (2) the nonsense (or missense) mutation of the *TAX* gene; and (3) epigenetic change in the 5'-LTR, resulting in DNA hypermethylation and histone modification that silence the transcription of viral genes.¹⁰⁷

HBZ is transcribed from the 3'-LTR, which is conserved and hypomethylated in all ATL cases; its persistence is likely central to leukemogenesis. *HBZ* promotes T cell proliferation in its RNA form by the regulation of the e2F-1 pathway; the HBZ protein interacts with CREB-2 and suppresses Tax-mediated viral transcription.¹⁰⁸ Initially, the T lymphoproliferation is polyclonal and controlled by host defense mechanisms; however, as Tax expression diminishes, an oligoclonal or monoclonal T cell proliferation that is interleukin-2 independent emerges, resulting in the clinical manifestations of ATLL. HTLV-1 contributes to a multi-step process of worsening genetic instability characterized by mutation of *p53*, deletion of tumor-suppressor genes *p15* and *p16*, and DNA methylation.

There is a high degree of diversity and complexity in the cytogenetic abnormalities of ATLL, and neither specific translocations nor genes have been identified. In a series of 107 ATLL cases in Japan, translocations involving 14q32 (28%) or 14q11 (14%) and deletion of 6q (23%) were the most frequent chromosomal abnormalities.¹⁰⁹ Array-based CGH has revealed gains in 1p, 2p, 4q, 7p, and 7q, and losses in 10p, 13q, 16q, and 18p.¹¹⁰ Spectral karyotyping (SKY) and high-resolution single nucleotide polymorphism (SNP) array-CGH identified frequent breakpoints at 10p11, 14q11, and 14q32.¹¹¹ A candidate tumor suppressor gene, transcription factor 8 (*TCF8*), was found at 10p11. Downregulation of *TCF8* expression in ATLL cells in vitro has been associated with resistance to transforming growth factor beta1 (TGFβ1).¹¹¹ Aneuploidy, multiple chromosomal breaks, and loss of tumor suppressor genes are associated with an aggressive course.¹¹²

Forkhead box p3 (FOXP3) is a nuclear protein that functions as a transcriptional repressor that is implicated in T cell regulation and has been identified uniquely to a variable percentage (36–68%) of ATLL cases.^{113,114} FOXP3 expression correlates with clinicopathologic features of ATLL and is more commonly associated with pleomorphic morphology (as opposed to anaplastic), EBV association, immunodeficiency, and simpler chromosomal abnormalities than FOXP3-negative cases.¹¹⁴ Chemokine receptors, such as chemokine receptor 4 (CCR4) and CCR8, expression on ATLL cells may contribute to epidermotropism and antiapoptosis.¹¹⁵ Survival differences have been negative to variable based on FOXP3 expression; however, the more

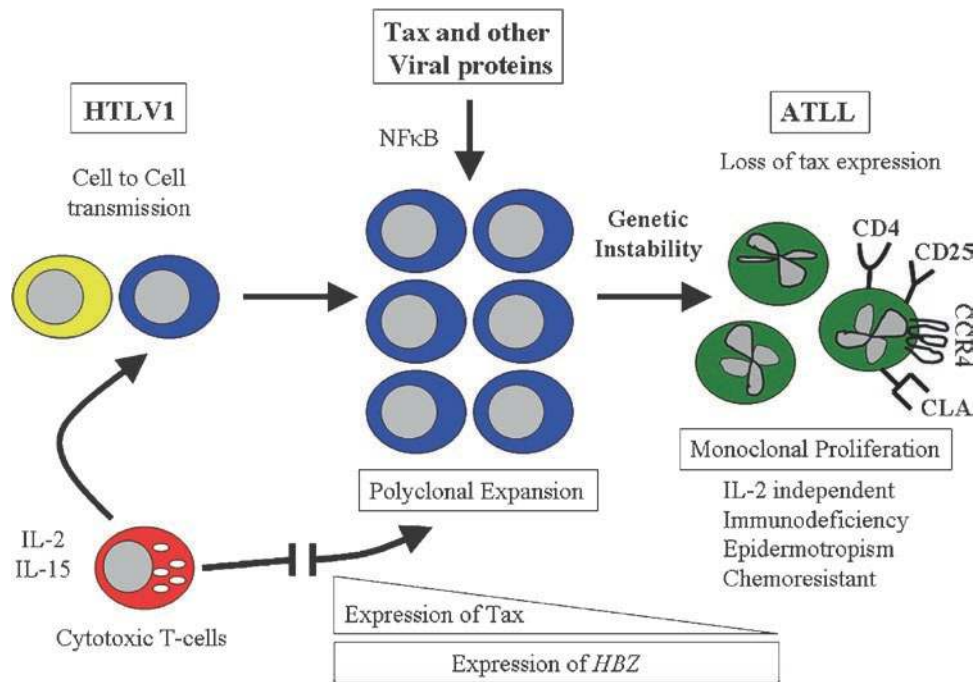


FIG. 25.12. Course of HTLV-1 infection. After infection of a mature T-helper cell, there is long latency period (decades), which can be controlled by cytotoxic T cells and an autocrine IL-2 loop. Clonal proliferation is promoted by pleiotropic actions of Tax and other viral proteins that inhibit apoptosis and induce $\text{I}\kappa\text{B}\alpha$, which activates the NF κB pathway. HBZ promotes T cell proliferation and the HBZ protein suppresses Tax-mediated viral transcription.

As Tax expression is lost, there is emergence of a monoclonal T-cell population that is independent of IL-2. CC chemokine receptor 4 (CCR4) and cutaneous lymphocyte antigen (CLA) on ATLL cells interact with endothelial cells in the skin and contribute to epidermotropism. ATLL follows a multistep process of worsening genetic instability and is subdivided into clinical syndromes characterized by immunodeficiency and chemoresistance.

complex cytogenetics associated with FOXP3-negative cases suggest that FOXP3 expression could be lost with disease progression.¹¹⁴

On-going genetic mutations are necessary for the progression of ATLL. DNA microarray analysis has identified recurrent gain of chromosomes at 3/3p among patients with the acute form of ATLL.¹¹⁶ Expression of the gene for MET, a receptor tyrosine kinase for hepatocyte growth factor (HGF), is associated with the acute stage, and increased plasma concentrations of HGF have been shown to precede the expression of MET.¹¹⁶ A hypothesis suggests that ATLL cells secrete cytokines, including tumor necrosis factor- α and interleukin-1 β , which induce HGF in fibroblasts and that the HGF-MET signaling pathway is a candidate molecular mechanism for the progression of ATLL.

Despite combination chemotherapy, which may yield brief responses, median survivals for the acute and lymphomatous forms of ATLL are less than 1 year.¹⁰⁷ Adverse prognostic factors include age >40 years, poor performance status, tumor bulk, elevated LDH, and hypercalcemia. The major causes of death are opportunistic pneumonia and progressive disease.¹¹⁷

Because of its chemo-resistance and viral origin, ATLL provides unique targets for investigational therapy. Interferon plus zidovudine (AZT) has been as effective as chemotherapy, and has been combined with it.¹¹⁸ NF κB inhibition has been proposed as a therapeutic target. See the discussion below in the section “Therapeutic Implications of T-Cell Receptors and Molecular Pathways.” Trials have shown activity of conjugated and unconjugated monoclonal antibody therapy directed at the IL-2 receptor, the proteasome inhibitor (bortezomib), and the histone deacetylase inhibitor (romidepsin (depsipeptide)).^{107,119} Allogeneic transplant has been utilized in selected cases and may offer the best prospect for a long-term survival.¹²⁰ Other proposed methods to prevent the development of ATLL include antiretroviral therapy and a Tax-targeted vaccine.

T-Large Granular Lymphocyte Leukemia

T-LGL involves effector-memory cells of cytotoxic T lymphocyte (CTL) origin (CD3+ CD8+), which are resistant to apoptosis, despite expressing high levels of Fas and Fas

ligand. Molecular profiling shows dysregulation of apoptotic genes.¹²¹ Genes, such as *TNFAIP3* (an NFκB inducible gene) and myeloid cell leukemia 1 (*MCL1*), that are upregulated in LGL are antiapoptotic; whereas, those that are downregulated, such as *BAX*, are proapoptotic. Inhibition of STAT3 pathway is associated with decreased *MCL1* and increased apoptosis of LGLs.¹²² Inhibition of acid ceramidase, an enzyme central to sphingolipid-mediated signaling, also leads to apoptosis of LGLs.¹²¹

Therapeutic Implications of T-Cell Receptors and Molecular Pathways

The mature T/NK neoplasms are relatively chemoresistant to anthracycline-based regimens; and novel therapies, some of which have been addressed in the disease descriptions above, are needed to improve prognosis. Stem cell transplantation, both autologous and allogeneic, has been advocated for patients with poor prognostic factors, but its use should not be overestimated, because most reports in PTCL are small retrospective series and involve highly selected patients.^{2,123,124} Agents directed toward T-cell receptors (or molecular pathways that are unique to subtypes of PTCL) are under investigation (Table 25.3).¹²⁵

Mycosis fungoides has been a disease that has led to the approval of agents directed at T-cell receptors and of new chemotherapy drugs, which are being evaluated in other types of PTCL. Denileukin difitox, a fusion protein

comprised of IL2 ligand and diphtheria toxin, is being used as a single agent in relapsed/refractory PTCL.¹²⁶ Alemtuzumab, a humanized anti-CD52 antibody, is active in T-PLL and is under investigation in T-cell lymphomas.^{127,128} Both denileukin difitox and alemtuzumab are being utilized with combination chemotherapeutic regimens for PTCL.

Monoclonal antibodies, Zanolimumab and Siplizumab, are humanized antibodies directed at cell surface receptors CD4 and CD2, respectively.^{129,130} SGN-30 (a chimeric monoclonal antibody) and MDX-060 (iratimumab; a humanized monoclonal antibody) have shown efficacy in CD30+ ALCL.^{131,132} Another humanized antibody (KW-0761) targets the chemokine receptor 4 (CCR4), which is expressed by ATLL and a subset of PTCL.¹³³

Gemcitabine, a pyrimidine analog, and purine analogs (i.e., pentostatin, fludarabine, cladribine, and forodesine) have activity as single agents in T-cell lymphomas, and are being combined with other agents.² Pralatrexate is a novel folate antagonist that shows a high response rate in PTCL, perhaps because of its high affinity for reduced folate carrier type I and greater intracellular accumulation than other antifolate drugs.¹³⁴

Histone deacetylase (HDAC) inhibitors induce histone acetylation and chromatin remodeling and activate or repress genes that control proliferation, apoptosis, and immune modulation. Vorinostat and romidepsin (depsipeptide) have activity in cutaneous T-cell lymphoma (CTCL), but their ability to induce apoptosis varies according to the type of prosurvival protein expressed.¹³⁵ Panobinostat is another HDAC inhibitor, which has been shown to down regulate genes affecting angiogenesis in patients with CTCL.¹³⁶

Molecular pathways are being targeted for therapy in lymphomas. NF-κB positively regulates gene transcription that induces several antiapoptotic proteins, as well as proteins that affect cell cycle progression, and is regulated by the ubiquitin–proteasome pathway.¹³⁷ Bortezomib inhibits both activation pathways for NF-κB, by inhibiting proteasome-mediated degradation of IκB proteins (multifactorial regulators of NF-κB transcription factors) and processing of p100.

Inhibitors of protein kinase C (PKC), PI3K-AKT, mTOR, and cyclin-dependent kinases are in clinical trials. The PKC inhibitor enzastaurin acts through the AKT pathway and induces apoptosis in CTCL cell lines.¹³⁸ UCN-01 (hydroxystaurosporine), an inhibitor of PKC and cyclin dependent kinases, is also an inhibitor of ALK and is in a phase II trial for ALCL and PTCL.¹²⁵ Genomic profiling will likely better subdivide types of T-cell lymphomas, identify specific molecular targets and assist in the selection of therapy.^{139,140}

TABLE 25.3 Therapies for peripheral T/NK cell neoplasm: cell surface targets, chemotherapy, and small molecules targeting molecular pathways.

Therapy	Target
<i>Cell surface targeted therapy</i>	
Denileukin difitox	IL-2 receptor
Alemtuzumab	CD52
Zanolimumab	CD4
Siplizumab	CD2
SGN-30, MDX-060	CD30
kW-0761	CCR4
<i>Chemotherapy agents</i>	
Gemcitabine	Pyrimidine analog
Pentostatin, fludarabine, cladribine, forodesine	Purine analogs
Pralatrexate	Anti-folate
Vorinostat, romidepsin, panobinostat	Histone deacetylase inhibitors
Bortezomib	Ubiquitin–proteasome inhibition; NFκB
<i>Small molecule agents</i>	
Enzastaurin	Protein kinase C and AKT pathway
UCN-01	Protein kinase C and cyclin dependent kinase

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Precursor T-Cell Neoplasms

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Introduction

Precursor T-cell lymphoblastic leukemias and lymphomas represent 15% of childhood acute lymphoblastic leukemias (ALLs) and one third of pediatric non-Hodgkin lymphomas, respectively. T-cell ALLs are characterized by prominent (>30%) bone marrow (BM) infiltration with or without mediastinal mass, while T-cell lymphoblastic lymphomas show mediastinal masses in the context of limited or no BM involvement. These two clinical entities share a similar spectrum of molecular and cytogenetic abnormalities, and most probably represent different manifestations of the same disease, commonly designated here as T-ALL.^{1,2}

Oncogenic transformation of T-cell precursors in T-ALL is driven by a combination of specific genetic abnormalities found exclusively or most prominently in this disease, and more general oncogenic lesions in oncogenes and tumor suppressors involved in the pathogenesis of a broader spectrum of human cancers. The most prominent T-cell specific abnormality is the presence of activating mutations in *NOTCH1*, which are detected in over 55% of T-ALL cases.^{3–5} However, the most prevalent genetic abnormality in this disease, occurring in about 70% of the cases, is the deletion of the 9p21 chromosomal region, which results in the loss of the p16/INK4A and p14/ARF tumor suppressor genes.^{6–8} These highly prevalent mutations constitute the core of the mechanism of transformation in most T-ALLs, and their transforming effects are modulated by the aberrant expression of T-cell specific transcription factor oncogenes, including: basic helix-loop-helix (bHLH) family members, such as *TAL1*,^{9–12} *TAL2*,¹³ *LYL1*¹⁴ and *BHLHB1*;¹⁵ LIM-only domain (LMO) factors, such as *LMO1* and *LMO2*,^{16–20} the *TLX1/HOX11*,^{21–24} *TLX3/HOX11L2*,^{8,25} *NKX2.5*,^{26,27} and *HOXA* homeobox genes;^{28,29} and *MYC*,^{30–34} *MYB*,³⁵ and *TANI*, a truncated and constitutively activated form of the NOTCH1 receptor³⁶ (Table 26.1).

These oncogenic transcription factors are frequently activated by chromosomal translocations juxtaposing them to the enhancers of T-cell receptor (TCR) genes, *TCRB* and *TCRA/D* in chromosome bands 7q34 and 14q11, respectively,

which are generated by errors in the recombination process that generates functional T-cell antigen receptors during normal thymocyte development.^{2,37,38} In addition, alternative chromosomal rearrangements, resulting in aberrant expression of some of these oncogenes (that do not involve the *TCR* loci) may be found in a number of T-ALL cases. A prominent example of this alternative mechanism is the *TAL1d* deletion, a small intrachromosomal rearrangement in chromosome 1p32, that places the *TAL1* gene under the control of the promoter of *SIL*, a nearby gene highly expressed in T-cell precursors.³⁹ Similarly, *LMO2* may be activated by translocations into the *TCR* loci or by small deletions in chromosome 11p13.⁴⁰ Moreover, both *TAL1* and *LMO2* are frequently found to be biallelically expressed in T-ALL that lack structural alterations in their respective loci, suggesting that alternative mechanisms most probably involving the activation of upstream regulatory factors controlling the expression of these transcription factor oncogenes are also involved in the pathogenesis of T-ALL.^{41,42}

Each of these T-cell specific transcription factor oncogenes defines different molecular subgroups of T-ALL associated with specific patterns of gene expression, a specific block in T-cell differentiation, and distinct clinical characteristics.^{8,43} Overall, these results suggest that aberrant expression of these oncogenic transcription factors contributes to the pathogenesis of T-ALL by disrupting the normal circuitry that controls cell proliferation, differentiation, and survival during T-cell development.^{8,28,43}

The complexity of genetic alterations associated with T-cell transformation is completed with a number of rare (but recurrent) cytogenetic and molecular alterations resulting in: (1) expression of fusion transcription factor oncogenes, such as *PICALM-MLLT10/CALM-AF10*,^{44–46} *MLL-MLLT1/MLL-ENL*,^{47,48} *SET/NUP214*,⁴⁹ and *NUP98-RAP1GDS*;^{50,51} (Table 26.2); (2) activation of genes involved in cell proliferation, such as *LCK*,⁵² *CCND2*,^{53,54} *JAK1*,⁵⁵ *NUP214-ABL1*,⁵⁶ *EML1-ABL1*,³⁸ and *NRAS*,^{57,58} and (3) inactivation of tumor suppressor genes responsible for control of cell growth, including *NFI*⁵⁹ and *PTEN*.⁶⁰

TABLE 26.1. T-cell receptor gene clusters and their involvement in chromosomal aberrations affecting transcription factors in T-ALL.

T-cell receptor gene cluster			Partner gene	
Gene cluster	Gene symbol	Chromosome location	Gene symbol	Chromosome location
<i>T-cell receptor</i> α/δ	<i>TCRA/D</i>	14q11	<i>OLIG2</i>	21q22
			<i>CCND2</i>	12p13
			<i>LMO1</i>	11p15
			<i>LMO2</i>	11p13
			<i>MYC</i>	8q24
			<i>NKX2-5</i>	5q35
			<i>TAL1</i>	1p32
			<i>TLX1</i>	10q24
<i>T-cell receptor</i> β	<i>TCRB</i>	7q34-35	<i>TLX3</i>	5q35
			<i>CCND2</i>	12p13
			<i>HOXA cluster</i>	7p15
			<i>LCK</i>	1p34
			<i>LMO1</i>	11p15
			<i>LMO2</i>	11p13
			<i>LYL1</i>	19p13
			<i>NOTCH1</i>	9q34
			<i>TAL2</i>	9q32
			<i>TLX1</i>	10q24
			<i>MYB</i>	6q23

TABLE 26.2. Translocation-associated fusion oncogenes in T-ALL.

Translocation	Fusion transcript
t(11;19)(q23;p13.3)	<i>MLL-MLLT1</i>
t(10;11)(p13;q14)	<i>PICALM-MLLT10</i>
t(4;11)(q21;p15)	<i>NUP98-RAP1GDS1</i>
t(11;18)(p15;q12)	<i>NUP98-SETBP1</i>
t(10;11)(q25;p15)	<i>NUP98-ADD3</i>
t(3;11)(q29;p15)	<i>NUP98-IQCG</i>
t(6;11)(q24;p15)	<i>NUP98-CCDC28A</i>
t(11;18)(p15;q12)	<i>NUP98-SETBP1</i>
del(9)(q34.11q34.13)	<i>SET-NUP214</i>
Episomal amplification/inv 9q34	<i>NUP214-ABL1</i>
t(9;14)(q34;q32)	<i>EML1-ABL1</i>
t(9;12)(p24;p13)	<i>ETV6-JAK2</i>

Activating Mutations in the NOTCH1 Signaling Pathway

The NOTCH signaling pathway is an evolutionary-conserved signaling mechanism responsible for the direct transduction of developmental signals at the cell surface into changes in gene expression in the nucleus^{61,62} and plays a critical role in lineage specification decisions that enable multipotential precursor cells to become committed to specific cell lineages during development.^{63,64} In the hematopoietic system, NOTCH1 signaling plays a critical role in T-cell development^{62,65}, by driving the initial commitment of undifferentiated hematopoietic

progenitors to the T-cell lineage,^{66–69} and then by promoting thymocyte maturation during the early stages of intrathymic T-cell development.⁷⁰

The basic components of the NOTCH pathway include the Delta and Serrate (DSL) family of ligands (Delta-like 1, 3 and 4; and Jagged 1 and 2), the NOTCH receptors (NOTCH1–4), and the CSL (CBF1/Su(H)/LAG-1) DNA binding protein, a transcription regulator that binds to the promoter of NOTCH target genes and mediates the activation of gene expression upon interaction with the nuclear/activated forms of NOTCH.⁶²

Resting mature NOTCH receptors are heterodimeric transmembrane proteins generated by proteolytic cleavage from a single precursor polypeptide. The N-terminal fragment of the receptor contains multiple EGF repeats responsible for ligand interaction and a series of LNR repeats, which stabilize the heterodimeric association between the N-terminal and C-terminal fragments. The C-terminus (membrane-bound) portion of the receptor constitutes a membrane-anchored transcription factor (containing nuclear localization signals, a RAM, and six ankyrin repeat protein–protein interaction domains required for the interaction with CSL) and a carboxy-terminal PEST sequence responsible for turning off NOTCH signaling via proteasomal degradation of the activated receptor in the nucleus.⁶²

Physiologic activation of NOTCH1 signaling is triggered by the interaction of NOTCH1 in the membrane with a DSL ligand expressed on the surface of a neighboring cell. This ligand-receptor interaction induces two consecutive proteolytic cleavages in the C-terminus membrane-anchored subunit of NOTCH1, first by an ADAM metalloprotease, and subsequently by the γ -secretase complex, and results in the release the intracellular domains of the receptor (ICN1) from the cell membrane^{71,72} (Figure 26.1). The γ -secretase complex is involved in the processing of a number of class I transmembrane proteins, including all four NOTCH receptors and APP, the amyloid precursor protein, playing a critical role in the activation of NOTCH1–4 and in the generation of the amyloidogenic A β peptides, which accumulate in the brains of Alzheimer's disease patients.^{73,74} After γ -secretase cleavage, ICN1 rapidly translocates to the nucleus, and triggers the expression of NOTCH targets by binding to the CSL DNA binding protein.⁷⁵ Most notably, recruitment of the MAML1 coactivator and the RNA polymerase complex to NOTCH-CSL target promoters results in the termination of NOTCH1 signaling by CDK8-mediated phosphorylation of the C-terminus PEST domain of the receptor, leading to proteasomal degradation of activated NOTCH1 by the FBXW7/Se110-CSF ubiquitin ligase complex^{76–78} (Figure 26.1).

The first evidence connecting aberrant NOTCH1 signaling to the pathogenesis of T-ALL came from the characterization of the t(7;9)(q34;q34.3) translocation, a rare chromosomal

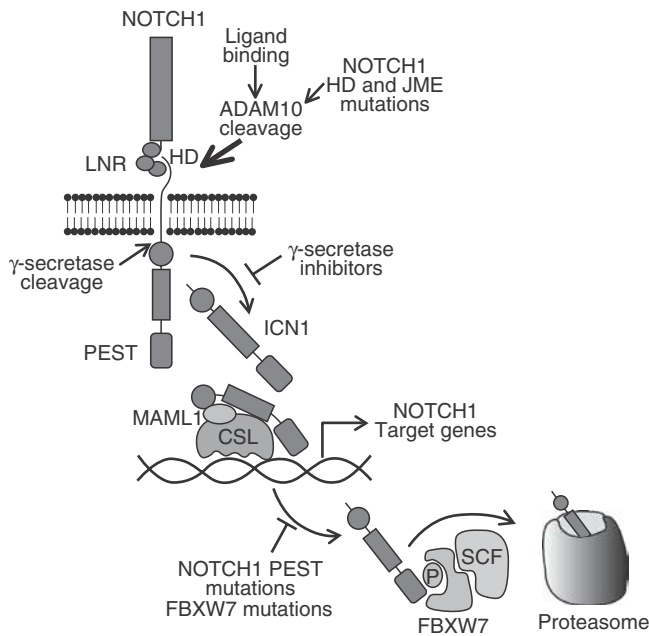


FIG. 26.1. Schematic representation of the NOTCH1 signaling pathway. Binding of delta serrate ligand (DSL) induces consecutive cleavages of the NOTCH1 receptor by the ADAM10 and by the γ -secretase proteases, causing release of the intracellular domains of NOTCH1 (ICN1) from the membrane. ICN1 then translocates to the nucleus where it associates with CSL and MAML1 to activate the expression of target genes. The NOTCH1 signaling cascade is terminated by FBXW7/SCF mediated ubiquitination and subsequent proteasomal degradation of ICN1.

rearrangement present in about 1% of human T-ALL cases.³⁶ This translocation juxtaposes a truncated *NOTCH1* gene next to the *TCRB* locus, leading to the aberrant expression of an intracellular constitutively active form of NOTCH1.³⁶ However, the most prevalent mechanism inducing constitutive activation of NOTCH1 in human leukemias are activating mutations in the *NOTCH1* gene, which are present in 50–60% of T-ALLs.^{3–5,79}

Activating mutations in *NOTCH1* (located in the heterodimerization domain (*HD* alleles) and the juxtamembrane extracellular region (*JME* alleles) of the receptor) induce ligand-independent activation of NOTCH1 signaling.^{3,79} In contrast, truncating mutations in the C-terminal region of the protein, which delete the PEST domain, extend NOTCH1 signaling by impairing the proteasomal degradation of ICN1.³ In addition, homozygous deletions (or heterozygous mutations) in *FBXW7* involving three critical arginine residues, that mediate the interaction of this F-box protein with the phosphodegron moiety in the NOTCH1 PEST domain, also extend NOTCH1 signaling, by impairing the proteasomal degradation of ICN1 in 15% of T-ALL cases.^{80–83} Moreover, FBXW7 mediates the proteasomal degradation of JUN, MYC, and cyclin E in addition to ICN1.⁸⁴ Thus, increased

MYC, JUN, and cyclin E stability may cooperate with increased ICN1 levels in the transformation of T-ALLs with FBXW7 mutations and deletions. Importantly, about 15–25% of these leukemias harbor two concurrent lesions activating NOTCH1: the first one inducing ligand-independent activation of NOTCH1 – an *HD* or *JME* allele, and a second one leading to increased protein stability and extended duration of NOTCH1 signaling – a *PEST* truncation or *FBXW7* mutation.^{3,80,82,83} Interestingly, activating mutations in NOTCH1 may act as primary initiating events in the pathogenesis of T-ALL and may even be detected at birth in preleukemic clones originated during prenatal development,⁸⁵ but also as subclonal and secondary mutations acquired during disease progression.⁸⁶

The clinical relevance of mutations activating the NOTCH1 pathway is emphasized by the potential role of NOTCH1 as a therapeutic target in T-ALL.⁶² Given the strict requirement of γ -secretase cleavage for the activity of transforming NOTCH1 mutants, inhibition of the γ -secretase complex may be exploited to abrogate the function of oncogenic NOTCH1 in T-ALL lymphoblasts. Notably, small molecule γ -secretase inhibitors (GSIs), originally developed for the treatment of Alzheimer's disease, effectively block NOTCH1 signaling and impair the growth and proliferation of T-ALL cells harboring activating mutations in NOTCH1 by inducing cell cycle arrest in G1.^{3,87–90} These observations have prompted the investigation of GSIs for the treatment of relapsed and refractory T-ALL.⁹¹

Aberrant Expression of Transcription Factor Oncogenes

As mentioned earlier, the activation of transcription factor oncogenes plays a critical role in the pathogenesis of T-ALL. Most of these transcriptional regulators represent developmentally important genes involved in the specification of cell fate decisions during embryonic development and are aberrantly expressed at high levels in thymocyte progenitors because of chromosomal translocations.

Basic Helix-Loop-Helix Transcription Factor Oncogenes *TAL1*, *TAL2*, *LYL1*, and *BHLHB1*

Basic helix-loop-helix (bHLH) transcription factors are characterized both by the presence of a basic domain involved in DNA binding and by two helices, separated by a loop, that mediate the formation of homodimeric and heterodimeric complexes. Based on their structure and dimerization potential, bHLH proteins may be subdivided into several classes, among which class I and class II are the most relevant in the pathogenesis of T-ALL. Class I bHLH factors, also known as E proteins because of their capacity to bind

to E-box sequences (CANNTG), include E47 and E12 and are generated in vertebrates by alternative splicing of the E2A locus, HEB, and E2-2. E proteins form homodimers with strong transactivation activity, but may also heterodimerize with class II bHLH proteins, which modifies their pattern of promoter targets and typically results in decreased levels of transcriptional activation.⁹² Class II bHLH factors, including the T-ALL oncogenes *TAL1*, *TAL2*, *LYL1*, and *OLIG2/BHLHB1*, modulate gene expression by forming heterodimeric DNA binding complexes with class I proteins.⁹²

The *TAL1* gene in chromosome band 1p32 plays an important role in the development of the earliest hematopoietic progenitors and in the differentiation of the erythroid and megakaryocytic cell lineages.⁹³ Aberrant expression may be detected in 60% of T-ALL cases.^{8,42} In 3% of childhood T-ALLs, the t(1;14)(p32;q11) places the *TAL1* locus in chromosome 1p32 under the control of the strong T-cell specific enhancers driving the expression of *TCRA/D* in chromosome 14q11.^{9-12,94} In 16–30% of T-ALL cases, aberrant expression of *TAL1* is due to a small intrachromosomal rearrangement (*TAL1d*), which deletes a 90Kb sequence upstream of the *TAL1* locus and places the *TAL1* gene under the control of the promoter of *SIL*, a nearby gene expressed at high levels in T-cells.³⁹ In addition to these cis-acting chromosomal alterations, which result in monoallelic *TAL1* expression, *TAL1* is biallelically expressed in a significant fraction of T-ALL cases, suggesting that additional trans-acting mechanisms contribute to *TAL1* activation in T-ALL.^{41,42} *TAL1* expressing T-ALLs typically are $\alpha\beta$ T-cell tumors and are characterized by a maturation arrest at the late cortical, double-positive stage of thymocyte development.^{8,42} The oncogenic potential of *TAL1* is illustrated by the induction of T-cell lymphoblastic tumors in transgenic mice expressing *TAL1* in developing thymocytes.^{95,96}

In T-ALL cells, TAL1 forms primarily inactive transcriptional complexes that contain E2A and HEB, and LIM only domain factors, LM01 or LM02, resulting in decreased expression of E2A/HEB target genes. Thus, although TAL1 may be present in transcriptional complexes activating the expression of some target genes⁹⁷; the oncogenic activity of TAL1 seems to be mediated primarily by reducing the level of transcriptional activity of promoters normally controlled by E12, E47, and HEB. Consistent with this model, genetic inactivation of one copy of *E2A*, which encodes both E12 and E47, or *HEB* results in an increased tumor formation in transgenic mice expressing *TAL1* in T-cell progenitors.⁹⁸

TAL2, LYL1, and BHLHB1 are bHLH factors closely related to TAL1 and are ectopically expressed in rare T-ALL cases harboring the t(7;9)(q34;q32), t(7;19)(q35;p13), and t(14;21)(q11;q22), respectively.^{13-15,99} As in the case of TAL1, these bHLH factors form inactive transcriptional complexes with E2A, suggesting that they may promote T-ALL transformation by inhibiting the expression of E2A target genes.⁹⁹

LIM Only Domain Factors LM01 and LM02

The genes encoding LM01 (in chromosome band 11p15) and LM02 (in chromosome band 11p13) are frequently rearranged in T-ALL cases. Chromosomal abnormalities involving LM01 and LM02 include intrachromosomal deletions in the short arm of chromosome 11, including del(11)(p12;p13), which delete negative regulatory sequences controlling the expression of *LM02* in T-cell precursors and translocations, which place the *LM01* or *LM02* genes under the control of strong enhancers in the *TCR* loci.^{16-20,40} Altogether, these rearrangements account for ~9% of pediatric T-ALL cases. However, as in the case of *TAL1*, biallelic expression of *LM02* may be detected in additional T-ALL cases, suggesting that additional trans-acting mechanisms contribute to *LM02* activation in T-ALL.^{8,41}

LMO proteins are transcriptional regulators devoid of DNA binding activity which associate with bHLH transcriptional complexes via protein–protein interactions.^{100,101} LMO factors may contribute to T-cell transformation via their association with the TAL1 and LYL1 bHLH factors in transcriptional complexes that disrupt the transactivation function of E2A.^{101,102} In agreement with this model, *LM01* and *LM02* are frequently expressed in cases harboring deregulated *TAL1* or *LYL1* expression.⁸ Moreover, the oncogenic activity of *Lm01* or *Lm02* in transgenic mice^{103,104} is enhanced in double transgenic animals expressing TAL1 in developing thymocytes.^{102,105}

Homeobox Transcription Factors TLX1, TLX3 and HOXA9

The Homeobox (HOX) family of transcription factors genes is structurally and functionally conserved through evolution from *Drosophila* to vertebrates, and plays a critical role in body patterning and organogenesis during development.¹⁰⁶ Numerous *HOX* genes have been implicated in the pathogenesis of murine and human leukemias,¹⁰⁷ and although their mechanisms of action have not been fully characterized, they are thought to promote cell survival and proliferation by activating transcriptional programs that interfere with normal hematopoietic development.

TLX1/HOX11 is the founding member of a family of *HOX* genes that includes *TLX2/HOX11L1* and *TLX3/HOX11L2*.¹⁰⁸ All three members of this family are characterized by the presence of a threonine in the third helix of the homeodomain, which confers specific DNA binding properties. *TLX1* was originally identified as the gene translocated into the *TCRA/D* locus in the recurrent t(10;14)(q24;q11) in T-ALL.²¹⁻²⁴ *TLX1* is rearranged and aberrantly expressed in 5% to 10% of pediatric and up to 30% of adult T-ALL cases.^{8,109-111} Similarly to other *HOX* genes, *TLX1* plays a key role during embryonic development and organogenesis.

Specifically, *TLX1* acts as a master transcriptional regulator necessary for the genesis of the spleen.^{112,113}

A second TLX family member, *TLX3*, is aberrantly expressed in T-ALL cases harboring a t(5;14)(q35;q32).²⁵ This cryptic chromosomal rearrangement, induces ectopic expression of *TLX3* in 5q35, by bringing it under the influence of strong transcriptional regulatory elements in the *CTIP2/BCL11B* gene in 14q32, which is highly expressed during T-lymphoid differentiation.^{25,114} In contrast to the higher prevalence of *TLX1* expression in adult T-ALL, the t(5;14) translocation and *TLX3* expression are present in 20–25% of pediatric (but in only 5% of adult) T-ALL cases.^{8,109,111,115,116}

As in the case of *TLX1*, the role of *TLX3* as a master transcriptional regulator acting upstream of important pathways involved in cell fate determination is supported by its importance during embryonic development.¹¹⁷ In mice, *TLX3* expression is required for the development of the ventral medullary respiratory center,¹¹⁷ and animals deficient in this protein show congenital central hypoventilation and die soon after birth because of respiratory failure.

The *TLX1* and *TLX3* proteins share a high degree of sequence identity at the amino acid level, especially in their DNA-binding homeobox domain, where they differ in only three amino acids. This high level of structural homology strongly suggests that *TLX1* and *TLX3* may share common transcriptional targets and have a common mechanism of T-ALL transformation. Consistent with this hypothesis, immunophenotypic analysis has demonstrated that *TLX1* and *TLX3* T-ALLs share a common early cortical arrest in thymocyte development characterized by the expression of CD1a and CD10.^{8,111} Similarly, gene expression profiling studies showing a similar pattern of gene expression in *TLX1*- and *TLX3*-induced leukemias. Moreover, the identification of the *NUP214-ABL1* fusion oncogene⁵⁶ as an oncogenic event in T-ALL strictly associated with the overexpression of these two transcription factor oncogenes further supports the hypothesis that *TLX1* and *TLX3* share a common leukemogenic pathway. In contrast with these similarities, *TLX1* and *TLX3* show different clinical outcomes. Thus, while *TLX1* is associated with a favorable prognosis both in childhood and in adult T-ALL,^{2,8,110} aberrant expression of *TLX3* may be associated with a higher incidence of relapse.^{8,116,118,119}

In contrast with orphan homeobox genes, such as *TLX1* and *TLX3*, which are encoded in isolated and independent loci in the genome, canonical homeobox genes involved in the specification of body patterning have a conserved structural organization and show tightly regulated expression. This genomic organization consists of four paralogous clusters, named *HOXA-D* and containing 9–11 genes each.^{120,121} Chromosomal rearrangements inv(7)(p15q34) and t(7;7)(p15;q34) involving the *TCRB* locus (7q34–35) and the *HOXA* locus (7p15) are found in 3% of T-ALL patients and cause overexpression of several *HOXA* cluster genes.^{28,29,37} Interestingly, the chromosomal breakpoints in these rearrangements cluster tightly at the vicinity of the

HOXA10 and *HOXA9* genes.^{28,37,122} Importantly, ectopic expression of *HOXA* cluster genes is also observed in *MLL-MLLT1*-,^{28,123} *SET-NUP214*-,⁴⁹ and *PICALM-MLLT10*-,^{28,124} rearranged leukemias, suggesting a more general pathogenic role of *HOXA* dysregulation in the pathogenesis of T-ALL.

MYB

The *MYB* oncogene is the cellular counterpart of *v-Myb*, a leucine zipper transcription factor oncogene responsible for a fatal monoclastic leukemia syndrome induced by the avian myeloblastosis virus in chickens.^{125,126} The oncogenic potential of *MYB* dysregulation was shown by the induction of lymphoid or myeloid tumors in transgenic mice expressing *v-Myb*.¹²⁷ Moreover, the murine *c-Myb* locus is also frequently activated in lymphoid leukemias induced by retroviral insertional mutagenesis.^{128–132}

In the hematopoietic system, *MYB* is normally expressed in immature and proliferative progenitor populations and is turned off during cell maturation and lineage differentiation.^{133,134} Consistent with this pattern of expression, *Myb* is essential for hematopoietic development and plays a role in lineage commitment, proliferation, and differentiation.^{135–138}

In humans, somatically acquired single locus duplications of *MYB* are present in about 10% of T-ALL cases.^{35,81,139} These *MYB* copy number alterations are generated by homologous recombination between *Alu* elements in T-ALL and result in an increased expression of *MYB* in leukemic lymphoblasts.⁸¹ In addition, the *MYB* locus is rearranged in rare T-ALL cases harboring the t(6;7)(q23;q34) translocation, which juxtaposes the *MYB* gene and *TCRB* regulatory sequences and results in high levels of *MYB* expression in T-cell progenitors.³⁵ The presence of the t(6;7)(q23;q34) translocation defines a distinct clinicobiologic group of T-ALL cases, characterized by a very young age (<2 years) and a distinct gene expression signature (characterized by high level of expression of genes involved in proliferation and mitosis).³⁵

MYC

The *MYC* oncogene encodes a pleiotropic basic-helix-loop-helix leucine zipper family transcription factor involved in the control of growth and metabolism, proliferation, and cell cycle progression.¹⁴⁰ *MYC* plays a central role in the pathogenesis of T-ALLs transformed by the t(8;14)(q24;q11) translocation, which induces high levels of *MYC* expression in T-cell progenitors.^{9,30} In addition, the identification of *MYC* as an important direct transcriptional target of *NOTCH1* in T-ALL supports a more general role of *MYC* in the pathogenesis of T-ALL.^{90,141,142}

Transcription Factor Fusion Oncogenes

In addition to chromosomal rearrangements resulting in aberrantly high levels of expression of oncogenic factors, a

number of recurrent translocations found in T-ALL result in the expression of fusion transcripts encoding chimeric oncoproteins, such as *MLL-MLLT1*, *PICALM-MLLT10*, *SET-NUP214*, and *NUP98-RAP1GDS1*.

MLL-MLLT1

The *MLL* gene located in chromosome band 11q23 is the human homolog of trithorax, a *Drosophila* gene responsible for developmental patterning and cell fate decisions via the regulation of *hox* gene expression.^{143,144} Translocations involving chromosome band 11q23 are characteristically found in most infant leukemias and in secondary leukemias occurring after chemotherapy treatment with topoisomerase inhibitors, and are typically associated with a very poor prognosis.^{145–147} Overall, more than 50 translocations associated with the pathogenesis of ALLs, acute myeloid leukemias (AMLs), and myelodysplastic syndrome involving the *MLL* locus have been described.^{146,147} These rearrangements generate fusion oncogenes encoding chimeric proteins consisting of the 2 AT-hook and DNA methyltransferase domains of *MLL* fused to the carboxyterminal portion of a number of different proteins with little structural resemblance to each other.¹⁴⁷ The two most common *MLL* rearrangements associated with the pathogenesis of ALL are t(4;11)(q21;q23) and t(11;19)(q23;p13.3) translocations, which encode the *MLL-AFF1* (*MLL-AF4*) and *MLL-MLLT1* (*MLL-ENL*) fusion genes, respectively.² *MLL-MLLT1* (*MLL-ENL*) rearranged tumors represent a distinct biological and molecular group of T-ALLs characterized by an increased expression of *HOXA9*, *HOXA10*, and *HOXC6*, and also of the *MEIS1* *HOX* coregulator;^{29,123} in contrast with other *MLL* rearranged leukemias, these seem to be associated with a favorable prognosis.^{8,148}

PICALM-MLLT10 (*CALM-AF10*)

The t(10;11)(p13;q14) translocation is found in 5–10% of T-ALL cases and in rare AMLs.^{149–152} This chromosomal rearrangement results in the expression of the *PICALM-MLLT10* (*CALM-AF10*) fusion oncogene in up to 5–10% of T-ALL cases, of which only half show the translocation in their karyotype.^{28,46,124} *PICALM-MLLT10* rearranged T-ALLs are associated with an immature immunophenotype, or differentiation into the TCR $\gamma\delta$ lineage and show a characteristic gene expression signature dominated by the upregulation of *HOXA* genes.^{28,46,124}

The *PICALM* (phosphatidylinositol binding clathrin assembly protein; also known as *CALM*) gene encodes a ubiquitously expressed protein involved in endocytosis.¹⁵³ Mutations in the mouse *Picalm* gene result in functional iron deficiency, ineffective hematopoiesis, and impaired growth, suggesting a role of *PICALM* in endocytosis-mediated iron transport.¹⁵⁴ The *MLLT10* (also known as *AF10*) gene was initially cloned as an *MLL* partner gene in the recurrent t(10;11)(p13;q23) translocation,^{155,156} and encodes

a protein involved in transcriptional regulation.¹⁵⁷ At least four *MLLT10* fusion sites have been described in *PICALM-MLLT10* rearranged leukemias. Fusion transcripts which retain most of the *MLLT10* coding sequence seem to be associated with more mature immunophenotypes; whereas 3' fusion transcripts, which contain less *MLLT10* sequence, lack TCR expression on the cell surface and are associated with a more immature T-cell immunophenotype.^{46,158} Notably, patients harboring leukemias with a *PICALM-MLLT10* fusion and showing an immature phenotype may have a poor prognosis.⁴⁶

SET-NUP214

The del(9)(q34.11q34.13) rearrangement, a cryptic and recurrent deletion, has been identified in three T-ALL patients with aberrant expression of *HOXA* genes, but lacking any of the known chromosomal lesions previously reported to induced *HOXA* gene dysregulation, such as *HOXA* rearrangements, or expression of the *MLL-MLLT1* and *PICALM-MLLT10* fusion oncogenes.⁴⁹ The del(9)(q34.11q34.13) results in a *SET-NUP214* fusion gene, which encodes a chimeric protein consisting of the N-terminal domains of SET, a protein with homology with the yeast nucleosome assembly protein NAP-I, and the C-terminal domains of the NUP214, a nuclear pore complex protein.⁴⁹ The *SET-NUP214* oncogene has also been isolated in single cases of undifferentiated leukemia and AML, showing that this rearrangement is not strictly associated with tumors of the T-cell lineage.^{159,160} Functional studies on the role of SET-NUP214 in T-ALL have demonstrated that this oncogenic transcription regulator binds to the promoter regions of specific *HOXA* genes and activates the expression of members of the *HOXA* cluster.

NUP98 Fusion Oncogenes

The *NUP98* gene, located in chromosome band 11p15.4, was originally identified at the site of a t(7;11)(p15;p15.4) chromosomal translocation, resulting in the fusion of *NUP98* with the homeobox gene *HOXA9* in AML.^{161,162} Subsequently, *NUP98* has been shown to be fused to the homeobox genes, *HOXD13* (2q31), *HOXD11* (2q31), *HOXA13* (7p15), *HOXA11* (7p15), and *HOXC11* (12q13); to *PMX1* (1q23); and to nonhomeobox genes, such as *NSD1* (5q35), *NSD3* (8p11), *LEDGF* (9p22), *DDX10* (11q22), and *TOP1* (20q11) in myeloid tumors.¹⁶³ Rare *NUP98* rearrangements found in T-ALL coexpressing myeloid markers include *NUP98-RAP1GDS1*, *NUP98-SETBP1*, *NUP98-ADD3*, *NUP98-IQCG*, *NUP98-CCDC28A*, and *NUP98-SETBP1*, resulting from the t(4;11)(q21;p15), t(11;18)(p15;q12), t(10;11)(q25;p15), t(3;11)(q29;p15), t(6;11)(q24;p15), and t(11;18)(p15;q12) translocations, respectively.^{50,164–167}

The *NUP98* fusion transcripts join the N-terminal NUP98 FG repeat motifs with the 3' region of the partner genes,

consisting of a homeodomain in the case of *HOX* partner genes, or domains with significant probability of adopting a coiled-coil conformation in the case of non-*HOX* partner genes. The NUP98 FG repeats have strong transcriptional transactivation potential through interaction with CBP/p300,¹⁶⁸ and their fusion with homeobox domains probably results in oncogenic dysregulation of *HOX* target genes. In the case of non-*HOX* partners, although coiled-coil domains are devoid of DNA binding activity, they may facilitate the interaction of the chimeric protein with transcription factors, leading to dysregulated gene expression.¹⁶³

Aberrant Activation of Tyrosine Kinase Oncoproteins

NUP214-ABL and *EML1-ABL*

The *ABL1* gene in chromosome band 9q34 encodes a tyrosine kinase, typically localized in the nucleus and in the cytoplasm of proliferating cells.¹⁶⁹ Although the functions of *ABL1* are not yet fully established, *ABL1* is normally activated by DNA damage downstream of ATM¹⁷⁰ and may be involved in the induction of p53-independent apoptosis.¹⁷¹ In the cytoplasm, *ABL1* regulates F-actin dynamics in response to growth factor and adhesion signals,¹⁷² while in the nucleus, *ABL1* kinase activity is regulated through its interaction with the retinoblastoma protein.¹⁷³ Mice deficient in *ABL1* develop a wasting syndrome and die soon after birth.^{174,175} The rearrangement of *ABL1*, resulting in the expression of the BCR-*ABL1* fusion oncogene is characteristic and almost universal in chronic myeloid leukemias (CML) harboring the t(9;22) translocation.¹⁷⁶ BCR-*ABL1* encodes a chimeric oncoprotein with cytoplasmic subcellular localization and constitutively active tyrosine-kinase activity.^{177,178} In precursor-B ALL, aberrant activation of *ABL1* is mostly found in adult cases, 25% of which show the t(9;22) rearrangement and express the BCR-*ABL1* fusion oncogene.^{2,179} The t(9;22) translocation is rare in T-ALL; however, approximately 6% of children and adults with T-ALL harbor a complex rearrangement of the *ABL1* locus, resulting in expression of a *NUP214-ABL1* fusion oncogene.⁵⁶ Both *NUP214* and *ABL1* are contiguous loci with a head to tail configuration in chromosome band 9q34. As a result, the *NUP214-ABL1* fusion gene is produced by a complex rearrangement that results in a circularization of this chromosomal region and episomal amplification of the resulting fusion oncogene.⁵⁶ *NUP214* is a component of the nuclear pore complex, and the *NUP214-ABL1* oncoprotein is localized at the nuclear pore in an oligomeric configuration, which induces constitutive activation of *ABL1*.¹⁸⁰

In addition to *NUP214-ABL1*, a cryptic translocation (i.e., t(9;14)(q34;q32)), resulting in the expression of an *EML1-ABL1* fusion gene and the formation of EML1-*ABL1* protein containing the coiled-coil domain of EML1 and the kinase domain of *ABL1*, has been reported in T-ALL.³⁸ In contrast

with the poor prognosis associated with expression of BCR-*ABL* in ALL, *NUP214-ABL1*-positive and -negative patients do not differ significantly in their major clinical features; *NUP214-ABL1* has not been associated with significant differences in survival.^{181,182}

When expressed in murine hematopoietic precursors, *NUP214-ABL1* transforms hematopoietic cells in vitro and induces a syndrome similar to CML and T-ALL in mice.¹⁸⁰ Notably, both *NUP214-ABL1* and *EML1-ABL1* are tightly associated with the rearrangement and aberrant expression of *TLX1* or *TLX3* genes, which suggest a specific mechanism of interaction between the transcriptional network controlled by these transcription factors and the oncogenic program activated by constitutive activation of *ABL1* in T-ALL.^{38,56}

The development of tyrosine kinase inhibitors targeting the *ABL1* kinase has opened novel therapeutic opportunities for the treatment of BCR-*ABL1* rearranged leukemias.^{183,184} Most notably, both *NUP214-ABL1* and *EML1-ABL1* fusion proteins are effectively inhibited by small molecule tyrosine kinase inhibitors active against BCR-*ABL1*, such as imatinib mesilate, suggesting that incorporation of small molecule tyrosine kinase inhibitors may improve the therapeutic outcome of *ABL1* rearranged T-ALL cases.^{38,181,185}

JAK1 Mutations

The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway plays an essential role in the control of cell proliferation, differentiation, and apoptosis in the hematopoietic system. In particular, the JAK family of tyrosine kinases (JAK1, JAK2, JAK3, and TYK2) mediates the cell growth and survival effects of interferons and numerous cytokines, including IL2, IL3, IL4, IL5, IL6, IL7, IL13, granulocyte monocyte colony stimulating factor (GM-CSF), growth hormone (GH), prolactin (PRL), erythropoietin (EPO), and thrombopoietin (TPO).¹⁸⁶

The fundamental role of the JAK-STAT signaling pathway in the development and function of the hematopoietic system is highlighted by the phenotypes of *JAK* knockout mice. Thus, *JAK1* null mice exhibit profound defects in lymphoid cell development, because of the inability of *JAK1* null T and B cells to respond to cytokine signals, leading to defects in thymocytes, pre-B cells, and mature T and B lymphocytes, and a severe combined immunodeficiency (SCID) phenotype.¹⁸⁷ *JAK2* null mice are embryonically lethal due to the lack of erythropoiesis, a phenotype explained by the lack of effective EPO, TPO, IL-3, and IL-5 signaling.¹⁸⁸ Genetic inactivation of *JAK3* induces loss of cytokine signals mediated by the IL2R γ C chain (IL2, IL4, IL7, IL21), and results in profound defects in the lymphoid system.^{189,190} Finally, *Tyk2* null mice display only reduced responses to interferons and IL-12.¹⁹¹

Given the important role of cytokines and growth factors in providing cell proliferation and survival signals for the development and function of the hematopoietic system, it is

not surprising the JAK-STAT pathway, when dysregulated, may contribute to the pathogenesis of leukemias and lymphomas. Constitutive activation of JAK2 has been found in >95% of patients with polycythemia vera and 50–60% of patients with essential thrombocythosis or myelofibrosis with myeloid metaplasia and in rare cases of AML which harbor the JAK2 V617F mutation, a single amino acid substitution in JAK2 which results in dysregulated kinase activity.^{192–196}

The first indication of a direct involvement of the JAK-STAT pathway in ALL came from the characterization of the t(9;12)(p24;p13) translocation, a rare but recurrent chromosomal rearrangement found in T and pre-B ALLs and rare cases of atypical CML.^{197,198} The resultant *ETV6-JAK2* (*TEL-JAK2*) fusion oncogene has deregulated and constitutively active JAK kinase activity^{198,199} and induces rapidly fatal leukemia, characterized by a selective expansion of CD8-positive T-cell lymphoblasts in transgenic mice.²⁰⁰

A broader role for JAK activation in the pathogenesis of T-ALL has recently been demonstrated with the identification of activating point mutations in the *JAK1* gene in 18% of adult T-ALL cases with a much lower frequency in childhood T-ALL.⁵⁵ *JAK1* mutations are characteristically present in association with activating mutations in *NOTCH1*, suggesting a synergistic interaction between NOTCH1 signaling and JAK-STAT activation in T-cell transformation.⁵⁵ An important feature of *JAK1* activating alleles is that they seem to arise late in the pathogenesis of T-ALL, and may be primarily associated with disease progression.⁵⁵ Even so, the presence of activating mutations in *JAK1* has been associated with a poor response to therapy and with a conferred poor prognosis in adult T-ALL.⁵⁵

FLT3 Mutations

FLT3 encodes a receptor tyrosine kinase with an important role in the development of hematopoietic stem cells.^{201,202} Activating mutations of *FLT3* are common in AML, but rare in ALL, where they are mostly restricted to *MLL* rearranged and hyperdiploid tumors.^{203–208} These mutations typically consist of internal tandem duplications in the juxtamembrane domain of the receptor and point mutations in the activation loop of the kinase domain, and lead to constitutive *FLT3* kinase activity in the absence of ligand.^{203,205,206,209} Importantly, small molecule *FLT3* kinase inhibitors may induce programmed cell death against AML lymphoblasts in vitro, and are currently undergoing phase I and II testing.^{210,211} *FLT3* mutations have been reported in occasional cases of T-ALL, and may be associated with surface expression of CD117/KIT and an immature immunophenotype.^{212,213}

LCK Translocation and Overexpression

The lymphocyte-specific tyrosine kinase, *LCK*, is a critical mediator of signals driving proliferation and survival downstream of the preT-cell receptor (preTCR) in developing thymocytes and the TCR in mature lymphocytes.²¹⁴ Characterization of rare T-ALL cases with the t(1;7)

(p34;q34) has demonstrated that this rearrangement results in aberrantly high levels of expression of *LCK*, showing that aberrant activation of preTCR/TCR signals may contribute to the transformation of T-cell progenitor cells.⁵²

RAS Gene Mutations and Loss of *NF1*

Proto-oncogenes of the RAS family – *HRAS*, *KRAS*, and *NRAS* – encode 21-kDa proteins that are associated with the inner surface of the cytoplasmic membrane and transmit proliferation stimulating signals from tyrosine kinase, nontyrosine kinase, and G protein coupled receptors.^{215,216} The RAS proto-oncogenes are frequently activated in human cancer by somatic mutations that alter the amino acids specified by codons 12, 13, or 61, which result in the accumulation of RAS proteins in their active, GTP-bound conformation in the absence of growth factor binding to upstream surface receptors.^{215,216} *NRAS* mutations have been reported in 5–10% of T-ALL cases.^{57,58,217} In addition, about 3% of T-ALL cases show a recurrent cryptic deletion in chromosome 17, del(17)(q11.2), leading to biallelic loss of the neurofibromatosis type 1 (*NF1*) gene, which encodes a negative regulator of the RAS pathway.⁵⁹

Mutational Loss of PTEN

The PI3K-AKT signal transduction pathway mediates increased cell growth, proliferation, and survival downstream of tyrosine kinases and G protein-coupled growth factor receptors.^{218–223} Activation of PI3K in the vicinity of the activated membrane bound receptor triggers the generation of phosphatidylinositol triphosphate (PIP3). Accumulation of PIP3 recruits AKT at the plasma membrane and induces its phosphorylation and activation by the PDK1 and the mTOR-Rictor kinases.^{224,225} In turn, AKT phosphorylates different substrates, which promote increased glucose metabolism, cell cycle progression, and cell survival by multiple direct and indirect mechanisms.^{218–221} Termination of the PI3K-AKT signaling is mediated by PTEN, a lipid phosphatase which inactivates PIP3.^{218–221}

The first indication of the oncogenic properties of AKT was the identification of the v-Akt oncogene in a transforming retrovirus isolated from an AKT mouse T-cell lymphoma.^{226,227} The transforming effects of v-Akt are dependent on a myristoylation site that localize the protein into the plasma membrane and on constitutive activation of its kinase function.²²⁸ Over the last decade, numerous findings have established a prominent role for hyperactivation of AKT signaling in the pathogenesis of many human cancers.^{218–223} Aberrant PI3K-AKT signaling in tumor cells may result from direct mechanisms (such as activating mutations in *PI3KCA* (which encodes for p110 α) or amplification of *AKT2*), or from indirect means (including alterations of upstream factors, such as the RAS oncogenes or growth factor receptors). However, the most frequent molecular lesion associated with constitutively-active AKT signaling

in human cancer is the loss of *PTEN* tumor suppressor gene. Homozygous and heterozygous somatic mutations in *PTEN* have been reported in a very broad range of cancer types, including advanced glial and prostate tumors, endometrial carcinomas, and melanomas,^{229–231} and at lower frequency in human leukemias and lymphomas.^{232–237}

Most notably, loss of *PTEN* has been shown to promote the self-renewal of leukemic stem cells.²³⁸ Mutation analysis in T-ALL has shown biallelic truncating mutations in *PTEN* and chromosomal deletions encompassing the *PTEN* locus in 5–10% of cases, with additional mutations occurring at relapse.^{60,239} In addition, detailed analysis of *PTEN* expression has shown complete loss of *PTEN* protein in 17% of T-ALL samples.⁶⁰ Overall, these results show that mutational loss of *PTEN* is relatively common in human T-ALL at diagnosis, and may also occur as a secondary event during disease progression.

Alterations in Cyclin-Dependent Kinase Inhibitors and Cyclin D2 Overexpression

The *INK4A* locus in the short arm of chromosome band 9p21 contains two tumor suppressor genes, *p16INK4A* and *p14ARF*, which have unique promoters and first exons and share a common second and third exon. Despite this common sequence, p16 and p14 have distinct amino acid sequences; their common second and third exons are translated using different reading frames. In addition to *p16INK4A* and *p14ARF*, a third cyclin-dependent kinase inhibitor, *p15INK4B*, is also located in this region.^{240–245}

Type D cyclins are key factors in promoting the progression from G1 into S phase of the cell cycle. Cyclin D-CDK4/6 complexes phosphorylate and inactivate the retinoblastoma protein, leading to the release of E2F transcription factors, which promote entry into S phase. p16INK4A and p15INK4B interfere with cell cycle progression working as direct inhibitors of cyclin D-CDK4/6 complexes. In contrast, p14ARF, the third tumor suppressor located in 9p21, functions as an antagonist of MDM2, a critical posttranslational regulator of the p53 tumor suppressor.²⁴⁶ In resting conditions, MDM2 ubiquitinates and degrades p53. However, upon activation of oncogenic stress, p14 ARF is upregulated, leading to MDM2 inactivation and p53 stabilization, triggering G1 cell cycle arrest and apoptosis.^{246,247}

Chromosomal deletions of the short arm of chromosome 9, involving both the *p16INK4A/p14ARF* and the *p15INK4B* loci, are the most frequent genetic abnormality and are present in over 70% of T-ALL cases.^{6,248}

Aberrant activation of cyclinD/CDK complexes also seems to be the underlying mechanism contributing to T-cell transformation in leukemias with the t(12;14)(p13;q11) and t(7;12)(q34;p13) translocations, resulting in high levels of *CCND2* expression via the juxtaposition of the *CCND2* locus at 12p13 with strong enhancers in the *TCRA/D* and the *TCRB*, respectively. *CCND2* rearrangements have been observed in association with aberrant expression of *TAL1*, *HOXA* cluster

genes, and *TLX3* (activating mutations in *NOTCH1*), and also interestingly with deletion of *p16INK4A/p14ARF*.^{53,54}

Clinical Implications for Therapy Stratification and Molecularly Targeted Drugs

T-ALL is an aggressive hematologic cancer that accounts for 10–15% of pediatric and 25% of adult ALL cases.^{2,249} Patients with T-ALL often present aggressive features, including very high circulating blast cell counts and infiltration of the central nervous system (CNS) at diagnosis.²⁵⁰ In the early days of ALL therapy, T-ALL was recognized as a poor prognostic group leading to the introduction of more intensive chemotherapy treatments. Consequently, T-ALL patients have gradually achieved remarkable improvements in outcome over the last two decades. Even so, 25% of children and adolescents^{251–255} and 50% of adults²⁵⁶ with T-ALL still fail to respond to these therapies. The limited therapeutic options available for those patients who present with primary resistant disease or relapsed T-ALL (developing after the induction of complete remission), underscore the need to develop better treatment stratification protocols and to identify more effective antileukemic drugs.^{257–261} This imperative is further supported by studies of the long-term effects of intensified chemotherapy in survivors of T-ALL, showing that gains in leukemia-free survival have been achieved at the cost of significant increases in rates of acute and chronic life-threatening and debilitating toxicities.²⁶²

Clinical and biological prognostic factors in precursor-B-ALL such as age, sex, white blood cell counts at diagnosis, presence of mediastinal mass or CNS involvement have failed to demonstrate a prognostic value in T-ALL, emphasizing the importance of molecular and cytogenetic prognostic markers in this disease.²⁶³ Analysis of cytogenetic alterations and expression of T-ALL oncogenes has shown that *TLX1* translocations and high levels of *TLX1* expression are associated with favorable prognosis in children and adults with T-ALL.^{8,109,264} Similarly, the presence of *MLL-ENL* fusion transcripts seems to be associated with a reduced risk of relapse, which is in contrast with the dismal prognosis of precursor-B-ALL cases harboring *MLL* fusion transcripts. In contrast, aberrant expression of *TLX3* or *TAL1* seems to be associated with less favorable outcomes, although the prognostic value of these transcription factor oncogenes is less clear and may be influenced by different treatments.^{8,109,119,265}

Several studies have addressed the prognostic significance of *NOTCH1* and *FBXW7* mutations in T-ALL with conflicting results. Two of these studies found an association of *NOTCH1* mutations with favorable outcome in pediatric T-ALL.^{5,82} However, these results could not be confirmed in a separate series of pediatric T-ALL cases, which showed no association of *NOTCH1* mutations with prognosis.²⁶⁶ Moreover, a strong association of activating *NOTCH1* mutations

with poor prognosis has been reported in adult T-ALL.²⁶⁷ Overall, the prognostic significance of alterations in the NOTCH1 signaling pathway in T-ALL remains to be elucidated and may be influenced by differences in age groups and treatment.

Molecular studies of ALL clonality have shown that distinctive DNA sequences, corresponding to the junctional regions of Ig and *TCR* gene rearrangements, may be identified in most ALL patients and provide unique markers for minimal residual disease (MRD) analysis by quantitative PCR. Several prospective studies have demonstrated the prognostic value of MRD detection in BM.^{268–270} Thus, children with undetectable MRD at the end of induction (monitored using quantitative polymerase chain reaction (PCR) techniques) have a very good prognosis. In contrast, patients with high MRD levels at the end of induction treatment have a poor prognosis and should be considered candidates for treatment intensification particularly, if high levels of MRD persist after consolidation treatment.

Our improved understanding of the molecular basis of T-ALL has also facilitated the initiation of studies testing the effectiveness of molecularly-targeted therapies in this disease. In this context, the identification of activating mutations in *NOTCH1* present in over 50% of T-ALL patients at diagnosis³ has brought enormous interest for the development of molecularly-tailored therapies in T-ALL and prompted the initiation of clinical trials to test the effectiveness of blocking NOTCH1 signaling with γ -secretase inhibitors (GSIs) in this disease. These small molecules inhibit a critical proteolytic cleavage required for the activation of the NOTCH1 receptor and induce cell cycle arrest in T-ALL cell lines *in vitro*.^{3,89,271} Yet, the development of anti-NOTCH1 therapies for T-ALL has been questioned, based on the observation that only a minority of T-ALL cell lines harboring mutations in the *NOTCH1* gene respond to NOTCH1 inhibition with GSIs. *In vitro* resistance to GSI therapy in these tumors is associated with the mutational loss of PTEN, highlighting a close interaction between NOTCH1 and PI3K-AKT signaling in the control of cell growth and proliferation in T-ALL lymphoblasts.⁶⁰

Identification of activating mutations on oncogenic kinases in subsets of T-ALL has opened the opportunity to incorporate kinase inhibitors in the therapy of these patients. The efficacy of ABL1 kinase inhibitors for treatment of patients with BCR-ABL1 positive leukemias and the sensitivity of NUP214-ABL1 to imatinib^{56,181,185} strongly suggest that NUP214-ABL1 positive T-ALL patients may benefit from treatment with imatinib and second generation ABL1 kinase inhibitors. Similarly, the presence of activating mutations in *JAK1* in 18% of adult T-ALL cases suggests that JAK inhibitors with significant inhibitory activity against JAK1 (currently in clinical trials for the treatment of myeloproliferative neoplasms) may be effective in the treatment of this group of patients.⁵⁵

Overall, the identification of a multiplicity of molecular abnormalities in T-ALL has significantly improved our understanding of the mechanisms that contribute to the malignant transformation of T-cell precursors and uncovered a high level of heterogeneity and molecular complexity in these tumors. The identification of clinically-relevant prognostic markers and potential targets for the development of molecularly tailored therapies warrant a new generation of clinical trials, aiming to integrate these findings in the treatment of T-ALL.

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Classical Hodgkin Lymphoma and Nodular Lymphocyte Predominant Hodgkin Lymphoma

Michele Roulet and Adam Bagg

Background

Hodgkin lymphoma (HL) is a rather unique neoplasm. In contrast to most other lymphomas, and indeed malignancies in general, the bulk of the infiltrate in tissues affected by HL is comprised of non-neoplastic T cells, B-cells, macrophages, eosinophils, neutrophils, and plasma cells, while the neoplastic cells are rare, accounting for only approximately 1% of the tumor mass.¹ The neoplastic cells include the hallmark binucleated large Reed–Sternberg cells, and morphologic variants thereof, which are collectively referred to as Hodgkin/Reed–Sternberg (HRS) cells. Intricate bi-directional signaling between the neoplastic cells and this pleomorphic, reactive background is integral to the tumor's pathobiology and clinical features, with an evaluation of the various neoplastic and reactive cells being central to contemporary diagnosis and classification. Another unusual feature is that whereas the cell of origin in other lymphomas can almost always be correlated with a specific stage of lymphoid maturation, HRS cells do not have a morphologically and immunophenotypically identifiable normal hematopoietic counterpart. In fact, the unusual but characteristic immunophenotype of HRS cells includes antigens typically found on a spectrum of cells, such as dendritic cells, granulocytes, monocytes, B-cells, and plasma cells.^{2,3} For these reasons (rarity of neoplastic cells within the tumor and confusing immunophenotype), determining the ontogeny of HRS cells had been technically challenging. Additional impediments to characterizing these cells included the presence of only several cell lines⁴ and no animal model.

Hodgkin “Disease” Is a B-Cell “Lymphoma”

Discovering the true B-cell origin and monoclonality of HRS cells only became possible when single cells could be procured for analysis. Microdissection of these single cells from tissue sections and subsequent polymerase chain

reaction (PCR) amplification of both genomic (DNA) and expressed (mRNA) sequences have allowed for the analysis of the configuration of the immunoglobulin heavy chain gene (*IGH*). These studies have demonstrated that the HRS cells contain rearranged *IGH* genes in most cases, thus indicating that their likelihood of being B-cells. Furthermore, the rearrangements have been shown to be identical in all the cells in one specimen, indicating monoclonality and essentially confirming the neoplastic nature of this “disease”.^{5,6} It has been subsequently shown that these monoclonally rearranged *IGH* genes are stably, but mostly heavily, somatically mutated, indicating their germinal center (GC), or post-GC origin. Approximately 25% of cases harbor crippling mutations (i.e., the generation of stop codons),⁷ which under normal physiologic conditions, would elicit apoptosis in these cells. However, this does not occur in HRS cells and a pre-apoptotic GC B-cell origin of HRS cells, at least in classical HL, has been hypothesized.⁸ Only in rare reports do the HRS cells appear to be of T-cell lineage.^{9,10}

In light of these seminal discoveries, Hodgkin “disease” has been appropriately renamed Hodgkin “lymphoma” in the 2001 WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. HL is currently categorized as either classical (CHL) or nodular lymphocyte predominant (NLPHL), with the much more common former group further subclassified into nodular sclerosis (NS), mixed cellularity (MC), lymphocyte-depleted (LD), and lymphocyte-rich (LR) subtypes.¹¹ Although both CHL and NLPHL are neoplasms of GC B-cells, in which there is a paucity of malignant cells within a dominant reactive background, there are many important biological, morphological, and clinical differences between these major forms of HL (Table 27.1). For example, in NLPHL the neoplastic cells are morphologically distinctive lymphocytic and histiocytic (L&H) or “popcorn” cells, and they are more likely to express B-cell antigens and lack CD15 and CD30, two antigens prototypically expressed on HRS cells of CHL.¹² In contrast to CHL, the neoplastic cells in NLPHL demonstrate ongoing somatic hypermutation of their *IGH* genes, with intraclonal diversity, indicative of the derivation from an antigen-selected GC B-cell.^{13,14} Clinically, NLPHL typically

TABLE 27.1. Features that distinguish classical Hodgkin lymphoma (CHL) from nodular lymphocyte predominant Hodgkin lymphoma (NLPHL).

	CHL	NLPHL
<i>Putative cell of origin</i>		
Germinal center B-cell (GC B-cell)	Pre-apoptotic GC B-cell	Antigen selected, mutating GC B-cell
<i>Clinical</i>		
Age distribution	Bimodal	Unimodal
Sites involved	Mediastinum, abdomen, cervical, spleen	Peripheral lymph nodes
Stage at diagnosis	Typically II or III	Often I
B symptoms	~40%	<20%
Clinical course	Aggressive, but curable	Indolent, late relapses
Transformation to large cell NHL	<1%	2–3%
<i>Pathology</i>		
Growth pattern	Diffuse or nodular	Nodular (mostly)
Tumor cell morphology	Classic RS cells	“Popcorn” or lymphocytic and histiocytic variants
	Mononuclear variants	
	Lacunar cells	
Cellular background	Variable	Small lymphocytes
Background lymphocytes	Predominantly T cells	Predominantly B-cells
<i>Immunophenotype</i>		
Prototypic surface markers		
CD15	+ (~85%)	+
CD30	+	+
CD20	+ (~40%)	+
CD45	–	
EMA		
B-cell transcription factors		
BOB1	+ (weak)	+
OCT2	–	+
PAX5		+
PU1		+
Germinal center markers		
CD10	+ (~30%)	+
BCL6	+ (~75%)	+
AID		+
HGAL		
Plasma cell markers		
MUM1	+	+ (~85%)
CD138		+ (~80%)
J chain	–	
Immunoglobulin		
Additional non-B-cell markers		
Fascin	+	+
ATF3	+	+
Signaling molecules	+ (~10%)	+
SYK	+ (~40%)	+
BLNK		
PLCγ2		
LYN		
FYN		
EBV antigens		
LMP1	+ (~40%)	–
<i>Clonally rearranged immunoglobulin genes</i>		
SHM	Static	Ongoing
Consequences of SHM	“Crippled” (~25%)	Functional

NHL non-Hodgkin lymphoma, RS Reed–Sternberg cells, EMA epithelial membrane antigen, BOB1 B-cell Oct-binding protein 1, OCT2 octamer binding transcription factor 2, PAX5 paired box gene 5, AID activation-induced cytidine deaminase, HGAL human germinal center associated lymphoma, MUM1 multiple myeloma-1, ATF3 activating transcription factor 3, EBV Epstein–Barr virus, LMP1 latent membrane protein 1, SHM somatic hypermutation.

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presents at an early stage and has a good prognosis, albeit with frequent relapses.¹⁵ Since CHL is much more common, and somewhat better understood than NLP HL, this chapter will focus primarily on the current understanding of the genetics and pathobiology of CHL.

Oncogenic Events in Classical Hodgkin Lymphoma

Unlike numerous other hematologic malignancies that are associated with fairly specific genetic mechanisms, there is no known single disease-defining neoplastic event in HL. Nevertheless, and although not unique in the context of lymphomas, constitutive activation of the NF κ B pathway is central to its pathogenesis.^{16,17} NF κ B transcription factors, homo or heterodimers composed of c-REL, p50, p52, RELB, or p65/RELA, are located in the cytoplasm and bound in an inactive form to inhibitors, the I κ Bs. Phosphorylation of the inhibitors results in polyubiquitination and proteasomal degradation, releasing NF κ B, which then translocates to the nucleus and facilitates transcription of a number of genes.¹⁸ Activation of this pathway has proliferative and anti-apoptotic effects, as well as leading to increased expression of chemokines and cytokines, that are central to the inflammatory response.

Detailed below are some of the quite heterogeneous genetic abnormalities described in HL; these include a spectrum of mutations, gains and losses of genetic material, translocations, and epigenetic phenomena, as well as microRNA and other expression profiles (Table 27.2).

Mutations

Acquired mutations are one of the numerous mechanisms leading to NF κ B activation. Cell lines and primary HRS cells have been found to harbor somatic mutations, leading to I κ B inactivation with mutations of NF κ B $B\alpha$ seen in as many as 25% of cases.¹⁹ Mutations of *NFKBIE* (I κ B ϵ), another NF κ B inhibitor, have also been demonstrated in fewer cases (~15%).²⁰ Activation of the JAK–STAT pathway occurs in HRS cells.^{21,22} This pathway is regulated by a negative feedback loop involving suppressors of cytokine signaling (SOCS).²³ Mutations in *SOCS1* have been identified in ~45% of CHL cases and three of five HL cell lines.²⁴

Chromosomal Gains/Genomic Amplifications

A number of specific and well-characterized gains and amplifications are central to the genesis of CHL. Despite technical challenges, conventional cytogenetics has demonstrated trisomies of chromosomes X, 8, and 20.²⁵ Fluorescence immunophenotyping and interphase cytogenetics as a tool for the investigation of neoplasms (i.e., FICTION) studies, employing centromeric DNA probes specific for seven different chromosomes (i.e., X, Y, 1, 8, 12, 15, 17), found that all cases

TABLE 27.2. Important oncogenic events in CHL.

Neoplastic event	Approximate frequency
Mutations	
<i>NFKBIA</i> (I κ B α)	~25%
<i>NFKBIE</i> (I κ B ϵ)	~15%
<i>SOCS1</i>	~45%
Gene amplifications	
<i>CREL</i>	~50%
<i>JAK2</i>	~30–35%
<i>MDM2</i>	~60–70%
<i>BCL3</i>	~15–50%
<i>STAT6</i>	~25%
Translocations involving <i>IGH</i>	
<i>BCL6</i> /t(3;14)	~10% ^a
<i>BCL3</i> /t(14;19)	~5%
<i>CMYC</i> /t(8;14)	~5%
<i>CREL</i> /t(2;14)	~5%
Tumor suppressor gene methylation	
<i>CDKN2A</i> (p16/ <i>INK4a</i>)	~35–40%
<i>CDKN2B</i> (p15/ <i>INK4b</i>)	~40–45%
<i>CDKN4C</i> (p18/ <i>INK4c</i>)	~20–25%
<i>RASSF1A</i>	~65%
EBV infection	~40%
NF κ B pathway activation	~100%

I κ B α inhibitor of NF κ B α , I κ B ϵ inhibitor of NF κ B ϵ , *SOCS1* suppressor of cytokine signaling 1, *JAK2* Janus kinase 2, *MDM2* Murine double minute 2, *INK* inhibitor of cyclin dependent kinase, *EBV* Epstein–Barr virus, NF κ B nuclear factor-kappa B.

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^a*BCL6* translocations are seen in ~50% of NLP HL, affecting either *IG* or non-*IG* loci.

analyzed showed chromosomal abnormalities, which were primarily gains. Correlation of classical cytogenetic and FICTION data was demonstrated in some of the cases; however, for the majority of cases, the changes were only detected using FICTION.²⁶ Studies using comparative genomic hybridization (CGH) showed genomic amplifications of 2p13–p16 involving *CREL*,²⁷ 9p23–p24 involving *JAK2*,²⁸ and 12q14 involving *MDM2*²⁹ gene loci. The likely consequences of these events include activation of the NF κ B and JAK–STAT pathways, and p53 inhibition, respectively. Amplification of *BCL3* and *STAT6* has also been demonstrated in CHL cell lines, with probable further affectation of the NF κ B and JAK–STAT pathways.^{30–32} CGH has also demonstrated chromosomal gains, including 9p, 16p, 17p, 17q, and 20q.²⁷ Array-CGH has confirmed many of these findings and, in addition, demonstrated gains of 7p and Xq, affecting the *FSCN1* and *IRAK1* loci, and their protein products are known to be expressed in CHL.³³ Sub-megabase resolution tiling (SMRT) has also both confirmed many of these findings, as well as identifying potentially novel amplified loci.³⁴

Chromosomal Losses

While overall less frequent than gains, a number of chromosomal losses have been reported. Losses seen by

conventional cytogenetics include deletions involving 7q and 6q.²⁵ CGH identified losses involving 4q32-qter, 13q31-qter, 17p, and 18p in approximately 10–15% of cases examined.^{27,28} FICTION studies have showed loss of chromosome X or Y in a subset of cases.²⁶ Array CGH analysis of CHL cells lines has shown a homozygous deletion in the region 15q26.2 and a 2.35 Mb deletion at 16q12.1.³⁰ The latter region contains *CYLD*, which encodes an enzyme important in suppression of the NFκB pathway and potentially affected by this deletion. Losses of 4q and 11q have also been demonstrated using aCGH on CHL cell lines,³³ while regions of loss illustrated by SMRT include 13q12.13-q12.3, and 18q21.32-q23.³⁴

Epigenetic Events

Epigenetic phenomena also contribute to disease oncogenesis via loss of expression of genes (rather than actual genetic loss or functional loss due to a mutation). Epigenetic silencing typically occurs via methylation of the CpG regions in the promoters of putative tumor suppressor genes, leading to loss of their transcription and absence of protein expression. Numerous genes may be affected; however, the best studied are those that encode members of a family of cyclin-dependent kinase (CDK) inhibitors, such as *CDKN2A* (*p16INK4a*), *CDKN2B* (*p15INK4b*), and *CDKN2C* (*p18INK4C*), which are frequent targets of epigenetic silencing in HL.^{35–37} Candidate tumor suppressor genes (i.e., *RASSF1A* and *PCDH10*) may also be affected by such silencing.^{38,39} By contrast, the CD30 promoter in HRS cells is only rarely methylated. Rather, CD30 induction and overexpression partly result from the interaction of JunB with *hypomethylated* CD30 CpG islands.⁴⁰

Translocations

FISH and FICTION studies have demonstrated recurrent translocations involving the immunoglobulin genes, especially *IGH*, in approximately 20% of CHL cases. The multiple partners include *BCL3*, *BCL6*, *MYC*, and *CREL*.⁴¹ While translocations involving *BCL3* have also been demonstrated in CHL in another study,³¹ *BCL6* translocations could not be confirmed.⁴² In some cases of composite lymphoma, the HRS cells have been shown to harbor translocations involving the *IGH* gene and *CCND1* or *BCL2*, likely reflecting their derivation from the associated mantle cell and follicular lymphomas, respectively or from a common precursor that acquired a translocation early in B-cell development.^{43,44}

microRNAs (miRNAs)

As with numerous other neoplasms, miRNAs have also been shown to play a role in the pathogenesis of CHL. A 25-miRNA signature differentiates between CHL and reactive lymph nodes.⁴⁵ Some of the upregulated miRNAs correlate with cytogenetic changes known to occur in CHL. This includes miR-21 (on 17q), which has been reported to

upregulate anti-apoptotic genes.⁴⁶ Furthermore, MC and NS subtypes show differential patterns of miRNA expression. EBV infection, important in a subset of CHLs, influences the miRNA profile.⁴⁵

Gene Expression Profiling

Although lymphomas have traditionally been broadly divided into either non-Hodgkin (NHL) or Hodgkin categories, gene expression profiling has provided new insights into the relationship between HL and other so called NHLs. For instance, there are many important clinical and pathobiological similarities between NS CHL and primary mediastinal B-cell lymphoma (PMBL) that suggest a common pathogenetic pathway. These include expression of high levels of *IL13-R*, *TARC*, *TRAF1*, *PDL2*, and *MAL*^{47,48}; activation of NFκB; overexpression of receptor tyrosine kinases such as *JAK2*, *RON*, and *TIE1*⁴⁹; gains of 2p and 9p⁵⁰; and *SOCS1* mutations.²⁴ *PDL2* is normally found in T cells and is a regulator of T-cell activation. *MAL* (MyD88 adaptor like), which functions in membrane lipid raft organization, is only expressed in a subset of CHL cases, mainly of the NS type, where it may be associated with a worse prognosis.⁵¹ A subset of thymic medullary B-cells (i.e., asteroid cells) expresses *MAL*, which supports the notion that this is the putative cell of origin in both HL and PMBL.⁵² Expression profiling of anaplastic large cell lymphoma, another NHL that shares some morphologic and immunophenotypic features with CHL, shows that while these two lymphomas clustered separately, the two entities were still more closely related to each other than to B-cell NHL or normal cells. Curiously, *TBET*, one of the T-cell transcription factors expressed in CHL, is not expressed in ALCL, a lymphoma typically of T-cell lineage.⁵³

Proteomics (Also See Chap. 14)

Studying protein expression in CHL is uncovering patterns that may further help in understanding this disease and possibly, in the future, diagnosis. Mass spectrometry has demonstrated different protein expression patterns in the sera from HL patients versus healthy controls.⁵⁴ Examining two HL cell lines by liquid chromatography–tandem mass spectrometry showed not only proteins known to be expressed in HL, but revealed new potential contributors to the pathogenesis of HL. These include BRAF, PIM1, and ErbB-3, which are expressed in other cancers.⁵⁵ ALCAM, Cathepsin S, CD26, CD44, IL1R2, MIF, and TARC are seven proteins that are significantly elevated in HL patients' plasma compared with healthy controls.⁵⁶

Genetic Predisposition

HL may rarely be familial, and epidemiologic studies have proposed that hereditary factors may predispose to its development.^{57–59} These observations suggest that there may be

an additional germline or somatic genetic contribution to the development of CHL in some cases, over and above those that are acquired in the tumor cells. The hypothesis for a gene for CHL that resides in the pseudoautosomal region of a sex chromosome is based on the observation of gender concordance among siblings with CHL.⁶⁰ A variety of studies have suggested a role for certain human leukocyte antigen (HLA) genes, with single nucleotide polymorphism (SNP) analyses confirming this role, particularly in the context of EBV-positive CHL.^{58,61} These genetic and ultimately protein variants may influence the presentation of viral antigens to cytotoxic T cells. SNPs in the promoters for various interleukin genes might influence susceptibility to CHL, as has been reported with *IL6*⁶² and *IL10*.⁶³ *IL6*, *IL10*, and *TNF* SNPs may predict clinical outcome.⁶⁴ Killer cell immunoglobulin-like receptors (KIRs) are important in activating or inhibiting natural killer cell responses. Strong linkage disequilibrium of five *KIR* genes has been associated with CHL, and a protective effect of two activating receptors, *KIR3DS1* and *KIR2DS1*, has been observed in CHL patients with detectable EBV.⁶⁵ While rare, the development of CHL in a patient with ICF syndrome (immunodeficiency, centromeric heterochromatin instability, and facial abnormalities) puts forth the possibility that hypomethylation resulting from mutations of *DNMT3B* may predispose to CHL.⁶⁶ Together, these studies on the genetic predisposition to HL underscore the central role that the immune system plays in its genesis and maintenance.

Role of EBV (Also See Chap. 7)

Epstein–Barr virus (EBV) has a role in the oncogenesis of several malignancies, including hematologic malignancies. This is particularly well established in a subset of patients with CHL; however, there appears to be no causal role for EBV in NLP HL, which is uniformly negative for the viral genome. Epidemiological studies have reported an increased risk of developing CHL in subjects with a previous history of infectious mononucleosis.^{67,68} In the western world, HRS cells are infected in approximately 40% of cases of CHL^{69,70} with a higher percentage of EBV positivity among the MC subtype.⁷¹

EBV proteins have differing roles leading to proliferation and immortalization of the infected cells. Different expression patterns, or “latencies”, of 9 key proteins are seen in various neoplastic and infectious scenarios. The latency type IIa expression pattern, defined by the expression of EBNA1, LMP1, and LMP2a is seen in EBV-positive CHL.^{72,73} Each of these proteins has a role in the pathogenesis of CHL. LMP1 facilitates survival by mimicking an activated CD40 receptor, upregulating *BCL2* and *BMI1*, and activating NFκB.^{74–77} The immunosuppressive environment of the HRS cells is partly molded by LMP1, which stimulates cytokines that recruit T regulatory cells.⁷⁸ LMP1 may also down-regulate CD99, causing induction of HRS-like cells in vitro.⁷⁹ HRS cells characteristically

lack expression of the B-cell receptor (BCR) complex, which should result in apoptosis due to the absence of positive selection signals; however, LMP2a acts as a substitute for the BCR in EBV-positive cases.⁸⁰ Thus, the majority of cases (92%) of CHL that harbor crippling mutations, resulting in lack of a functional BCR, are EBV-positive, while fewer (44%) cases without crippling mutations are EBV-positive.⁸¹ EBNA1-mediated decreases in Smad2 protein results in downregulation of the TGFβ target gene protein tyrosine phosphatase receptor K (PTPRK), leading to increased cell growth and survival.⁸²

While a role for the measles virus has been proposed,⁸³ more rigorous analysis failed to confirm this association.⁸⁴ HHV6 genome DNA has recently been detected in both EBV+ and EBV– cases of CHL, most frequently in the NS subtype⁸⁵; however, a causal role for HHV6 remains to be established.

The Peculiar Phenotype of HRS Cells

Despite their bona fide mature B-cell origin, HRS cells in CHL show absent or low expression of immunoglobulin and many other B-cell antigens, including surface markers and multiple B-cell-specific transcription factors (Figure 27.1). Examination of approximately 12,000 genes through gene expression profiling proved that HRS cells have an aberrant GC phenotype with activated NFκB and resistance to apoptosis.⁸⁶ Serial analysis of gene expression (SAGE) analyzing the HRS cell line L1236 and tonsillar GC centroblasts, as well as Affymetrix microarrays comparing HL cell lines with four normal B-cell subsets, confirmed the downregulation of almost all B-cell-specific genes.^{87,88} Affected genes include *CD19*, *CD20*, *CD79*, *IG*, *OCT2*, *BOB1*, *PUI1*, the tyrosine kinase *SYK*, and *SLP-65 (BLNK)*, an intracellular adaptor protein, important for BCR signaling. HRS cells maintain expression of *CD80*, *CD86*, and *MHCII*, which are central mediators in B-cell and T-cell interactions. Similar results were obtained in a separate study using SAGE, which compared CHL cell lines with germinal center B-cells. Epigenetic events contribute to the HRS cells' loss of B-cell phenotype. Hypermethylation of the promoters for *BOB1*, *PUI1*, *LYK*, *SYK*, *CD19*, *CD20*, *CD79B*, and *TCL1* has been described.^{89,90} Naive B-cells' survival depends on positive selection signals that take place in the germinal center. These happen via the BCR, which includes CD79 and surface immunoglobulin. HRS cells lack a BCR and have crippling immunoglobulin gene mutations (in ~25%), yet apoptosis does not occur as expected. HRS cells' loss of a B-cell phenotype is hypothesized as critical in evading apoptosis, by escaping negative selection.

Lack of activation of the immunoglobulin gene promoter is partly responsible for the HRS cell phenotype. Octamer binding transcription factor 2 (OCT2), a POU family member, along with the cofactor B-cell Oct-binding protein (BOB1)

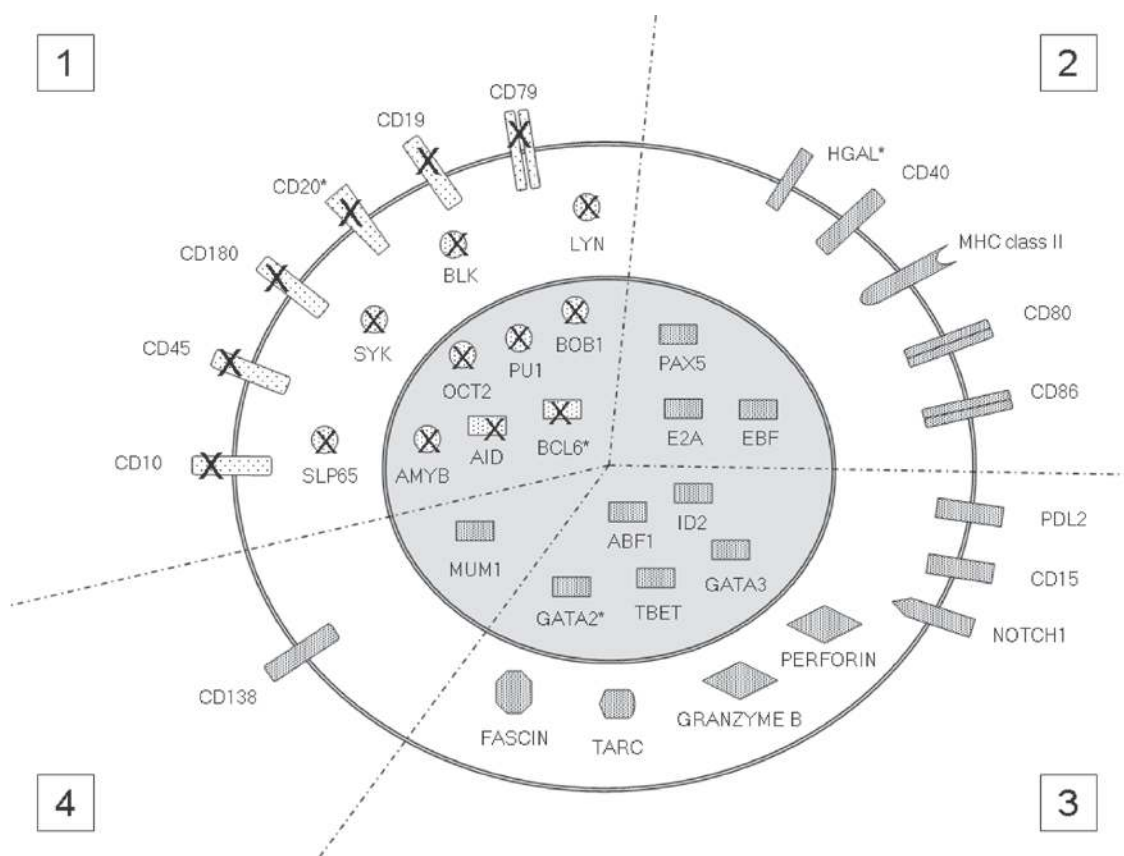


FIG. 27.1. The unusual phenotype of HRS cells in CHL. These unusual features include: (1) decreased or absent lymphoid, B-cell, and germinal center proteins; (2) retained expression of selected B-cell proteins and molecules involved in antigen presentation; (3) “aberrant” expression of molecules not normally expressed on B-cells; and (4) expression of plasma cell associated antigens. *CD20 and BCL6 expression is not absent in all cases, and is expressed in ~40% and ~30% of cases, respectively; HGAL is expressed in ~75% of cases, and GATA2 in ~50%. Reproduced from Expert

Rev. Mol. Diagn. 7(6), 805–20 (2007), with permission of Future Drugs Ltd. *SLP65* Src homology 2 domain-containing leukocyte protein 65, *OCT2* octamer binding transcription factor 2, *BOB1* B-cell Oct-binding protein 1, *AID* activation-induced cytidine deaminase, *HGAL* human germinal center associated lymphoma, *PAX5* paired box gene 5, *EBF* early B-cell factor, *ABF1* activated B-cell factor 1, *ID2* inhibitor of differentiation and DNA binding, *TARC* thymus and activation regulated chemokine, *MUM1* multiple myeloma-1.

are important in octamer-dependent immunoglobulin promoter transactivation among other B-cell-specific gene functions.^{91,92} ETS family member PU1 is another transcription factor important in B-cell differentiation and immunoglobulin expression.⁹³ OCT2, BOB1, and PU1 are not expressed in CHL.^{92,94,95} While the expression of many B-cell-specific genes is lost, expressions of some transcription factors important for B-cell phenotype are retained, although at levels lower than in normal B-cells.⁹⁶ For example, both E2A and PAX5 are expressed. However, PAX5 is weakly expressed in HRS cells in CHL and some of its target genes are not expressed. In contrast to some other B-cell malignancies, mutations of the *PAX5* gene have not been demonstrated in CHL.⁸⁷ Although E2A is expressed in HRS cells, two of its inhibitors [inhibitor of differentiation/DNA binding (ID2), which is not normally expressed in B-cells, and activated B-cell factor 1 (ABF1)] are also

expressed.⁹⁷ The expression of these inhibitors in HRS cells is another factor adding to the loss of expression of B-cell-specific genes.

Expression of several T-cell-associated genes may also help conceal the B-cell phenotype in CHL. Activation of NOTCH1, a protein not normally expressed in B-cells,⁹⁸ favors T-cell differentiation and suppresses B-cell differentiation early in lymphocyte development, perhaps by inhibiting E2A.⁹⁹ In HRS cells, activated NOTCH1 leads to proliferation and resistance to apoptosis. NOTCH1 may also contribute to activation of the NFκB pathway. Additionally, GATA2 may mediate the effects of NOTCH1 and is expressed in 50% of CHL cases.¹⁰⁰ Of note, the T-cell transcription factors T-BET and GATA3, which guide Th1 and Th2 cytokine production respectively, are present in HRS cells. A role in cytokine production and possibly apoptosis resistance has been proposed⁵³; however, the exact significance of their

expression is still unclear. HRS cells also express the receptors, transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), B-cell maturation antigen (BCMA), and heparan sulfate proteoglycan (HSPG). However, their expression pattern is different from that of GC B-cells, and rather resembles that seen in normal plasmacytoid B-cells.¹⁰¹

Disrupted Cellular Pathways

A variety of intra- and inter-cellular pathways are crucial to the biology of HL, and they may be activated in a number of ways (Figure 27.2). NFκB may be activated via a number of upstream mechanisms. CD30, CD40, and receptor activator of NFκB (RANK), all members of the tumor necrosis factor (TNF) receptor family, are involved in the activation

of NFκB.^{102–104} As described earlier, LMP-1 in EBV-positive cases may activate NFκB, by simulating an activated CD40 receptor.⁷⁷ NOTCH1⁹⁸ and signaling via TNF receptor associated factor 1 or 2 (TRAF1 or TRAF2)¹⁰⁵ also contribute to NFκB activation. NFκB targets numerous genes, including those encoding various cytokines, surface receptors, transcription factors, and anti-apoptotic mediators.

Dysregulation of other pathways in HRS cells also occurs, resulting in enhanced survival and proliferation. The MAP/ERK pathway becomes activated via CD30, CD40, or RANK, and induces *JUNB* expression.¹⁰⁶ Activation of the phosphatidylinositol 3 kinase pathway also induces proliferation, while inhibiting apoptosis in CHL.¹⁰⁷ Constitutive activation of the ERK5 pathway results in proliferative and anti-apoptotic effects.¹⁰⁸ Normally, activation of the CD95 (FAS) death receptor pathway removes defective GC B-cells. Although HRS cells express intact CD95, they are resistant

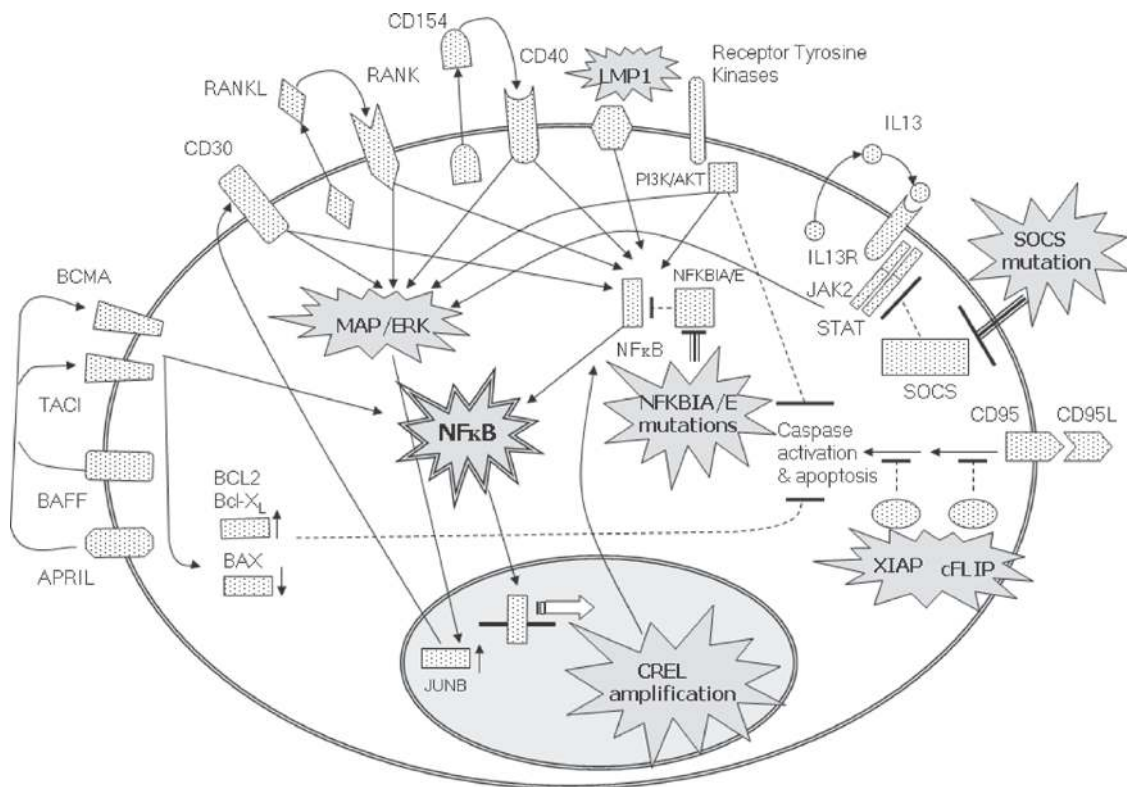


FIG. 27.2. HRS cell showing primary pathways central to its biology and neoplastic transformation. A complex network of pathways is activated, due to both extracellular factors and intracellular events. Key phenomena are highlighted, with the activation of the NFκB pathway being an essential component. This pathway may be activated via CD30 overexpression, the lack of inhibition due to mutant NFKBIA (IκBα) and NFKBIE (IκBε), CREL amplification and EBV-LMP1, amongst other mechanisms. Once NFκB is translocated from the cytoplasm to the nucleus, a host of different genes central to the biology of the HL and neoplastic transformation is activated (not shown, see text for details). Activation of the MAP/ERK pathway (via a number of upstream events) and JAK/STAT pathway (via IL13 and/

or loss of SOCS inhibition) are also important. A variety of different mechanisms, including increased expression of XIAP and cFLIP, contribute to the anti-apoptotic phenotype. Reproduced from Expert Rev. Mol. Diagn. 7(6), 805–20 (2007), with permission of Future Drugs Ltd. RANK receptor activator of NFκB, RANKL receptor activator of NFκB ligand, LMP1 latent membrane protein 1, BCMA B-cell maturation antigen, TACI transmembrane activator and calcium modulator and cyclophilin ligand interactor, BAFF B-cell-activating factor of the TNF family, APRIL a proliferation inducing ligand, JAK2 Janus kinase 2, STAT signal transducer and activator of transcription, SOCS suppressor of cytokine signaling, XIAP X-chromosome-linked inhibitor of apoptosis protein, cFLIP cellular FLICE-inhibitory protein.

to CD95-induced apoptosis.¹⁰⁹ NF κ B-induced expression of anti-apoptotic factors, including *cFLIP*, *XIAP*, and *BCL-X_L*,¹¹⁰ is partly responsible.^{21,111–113}

HRS cells also demonstrate activation of a number of members of the signal transducers and activators of transcription (STAT) family of transcription factors including STAT1, STAT3, STAT5, and STAT6.^{114,115} Multiple factors may lead to activation, resulting in proliferative effects, with activation of JAKs leading to phosphorylation and activation of STATs. In addition, *JAK2* kinases are amplified in some HRS cells, providing a mechanism for constitutive STAT activation.²⁸ HRS cells express IL-13 and its receptor, IL-13R. Stimulation of the IL-13R results in JAK activation of STAT6 and HRS cell proliferation.²² STAT6 activates IL-4 and IL-13 genes, promoting its own expression. Lastly, HRS cells express IL-21 and its receptor IL-21R; IL-21 contributes to HRS proliferation and survival via phosphorylation of STAT3 and STAT5.¹¹⁶

Unlike normal B-cells, in primary CHL, there is expression of up to 7 receptor tyrosine kinase (RTK) family members, including PDGFRA, DDR2, EPHRINB1, RON, TRKA, TRKB, and TIE1.^{117,118} Stimulation of these RTKs occurs via paracrine and autocrine mechanisms.¹¹⁹ RTK expression pattern varies among the different subtypes of HL and with EBV status. NS CHL averages the most (4 RTK/case) and NLP HL has the lowest numerical average (1 RTK/case). Analysis of EBV-positive and EBV-negative cases of NS and MC CHLs has revealed that co-expression of three or more RTKs is almost exclusively seen in the EBV-negative cases.⁴⁹ Interestingly, although LMP2a mimics the BCR (see above), there was no demonstrable increase in RTK activation in the EBV-positive cases. It has been suggested that the RTKs may exert effects via triggering the PI3K/AKT pathway, which activates NF κ B, which is also stimulated by LMP1 in EBV-positive cases. In contrast to other malignancies,¹²⁰ activating mutations of RTKs have not been described in CHL, at least at the level of HL cell lines.

Recruitment of PI3K-like protein kinase kinases (PIKK), such as ATM and ATR, is required for PIKK-dependent DNA damage signaling.¹²¹ These kinases respond to double strand DNA breaks by phosphorylating multiple substrates, ultimately resulting in cell cycle arrest with DNA repair or alternatively, apoptosis. The ATM protein is significantly downregulated in HRS cells of CHL. Reduced ATM transcription occurs in the absence of allelic loss, promoter hypermethylation, or ATM mutations; mechanistically, deregulation of factors acting upstream of ATM is postulated.¹²² By contrast, ATR may be directly genetically altered in CHL.¹²³

Nodular Lymphocyte Predominant Hodgkin Lymphoma

The major clinical, morphological, and immunophenotypical features of NLP HL, and a comparison with CHL, were noted previously and are demonstrated in Table 27.1.

There are few cytogenetic data from primary NLP HL cases, due to similar technical issues that have limited the study of the neoplastic cells in CHL. However, in one recent study it was shown that conventional cytogenetics in NLP HL frequently demonstrates a complex karyotype with recurrent chromosomal defects, including gains or partial gain of 1q, 3q27 rearrangements, loss of chromosome 4 (or 4q28 rearrangements resulting in partial loss), loss of chromosome 7 (or 7q23–q33 rearrangements), chromosome 9 imbalances, loss or partial deletion of chromosome 13, and 14q32 rearrangements.¹²⁴ Translocations involving *BCL6* are recurrent in NLP HL, occurring in 48% of cases by interphase FISH. Partners include immunoglobulin and non-immunoglobulin loci, such as 7p12 potentially affecting the *IKZF1* (Ikaros) gene, as well as 9p13 and 4q32, each with several candidate genes.¹²⁵ A separate study using FISH and FICTIO has demonstrated recurrent *IGH-BCL6* translocations in ~20% of cases.¹²⁶

CGH has demonstrated chromosome gains of 1q, 3p, 5q, and Xq and losses of 11q and 17p.¹²⁷ Deletions involving chromosome 17 have also been reported in separate studies using conventional cytogenetics and in an NLP HL cell line.^{25,128} While the exact significance of this loss is uncertain, several tumor suppressor genes map to this region.

Summary

Despite the challenges posed by the rarity of malignant cells and their deceptive immunophenotype, microdissection with PCR analysis of single cells has finally resulted in the realization that HL is a lymphoma of B-cell origin, albeit one that conceals its own identity, presumably in order to ensure its survival. Although many of the recent studies have led to our learning a great deal about the molecular biology and pathogenesis of HL, none of these elegant molecular studies are currently actually necessary in routine diagnostic clinical practice.¹²⁹ Nevertheless, translating this knowledge into useful targeted therapeutic intervention is essential, as treatment-related malignancies and complications remain a real concern for these patients, even in the face of increasing survival rates.¹³⁰ Additionally, as current methods to predict prognosis are fairly unreliable, within this accruing body of knowledge, there are genes, proteins, or perhaps a cytokine signature that may aid in predicting clinical course and outcome. Tissue-based factors (i.e., *BCL2*,¹³¹ *HLA-DR*,¹³² and phosphorylated STAT5¹³³ expressions) and less-invasive serum- and plasma-based assays [i.e., *TARC/CCL17* and *MDC/CCL21*^{134,135} *IL1-RA*, *IL-6*, *sCD30*¹³⁶ and *sBAFF*¹³⁷], are among those already showing potential.

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Posttransplant Lymphoproliferative Disorder

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Introduction

Posttransplant lymphoproliferative disorder (PTLD) is a rare, life-threatening neoplasm occurring after hematopoietic stem cell or solid organ transplant. The pathobiology of PTLD is distinct from conventional lymphoma, in that immunosuppression and Epstein–Barr virus (EBV) are critical cofactors in tumor development, and these features also afford unique strategies for targeted therapy.

The disease begins when iatrogenic immunosuppression leads to diminished T cell immunity and then massive proliferation of EBV-infected B lymphocytes. Acquired genetic change appears to be responsible for neoplastic transformation and disease progression. Because morbidity and mortality are high, early recognition of PTLD is important for prompt clinical management. Several new tools are available to assist in early diagnosis and preemptive therapy.

Clinical Setting

Risk factors for PTLD include the type and intensity of the immunosuppressive regimen (especially agents such as fludarabine, azathioprine, or antithymocyte globulin that deplete T cells), primary EBV infection occurring after transplant in a recipient who was seronegative prior to transplant, genetic constitution (i.e., cytokine polymorphisms, HLA type), the type of organ that was grafted, whether grafted marrow is HLA-mismatched or from an unrelated donor, coinfection with other pathogens, and reduced intensity conditioning.^{1,2}

PTLD often presents with constitutional symptoms, organ dysfunction, or a rapidly enlarging mass. The most common anatomic sites of disease are the graft itself, hematopoietic organs (including bone marrow or lymphoid tissue – especially in Waldeyer’s ring), and extranodal sites (especially visceral). Biopsy is recommended to confirm a diagnosis of PTLD and to rule out infection, graft-versus-host disease, and rejection, each of which is treated quite differently from PTLD.³

Histopathology

A spectrum of histologies are seen, and varying clinical and molecular features are reflected in the histopathologic subtypes.^{4–6} EBV is usually present within the proliferating B cells by in situ hybridization to EBV-encoded RNA (*EBER*). Although the clinical presentation and histopathology may resemble infectious mononucleosis, it is important to avoid using diagnostic terminology implying a self-limited process. Instead, any immunosuppressed transplant patient who meets the histopathologic criteria should receive a diagnosis of PTLD with further grouping into one of four subtypes as outlined in the World Health Organization (WHO) subclassification scheme.⁶ Without accurate diagnosis and prompt intervention, PTLD often progresses rapidly to a fatal conclusion.

Early lesions tend to occur soon after transplant in seronegative recipients, and a key microscopic feature is preservation of tissue architecture. Because these lesions are often polyclonal, they are thought to represent an initial phase of disease pathogenesis that may progress to frank neoplasia. Treatment involves reducing or terminating immunosuppression, so that the body’s own immune system may control EBV is detectable in the lymphoid infiltrate by *EBER* in situ hybridization.

Polymorphic PTLD is characterized by a polymorphous mix of small lymphocytes and larger immunoblasts, including clonal EBV-infected B lymphocytes, plasma cells, and reactive CD4- and or CD8-expressing T lymphocytes. The proliferating lymphocytes usually express CD20 and are light chain-restricted (Figure 28.1). The lymph node architecture is often effaced; the mitotic rate is high; and the clinical behavior is aggressive. Reconstituting natural immunity is often, but not always, successful in reversing tumor growth.

Monomorphic PTLD mimics the histopathology and immunophenotype of conventional lymphomas, typically diffuse large B cell lymphoma, myeloma, plasmacytoma, or Burkitt lymphoma. Much less commonly, the tumor resembles anaplastic large cell lymphoma, peripheral T cell lymphoma, or NK cell lymphoma. Monomorphic lymphoma is monoclonal

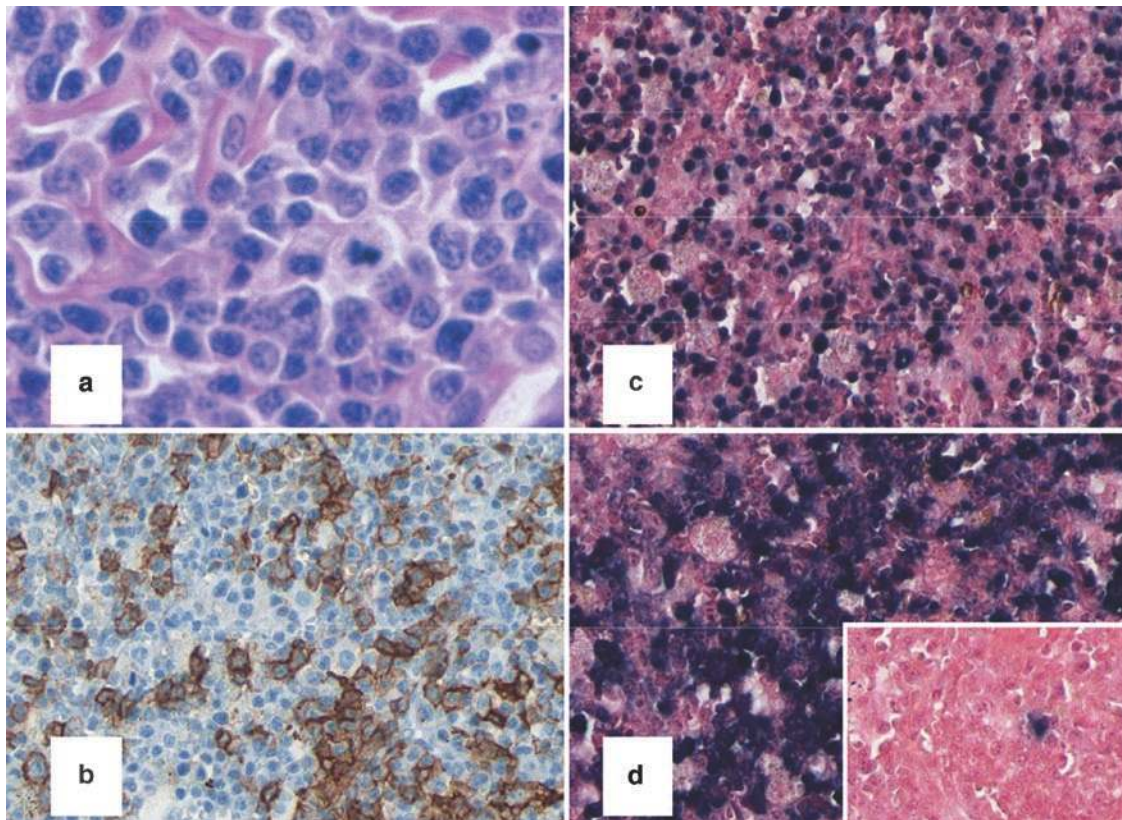


FIG. 28.1. A polymorphic PTLD (a) is comprised of a mixture of plasma cells and lymphocytes. The proliferating lymphocytes express EBV-encoded RNA (b) and CD20 (c) and they are clonal based on expression of kappa transcripts (d) but not

lambda transcripts (*inset*). (a, H&E stain, $\times 400$; b, *EBER* in situ hybridization, $\times 100$; c, CD20 immunohistochemistry, $\times 100$; d, kappa in situ hybridization, $\times 100$; inset, lambda in situ hybridization, $\times 100$).

as shown by light chain restriction, antigen receptor gene rearrangement, karyotype, and/or EBV genomic structure analysis. Clonality is further supported by *EBER* in situ hybridization, showing viral localization to all the malignant-appearing cells. A minority of tumors lack EBV, and these tend to arise more than a year after transplant, so it is speculated that they represent lymphomagenesis of the conventional type. Nevertheless, some EBV-negative tumors respond to withdrawal of immunosuppression, implying that their pathogenesis is akin to EBV-related PTLDs.

Classical Hodgkin lymphoma-type PTLD is a rare variant, occurring late after transplant.⁷ It has a classical immunophenotype, consistently expresses *EBER* within the malignant Reed–Sternberg/Hodgkin cells, and it responds well to therapy. Another rare variant of PTLD is primary effusion lymphoma coinfecting with EBV and HHV8. Well-differentiated lymphomas of the follicular, marginal zone, or mucosa-associated lymphoid tissue (MALT) subtypes may be considered PTLDs when they arise in transplant recipients, but they are generally EBV-negative, are not clinically aggressive, and do not require treatment as an aggressive lymphoma. Florid follicular hyperplasia is proposed as an

early histologic manifestation of PTLD by some (but not all) investigators.⁸

The vast majority of PTLDs are of B cell origin. Rarely, PTLD arises from naïve B lymphocytes, but more commonly there is somatic mutation of *IGH* indicating germinal center origin (*BCL6* expression) or postgerminal center origin (*MUM1* and/or *CD138* expression).⁹ It is important to document whether CD20 is expressed since therapy using antibody to CD20 is commonly contemplated. After CD20 therapy is initiated, outgrowth of a CD20-negative subclone may occur, conferring resistance to CD20 antibody therapy. EBV infection may contribute to downregulation of CD20, and EBV infection may also account for lack of CD20 in *de novo* PTLD resembling anaplastic large cell or Hodgkin lymphoma.

Clonality and Clonal Evolution

Clonality is typically measured by immunophenotype, by in situ hybridization to kappa and lambda transcripts, or by polymerase chain reaction (PCR) across the rearranged *IGH* gene.^{10,11} EBV-infected tumors may also be evaluated for

clonality with respect to the structure of the EBV genome, since each infected cell tends to have a unique fused terminal repeat structure once the viral DNA circularizes within the cell nucleus (Figure 28.2).^{12,13} PTLDs tend to be monoclonal, with the exception of early lesions that are frequently polyclonal. Occasional tumors have two clonal *IGH* genes that may represent biallelic rearrangement in a monoclonal lesion, or two separate monoclonal neoplasms. Oligoclonal *IGH* genes have also been described, implying that several independent tumors arose synchronously in a host who was quite susceptible to EBV-related neoplasia. Monoclonal PTLDs tend to behave more aggressively than do polyclonal early lesions.^{4,14}

In cell culture models, normal B lymphocytes that are EBV-infected progress to monoclonality within a couple of months of in vitro growth, implying that a single cell had a growth advantage over the others.¹⁵ The factors influencing which clone dominates are uncertain, but possible contributors include immunologic characteristics and/or secondary genetic changes in the human or viral genome.

Mechanisms of Viral Lymphomagenesis

In immunosuppressed transplant recipients, it is likely that uncontrolled EBV infection is primarily responsible for many polyclonal “early lesions”; other early lesions, as well as monoclonal PTLDs, have probably acquired one or more genetic defects driving neoplastic behavior.⁸ Early diagnosis and intervention is a worthy strategy for minimizing cell divi-

sion to prevent accumulation of genetic defects that may render the PTLD more resistant to therapy. About half of PTLDs have crippling *IGH* mutations that should normally result in apoptosis; however, EBV may rescue crippled cells, so they not only survive, but proliferate.

EBV is a ubiquitous virus that infects most humans before adulthood and thereafter persists for the duration of life, usually without adverse health effects. Immunosuppression may render the immune system incapable of controlling the infection, permitting expression of a wider range of viral proteins than would be possible in a healthy host.¹⁶ Viral LMP1 is oncogenic, as evidenced by its ability to signal through NFkB to promote proliferation, and to upregulate cytokines promoting survival.¹⁷ Another viral gene product, EBNA2, operates through MYC to promote cell proliferation.¹⁸ LMP1 and EBNA2 are commonly expressed in PTLD, although not necessarily in the same cells and not reliably enough to be used as routine markers of infection.^{17,19}

EBER is a reliable marker of latent EBV infection, because non-polyadenylated *EBER* transcripts are present at high levels in every latently infected cell where they contribute to immune evasion by regulating interferon response.¹⁶ Thus, *EBER* in situ hybridization is the gold standard assay for assigning whether a given tumor is EBV-related.¹⁹ *EBER* is localized to nuclei, often sparing the nucleoli. To avoid false-negative interpretations resulting from *EBER* degradation, a control hybridization should be done to assure that RNA is preserved and available for hybridization. Commercial probes and automated systems now are available to facilitate implementation of *EBER* histochemistry in clinical histology laboratories.²⁰

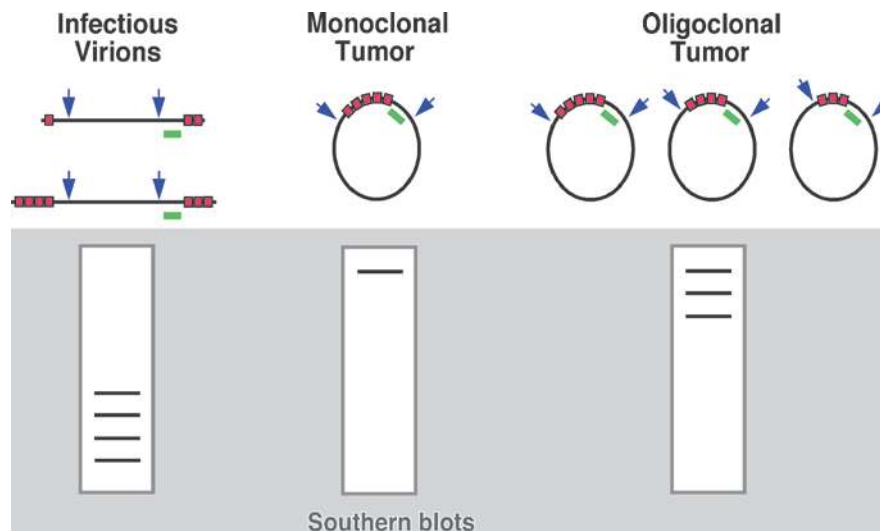


FIG. 28.2. Clonality of EBV terminal repeat sequences reflects the clonal nature of an infected neoplasm. The clonality assay relies on the presence of variable numbers of tandem repeat sequences (red boxes) at the ends of the linear EBV genome. Upon infection, end-joining creates an episome fusing up to 20 terminal repeats. When an infected cell divides, the relatively unique terminal repeat structure is inherited by all cellular progeny. A monoclonal neoplasm is characterized by

monoclonal, episomal EBV DNA as demonstrated by a single band on Southern blot analysis of BamH1 digested DNA. BamH1 cuts the EBV genome at sites flanking the terminal repeats (blue arrows), resulting in restriction fragment(s) that are recognized by a DNA probe (green bar). On Southern blots, infectious virions produce a ladder array of small bands while a monoclonal tumor exhibits a single large band and an oligoclonal tumor has several large bands.

Immunohistochemistry often reveals LMP1, EBNA1, and EBNA2 expression in a variable proportion of *EBER*-expressing lymphocytes.²¹ When positive, these stains may be used as evidence of EBV-relatedness, but negative results should not be relied upon to exclude EBV infection. Instead, *EBER* in situ hybridization (or high EBV viral load in blood or plasma) is used to complement immunostains in defining EBV-related disease. A small proportion of PTLT cells may express lytic viral proteins, indicating that some cells are on a trajectory to produce infectious virions. In the lytic phase, EBV upregulates IL10 and its own virally encoded IL10-like protein (BCRF1) to suppress interferon and macrophage responses to viral replication, while upregulating BCL2 and its own BCL2-like protein (BHRF1) to suppress apoptosis.

In SCID mouse models, growth of EBV-infected B cells is more robust in the presence of T cells, indicating that T cells not only control, but also abet B cell growth. Another surprising finding is that that latently infected neoplastic B cells grow more robustly when the B cells are permissive for lytic viral replication.

Host Chromosomal Genetic Alteration

Cytogenetics reveals that about half of PTLTs harbor gross chromosomal rearrangements or numeric abnormalities.^{8,22,23} A range of defects have been described, including mutation or rearrangement of *BCL6* (3q27), *MYC* (8q24), *PAX5* (9p13), *PIMI* (6p21), *RHOH* (4p13), and *NRAS* (1p13).^{8,22,23} Some of these genes are familiar because of their association with sporadic lymphoma and because of their well-known effects on cell cycle regulation, resistance to apoptosis, and immune evasion. Mutations in *BCL6*, *MYC*, *PAX5*, and *RHOH* have been attributed to the process of somatic hypermutation, which is primarily intended to create antibody diversity in *IGH* variable segments.²⁴

Comparative genomic hybridization reveals numeric chromosomal abnormalities in about half of PTLTs^{23,25}; and in vitro studies confirm that EBV contributes to abnormal chromosomal segregation.²⁶ Inactivation of the tumor suppressor genes, *DAPK1* and *MGMT*, by promoter methylation is also extremely common in PTLTs.²⁷ Epigenetic control of gene expression might be attributable to EBV, since it is known that EBV controls its own viral transcriptome, as well as host gene expression by promoter methylation.⁹ The various genetic and epigenetic changes (along with the effects of foreign genes brought in by EBV) may account for the unique gene expression profiles found in EBV-positive PTLT.²⁸

PTLT patients may be predisposed to acquire genetic defects, as a consequence of chronic inflammation related to rejection, graft versus host disease, and infection. The propensity of PTLT to occur in the grafted organ or in mucosal surfaces suggests that chronic antigenic stimulation may contribute to neoplastic transformation, perhaps via reactive oxygen species damaging DNA.

Few genetic studies have been done on large enough cohorts of patients to identify prognostic genetic factors. In one study, poor outcome was found in association with *BCL6* mutation.²⁹

EBV-Negative PTLT

EBV-negative PTLT that tends to occur late after transplant is more likely to be of T or NK lineage, and it tends to have an unfavorable outcome.^{30,31} While some of these late-occurring, EBV-negative tumors undoubtedly represent malignant lymphoma of the conventional type,³² anecdotal evidence of response to withdrawal of immunosuppression supports a PTLT-like therapeutic approach.³³ The somewhat unexpected response of these EBV-negative lymphomas to immune reconstitution implies that either (1) the laboratory assay used to detect EBV was falsely negative, (2) biopsy failed to sample a concurrent EBV-driven tumor in the patient, or (3) restoration of immunity may control cancer, even when the tumor is driven to proliferate by EBV-independent mechanisms. Rarely, PTLT has been linked to HHV8. Other herpes family viruses have not been implicated, nor have they been evaluated in a systematic fashion.

Blood Tests to Diagnose, Predict and Monitor PTLT

EBV-related PTLT patients have high levels of EBV DNA in whole blood and in plasma.³⁴ Furthermore, EBV testing serves as a harbinger of progression to PTLT, since blood levels of EBV DNA rise before the patient becomes symptomatic.^{35–38} Frequent monitoring of high risk patients permits preemptive therapy to be used in disease prevention.^{2,36,39–42} In clinical trials, solid organ recipients should have EBV loads measured at least once monthly during the first year, and continued monitoring should be considered in high risk patients, as judged by prior EBV loads and the degree of immunosuppression.^{2,3,33,43} Antiviral prophylaxis is typically used as a preventive measure.³ Viremia implies a transplant recipient is at higher risk for progression to PTLT and also at risk for other adverse outcomes, such as graft dysfunction and acute rejection.^{44–46} Since viremia alone is an imperfect predictor of progression to PTLT, some investigators have proposed using complementary tests such as CD8 or CD4 T cell counts, EBV-specific T cell counts as measured by a peptide tetramers or ELISPOT, ATP release, EBV serology, cytokine gene polymorphism, and rtPCR targeting EBV transcripts.^{47–55}

Serology is not reliable for identifying infection in transplant recipients having a dysfunctional immune system. However, EBV seropositivity prior to allogeneic transplant implies that the patient has established humoral and cellular immunity, which offers some protection against subsequent PTLT. For those patients who are EBV seronegative prior to transplant,

vaccines are being developed in hopes of establishing such protection. Patients with active primary EBV infection should not undergo transplantation, according to the European Best Practice Guideline for Renal Transplantation.³

Therapy

The presence of EBV within every neoplastic cell of most PTLDs has led to novel virus-directed strategies for treating this neoplasm. Restoring natural immunity by withdrawal of immunosuppressive drugs is the first line strategy. Antiviral therapy is only partially effective, since nucleoside analogs (i.e., ganciclovir) are capable of killing cells in lytic infection, but not in latently infection. Strategies have been explored to switch cells from latent to lytic phase using either differentiating agents, demethylating drugs, or more traditional chemo- or radiotherapy, that may work synergistically with ganciclovir.

Anti-CD20 antibody therapy (i.e., rituximab) eliminates mature B cells, explaining why it is important to document CD20 expression in PTLD specimens. After therapy with rituximab, CD20 immunohistochemical stains may remain informative since the antibody typically used in histology laboratories targets a different epitope from the one used therapeutically. In addition, some patients relapse with CD20-negative PTLD, and it appears that EBV promotes survival of aberrant CD20-negative B cells, in an analogous fashion to what is proposed in the pathogenesis of Hodgkin lymphoma.^{56–58}

Infusion of donor cells may be effective, in part because healthy individuals have abundant EBV-directed T cells comprising about 5% of all circulating mononuclear cells.⁵⁵ In selected centers, cell culture systems are being explored to enrich for EBV-directed cytotoxic T lymphocytes from either autologous or allogeneic sources by growing blood cells in the presence of viral antigens. These EBV-specific T cells are then infused into the patient where they can recognize and target infected neoplastic cells.^{59,60}

Monomorphic PTLD are least likely to respond to EBV-directed therapies, implying that they harbor EBV-independent drivers of cell growth that may require more traditional multidrug chemotherapy, radiation, and/or surgical intervention. Nevertheless, the presence of EBV still serves as a tumor marker that may assist in monitoring the efficacy of therapy. Successful therapy is accompanied by a rapid drop in whole blood or plasma EBV levels reflecting diminished disease burden.^{41,61}

Conclusion

PTLDs are unique forms of lymphoid neoplasia united by their clinical presentation after transplantation and by their strong association with EBV. Based on their diverse

microscopic appearance and host genetics, four major histopathologic subtypes have been defined as useful in predicting clinical outcome in response to therapy. Clinicians and pathologists have an important role in early recognition of PTLT, so that prompt management may be provided using immunotherapeutic strategies and EBV-directed approaches with or without more conventional chemo- and radiation therapies. Increasingly, noninvasive blood tests are being used along with preemptive therapy to thwart tumor progression.

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AIDS-Related Lymphomas

Amy Chadburn and Ethel Cesarman

Introduction

Individuals who are immunodeficient, including those who are HIV infected, have an increased risk of developing lymphoproliferative disorders (LPDs). These lesions in the HIV-infected patient population are somewhat heterogeneous, but are more often clearly benign or malignant than the LPD lesions that arise in other immunodeficient states. Like those occurring in the setting of posttransplantation (also see Chap. 28), methotrexate therapy, primary immune disorders and advanced age, the AIDS-related LPDs are often associated with herpesvirus infection, particularly Epstein–Barr virus (EBV; HHV-4) and Kaposi sarcoma herpesvirus (KSHV; HHV-8). Also similar to the other immunodeficiency-related lymphoproliferations, if the HIV positive patient's immune status can be restored, such as by highly active antiretroviral therapy (HAART), the lesions may regress.^{1–3} However, in many instances, a secondary genetic alteration, not all of which have been characterized, has occurred resulting in transformation to an irreversible neoplastic process.

Non-Hodgkin lymphoma (NHL) is the second most common AIDS-related malignancy with an incidence estimated to be between 4 and 10%.^{4,5} While the incidence has decreased since the implementation of HAART, most studies indicate that the risk remains at least ten times higher in HIV-infected individuals than in the general population. The underlying pathogenetic mechanisms associated with the development of AIDS-related NHLs are complex and are thought to be related to disrupted immune surveillance, chronic antigenic stimulation, genetic alterations, cytokine dysregulation, and herpes virus infection.^{6,7} Furthermore, approximately 50% of the AIDS-related NHLs contain somatic hypermutations in the regulatory region of a number of proto-oncogenes such as *MYC*, *BCL6*, *PAX5*, *RhoH/ITF*.⁸ These genetic changes are thought to be a result of the germinal center reaction, and suggest a role in the pathogenesis of lymphoid neoplasia in the HIV-positive patients.

Oncogenic Viruses (Also See Chap. 7)

The role of herpesviruses, specifically the Epstein–Barr virus (EBV; HHV4) and the Kaposi sarcoma herpesvirus (KSHV; HHV8), in the development of a significant percentage of AIDS-related lymphomas cannot be underestimated. These viruses encode for a variety of oncogenic gene products that are important in the development of these tumors.

Epstein–Barr Virus (EBV; HHV4)

The Epstein–Barr virus in lymphoid malignancies is usually latent. The different stages of EBV latency are defined by the expression of nine latent genes (Table 29.1).⁹ Some of these EBV latent genes, such as latent membrane protein-1 (LMP1), LMP2A, and EBV nuclear antigen-2 (EBNA2), are important in the pathogenesis of a proportion of AIDS-related lymphoma.

Latency Membrane Protein-1 (LMP1)

This oncogenic EBV gene product, expressed during latency II and III, plays a significant role in B cell transformation driving cell growth by acting as a constitutively active TNF-family receptor initiating the NF-kappaB signaling pathway through the TRAF (tumor necrosis factor [TNF] receptor associated factor) and TRADD (TNF-receptor 1-associated death domain protein) molecules.^{9–15} LMP1 also activates a variety of other signaling pathways (i.e. p38/MAP kinase and PI3K/Akt), influences the expression of chemokines (i.e. CCL17 and CCL22), cytokines (i.e. IL6 and hIL10), antiapoptotic proteins (i.e. A20 and BCL2), and adhesion molecules (i.e. CD54/ICAM-1), as well as the cell cycle regulator, p27^{Kip1}, and molecules involved in antigen processing/presentation (reviewed in Brinkmann and Schulz and in Middeldorp and Pegtel).^{16–22} LMP1 represses p53 function possibly through the NF-kappaB pathway,²³ or perhaps by modulating the phosphorylation p53 by MAP kinases.²⁴ Furthermore, LMP1

TABLE 29.1. Epstein–Barr virus latency gene expression patterns.^{9,99}

Latency I	EBER1/2, EBNA 1
Latency II	EBER1/2, EBNA1, LMP1, LMP2A, LMP2B
Latency III	EBER1/2, EBNA1, LMP1, LMP2A, LMP2B, EBNA2, EBNA3A, EBNA3B, EBNA3C

induces, primarily through the p38/MAP kinase pathway, the precursor form of miR-155, BIC, which may play an additional role in the development of AIDS-related DLBCLs.²⁵ Knock-down of LMP1 in EBV-positive AIDS-related lymphoma cell lines results in apoptosis, showing an essential role for this viral protein in continued tumor cell survival.²⁶

Latency Membrane Protein-2 (LMP2A)

This EBV gene product, expressed during latency stages II and III, also plays an important role in B cell lymphomagenesis. In the context of AIDS lymphomas, this viral gene product controls expression of TRAF2 mRNA, which is important in LMP1 mediated NF-kappaB signaling.²⁶ In addition, LMP2A may mimic the B cell receptor, allowing B cells with crippling mutations in the immunoglobulin genes to survive.^{27,28}

EBV Nuclear Antigen 2 (EBNA2)

The EBV gene product EBNA2 is a transcription factor that induces expression of LMP1 and LMP2.²⁹ EBNA2 may also play a role in lymphomagenesis, by inhibiting activation-induced cytidine deaminase (AID) expression, downregulating BCL6 expression, causing chromosomal instability, as well as enhancing STAT3 transcriptional activity.^{30–32}

Kaposi Sarcoma Herpesvirus (KSHV; HHV8)

Like EBV, KSHV in AIDS-related lymphomas is usually a latent infection although some lytic cells may be present.³³ Relatively unique to KSHV, in comparison to other herpesviruses, is the presence of a large number of human homologue genes. These “hijacked” genes are thought to help enable KSHV to take over host cellular functions and avoid the host’s antiviral mechanisms.^{34,35} These include (but are not limited to) genes encoding for viral G-coupled protein receptor/viral interleukin-8 receptor (v-GPCR/v-IL-8R), v-FLIP (latent gene; viral Fas-associated death domain [FADD] interleukin-1b-converting enzyme [FLICE] inhibitory protein), viral interleukin-6 (v-IL-6), viral cyclin (v-cyclin), viral interferon regulatory factor (v-IRF3), and latent nuclear antigen-1 (LANA; LNA-1),

Latent Nuclear Antigen-1 (LANA; LNA-1; Open Reading Frame [ORF] 73)

This KSHV/HHV8 encoded gene product tethers the viral genome to human chromosomes and is important for episome

segregation in dividing cells. In addition, it possesses a variety of oncogenic functions, including the ability to bind to p53, thereby repressing the latter gene’s transcriptional activity and ability to induce apoptosis. LANA transactivates pRb-E2F dependent promoters and binds to the hypophosphorylated form of pRb. This allows KSHV to promote cell cycle progression, while inhibiting apoptosis. LANA also regulates both viral and cellular gene transcription.^{35–41} Immunohistochemical staining for LANA, which shows a speckled pattern of nuclear reactivity, is a relatively fast and reliable method of identifying KSHV/HHV8 infected cells.

Viral Cyclin (v-Cyclin; ORF 72)

This viral gene product activates the cyclin dependent kinases CDK4 and CDK6, freeing E2F to activate transcription of S-phase genes. When complexed to CDK6, viral cyclin is also able to phosphorylate pRb, which is then insensitive to inhibition by CDK inhibitors, such as p16^{INK4a} and p27^{KIP1}.^{42–44}

Viral Fas-Associated Death Domain Interleukin-1b-Converting Enzyme Inhibitory Protein (v-FLIP; ORF71/K13)

Viral FLIP is one of three viral proteins, along with LANA and v-cyclin, expressed in all KSHV infected cells.³⁵ Viral FLIP inhibits FAS-mediated apoptosis and is able to activate the NF-kappaB pathway, crucial for primary effusion lymphoma cell survival.^{45–47}

Viral Interleukin 6 (v-IL-6; K2)

Viral IL-6 is an early lytic gene. Viral IL-6 signals through the gp130 receptor subunit of the human IL-6 receptor activating the Stat–Jak pathway.⁴⁸ As B cells, including PEL cells, have IL-6 receptors, the production of v-IL-6 by PEL cells results in an autocrine growth cycle.^{49,50} In addition, v-IL-6 induces endogenous human IL-6 secretion, also contributing to the pathogenesis of primary effusion lymphoma and extracavitary primary effusion lymphoma.⁵¹

Viral Interferon Regulatory Factor (v-IRF3; LANA2; ORFK10.5)

This latency protein is expressed in KSHV/HHV8 infected hematopoietic tissues. It is thought to contribute to KSHV tumorigenesis in hematopoietic tissues, by inhibiting p53 driven apoptosis and transcriptional activation and by inhibiting apoptosis induced by the activation of interferon-activated protein kinase (PKR).^{52,53}

Classification of AIDS-Related Lymphomas

The vast majority of AIDS-related lymphomas are of B cell origin, and are of either germinal center or postgerminal center origin.^{54,55} These lesions are morphologically diverse.

TABLE 29.2. Morphologic classification of AIDS-related (AR)-lymphomas (derived from WHO classification – 2008).

Lymphomas also occurring in immunocompetent individuals

Burkitt lymphoma (BL)
 Diffuse large B-cell lymphoma (DLBCL)-centroblastic and immunoblastic
 Hodgkin lymphoma
 Other lymphomas:
 MALT lymphoma
 Peripheral T-cell lymphoma
 Natural killer (NK)-cell lymphoma

Lymphomas occurring more specifically in HIV-positive individuals

Primary effusion lymphoma (PEL)/extra-cavitary PEL
 Large B-cell lymphoma arising in KSHV/HHV-8-associated multicentric Castleman disease
 Plasmablastic lymphoma

Lymphomas occurring in other immunodeficient states

Polymorphics lymphoid proliferations

- Burkitt Lymphoma
- Diffuse Large B-cell Lymphoma-Centroblastic and Immunoblastic
- Hodgkin Lymphoma
- Other Lymphomas (relatively rare)
 - MALT Lymphoma
 - Peripheral T-cell Lymphoma
 - Natural killer (NK)-cell Lymphoma

Burkitt lymphoma and diffuse large B cell lymphoma are covered in depth in other chapters (see Chaps. 23 and 22, respectively); hence, the discussion in this chapter (below) will be focused on the HIV-related lesions. HIV-associated Hodgkin lymphoma, like many other immunodeficiency-related lymphoproliferative disorders, is highly associated with EBV infection.⁶⁰ Although Hodgkin lymphoma is not considered an AIDS defining illness, the incidence of this malignancy appears to be increasing in HIV-positive patients,⁶¹ and is associated with a more aggressive clinical course.⁶² However, Hodgkin lymphoma and the remaining other non-Hodgkin lymphomas, except Burkitt lymphoma and diffuse large B cell lymphoma, which also occur in immunocompetent individuals will not be discussed in this chapter.

Burkitt Lymphoma (BL)

AIDS-related Burkitt lymphoma is one of the largest groups of AIDS-related lymphomas, accounting for between 20 and 50% of cases and is one of three clinical categories of BL, namely endemic BL, sporadic BL, and AIDS-related BL (see Chap. 23).^{7,57–59,63–65} Morphologically, AIDS-related BL may show features as seen in immunocompetent patients or show plasmacytoid differentiation.^{56,58} As in immunocompetent patients, AIDS-related BLs are morphologically composed of medium sized cells associated with a high mitotic rate and numerous tingible body macrophages (Figure 29.1). The nuclei, which are approximately the size of a histiocyte nucleus, are relatively round with clumped chromatin and multiple, centrally placed, nucleoli. In the plasmacytoid variant, the cells have nuclei containing a single central nucleolus and eccentric cytoplasm. Often in this latter variant, immunostaining shows monotypic immunoglobulin within the cytoplasm.^{58,65} Although these features are “characteristic” of BL, in practice it is often difficult to separate BL morphologically from diffuse large B-cell lymphoma, particularly in the setting of HIV infection.⁵⁹

Immunophenotypically AIDS-related BLs are composed of monotypic B cells which express CD10 and BCL6, but lack CD21.^{7,66,67} The tumor exhibits a high proliferation rate; nearly all the cells express the proliferation-associated marker, Ki67 (MIB-1). The tumor cells, however, are BCL2 negative (Figure 29.2).

Although all cases show a monoclonal rearrangement and somatic mutations of the immunoglobulin chain

Although many of the AIDS-related lymphomas resemble NHLs occurring in immunocompetent individuals, others are relatively unique and preferentially develop in the setting of HIV infection. These latter lesions are highly associated with herpesvirus infection. In addition, some of the proliferations, the majority of which are EBV positive, resemble morphologically, immunophenotypically, and genetically those occurring in other immunodeficiency settings. AIDS-related NHLs may be classified based on morphology (which most closely reflects the World Health Organization Classification) and/or based on primary site of presentation (i.e. systemic, primary central nervous system, or body cavity; Table 29.2).^{7,56–58} In contrast to HIV-negative patients, HIV-positive individuals with NHL tend to present with advanced stage disease and high serum lactate dehydrogenase (LDH) levels. Furthermore, these lesions exhibit a predilection to involve extra-nodal sites, particularly the central nervous system, gastrointestinal tract, liver, and bone marrow. In addition, AIDS-related lymphomas tend to occur in sites that are only infrequently involved by disease in immunocompetent patients, such as the oral cavity, anus, heart, and body cavities.^{7,56–59} As our knowledge of lymphomagenesis has increased, it has become increasingly apparent that while morphology and clinical presentation are important, they alone are insufficient for the characterization of non-Hodgkin lymphomas, regardless of HIV status. Hence, knowledge of the defining molecular genetic characteristics and expression patterns of gene products, in addition to morphology and clinical presentation, is required for the correct classification and prediction of biologic behavior of these lesions.

Lymphomas Also Occurring in Immunocompetent Individuals

These neoplasms, as per the WHO, should be classified based on the criteria used for similar lesions in immunocompetent patients.^{56,58}

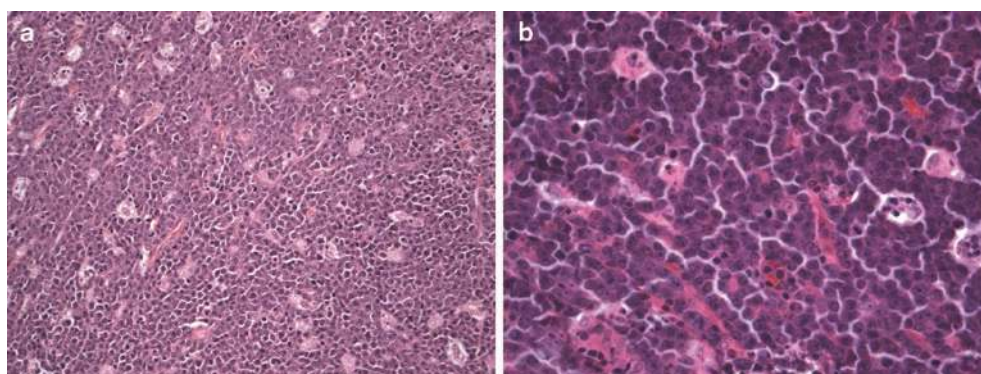


FIG. 29.1. (a) AIDS-related Burkitt lymphoma is morphologically composed of medium sized cells associated with a high mitotic rate and numerous tingible body macrophages imparting a “starry sky” appearance at low power. (b) At low power, the nuclei of the malig-

nant cells are approximately the size of a histiocyte nucleus and are relatively round. (a Hematoxylin and eosin $\times 20$ original magnification; b Hematoxylin and eosin $\times 40$ original magnification; b Courtesy of AIDS Cancer Specimen Resource).

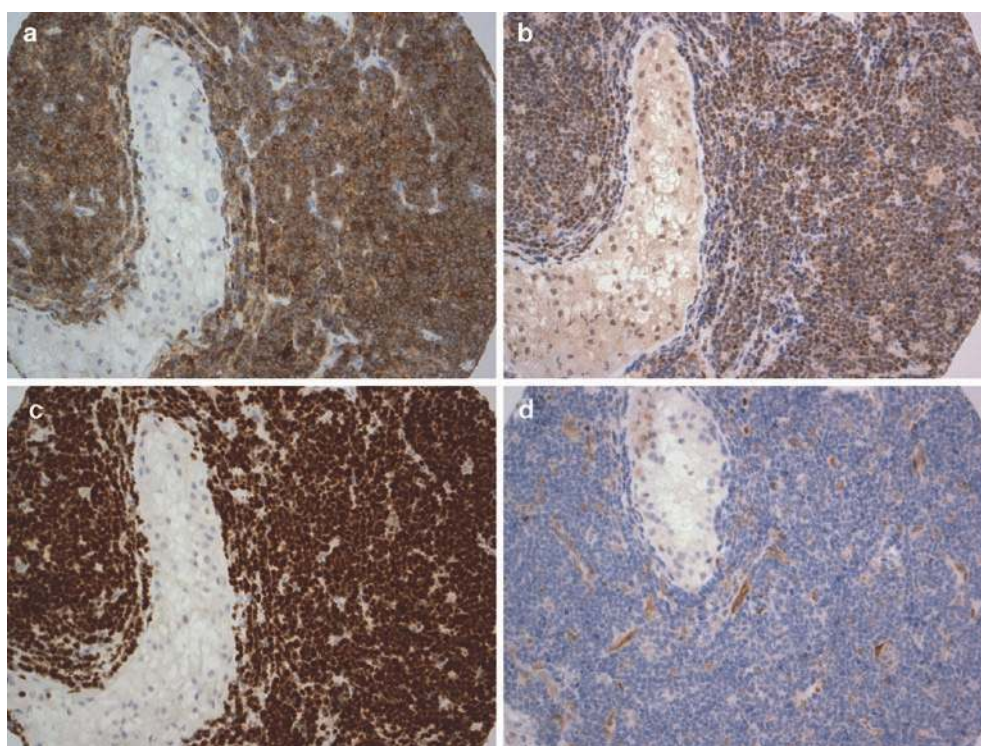


FIG. 29.2. The classic immunophenotype of AIDS-related Burkitt lymphoma: the cells are of B cell origin (CD19 and CD20 positive) and are also (a) CD10 and (b) BCL6 positive. (c) Virtually all of the tumor cells are positive for the proliferation-associated marker

Ki-67 (MIB-1) and (d) lack expression of the BCL2 protein. Note the scattered, rare BCL2 positive cells that most likely represent residual benign T cells (a–d immunoperoxidase $\times 20$ original magnification; courtesy of the AIDS Cancer Specimen Resource).

genes.^{63,68,69} BLs by definition demonstrate translocation of the *MYC* gene on chromosome 8 to one of the immunoglobulin genes on either chromosome 14 (most commonly; heavy chain; Figure 29.3), 2 or 22 (light chains).^{55,66,68} In the

t(2;8) and t(8;22) translocations involving the kappa and lambda immunoglobulin light chains, respectively, the *MYC* gene is translocated some distance from the immunoglobulin light chain sequence. However, when the *MYC* gene

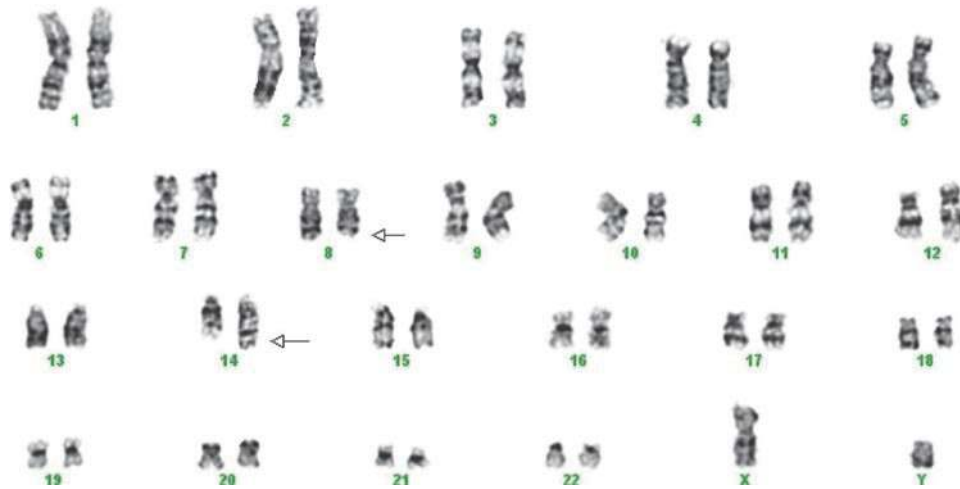


FIG. 29.3. Metaphase spread showing the t(8;14) characteristic of Burkitt lymphoma. The arrows indicate the translocation sites (figure courtesy of Dr. Susan Mathew, Director, Cytogenetics Laboratory, Weill Cornell Medical Center).

translocates to the immunoglobulin heavy chain gene on chromosome 14, one of two major translocation breakpoints is seen, which correlate with either the endemic or sporadic/HIV clinical category of BL. Specifically, in endemic BL the *MYC* breakpoint, which usually occurs more than 100 kb upstream from the first coding exon, is juxtaposed to a breakpoint in the joining region of the immunoglobulin heavy chain, while in the sporadic/HIV-associated BL, the *MYC* breakpoint is in-between the first and second exons, and the immunoglobulin heavy chain gene breakpoint occurs in the switch region.^{70,71} Although the significance of these different breakpoints is not yet clear, it is thought that the endemic type of translocation, involving the joining region, occurs in pre-B cells during VDJ joining. In contrast, the sporadic/HIV-associated type of translocation occurs later in B cell development, probably during isotype switching. In this latter scenario, the *MYC* translocation, based on mouse model studies, may result from double-stranded DNA breaks created by activation-induced cytidine deaminase (AID), an enzyme expressed primarily by germinal center B cell cells, important for class switch recombination and somatic hypermutation.^{71–75} The oncogenicity of *MYC*, an important regulator of many cellular functions including proliferation, differentiation, and apoptosis, is thought in BL to be due to its constitutive expression following translocation to one of the immunoglobulin genes. The translocation results in increased cellular metabolism and proliferation.^{71,76–79} The *MYC* translocation may be identified by molecular studies, including Southern blot hybridization studies and polymerase chain reaction (PCR) analysis,^{70,80} as well as in chromosomal metaphase spreads (Figure 29.3) and FISH (fluorescent in situ hybridization) analysis (Figure 29.4).⁸¹

MYC, amongst its varied functions, induces apoptosis. Anti-apoptotic mechanisms are in place in BL cells to coun-

ter this effect, including TP53 gene mutations that are identified in approximately 60% of cases.^{55,82,83} The relatively high percentage of cases with TP53 mutations, as well as alterations in other genes in the p53 and/or RB pathways, such as in *MDM2* or *p16INK4A*, suggests that disruption of these pathways has a cooperative effect on *MYC* driven neoplastic transformation.^{7,71,84} Furthermore, it has been shown that mutant p53 may regulate the expression of the *MYC* gene and is a potent activator of the *MYC* promoter.⁸⁵

Differences in the retinoblastoma (*RB*) gene and/or protein expression and expression of another growth related protein, p107, have been found in BL, which segregate with the different clinical categories. In the endemic form of BL, the *RB2/p130* gene is usually mutated and exhibits cytoplasmic p130 and high nuclear p107 protein expression; in sporadic BL, the *RB2/p130* gene is frequently mutated, but these cases (which also exhibit cytoplasmic p130 expression) have low levels of nuclear p107. In contrast, *RB* mutations have not been identified in AIDS-related BL. However, the wild-type *RB2/p130* gene is highly expressed and both the p130 and p107 proteins are present in large amounts in the nucleus. Although the *RB* gene product is normal in AIDS-related BL, it has been proposed that the HIV-associated soluble TAT protein may be secreted from infected cells, and then enters uninfected lymphoma cells, where it may bind and inactivate p130, thereby possibly contributing to lymphomagenesis.^{71,86–88}

Although BL is thought to be of germinal center cell origin, only very rare cases contain rearrangements of the *BCL6* gene, while *BCL2* gene rearrangements have not been identified.^{6,7,63,66,89–92} However, more than 50% of BL cases contain mutations in the *BCL6* gene.^{66,91} Mutations in the *RAS* gene are also found in about 20% of cases,⁹² which may be important in the pathogenesis of BL, as *RAS* has been shown

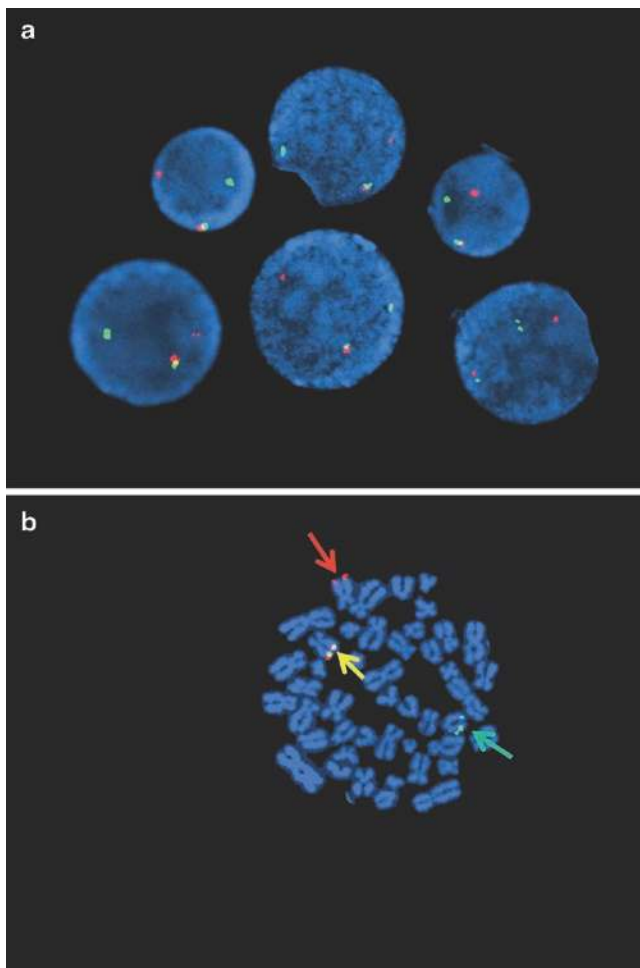


FIG. 29.4. FISH analysis for t(8;14) translocation. The spectrum orange and spectrum green labeled dual color break-apart CMYC probe was used for detecting the t(8;14) translocation. (a) Interphase cells with a normal copy of CMYC (red and green together resulting in an yellow signal), and one red and one green signal split apart due to the t(8;14) translocation. (b) Metaphase cell showing the normal chromosome 8 with the intact MYC gene (yellow signal; yellow arrow) and the der(8)t(8;14) chromosome with the red signal (red arrow) and the der(14)t(8;14) chromosome with the green signal (green arrow). The der(8) and der(14) indicate the rearranged chromosome, resulting in the t(8;14) translocation (figures courtesy of Dr. Susan Mathew, Director, Cytogenetics Laboratory, Weill Cornell Medical Center).

to contribute to the neoplastic transformation of EBV-infected B cells in vitro.^{55,93} Furthermore, approximately 30–40% of HIV-related BL cases contain somatic hypermutations in proto-oncogenes, such as *PIM-1*, *PAX-5*, and *RhoH/TTF*.⁸ These latter genes have been implicated in lymphoma-associated chromosomal translocations and may have relevant functional consequences in the lymphomagenesis of BL in particular.⁸ Furthermore, as somatic hypermutations are found in a variety of proto-oncogenes, it is quite possible that additional

somatic hypermutations in as yet undiscovered genes may contribute to the pathogenesis of HIV-related BL.⁸ In addition, examination of the IgH genes shows, in contrast to sporadic BL, but similar to endemic BLs, that AIDS-related BL have a relatively large number of somatic mutations, and at least some cases show evidence of antigen selection.^{69,94} However, there has been no evidence of on-going mutations.⁶⁹ These findings suggest that HIV-related BL are derived from cells that have terminated the germinal center reaction, and thus are late germinal center (or memory) B cells.^{69,94} Recently, genetic microarray studies, using primarily non-AIDS-related BLs, have defined “signature profiles” for BL.^{95,96} Although signature profiles identify cases as “molecular” BL, there is a “gray” zone of cases that are intermediate between BL and diffuse large B-cell lymphoma.⁹⁷ However, the impact of these molecular profiles with respect to the diagnosis, prognosis, and treatment of AIDS-related BL has yet to be defined.

The Epstein–Barr virus (EBV) is found in 25–85% of HIV-associated BL, depending on the study cohort.^{7,55,56,58,59,63,67,82,90,98} The percentage of EBV-positive cases also varies based on the morphologic subtype, with BL with plasmacytoid differentiation exhibiting the highest rate of EBV positivity.^{58,59} In contrast to Hodgkin lymphoma and diffuse large B-cell lymphoma-immunoblastic, BL tends to exhibit the EBV latency I gene pattern (Table 29.1), expressing only EBER (Figure 29.5) and EBNA1, but not the oncogenic genes LMP1, LMP2A, or EBNA2,^{26,32,59,71,99–101} although heterogeneous latency patterns have also been reported.¹⁰⁰ EBV is thought to contribute to the pathogenesis of BL by acting as an anti-apoptotic agent and counteracting the pro-apoptotic effect of *MYC*.^{71,100,102,103} Upregulation of *BCL2* by EBER or reduced expression of the pro-apoptotic *BCL2* family protein, Bim, has been suggested as potential mechanisms.^{71,101–103} In addition, in at least one study, the number of somatic mutations was higher in the EBV positive BL cases than in the EBV negative ones, suggesting an additional role of this virus in the mutational process, possibly via EBNA2, contributing to the development of the neoplastic proliferation.⁹⁴

Diffuse Large B-Cell Lymphoma (DLBCL)

Diffuse large B cell lymphoma consists of lesions composed of numerous centroblasts with a variable number of immunoblasts (25–30% of HIV-related NHLs) and those composed predominately (>90%) of immunoblasts, often with plasmacytoid features (immunoblastic variant; approximately 10% of cases).⁵⁸ The immunoblastic variant of DLBCL is highly associated with EBV infection, is associated with a lower CD4 count, and usually occurs late in the course of the disease. With the institution of HAART, the incidence of DLBCL has decreased as well as the relative percentage of the immunoblastic subtype.^{7,57,67,98,104–109} Morphologically, the cells comprising the immunoblastic variant tend, in

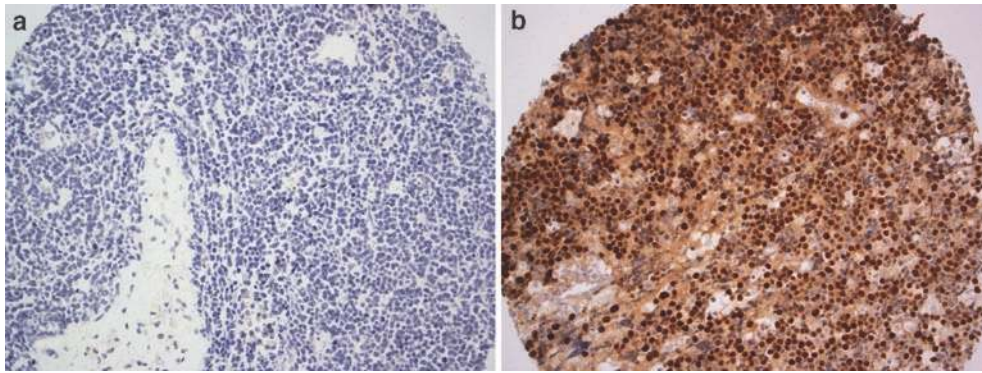


FIG. 29.5. (a) AIDS-related Burkitt lymphomas are usually EBV negative as indicated by *in situ* hybridization using an EBER1/2 probe, which detects small ribonucleotides. (b) Approximately 25–85% of AIDS-related Burkitt lymphomas are EBV positive as

indicated by the brown nuclear signal obtained by *in situ* hybridization using an EBER1/2 probe. (a and b *in situ* hybridization $\times 20$ original magnification; courtesy of the AIDS Cancer Specimen Resource).

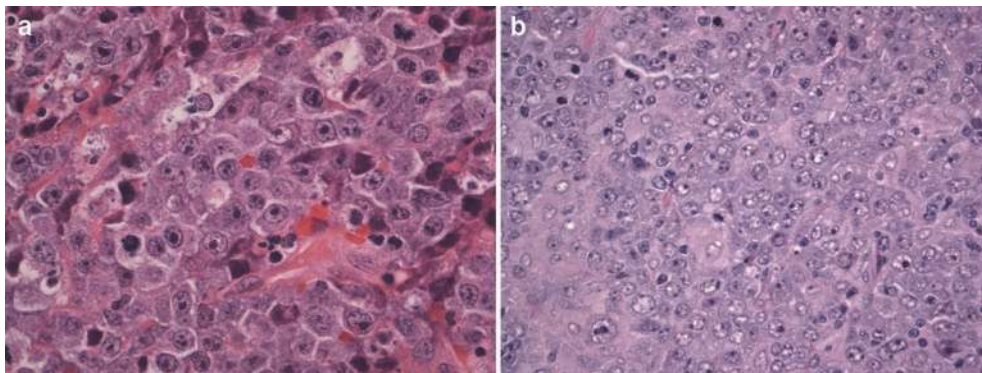


FIG. 29.6. AIDS-related diffuse large B cell lymphomas (DLBCLs): (a) AIDS-related immunoblastic lymphomas are composed of large cells with large nuclei, prominent, often central, nucleoli and abundant cytoplasm; perinuclear hofs are often seen. Mitotic figures and

the tingible body macrophages are frequent. (b) The AIDS-related centroblastic DLBCLs are more heterogeneous in appearance. The nuclei tend to be round and there are often multiple nucleoli that often abut the nuclear rim (a and b hematoxylin and eosin; $\times 60$).

comparison to more conventional centroblastic DLBCLs, to be larger and more pleomorphic and to have more cytoplasm, a perinuclear hof, and central nucleoli (Figure 29.6a). These neoplasms often have areas of necrosis; mitotic figures are usually numerous and tingible body macrophages may be present. The more centroblastic lymphomas have a variable number of immunoblasts, which may impart a more heterogeneous appearance to the neoplastic proliferation. The nuclei tend to be round with one to several nucleoli that often abut the nuclear membrane and are usually intermediate in size between those seen in BL and the immunoblastic DLBCLs (Figure 29.6b).^{57,110} As mentioned previously, AIDS-related DLBCL may be difficult to distinguish from BL based only on morphology.⁵⁹

Immunophenotypically, AIDS-related DLBCL is somewhat more heterogeneous than BL. The neoplastic cells are of B cell origin, expressing pan-B cell antigens.^{67,111} As with

DLBCLs in immunocompetent patients, AIDS-related cases may be subclassified as those that are of germinal center (GC; Figure 29.7) or nongerminal center (non-GC) origin (Figure 29.8). However, in contrast to immunocompetent DLBCLs where the number of non-GC cases is either equal to or greater than the number of GC cases,^{112,113} the HIV-related DLBCLs appear to be more often of GC origin.^{111,112,114–116} Furthermore, while two dimensional contour frequency plots of antigen expression has shown that immunocompetent DLBCLs segregate into two distinct populations, HIV-related DLBCLs appear to cluster into a single group intermediate between GC and non-GC phenotypes.^{117,118} This may be a reflection of the relatively high percentage (40–80%) of each of the histogenic defining antigens (i.e. CD10, BCL6, and MUM1) expressed by HIV-positive DLBCLs.^{54,111,112,114–116} There does, however, appear to be a correlation between EBV positivity and MUM1 expression.^{54,116}

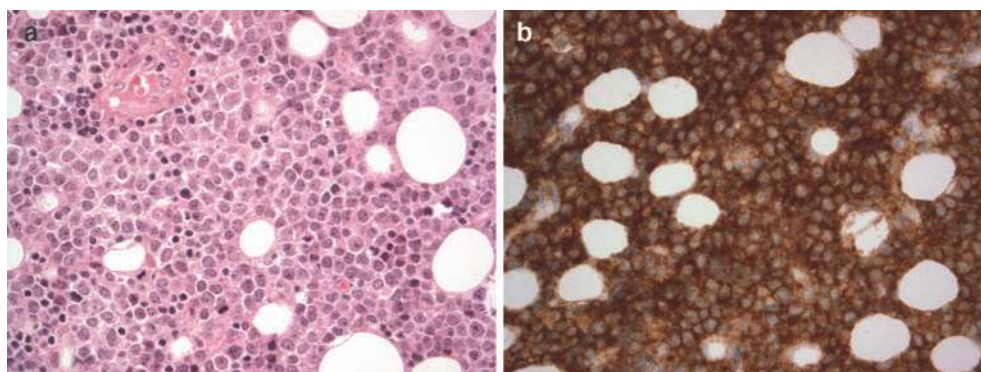


FIG. 29.7. AIDS-related diffuse large B cell lymphoma. (a) This DLBCL is of germinal center origin based on expression of (b) CD10; the tumor cells were also positive for BCL6 but lacked

expression of MUM1 (a hematoxylin and eosin $\times 40$ original magnification; b immunoperoxidase $\times 40$ original magnification; courtesy of the AIDS Cancer Specimen Resource).

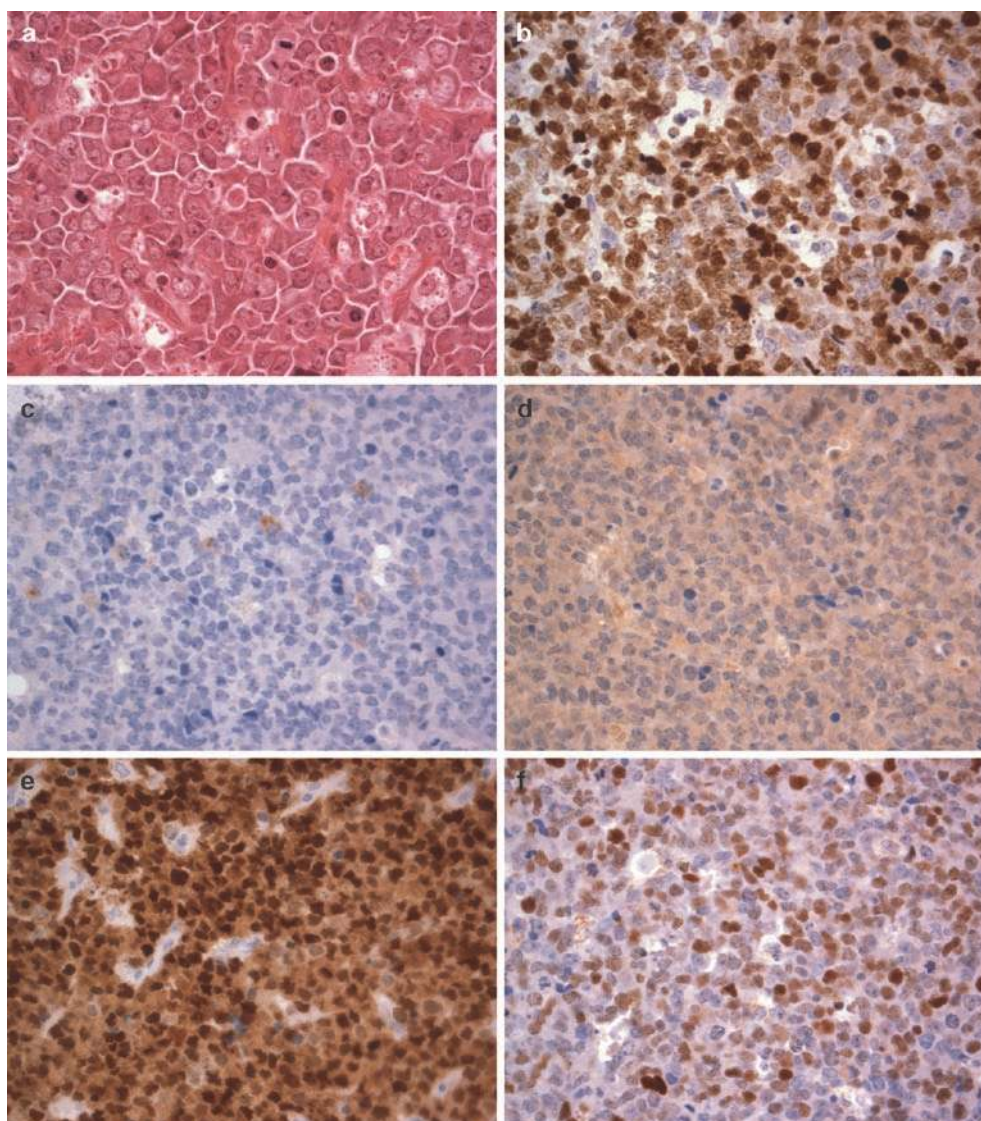


FIG. 29.8. AIDS-related diffuse large B-cell lymphoma. (a) This DLBCL is (b) EBV positive based on in situ hybridization. Based the pattern of antigen expression, specifically lack of (c) CD10 and (d) BCL6 and expression of (e) MUM1, the lymphoma is of non-germinal

center origin. (f) The tumor cells are also positive for p53 (a hematoxylin and eosin, $\times 60$ the original magnification; b in situ hybridization $\times 40$ original magnification; c–f. immunoperoxidase $\times 40$ original magnification; courtesy of the AIDS Cancer Specimen Resource).

Virtually all AIDS-related DLBCLs show clonal rearrangements of an immunoglobulin gene (Figure 29.9)^{7,55,57,67,69,104,119}; although, cases of polyclonal lymphoma, the majority of which are EBV negative, have been described.^{120,121} In greater than 90% of cases, the immunoglobulin genes show evidence of somatic hypermutation; the unmutated cases are predominantly primary central nervous system immunoblastic DLBCLs.⁶⁹ Furthermore, less than 20% of cases show evidence of ongoing somatic hypermutation.⁶⁹ Analysis of IgHV gene usage shows over-usage of IgHV4 family genes (particularly IgH4-34 and IgHV4-39) and under-usage of IgHV3 genes, in comparison to normal B cells.^{69,119}

AIDS-related systemic DLBCLs, in contrast to BL, are not pathogenetically associated with a specific genetic alteration. In general, the AIDS-related systemic DLBCLs lack *BCL1* and *BCL2* gene rearrangements and structural alterations of the *RB* and *BAX* genes, only rarely to occasionally contain *RAS* or *TP53* gene mutations, and variably have a *MYC* (10–50%) or a *BCL6* (25% or less) gene rearrangement.^{7,55,68,89–91,122,123} Approximately 60–70% of cases contain mutations in the regulatory region of the *BCL6* gene.^{54,91} There are a significant number of aberrant hypermutations in proto-oncogenes, such as *PIM-1*, *PAX-5*, *RhoH/TTF*, and *MYC* with over 50% of AIDS-related DLBCL cases containing a mutation in at least one of these genes.⁸ The rate of ongoing somatic hypermutation in these proto-oncogenes, as with immunoglobulin genes, appears to be low.⁸

In contrast to the systemic DLBCLs, the primary central nervous system (CNS) AIDS-related DLBCLs contain fewer genetic alterations. Only small numbers of cases, if any, have been found to contain rearrangements in the *BCL6*, *BCL2*, or *MYC* genes or to reveal *TP53* mutations or *BAX* gene altera-

tions. Furthermore, it appears that primary CNS DLBCLs only rarely contain aberrant somatic hypermutations in the proto-oncogenes *PIM-1*, *PAX-5*, *RhoH/TTF*, and *MYC*; although, only a small number of cases have been studied.⁸ Approximately 25–45% of primary CNS lymphomas contain mutations in the *BCL6* gene.^{7,54,122–125} Primary CNS DLBCLs, like the systemic cases, exhibit clonal immunoglobulin gene rearrangements, as well as frequently contain somatic hypermutations (approximately 80% of cases) in the immunoglobulin genes. However, in contrast to their systemic counterparts, primary CNS DLBCLs differ in that they do not exhibit preferential usage of the IgVH4 genes or any other VH gene family, but instead show preferential usage of IgLV6–57.^{7,69,124,126} Rare cases that do not contain somatic mutations have been identified; these tend to be lesions with immunoblastic morphology.^{69,126} Although analysis suggests that in the mutated cases the mutational process was antigen driven, it is no longer ongoing.^{69,126}

Genetic microarray studies of DLBCLs in immunocompetent patients have identified profiles that associate with clinical behavior.¹²⁷ Although similar studies analyzing AIDS-related DLBCLs have not been published, alternative types of genetic studies, such as suppression subtractive hybridization (SSH) have been used to identify differentially expressed genes in AIDS-related and immunocompetent DLBCLs.¹²⁸ This technique, however, has only definitively shown that *TCL1* is preferentially expressed in AIDS-related DLBCLs.^{128,129}

The Epstein–Barr virus is present in 30–40% of AIDS-related DLBCLs.^{67,104,112,115} This association with EBV varies based on morphology (conventional centroblastic vs. immunoblastic), primary site of disease (systemic vs. primary CNS), and histogenetic subtype (GC vs. non-GC). Approximately 35% of centroblastic DLBCLs are EBV positive (range: 0–50%), based primarily on Southern blot analysis, PCR analysis (Figure 29.10), and in situ hybridization (EBER probe) studies.^{7,54,55,67,82,90,91,116,124,130} In contrast, over 80% of the immunoblastic variants of AIDS-related DLBCLs are EBV-positive.^{7,54,55,67,90,124,130} Nearly all primary CNS DLBCLs (compared to only 30–40% of the systemic cases) are EBV-positive; the majority of the primary CNS cases also express the oncogenic gene, *LMP1*.^{7,83,114,116,124,125,130–132} In addition, less than 30% of the GC HIV-related DLBCLs are EBV positive, compared to nearly 70% of those that immunophenotypically are classified as non-GC.¹¹²

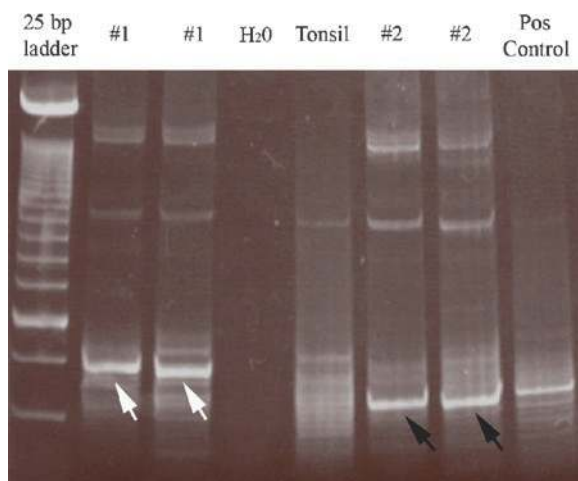


FIG. 29.9. PCR analysis. PCR analysis shows clonal rearrangements of the immunoglobulin heavy chain gene in case 1 (white arrows) and case 2 (black arrows). Controls included in the study show a sample without DNA amplification (H_2O), a sample showing a polyclonal pattern (Tonsil) and a sample showing a monoclonal population (Pos Control).

Lymphomas Occurring More Specifically in HIV-Positive Patients

There are three main types of lymphoma that preferentially occur in HIV-positive individuals. These neoplasms are associated with infection with herpesviruses and include primary effusion lymphoma (PEL)/extra-cavitary PEL (infection with KSHV and EBV), lymphomas arising in the setting of KSHV-associated multicentric Castleman disease (MCD; infection with KSHV), and plasmablastic lymphoma (infection with EBV).

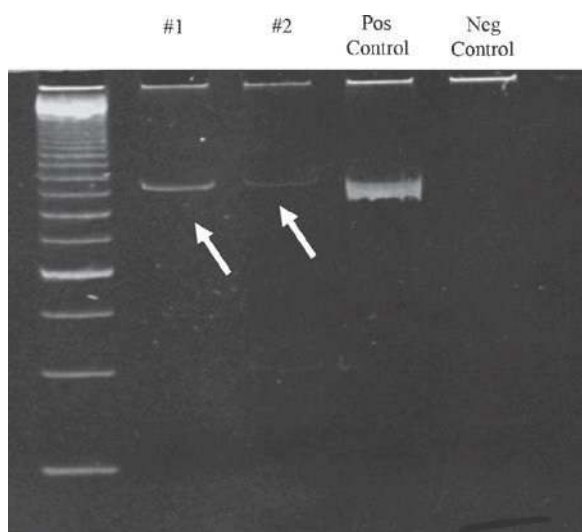


FIG. 29.10. PCR analysis for the Epstein-Barr virus (EBV). PCR analysis for EBV shows the presence of the virus in lanes labeled #1 and #2. Note that the locations of the bands are in the same location as the positive control (lane labeled Pos Control). Although PCR analysis identifies the presence of the virus, it does not indicate whether the infection is monoclonal or polyclonal.

Primary Effusion Lymphoma (PEL) and Extra-Cavitary PEL (EC-PEL)

This type of lymphoma occurs preferentially in HIV-positive patients and accounts for less than 5% of all AIDS-related lymphomas.⁵⁹ Although these lesions were reported in the late 1980s and early 1990s,^{133,134} PELs and EC-PELs were not recognized as distinct entities until the mid-1990s and the mid-2000s, respectively.^{135–138} PELs and EC-PELs are uniformly associated with the Kaposi sarcoma herpesvirus (KSHV); the vast majority of cases are also infected with EBV.^{135–139} PELs occur as an effusion involving primarily pleural, peritoneal, and/or pericardial spaces; in only 30% or less of the cases, the patients also have a solid tissue mass. In contrast, EC-PELs are solid lesions that do not have an associated malignant effusion, but are otherwise virtually indistinguishable from PELs, based on morphology, immunophenotype, and genotype.^{135–139} Although PELs and EC-PELs appear virtually identical, mouse model studies have shown some differences in gene expression profiles between KSHV-positive effusion tumor cells and solid KSHV-positive tumor cells, with the solid tumors more likely to express adhesion-like molecules and structural proteins.¹⁴⁰

PELs and EC-PELs have distinctive morphologic and immunophenotypic characteristics. In cytospin preparations, the malignant PEL cells are large with morphologic features that range from immunoblastic to plasmablastic. Some cells have a “jelly-fish” like appearance, while others have Reed–Sternberg-like features; often the cells are multinucleated (Figure 29.11a, b).^{133,134,136,137,139,141} The malignant cells from EC-PELs, when placed in suspension, exhibit similar

morphologic features as the PEL cells; in tissue sections, they are pleomorphic with centroblastic, immunoblastic, and/or plasmacytoid features (Figure 29.11c). As with classic PELs, EC-PELs cells with Reed–Sternberg-like features may also be seen.^{136,138,142} Immunophenotypically, the tumor cells are usually CD45 positive, but in general lack expression of B cell lineage associated antigens, such as CD19, CD20, CD22, CD79a, and PAX5; only a small percentage express immunoglobulin, preferentially of the lambda isotype. The malignant cells also lack expression of BCL6 and CD10, but express IFR4/MUM1, CD138, and the master regulator of terminal B cell differentiation, PRDM1/BLIMP1. In addition, they often express CD30, CD38, Vs38c, HLA-DR, and epithelial membrane antigen. Except for rare cases reported to be of T cell origin or exhibit aberrant antigen expression, the PEL and EC-PEL cells lack T and natural killer (NK) cell antigens.^{54,136–139,141–149} The PELs and EC-PELs, by definition, contain KSHV, and thus are positive for viral latency-associated nuclear antigen (LANA; ORF73). This virally encoded protein binds the virus to human DNA. Immunostaining for LANA shows a punctate nuclear pattern of immunoreactivity (Figure 29.11d).^{35,38,135,136,150–152} In addition, a small percentage of cells, usually between 5 and 20%, express viral-interleukin 6 (v-IL-6), a viral encoded protein thought to be important in the pathogenesis of both PEL and EC-PEL.^{49,50,136,142,153} Although the malignant cells are EBV positive in over 80% of cases, they are negative for LMP1 based on immunohistochemistry and real-time PCR (RT-PCR).^{54,154,155}

At the molecular-genetic level, PELs and EC-PELs virtually always contain clonal rearrangements of the immunoglobulin genes.^{135–138,141,156} The immunoglobulin genes usually contain somatic hypermutations, indicating that the majority of cases are of germinal center/postgerminal center origin.^{8,157–159} Furthermore, of the cases studied, the majority use the lambda light chain gene, usually from the IgVL4 family.¹⁵⁹ Gene expression profile analysis shows that the tumor cells exhibit a gene expression pattern that shares features of both immunoblasts and plasma cells, i.e. a “plasmablastic” profile. The PEL cells express genes encoding for proteins, such as IFR4/MUM1, CD30 (i.e., immunoblasts), PRDM1/BLIMP1, UPR, and CD138/syndecan-1 (plasma cells). Additional genes have been found to be specifically upregulated in PEL, including those encoding for IL-10, VEGF, PRGL-1/SELPLG, and vitamin D₃ receptor, while genes encoding for CD19, CD20, CD22, CD52, CD72, BLNK, BLR1/CXCR5, and CD79a/b are downregulated.^{160,161} These genetic features (in conjunction with the immunophenotypic findings) indicate that PELs and EC-PELs are derived from preterminally differentiated B cells, primarily of postgerminal cell derivation.¹³⁹ More recently, high-throughput quantitative reverse transcription PCR (qRT-PCR)-based assay studies have identified >60 PEL specific miRNAs, which may be able to define a biologic phenotype of the PELs, including some that contribute to the pathogenesis of KSHV-associated malignancies, such as PEL and EC-PEL.^{162,163}

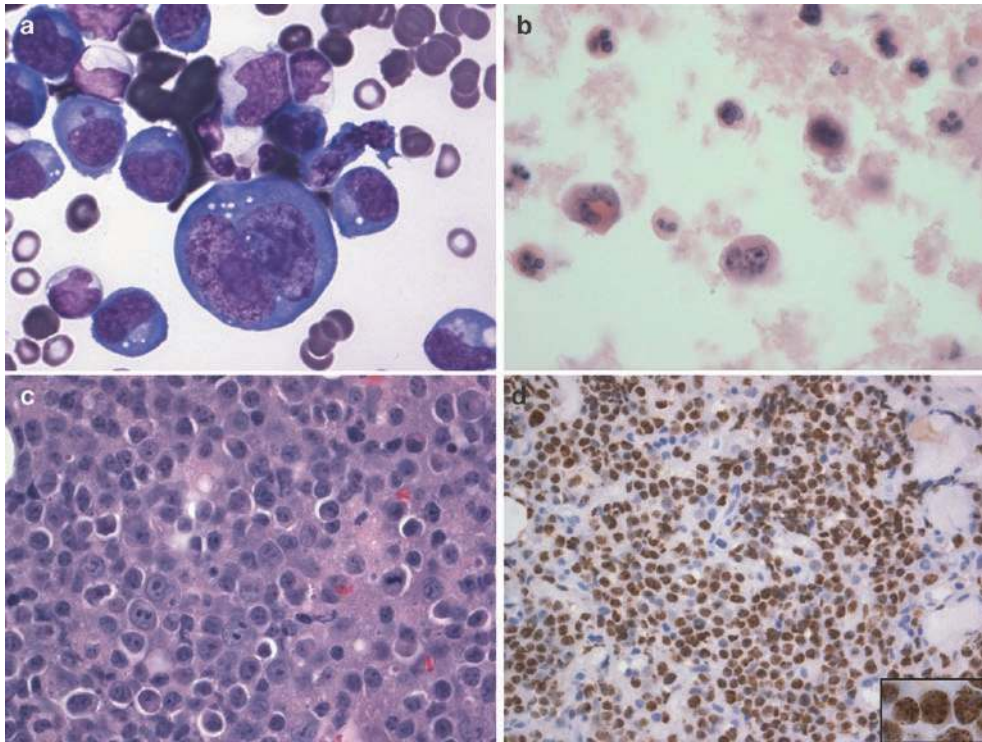


FIG. 29.11. Primary effusion lymphoma (PEL) and extra-cavitary PEL: (a) The PEL tumor cells are large and pleomorphic and often have prominent nucleoli. (b) Examination of a PEL cell block shows similar morphologic features of the tumor cells. (c) EC-PELs are virtually identical morphologically to PELs; they are also immunophenotypically and genotypically similar. Both PELs and EC-PELs, as in this case, are nearly always EBV positive. (d) The tumor cells

of both PELs and EC-PELs are uniformly positive for the latent nuclear antigen (LANA; LNA-1; ORF 73) of KSHV/HHV8 which tethers the virus to human DNA. Note the dot-like nuclear staining. (a Geimsa $\times 60$ original magnification; b and c hematoxylin and eosin $\times 60$ original magnification; d immunoperoxidase $\times 40$ original magnification; insert $\times 100$ original magnification; figures c and d courtesy of the AIDS Cancer Specimen Resource).

The PELs rarely, if ever, contain rearrangements involving the common B cell associated oncogenes and tumor suppressor genes, *BCL1*, *BCL2*, *BCL6*, or *MYC* genes; in addition, they only infrequently contain structural alterations in *H-ras*, *N-ras*, *K-ras*, or *TP53* genes.^{91,137,164} However, they frequently contain mutations in the noncoding 5' region of the *BCL6* gene and exhibit frequent aberrant somatic hypermutations in other proto-oncogenes, such as *PAX-5*, *PIM-1*, and *RhoH/TTF*, as well as in exons 1 and 2 of *MYC*.^{8,91,164} The significance of these latter alterations in the pathogenesis of PEL and EC-PEL, however, is not clear.

Both KSHV and EBV are present in the tumor cells. The EBV infection in the malignant cells is monoclonal, with the majority of cases expressing genes of latency pattern I.^{137,154,165–167} Although some cases contain EBV exhibiting latency pattern II/III, the malignant cells fail to express significant levels of the EBV transforming and immunogenic genes, LMP1 and EBNA2.^{138,154,165,168} Despite the fact that the pathogenetic effect of EBV in these tumors is not known, genetic microarray studies have shown differences in gene expression

patterns, including differences in the regulators of the MAP kinase pathway, between the EBV positive and EBV negative PELs.¹⁶⁹

KSHV is pathogenetically associated with the development of PEL and EC-PEL. The virus may be identified by a variety of methods, including electron microscopy, PCR analysis, immunofluorescence, and immunohistochemistry.^{135,170–174} A large number of copies (up to 200) of the KSHV genome are present within the tumor cells.^{175,176} The KSHV genome, like EBV, may be present as extrachromosomal circular DNA. However, in contrast to EBV, only some PELs contain monoclonal KSHV, based on examination of the number of terminal repeat regions; others contain biclonal or oligoclonal KSHV episomes, including those which are monoclonal by immunoglobulin and EBV-Southern blot terminal repeat studies.^{167,175} Like EBV, KSHV in PEL/EC-PEL is a latent infection.³³

Only a small number of studies examining for cytogenetic abnormalities in PELs and EC-PELs have been done. However, using FISH, metaphase spreads, and comparative

genomic analyses, the most frequent findings are increased copies or portions of chromosomes 12 and 7 and abnormalities in chromosome 1.^{59,136,164,177}

Large B-Cell Lymphoma Arising in HHV8 (KSHV)-Associated Multicentric Castleman Disease (MCD)

Multicentric Castleman disease, when it occurs in HIV-positive individuals, is almost uniformly associated with infection by KSHV; in addition, in HIV-negative individuals, KSHV is also identified in a significant number of cases.^{178–181} In the KSHV positive cells of MCD, the infected cells, which resemble plasma cells but also show features of immunoblasts, are preferentially located in the mantle cell zone, but occasionally may be seen outside the follicle.^{180–182} These cells may coalesce to form small confluent clusters of cells, i.e. “microlymphomas;” however, sheets of cells that obliterate the tissue architecture may also be seen; rarely, the tumors may exhibit a follicular growth pattern.^{180,183} The most frequent anatomic sites of large B-cell lymphoma arising in KSHV-associated MCD are the peripheral lymph nodes and spleen, which are also the most common sites of MCD. In addition, large B-cell lymphomas arising in KSHV-associated MCD may develop or present with a leukemic phase.^{180,184}

Morphologically, the KSHV infected cells are approximately twice the size of a small lymphocyte with a moderate amount of amphophilic cytoplasm and have a large vesicular nucleus with one or two relatively prominent nucleoli.¹⁸⁰ Immunophenotypically, the malignant cells usually exhibit weak or no expression of B cell antigens, such as CD20, only weakly express CD30, are negative for CD10, PAX5, BCL6, and CD138, exhibit variable expression of the memory associated antigen, CD27, and are IRF4/MUM1 positive, similar to the plasmablasts seen in MCD.^{143,180,181,183–185} The cells express monotypic cytoplasmic lambda light chain and IgM heavy chain, similar to the KSHV infected plasmablasts in MCD.^{143,180,183–185} The cells are uniformly LANA positive and also express v-IL-6.^{180,183,185}

By PCR analysis of the immunoglobulin genes, some (but not all) microlymphomas are monoclonal, although they all express monotypic lambda immunoglobulin light chain. However, the lesions that are larger, more diffuse, or extensively involving the peripheral blood are monoclonal.^{180,185} The immunoglobulin genes, in contrast to PELs and EC-PELs, show no evidence of somatic hypermutation, indicating that these lesions, in spite of CD27 expression, develop from naïve B cells.¹⁸⁵ The cells are positive for KSHV, but negative for EBV, based on PCR and/or in situ hybridization.^{180,183,185}

Plasmablastic Lymphoma

This is an uncommon type of lymphoma that occurs preferentially in HIV-positive individuals. These lesions occur most frequently in the oral cavity, but may also arise in other

extra-nodal sites and only rarely in lymph nodes.^{186–191} The cells are not too dissimilar from the cells seen in large B-cell lymphoma arising in KSHV-associated MCD, although they may be more immunoblastic-appearing. The tumor cells characteristically have round nuclei, a single prominent nucleolus, and a moderate to relatively abundant amount of cytoplasm.^{139,188,190,191} Immunophenotypically, they usually lack expression of CD20, PAX5, and BCL6, but express markers of the later stages of B cell differentiation, such as IRF4/MUM1, VS38c, CD138, and CD38. Expression of CD79a may be seen in a significant number of cases, although the number of positive cells in each case may be variable. Many cases are epithelial membrane antigen positive as well. These tumors, however, are either CD45 negative or weakly express this antigen. The plasmablastic lymphomas are EBV positive in approximately 75% of cases, in contrast to the EBV-negative large B-cell lymphomas arising in KSHV-associated MCD, although there is a higher rate of EBV positivity in the oral lesions. Furthermore, some cases are LMP1 positive, although the number of positive cells may be small. They are, however, KSHV-negative. A variable number of cases, ranging between 40 and 70%, express monotypic cytoplasmic immunoglobulin, including those that are positive only for the immunoglobulin heavy chain, IgG. The plasmablastic lymphomas express either kappa or lambda light chain, in contrast to the large B-cell lymphomas arising in KSHV-associated MCD, which are virtually all lambda light chain positive. Characteristically, plasmablastic lymphoma has a high proliferation rate with usually >80–90% of cells positive for Ki67.^{54,186–188,190,191} In addition, the cases are usually p53 positive and exhibit loss of expression of both p16 and p27.^{191,192}

Immunoglobulin molecular genetic studies show that the plasmablastic lymphomas are monoclonal, but only approximately 40% contain somatic hypermutations in the immunoglobulin heavy chain genes. Furthermore, the cases usually do not contain mutations in the *BCL6* gene.^{192,193}

Lymphomas Occurring in Other Immunodeficient States

This category includes the polymorphic lymphoid proliferations that resemble the polymorphic B cell posttransplantation lymphoproliferative disorders.

Polymorphic Lymphoid Proliferations

These lesions are very rare in the HIV-positive patient population, accounting for less than 5% of cases. These lesions occur in both HIV-infected children and adults.^{194–196} Morphologically, the lesions are composed of a heterogeneous (polymorphic) cell population consisting of small lymphocytes, plasma cells, histiocytes, and large transformed cells, including immunoblasts, exhibiting variable degrees of atypia; scattered Reed–Sternberg-like cells are often present. Areas of necrosis or individual necrotic cells may also be

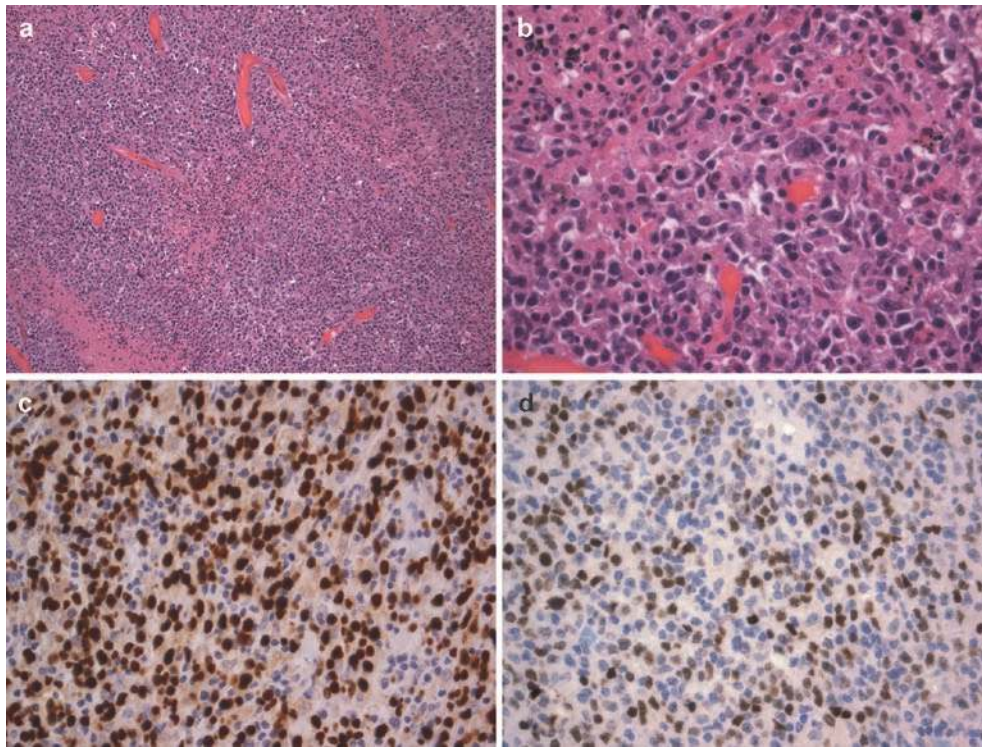


FIG. 29.12. Polymorphic lymphoid proliferation: (a) The polymorphic lesions are similar to the polymorphic post-transplantation lymphoproliferative disorders (PTLDs). There is destruction of the underlying architecture. Areas of coagulative necrosis may be present. (b) The cells comprising the lesions are heterogeneous in composition and show variable degree of cytologic atypia. (c) Usually a variable number of the cells within the lesions are

positive for the Epstein–Barr virus and in some lesions the cells, like in some polymorphic PTLs, are also LMP1 and (d) EBNA2 positive (a hematoxylin and eosin $\times 10$ original magnification; b hematoxylin and eosin $\times 40$ original magnification; c in situ hybridization $\times 40$ original magnification; d immunoperoxidase $\times 40$ original magnification; courtesy of the AIDS Cancer Specimen Resource).

identified (Figure 29.12a, b). These lesions often occur in extra-nodal sites.^{194–196} Immunophenotypically, the infiltrate usually contains a large number of CD20 positive B cells, although a significant number of CD3 positive T cells may also be present; the former are usually the larger atypical cells. The lesions may express monotypic immunoglobulin and/or show anomalous expression of CD43. Based on either Southern blot hybridization studies or PCR analysis, the majority of cases contain monoclonal rearrangements of the immunoglobulin genes, however, some may be polyclonal or oligoclonal. Some of the cases that are not monoclonal, based on immunoglobulin rearrangement studies, may be shown to be monoclonal, using Southern blot hybridization studies that examine the terminal repeat region of EBV. EBV is present in the majority of cases (Figure 29.12c); they may be positive for LMP1 or LMP1 and EBNA2 similar to many of the post-transplantation polymorphic lesions (Figure 29.12d).^{82,194–197} In general, these lesions lack structural alterations in oncogenes or tumor suppressor genes; however, those cases that do contain genetic alterations often exhibit more aggressive biologic behavior.^{82,194}

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Chronic Myelogenous Leukemia

Dan Jones

Introduction

Chronic myelogenous leukemia (CML) is the one of the most common chronic myeloproliferative disorders. It has become the paradigmatic disease for molecular diagnosis and monitoring for several reasons: (1) CML has a unitary molecular definition, requiring the demonstration of the t(9;22)(q34;q11) chromosomal translocation or its product, the BCR-ABL fusion gene; (2) The BCR-ABL chimeric protein is integral to CML leukemogenesis, as demonstrated in mouse transgenic leukemia models¹; (3) Blocking the BCR-ABL kinase, using the tyrosine kinase inhibitor (TKI) imatinib mesylate (Gleevec), results in regression of CML and durable clinical response in nearly all patients; (4) Daily lifetime therapy with imatinib (or similar second-generation TKIs) has become the standard therapy for CML, allowing standardized definitions of response and treatment resistance to be developed.

Diagnostic Criteria

Diagnosis of CML requires the demonstration of the BCR-ABL gene fusion, or the t(9;22)(q34;q11) chromosomal translocation which produces it. The t(9;22), also known as the Philadelphia chromosome (Ph) because of the city where it was first identified, is most commonly demonstrated by standard G-banded metaphase chromosomal preparations from bone marrow (BM) aspirate. Recently, however, there has been increasing use of fusion signal fluorescence in situ hybridization (FISH) probes that may be used in interphase preparations including blood samples. The incidence of “cryptic” t(9;22) (i.e., undetected by conventional karyotype but seen by other methods) will vary, depending on the experience of the laboratory, but is usually much less than 5% given the propensity of Ph+ cells to grow well in short-term culture. Use of FISH probes for diagnosis may also be useful for those cases where the ABL1 gene, at chr 9q34.1, or the BCR gene, at chr 22q11.23, are involved in complex 3- or 4-way chromosomal rearrangements. The effects of these

changes, seen in 2–5% of CML cases at diagnosis, may be clarified by parallel detection of the BCR-ABL fusion transcript type (Table 30.1).

Such reverse transcription quantitative polymerase chain reaction (RQ-PCR) detection of the BCR-ABL fusion transcript has also become more popular as a primary diagnostic tool. Use of this assay requires different primers and probes to detect each of the common chromosomal breakpoints and splice products involving BCR on chromosome (chr) 22. These include the *minor breakpoint cluster region*, fusing BCR exon e1 to ABL1 exon a2, producing the p190 BCR-ABL protein seen in Ph+ acute lymphoid leukemia (ALL), and the *major breakpoint cluster region* fusing BCR exons e13 and/or e14 with ABL1 exon 2, producing the p210 BCR-ABL protein seen in nearly all CML, and a minority of Ph+ ALL (Figure 30.1). Whether the e13a2, e14a2, or both transcripts is/are produced with the major breakpoint translocation appears to be influenced by polymorphisms that affect splicing efficiency.²

An extremely rare translocation between BCR exon 19 and ABL exon 2, producing a p230 BCR-ABL protein, has been reported.³ More common (but still rare) alternate breakpoints produce e1a3 or e13/e14a3 fusions,⁴ which are not detected by most RQ-PCR assays but would be detected by current FISH assays. CML-like myeloproliferative neoplasms lacking BCR-ABL gene fusion (Ph-negative) are no longer classified as CML and may be alternatively classified as atypical chronic myeloid leukemia, chronic neutrophilic leukemia, or myeloproliferative neoplasm, unclassifiable. The proteomics of CML are discussed in Chap. 14.

Clinicopathologic Features

CML affects a wide age range, with nearly all patients presenting with marked leukocytosis, including a range of immature myeloid forms, prominent basophilia, and variable splenomegaly. The BM biopsy in CML is almost always markedly hypercellular, with a predominance of myeloid forms at various stages of maturation. There is a great variation

TABLE 30.1. Comparison of conventional cytogenetics, FISH, and molecular studies for detection of BCR-ABL/t(9;22) rearrangement.

Feature	Methodology used for monitoring ^a		
	G-banded karyotype	BCR-ABL fusion FISH	BCR-ABL RQ-PCR
Marrow sample required	Yes	No	No
Equivalence of blood and marrow results	Not applicable	Yes ^b	Yes ^b
Sensitivity (% tumor)	5% (20 metaphases)	1–5% (200–1,000 cells)	0.001–0.01 ^c
Accuracy of measurement	±15%	±2–5%	±2–5-fold
False negative result	Yes, cryptic and 3-way translocations	Extremely rare	Yes, rare e19a2 and a3 fusions
False positive result	No	Yes, due to cell overlap on slide	Yes, mostly due to PCR contamination
Detection of other chromosomal abnormalities	Yes ^d	No ^d	No

^aFor additional details on monitoring intervals, see reference 33.
^bDiscordances can be seen in early relapses of Ph+ ALL.
^cDependent on amount of leukocytes present in pool used for initial RNA extraction/reverse transcription. Consensus recommendations favor use of 10 ml of peripheral blood for optimal sensitivity.
^dMost common secondary genomic changes in CML (trisomy 8, isochromosome 17q/monosomy 17) are usually well detected by karyotype. Extra copies of the Philadelphia chromosome and the derivative chromosome 9 may be more sensitively detected by BCR-ABL FISH probes.

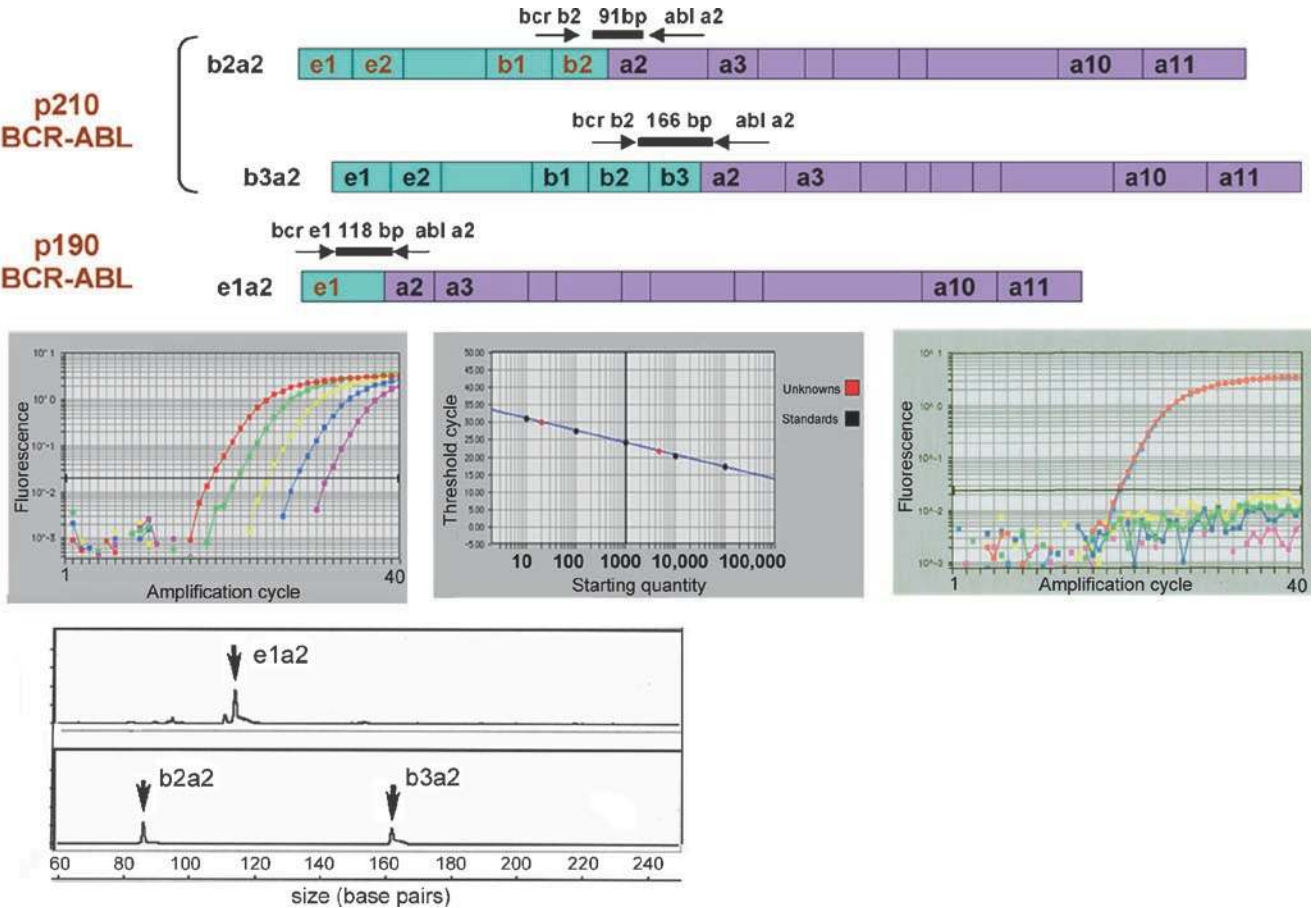


FIG. 30.1. BCR-ABL RQ-PCR assay. The locations of the primers and probes required for detection of the e13a2 (b2a2) or e14a2 (b3a2) (p210) and e1a2 (p190) BCR-ABL fusion transcripts are illustrated in the top figure. The middle panels show the result of the TaqMan-based assay with a BCR-ABL DNA standard diluted tenfold (left panel), log-plotted to derive the curve (middle panel), and a patient sample run in duplicate (right panel). Lower panel shows the results of capillary electrophoresis (CE) of the products after PCR is completed with different-sized products produced by the e1a2, e13a2, and e14a2 transcripts (one primer in each amplicon of the RQ-PCR reaction is labeled with a fluorochrome permitting CE detection). RQ-PCR reverse transcription quantitative polymerase chain reaction.

in the degree of BM fibrosis, basophilia, eosinophilia, and megakaryocytic proliferation at diagnosis, which may raise the differential diagnosis of other chronic myeloproliferative neoplasms, particularly primary myelofibrosis.

Most patients present in the chronic phase (CP) with few myeloblasts and less than 20% basophils; a small minority (~5–10%) present in accelerated phase (AP), which is defined based on having increased blasts and/or basophils, low platelet count,

or cytogenetic changes besides t(9;22). Patients presenting with t(9;22) and greater than 20% lymphoid blasts are usually diagnosed as Ph+ ALL, but some of these patients may recur with CP-CML, demonstrating that this actually represents cryptic blast phase (BP) of CML. Definitive diagnosis at the time of presentation of such transformed CML may be difficult but may be suggested by the presence of left-shifted myeloid hyperplasia, basophilia, and the presence of the major rather than the minor t(9;22) breakpoint (i.e. e13/14a2 BCR-ABL transcripts).

Given the variety of myeloid cell types present in CP-CML, routine flow cytometric (FCM) immunophenotyping is often not done. In AP or BP cases, FCM phenotyping reveals that the blasts usually have a myeloid phenotype [myeloperoxidase (MPO)+, CD117+] but may be lymphoid (10–20% of cases) or bilineal/biphenotypic (5–10%). In addition to expression of CD19 and TdT, cases of lymphoid BP-CML frequently express CD13, and CD33, and may have focal MPO positivity by FCM so extended immunophenotyping is recommended for precise classification.

Standard Therapy

Initial therapy for nearly all patients with CML is continuous daily oral imatinib (Gleevec), which acts to competitively displace ATP from its binding pocket (P-loop) in the ABL kinase domain, or to block ABL enzymatic action. Imatinib is a largely selective TKI, inhibiting BCR-ABL along with platelet-derived growth factor receptor (PDGFR) and KIT kinase, with relatively few reported off-target effects compared to other kinase inhibitors.⁵ The effectiveness of imatinib over the previous best therapy (i.e., interferon-alpha with or without cytarabine) was demonstrated in the pivotal phase III International Randomized Study of Interferon vs. STI571 (imatinib) (IRIS) trial, which has shown greater than 85–90% progression-free survival for patients on imatinib for 5 years or more.⁶ Although most patients receive 400 mg/day of imatinib, dose escalation to 800 mg/day has been shown to produce higher response rates and is recommended if optimal responses are not seen at lower doses.⁷ A minority of patients cannot tolerate standard imatinib therapy due to dose-limiting toxicities, which typically include rash and myelosuppression. There have been some pilot studies examining the effects of imatinib discontinuation or drug holidays once complete response has occurred, but the vast majority of patients continue TKI treatment indefinitely. A small number of patients are still treated with interferon and/or chemotherapy, or single agent hydroxyurea as initial therapy, and there have been some recent trials using other TKIs, particularly nilotinib and dasatinib, as frontline therapy.⁸

Molecular Monitoring of CML

The widespread use of single agent imatinib for frontline CML therapy has allowed the development of widely accepted criteria for assessing response (Table 30.2) and milestones for optimal, suboptimal, and failure responses to

TABLE 30.2. Definitions for assessing response in CML.

Parameters		
Complete hematologic response	Normalization of WBC counts	No circulating immature myeloid elements, platelet count in normal range (excluding treatment effects)
Cytogenetic response ^a	Minor	35–65% Ph+ metaphases
	Partial (PCyR)	<35% Ph+ metaphases
	Complete (CCyR)	0 Ph metaphases (20 cells counted)
Molecular response	Major (MMR)	>3-log reduction from baseline untreated levels ^b
	Complete (CMR)	None detectable in an RQ-PCR assay with at least 4.5-log sensitivity

WBC total blood white blood cells, RQ-PCR reverse transcription quantitative polymerase chain reaction, Ph Philadelphia chromosome.

^aMajor cytogenetic responses include CCyR and PCyR.

^bBaseline level often defined as the median or mean value from a group of newly diagnosed samples seen in the laboratory.

TABLE 30.3. Definitions of primary and secondary imatinib resistance in CML.

Time after diagnosis	Failure	Suboptimal response
0 months	NA	NA
3 months	No HR	Less than CHR
6 months	Less than CHR	Less than PCyR
	No cytogenetic response	
12 months	Less than PCyR	Less than CCyR
18 months	Less than CCyR	Less than MMR

HR hematologic response, CHR complete hematologic response, PCyR partial cytogenetic response, CCyR complete cytogenetic response, MMR major molecular response, NA not applicable.

^aCriteria largely derived from the European LeukemiaNet criteria.³⁴

imatinib (Table 30.3). Because of rapid analysis and widespread availability of the assay, BCR-ABL transcript levels, determined by RQ-PCR, have gradually replaced (or complemented) FISH and conventional cytogenetic as the routine monitoring method in CML. Although the response criteria outlined in Tables 30.2 and 30.3 still rely heavily on cytogenetic assessment of disease burden in follow-up samples, there are also benchmarks for use of BCR-ABL transcript levels that allow their application when only RQ-PCR data is obtained.

Routine Monitoring Algorithms

Consensus guidelines on the use of RQ-PCR have been promulgated and include information on required assay performance characteristics and monitoring algorithms.⁹ In most centers, standard monitoring for CML under imatinib therapy is BCR-ABL RQ-PCR every 3–6 months in blood and periodic BM sampling to assess cellularity. Conventional cytogenetic analysis of BM samples every 6 months to 1 year is also recommended (especially in suboptimal responders) to screen for clonal evolution that may signal transformation (Table 30.4). Blood monitoring is usually done every 3 months until optimal response is achieved and may then be done less frequently.

TABLE 30.4. Patterns of disease progression/transformation in CML.

Stage of disease	Clinical definition(s)	Molecular correlates
Secondary therapy resistance in chronic phase ("late CP")	Loss of CHR or CCyR	>tenfold rise in BCR-ABL RQ-PCR, ABL KD mutation ^a
Accelerated phase (AP)	Blasts > 15% Basophils > 20% Platelets < 100 × 10 ⁹ /L Clonal evolution (CE)	CE: Ph+ amplification, +8,+19 most common ABL KD mutation: P-loop sites most common
Blast phase (BP)	>30% blasts with myeloid (MBP), lymphoid (LBP) or biphenotypic markers	MBP: AML-type translocations, de117p13 (TP53) deletion LBP: Ph+ amplification, T315I ABL mutation, de17p13 (IKZF1) deletion

CHR complete hematologic response, CCyR complete cytogenetic response, RQ-PCR reverse transcription quantitative polymerase chain reaction, KD kinase domain, Ph Philadelphia.

^aThe significance of isolated finding of ABL KD mutation in the absence of clinical evidence of loss of response remains controversial.

By RQ-PCR criteria, optimal response is associated with a greater than 3-log reduction in BCR-ABL transcript levels from baseline values (i.e., major molecular response, MMR) within 18 months. Further reductions in BCR-ABL transcript levels, including attainment of complete molecular response (CMR) with undetectable transcript, may be associated with even better outcomes.¹⁰ Complete disappearance of the BCR-ABL transcript is the goal following stem cell transplantation.¹¹ The kinetics of PCR response may also be important, where failure to achieve 3-log or 4-log fold reductions in BCR-ABL transcript levels within 6 months may identify those at risk for secondary resistance.¹²

Assay Design and Quality Control Considerations for BCR-ABL RQ-PCR

Important considerations when validating and performing periodic QC on BCR-ABL RQ-PCR assays include ensuring adequate sensitivity, precision, and accuracy. For those laboratories starting a new test, use of a well-validated assay design, such as those published by the Europe Against Cancer (EAC) group, is recommended.¹³

Recommended features of an adequate RQ-PCR assay include at least 4–4.5-log dynamic range, measurement of enough cells to ensure adequate sampling, and 5–10 ml blood or 3 ml BM aspirate as input for RNA extraction/cDNA synthesis. Because attainment of major molecular response (MMR) is tied to clinical outcome, sensitivity controls should be included in every run to establish the lower level of analytical sensitivity. A method to assess and exclude low-level "false-positives" in RQ-PCR is also important and may include use of post-PCR sizing (Figure 30.1), or a separate qualitative PCR or high-sensitivity FISH assay.

To demonstrate reproducibility of an RQ-PCR assay, it is recommended that samples be run in duplicate, but at what step (i.e., blood, RNA, cDNA, or PCR) the sample should be split is not uniformly agreed upon. Since cDNA synthesis is usually the more variable step in the process, some guidelines recommend replicate reverse transcription.⁹ Assessing assay drift (by monitoring run-to-run variability in quantitation of calibrator samples) is recommended. Guidelines on

when to reject or to request repeat testing should be developed (i.e., less than fourfold variation in replicates down to the level of MMR).

Two issues related to standardization of BCR-ABL RQ-PCR assays across laboratories include how to report transcript levels and what gene to use as a normalizer.¹⁴ Given its relationship to the criteria for imatinib response, a common approach is to report absolute relationship of current BCR-ABL to the baseline newly diagnosed value (i.e., "log-fold reduction"). However, given the frequent absence of a baseline untreated sample for many patients, clinical laboratories also commonly report a normalized ratio of BCR-ABL transcript to a particular normalizer transcript. ABL1 itself is frequently used, as a normalizer, although BCR and GUSB are also frequently used¹⁴ since they do not show the nonlinearity in calculated ratios that it is often observed at high BCR-ABL levels (when ABL1 is used).¹⁵ As of 2010, given the use of different assays with different normalizers and the absence of a widely available standard, comparison between BCR-ABL levels obtained in different laboratories remains difficult.

Molecular Mechanisms of Therapy Resistance in CML (Also See Chap. 11)

Primary Imatinib Resistance

The principal milestones for optimal response to imatinib therapy are attainment of hematologic remission by 3–6 months and cytogenetic remission by 1 year. Primary resistance to imatinib, seen in 5–10% of patients, may be related to intrinsic features of a particular CML tumor (i.e., clonal evolution), pharmacodynamic considerations, dose-limiting toxicities, or therapy noncompliance. The initial response rates to imatinib are lower in those patients presenting with CML already in AP or BP, suggesting that factors mediating blast transformation compromise response to imatinib. Other postulated mechanisms of primary resistance among CML cases presenting in CP include low activity of an imatinib uptake cation transporter 1 (OCT1/SLC22A1)¹⁶ and increased activity of other efflux transporters.¹⁷ Recently, other drug metabolism genes such as PTGS1 have also been

identified as predictors of imatinib resistance based on gene expression studies.^{18,19} ABL kinase domain (KD) point mutations are rare in the setting of primary TKI resistance.

Secondary Imatinib Resistance and Blast Transformation

As CML progresses through late CP, AP, and BP, there are progressively shorter survivals and lower response rate to TKIs. Among imatinib-treated patients, approximately 1–4% per year demonstrate secondary imatinib resistance, usually first detected by rising BCR-ABL transcript levels but occasionally presenting with overt hematologic relapse or fulminant blast transformation (Table 30.5). Therapeutic options for imatinib resistance in CML include newer more powerful or less specific kinase inhibitors (e.g., dasatinib, bosutinib, and nilotinib), combination therapies, and stem cell transplantation. Therefore, detecting secondary resistance at an early stage and determining the mechanism of resistance are important for tailoring future therapies.

The most commonly identified factor mediating secondary imatinib resistance is the emergence of acquired point mutations in the ABL kinase domain, which occurs in approximately 45% of patients with secondary imatinib resistance, with other common molecular mechanisms of resistance including BCR-ABL gene amplification (extra Ph copies, usually detected by karyotype or FISH), clonal evolution, or activation of other growth-promoting kinases, including JAK2²⁰ and LYN.²¹

ABL KD Mutation

Among patients with chronic phase CML who develop secondary resistance to imatinib, 30–50% will have one or more BCR-ABL KD mutations detectable by direct DNA sequencing,^{22,23} whereas mutation frequencies are higher in those with AP or BP, especially for lymphoid BP.²⁴ BCR-ABL KD mutation screening in chronic phase CML is only recomm-

ended for those patients with inadequate initial response to TKIs, or those with evidence of loss of response. Some investigators recommend mutation screening even when small rises in BCR-ABL transcript levels are noted in sequential samples, but this is not the current standard practice.²⁵ Mutation screening is also recommended for all patients at the time of progression to accelerated or blast phase CML.

At least 73 different point mutations involving 57 different amino acids have been reported in the BCR-ABL kinase domain following TKI treatment, but 7 codons (G250, Y253, E255, T315, M351, F359, and H396) have been shown to account for 60–70% of all mutated sites (Figure 30.2).^{26,27} Mutations cluster within the ATP-binding P-loop (amino acids 248–256), the TKI binding region (amino acids 315–317), the catalytic domain (amino acids 350–363), and the activation (A)-loop (amino acids 381–402). The A-loop is a major regulator of BCR-ABL kinase activity, adopting either a closed (inactive) or an open (active) conformation that influences imatinib binding. A-loop mutations are thus rarely seen following treatment with newer conformation-independent kinase inhibitors. Splicing variants and deletions have also been identified in BCR-ABL in TKI-resistant samples, but their clinical significance is not yet clear.^{28,29}

To detect ABL mutations, most laboratories utilize direct (Sanger) sequencing of the entire KD to avoid bias in detection. However, this method is relatively insensitive and will miss low-level mutated CML clones. For this reason, various

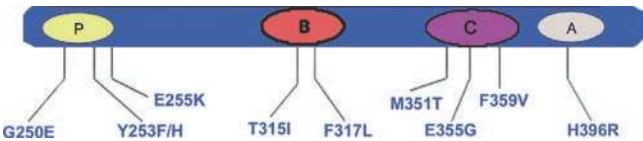


FIG. 30.2. Locations of the most common imatinib-resistance mutations in the ABL KD domain. Domain included the ATP-binding P-loop (P), the imatinib-binding pocket (B), the catalytic domain (C) and the activation loop (A). KD kinase domain, ATP adenosine triphosphate.

TABLE 30.5. Molecular mechanisms of secondary resistance.

Mechanisms	Frequency (%)	Association with phase of disease
<i>Imatinib resistance related to overcoming imatinib blockade (i.e., BCR-ABL dependent):</i>		
Point mutations in BCR-ABL kinase domain	45–50	CP: P-loop & A-loop ^a AP/BP: T315I, F317L, P-loop
Amplification of the BCR-ABL locus	5–10	All stages, increased in LBP
Transcriptional upregulation of BCR-ABL transcript or altered BCR-ABL transcripts	Unknown	All stages of disease
<i>Imatinib resistance related to bypassing imatinib (i.e., BCR-ABL independent mechanisms):</i>		
Activation of others kinases besides BCR-ABL	Unknown	LYN, JAK2
Translocated [inv3;3 inv16, t(8;21), t(3;21)] or dysregulated/mutated (RUNX1, EVI1) myeloid transcription factors	15–40	AP/BP
Loss of tumor suppressors (de117p14/TP53, de19p21/CDKN2A/B)	10–80	AP/BP
Widespread genomic instability	1–5	Mechanism unknown, mutagenic effect of BCR-ABL?

CP chronic phase, AP accelerated phase, BP blast phase.

^aCommon mutations in resistant CML-CP include G250E, Y253H, E255K, and H396R/P.

TABLE 30.6. Comparison of methods for BCR-ABL mutation detection.

Method	Sensitivity	Advantages	Disadvantages
Direct sequencing (dideoxy chain termination method)	DNA: 25% RNA: 10–20%	Can cover most of ABL KD in single amplicon from RNA/cDNA Bidirectional mutation confirmation	Expensive Time-consuming Not quantitative
Pyrosequencing	1–5%	Cheaper technique Faster technique Better sensitivity Quantitative	Requires multiple PCR amplicons due to shorter read lengths
Mutation-specific RQ-PCR	0.1%	Highest sensitivity Quantitative Rapid technique	Need different primers and/or probes for each mutation Need quantitative calibrators

RQ-PCR reverse transcription quantitative polymerase chain reaction, KD kinase domain.

TABLE 30.7. Common BCR-ABL KD mutation seen in imatinib-resistant CML and their comparative in vitro sensitivity to different TKIs.

Amino acid change	Prevalence in imatinib-resistant CML ^b	Nilotinib sensitivity ^c	Dasatinib sensitivity ^c
G250E	5–9%	High	High/intermediate
Y253F/H	11%	Intermediate	Intermediate
E255K/V	11–17%	Intermediate	Intermediate
T315I	13–16%	Low	Low
F317L	3–4%	Intermediate	Intermediate
M351T	10–13%	High	High
E355G	2–3%	High	High
F359V/C	5–6%	Intermediate	High
H396R/P	4%	High	High

^aOnly the most common imatinib-resistant BCR-ABL KD mutations are listed.

^bMutation prevalence data represent the percentage of a particular mutation relative to all mutated cases and are from references 26 and 27.

^cThe IC₅₀ values for each drug for in vitro inhibition of kinase activity of particular mutated BCR-ABL are somewhat variable depending on the study and the assay method.^{35–40} In vitro inhibition from a single study³⁸ was used to classify mutations into high, intermediate, and low sensitivity to dasatinib (IC₅₀ values ≤3 nM, 3–60 nM, and >60 nM, respectively) and nilotinib (IC₅₀ values ≤50 nM, 50–500 nM, and >500 nM, respectively) for this table.

other mutation detection methods have been developed including pyrosequencing,^{30,31} liquid bead array, and the highly sensitive mutation-specific quantitative and digital PCR methods,³² which may reliably detect a mutant transcript down to 1 in 100,000 BCR-ABL transcripts (Table 30.6). The particular methods used to detect BCR-ABL KD mutations will obviously have a great influence on the detection frequency, analytical sensitivity, and the clinical management. Because the clinical significance of low-level mutation burden is unclear, direct sequencing of the BCR-ABL transcript is likely still the most appropriate screening test in most clinical scenarios.

Interpretation of the clinical significance of particular ABL KD mutations may be complex. For the most common mutation sites, extensive data exists on the sensitivity of these ABL kinases to inhibition in vitro to different TKIs (Table 30.7).^{33–40} This data can then be used to infer in vivo response and aid in the selection of alternative therapy.⁴¹

Clonal Evolution

Secondary genetic changes are important in determining the mechanism of resistance and segregate with both the stage of disease and the phenotype of the blast transformation. Cytogenetic changes associated with transformation to AP/BP [besides an extra Ph or der22q] include isochromosome 17q (TP53 gene deletion), trisomy 8, and trisomy 19. Acquisition of AML-type translocations involving activation of dominant-negative myeloid transcription factors [most commonly inv3(q21q26)/RPN1-EVI1 and t(3;21)(q26;q22)/EVI-MDS1-RUNX1, but also t(3;3)(q21;q26), t(8;21)(q22;q22)/RUNX1-RUNX1T1, and inv(16)(p13q22)/CBFB-MYH11] is a feature associated with sudden myeloid blast transformation.^{31,42} Mutations⁴³ or transcription regulation^{44,45} of this family of transcription factors may also be observed with progression/transformation. Changes associated more specifically with lymphoid BP include deletion of chr 7p13 (IKZF1 locus)⁴⁶ and chr 9p21 (CDKN2A/B locus),⁴⁷ as well as the acquisition of ABL KD mutations with high-level kinase activity.^{4,31}

Finally, there is evidence that high-level uncontrolled BCR-ABL kinase levels may themselves be promutagenic due to the increased generation of reactive oxygen species (ROS) or effects on DNA repair.⁴⁸ This proposed intrinsic mechanism of disease progression adds to the rationale for careful molecular monitoring to detect and treat secondary resistance to TKIs as early as possible.

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Molecular Pathogenesis of Nonchronic Myeloid Leukemia Myeloproliferative Neoplasms

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Introduction

Nonchronic myeloid leukemia (CML) myeloproliferative neoplasms (MPNs), referred to as *BCR/ABL*-negative MPNs, have classically been categorized as polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). Each of these MPNs represents a multipotent hematopoietic stem cell-derived clonal myeloproliferation of one or more of the myeloid lineages with the variably common features of erythrocytosis, granulocytosis, and/or thrombocytosis in peripheral blood (PB) and/or variable bone marrow (BM) fibrosis. It is generally a disease of older individuals; however, ET and PMF have been reported in children. Other than the mentioned clinical characteristics of the PB, this category of disease also possesses a tendency toward organomegaly (i.e., hepatosplenomegaly), thrombosis, and bleeding. The BM is usually hypercellular with a blast count of <10%. Although these diseases are heterogeneous, they are characterized by increased blood cell production related to cytokine hypersensitivity, virtually normal cell maturation, and progressive evolution to BM failure with an end point of fibrosis or leukemia.

Current diagnosis of ET, PV, and PMF is based on a consensus-driven set of clinical and laboratory criteria that have undergone substantial modifications over the past decade, unlike CML, in which the *BCR/ABL* fusion gene resulting from t(9;22)(q34;q11) is invariably detected.¹ These changes are reflected in the current 2008 edition of the *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissue*. Revisions of this category were influenced by two factors. In contrast to CML, where the Bcr–Abl fusion protein leads to constitutive tyrosine kinase activity that plays a key role in the pathogenesis, the molecular pathogenesis of the *BCR/ABL*-negative chronic myeloproliferative diseases (CMPDs) has been poorly understood until the recent discovery of the somatic Janus kinase 2 mutations in 2005. The second factor that contributed to the 2008 revision is the

further appreciation of the morphologic and histologic features of this category of disease, which has allowed further subclassification of these neoplasms.¹ Table 31.1 reflects the current 2008 WHO classification for MPNs. Of note, mastocytosis, which has previously been separated into its own category is now under the category of MPN and is discussed in Chap. 32. With respect to the current literature, molecular aspects of PV, ET, and PMF will be discussed in this chapter. Those diseases associated with alterations in platelet-derived growth factor (PDGF) will be discussed in the MPN/MDS chapter (see Chap. 32).

Currently, the diagnosis and classification of MPN is primarily based on a combination of clinical findings, PB cell counts, morphologic examination of PB and BM trephine biopsies, and molecular genetic testing. Additional ancillary studies include cytogenetic analysis and immunophenotyping. As mentioned, molecular testing, especially JAK2 mutation, is becoming part of the routine work-up, due to the importance of the JAK2 nonreceptor tyrosine kinase in the diagnosis and pathogenesis of MPNs.^{1–6}

Clonality of MPNs has previously been established by X chromosome inactivation studies and later on by RNA transcription product analysis of active X chromosomes.^{7–13} Discovery of the JAK2 mutations provides further evidence to the clonal proliferation nature of MPN. Additionally, more recent studies have further suggested the possibility of an underlying somatic mutation prior to the acquisition of the JAK2 mutation.^{14,15} Thus, there are currently two hypotheses on the etiology of MPNs, which are important in contemplating the molecular findings of this category of neoplasms. The first theory establishes the JAK2 mutation as the single hit, which causes the onset of the MPN phenotype. The second theory suggests an underlying somatic mutation, which establishes clonality with later acquisition of the JAK2 mutation that establishes the MPN phenotype. Currently, neither hypothesis adequately explains every case of MPN, but they lay a foundation on which to contemplate the etiology of these entities.

TABLE 31.1. 2008 WHO classification of myeloproliferative neoplasms.

Chronic myeloid leukemia, BCR–ABL1 positive
Chronic eosinophilic leukemia, NOS (see Chap. 32)
Polycythemia vera
Primary myelofibrosis
Essential thrombocythemia
Chronic neutrophilic leukemia, NOS
Mastocytosis (see Chap. 32)
Myeloproliferative neoplasm, unclassifiable

From: 2008 Edition, *WHO Classification of Tumors of Haematopoietic and Lymphoid Tissue*.

Cytogenetics of Nonchronic Myeloid Leukemia Myeloproliferative Neoplasms

Cytogenetic abnormalities of non-CML MPNs comprise a heterogeneous group of generally nonspecific abnormalities that may aid in the initial diagnosis and clinical follow-up of patients (Table 31.2). The importance of these findings is supported by their inclusion in the WHO description of these lesions. They not only provide additional evidence as to the presence of a clonal malignancy, particularly in the absence of JAK2 or MPL mutation, but may also play a role in prognosis. In general, the presence of cytogenetic abnormalities at initial diagnosis is indicative of a poor prognosis.¹⁶

The incidence of cytogenetic lesions varies with the specific MPN entity. PMF is the most common MPN with cytogenetic abnormalities. Approximately, 33–43% of PMF cases possess some type of genetic lesion at diagnosis. The most common chromosomal abnormalities in PMF include del(13q), del(20q), partial trisomy 1q, and trisomy 8.^{17,18} Abnormalities of chromosomes 7, 9, and 12 are also reported.¹⁹ A recent study suggests that der(6)t(1;6)(q21-23;p21.3) is a specific chromosome abnormality in PMF and might harbor gene(s) specifically associated with PMF.²⁰ Deletions affecting chromosomes 7 and 5 also occur but may be associated with prior cytotoxic therapy.¹ At the time of leukemic transformation, 90% of PMF cases will have clonal cytogenetic abnormality(ies).²¹ The incidence of cytogenetic abnormalities is then followed by PV (33–42%), MPN-unclassifiable (20%), and ET(<5%).^{22–27} The chromosomal abnormalities in PV and ET will be further discussed later in this chapter. Common chromosomal aberrations that affect MPNs include deletions, additions, translocations, and ploidy involving chromosomes 1, 5, 8, 9, 13, and 20.²⁷

Al-Assar et al²⁸ studied comparative genomic hybridization (CGH), a relatively new molecular cytogenetic technique, versus conventional cytogenetics in patients with PMF and suggested that genomic aberrations were more common than previously reported by conventional cytogenetic analysis. According to this study, gain of 9p is the most common cytogenetic aberration (50% of the cases) and may play a crucial role in the pathogenesis of PMF.²⁸ Chromosome 9p contains JAK2, a gene recently identified to have a critical

TABLE 31.2. Common mutations and cytogenetic abnormalities in myeloproliferative neoplasms.

Polycythemia vera	>95%	JAK2 ^{V617F} mutation
	>20%	Cytogenetic abnormalities +8, +9, del(20)q, del(13)q, del(9p)
Primary myelofibrosis	≈50%	JAK2 ^{V617F} mutation
	5%	MPLW515K/L mutation
	30%	Cytogenetic abnormalities del(20)q, partial trisomy 1q, +8,+9 del(13)(q12-22) der(6)t(1;6)(q21-23;p21.3)
Essential thrombocythemia	40–50%	JAK2 ^{V617F} mutation
	1%	MPLW515K/L mutation
	5–10%	Cytogenetic abnormalities +8, abnormalities of 9q, del(20q)

gain-of-function mutation, which will be further discussed later in this chapter.

In regards to PV, a clonal genetic abnormality by JAK2 mutation is a major new criterion, but no specific karyotype has been identified. The absence of Philadelphia chromosome or BCR/ABL1 fusion gene is essential for exclusion of CML. Conventional cytogenetic analysis may reveal chromosomal abnormalities in the hematopoietic progenitor cells of PV patients, which increase with the stage of the disease, from less than 20% at diagnosis to 80–90% after 10 years of follow-up.^{22,29,30} Specific techniques, such as CGH and fluorescent in situ hybridization, appear to slightly increase the yield.^{31–35} Many chromosomal abnormalities exist and are similar to the abnormal karyotypes observed in patients with myelodysplastic syndrome and other MPDs. The most frequent abnormalities are deletion and translocation of chromosomal 20, trisomy 8, and trisomy 9. Other genetic aberrations involve abnormalities of chromosomal 13q, 5q, 7q, 1q, and monosomies 5 and 7.³⁶ More recently, array CGH (aCGH) studies with high density oligo-based microarrays have revealed that microdeletions and microduplications do not appear to play an essential role in the development of PV.^{37,38}

No consistent, specific, or universal recurring cytogenetic abnormality or molecular marker has been identified in ET. Random chromosomal abnormalities have been identified in 5% of ET patients.^{39–41} This finding may be due in part to the unexpected number of polyclonal cases (30–50%). Some authors have reported an increased frequency of trisomy 8 and trisomy 9 in ET, while others report no increased frequency of this cytogenetic abnormality.^{40,42} While recurring cytogenetic abnormalities in ET have not been established, some changes have been identified in association with transformation to acute leukemia. Cytogenetic abnormalities seen with ET in association with AML include t(2;17), t(3;17)(p24;q12), del(5)(q13q34), t(1;7), long-arm trisomy of chromosome 1, monosomy 7q, and deletion 17p.^{43–48} Deletion 17p (site of p53 gene) has a high association with previous hydroxyurea (HU) therapy. Pipobroman therapy is associated with long-arm trisomy of chromosome 1 and monosomy 7q.⁴⁷

Der(1;7)(q10;p10) has been associated with acute leukemic transformation in patients not treated with cytotoxic agents.⁴⁶ Using CGH, a gain of 18p was seen in one of eight patients with ET.³⁹ However, in 2006, bacterial artificial chromosome (BAC) aCGH studies revealed the absence of recurrent genomic abnormalities in ET, which has been further validated by recent aCGH studies with high density oligo-based microarrays.^{37,38} By definition, there should not be t(9;22) as seen in CML or del 5q-, t(3;3)(q21;q26), or inv(3)(q21q26) as seen in myelodysplastic syndromes that may have thrombocytosis.^{1,49}

With the advent of refined genomic microarray technologies, further evaluation of genomic alterations is possible – especially at the level of single nucleotide polymorphisms (SNPs). The SNP chip utilizes oligonucleotide microarray probes, which contain SNPs and 2 two types of probes at each genomic locus, allowing recognition of single nucleotide differences and each parental allele. This design allows detection of any allelic imbalance but cannot detect acquired somatic mutations. In addition, unlike conventional methods, SNP chips can detect loss of heterozygosity with neutral allelic dosage (uniparental disomy).^{50,51} Kawamata et al⁵⁰ analyzed MPNs by SNP chip microarray and demonstrated multiple aberrations in MPN. Deletions of RB1 and NF1, 9p uniparental disomy/JAK2 point mutations, and 1p uniparental disomy/MPL point mutations were identified. Although most of the genetic abnormalities in this cohort were identified in PMF, they were not specific for this disease and did not help in differentiating it from PV or ET. Rare aberrations, such as deletion at 5q23.1, involving a single gene, LOC51334, were identified in ET.^{50,52} Limited SNP array studies are currently reported in the literature; however, with the current expansion of molecular knowledge and technology, SNP data is likely to expand exponentially.

Overall, there are no cytogenetic abnormalities specific for non-CML MPNs; however, their presence may connote an unfavorable prognosis. Currently, there are no specific therapies related to the cytogenetics mentioned. However, at the molecular level, tyrosine kinase inhibitors are currently under investigation.

Molecular Pathways Involving MPN

As with many other hematopoietic and nonhematopoietic neoplasms, investigation at the molecular level is exceedingly producing new information concerning disease pathogenesis, as well as revealing possible targets for novel therapy (Table 31.3). The best example of this approach is the successful development and use of imatinib for CML, which has greatly impacted the way we approach modern medicine. This approach has also been applied to the investigation of non-CML MPNs in hopes of finding another “silver bullet.” Great strides have been made, especially recently, in the pathogenesis of classic non-CML with the

TABLE 31.3. Mutations/rearrangements of tyrosine kinase genes in myeloproliferative neoplasms.

Polycythemia vera	JAK2 ^{V617F} , JAK2 exon 12
Primary myelofibrosis	JAK2 ^{V617F} , MPLW151L/K
Essential thrombocythemia	JAK2 ^{V617F} , MPLW151L/K
Mastocytosis	KITD186V
Myeloid and lymphoid (or myeloid) neoplasms with eosinophilia	PDGFRA, PDGFRB, FGFR1

discovery of the JAK2^{V617F} mutation in the Janus family of nonreceptor tyrosine kinase domain on chromosome 9. Other mutations relevant to the non-CML MPNs include the JAK2 exon 12 mutations, MPLW151L/K, and KITD816V, as well as FIP1L1-PDGFRB, PDGFRB, and FGFR1.^{53,54} The mutations involved in systemic mastocytosis (KITD186V) and the abnormalities of the myeloid and lymphoid (or myeloid) neoplasms with eosinophilia (PDGFRA, PDGFRB, or FGFR1) are discussed in Chap. 32. The following section will focus on the JAK2 and MPL mutations.

JAK2^{V617F} Mutations

In approximately the first half of 2005, several groups reported a unique mutation in the JH2 domain of Janus kinase 2 (JAK2), due to the replacement of G to T in nucleotide sequences (1849G>T) and leading to a valine-to-phenylalanine substitution (V617F). This mutation occurs in the majority of the classic *BCR/ABL1*-negative (non-CML) MPNs patients, particularly PV. JAK2 is a cytoplasmic protein-tyrosine kinase, which catalyzes the transfer of the gamma-phosphate group of adenosine triphosphate to the hydroxyl groups of specific tyrosine residues in signal transduction molecules.^{55,56} The main downstream effectors of JAK2 are a family of transcription factors known as signal transducers and activators of transcription (STAT) proteins. This mutation in the pseudokinase autoinhibitory domain results in constitutive kinase activity and induces cytokine hypersensitivity, or independence of factor-dependent cell lines. Retroviral transduction of the mutant JAK2 into murine hematopoietic stem cells leads to the development of MPNs with polycythemia.^{55,56} These findings indicate that the JAK/STAT signal transduction pathway plays an important role in the pathogenesis of *BCR/ABL1*-negative MPNs.

With the application of adequately sensitive tests, it is now becoming evident that more than 90% of patients with conventionally defined PV carry the somatic JAK2^{V617F} mutation in their granulocytes.^{57,58} Although this mutation is a highly sensitive finding for PV, it is not specific. This mutation is also found in 35–95% (with the majority of reports at approximately 57%) of PMF patients and in 23–57% of ET patients.⁵⁷ Furthermore, this mutation may be infrequently identified in patients with chronic myelomonocytic leukemia (CMML) (3–20%), myelodysplastic syndromes

(1–5%), systemic mastocytosis (0–25%), Philadelphia chromosome (Ph)-negative CML (19%), chronic eosinophilic leukemia (0–2%), chronic neutrophilic leukemia (16–33%), juvenile MML (20%), and in rare cases of acute myeloid leukemia (AML), particularly those with a preceding MPN, or those with blasts showing megakaryocytic differentiation.^{2,57,59–61} Most importantly, the JAK2^{V617F} has been absent or very rarely observed in patients with reactive conditions, secondary polycythemia, chronic myeloid leukemia, lymphoid malignancies, and nonhematolymphoid malignancies, including colon, breast, and lung carcinomas.^{2,60,61} The myeloid disorder-specificity associated with this mutation is partially explained by a recent study showing that downstream signaling involving JAK2^{V617F} requires the presence of an associated type 1 cytokine receptor (the erythropoietin receptor (EpoR), the thrombopoietin receptor, or the granulocyte colony-stimulating-factor receptor).⁶²

Currently, most tests for JAK2 mutation apply molecular diagnostic technologies, which are available mostly at major reference laboratories, or medical centers. Recently, Aboudola et al⁶³ identified the possible use of phosphor-STAT5 expression in BM cells as an alternative method of detecting JAK2 mutation by immunohistochemistry (IHC). Although this has not yet been validated, its potential use may elicit the use of IHC for the detection of the JAK2 mutation by a much more widely available method.

In addition to being an excellent diagnostic marker for *BCR/ABL*-negative MPNs, JAK2^{V617F} mutant alleles may also have prognostic significance. In patients with ET, the presence of JAK2^{V617F} has been associated with advanced age at diagnosis, higher hemoglobin and leukocyte levels, and an increased rate of polycythemic transformation. However, the mutation does not appear to affect the incidence of thrombotic, leukemic, or fibrotic events.⁶⁴ Similarly, in patients with PMF, patients positive for JAK2^{V617F} had higher neutrophil and white cell counts ($P=0.02$) than did patients negative for JAK2^{V617F}; patients positive for JAK2^{V617F} were less likely to require blood transfusion ($P=0.03$). However, patients positive for JAK2^{V617F} had poorer overall survival, even after correction for confounding factors ($P=0.01$).⁶⁵

More recently, quantitative analysis of JAK2^{V617F} is gaining interest in the clinical setting for both prognosis and monitoring of MPNs. In 2007, Vannucchi et al⁶⁶ investigated whether the burden of JAK2^{V617F} allele correlated with major clinical outcomes in patients with PV. They found that individuals with >75% JAK2^{V617F} allele burden had a higher risk of suffering from pruritus, developing major cardiovascular events, and requiring chemotherapy. In the same year, another group investigated the usefulness of JAK2^{V617F} in monitoring residual disease after stem cell transplantation in patients with myelofibrosis.⁶⁷ Kroger et al⁶⁷ found that quantification of JAK2^{V617F} in patients with myelofibrosis allows monitoring of treatment response on a molecular level and may help to guide adoptive immunotherapy strategies.

The importance of the quantitative analysis of JAK2^{V617F} continues to evolve, and as evidenced by requisition for this assay by clinicians in our hospital, it may become part of the standard protocol for evaluation and follow-up of MPN patients.

Other JAK Mutations

Although the identification of the JAK2^{V617F} mutation provided an explanation for the pathogenesis of most PV cases, JAK2^{V617F}-negative cases of MPNs remained unexplained. In 2007, Scott et al identified four somatic gain-of-function mutations affecting *JAK2* exon 12 in 10 (90%) of 11 V617F-negative patients with PV.⁶⁸ Similar to the JAK2^{V617F} mutation, this category of mutations creates cytokine-independent hematopoiesis with downstream signaling of the STAT5, AKT, and MAP pathways.⁶⁹ Subsequent investigations by other groups confirmed these findings but also identified other differences as compared to the JAK2^{V617F} mutation. Unlike the JAK2^{V617F} mutation, which involves a single nucleotide, up to eight different mutations involving *JAK2* exon 12 have been identified.⁷⁰ Although the clinical phenotype of JAK2 exon 12 lesions is predominantly erythroid, there is significant overlap between JAK2^{V617F} and JAK2 exon 12 mutations.⁷¹ The prognostic significance of the *JAK2* exon 12 remains to be discerned. Other rare mutations that have been identified include the JAK2T875N kinase domain mutation⁷² and the JAK2ΔIREED deletion.⁷³

MPLW515 Mutations

The lack of a JAK2V617F mutation in a significant number of cases of ET and PMF has led to the investigation into other possible activating mutations and the discovery of the thrombopoietin receptor mutation, MPLW515L, on chromosome 1. This novel mutation was initially reported in 2006 by Pikman et al⁷⁴ in four cases of JAK2^{V617F}-negative PMF. Initially, the mutation appeared to be exclusive to cases of ET (~1%) and MF (~5%).^{74,75} However, like the JAK2 mutation, it does not appear to be specific to MPNs and has recently been identified in a case of refractory anemia with ringed sideroblasts (RAEB) with features of ET.^{76,77}

The MPLW515L mutation causes a single amino acid substitution in the transmembrane region of the thrombopoietin receptor; however, the specific pathogenesis of these alterations has not been elucidated. MPLW515L was found to activate the JAK–STAT signaling pathway and BM murine models demonstrated features of MPNs, including extramedullary hematopoiesis, splenomegaly, and megakaryocytic proliferation.^{74,75,78} Furthermore, cases of ET and PMF with the MPL mutation have a more severe anemia than those without MPL mutation.^{79–81} In addition, MPL and JAK2 mutations are not mutually exclusive in cases of ET and PMF. Up to 22% of cases studied in several groups have found the coexistence of MPL mutations with JAK2 mutations.^{75,76,79}

More recently, further investigation has led to the discovery of MPL mutation variants, including MPLW515K, MPLW515A, and MPLW515R. The latter two had not been previously described until recently, when Schnittger et al⁸² reported them in 35 (4%) of 869 cases of ET or PMF through DNA sequencing analysis. The relevance of these last two mutations remains to be seen, but the same group hypothesized that the size of the substituted amino acid may play a role in the pathogenesis of MPNs.

Another interesting finding related to the pathogenesis of this group of disease was discovered after investigation of endogenous hematopoietic colonies. Endogenous megakaryocytic colonies may be grown from MPL515-positive patient cells. Cells harboring the MPL mutation yielded virtually no endogenous erythroid colonies, in contrast to JAK harboring progenitors.^{80,82,83} The significance of these findings indicates that, although both mutations involve the JAK–STAT pathway in some fashion, they operate through different downstream pathways, which appear lineage-specific. The presence of the MPLW515 mutations has been reported in subsets of B and T lymphocytes and lends itself to the idea that the mutation occurs earlier in development than the JAK2 mutation in a myelolymphoid progenitor cell.⁸²

Expression Profiling Findings in MPNS

Gene expression profiling (GEP) yields important information about intrinsic cell function, by measuring the mRNA produced as a result of activation or inactivation of genes through the use of microarray technologies. Currently, the literature is limited on GEP studies focused on non-CML MPNs. However, some studies have provided important insights into the pathogenesis of non-CML MPNs, and the resulting data may play a further role in clinical evaluation of patients in the future.

Regarding PV, some of the first data uncovered by cDNA microarray studies identified upregulation and downregulation profiles of genes that have potential use as molecular signatures for this disease. Pellagati et al⁸⁴ revealed the upregulation of 147 genes in peripheral granulocytes of 11 patients diagnosed with PV, compared with healthy individuals, which included protease inhibitors and antiapoptotic factors. Eleven of these upregulated genes were identified in all the subjects analyzed and included: GYG, CMAP, SLPI, ADM, SFRS₃₁, FCER1G, S100 CAAF1, IP30, PYGL, GNG10, and ANX3. These results demonstrated that deregulation, especially by upregulation, may play a significant role in the pathogenesis of this disease. In 2005, Goerttler et al⁸⁵ conducted a similar study and found that a set of 64 genes could discriminate PV from secondary erythrocytosis by GEP. In addition, they identified overexpression of transcription factor NF-E2, in 93% of PV patients tested, and hypothesized that it is a key contributor in the pathogenesis

of PV and that its level of overexpression directly coincides with the amount of erythrocytosis and thrombocytosis seen clinically in PV patients.

GEP of ET cases has shed light into the pathogenesis of this disease, with the potential of subgrouping it based on molecular studies. Schwemmers et al⁸⁶ have demonstrated that non-JAK2^{V617F} ET patients express lower levels of several JAK/STAT target genes, most notably PIM and SOCS-2, and do not display constitutive STAT3 phosphorylation.⁸⁶ These findings help support the idea that ET, as well as other MPNs, is more heterogeneous than previously thought, and that some other unidentified molecular derangement is present. Puigdecane et al⁸⁷ expanded this study and identified 30 genes in PB granulocytes of ET patients, which differentiated JAK2^{V617F} negative and JAK2^{V617F} positive cases. Of these genes, 14 (i.e., CISH, C13orf18, CCL3, PIM1, MAFF, SOCS3, ID2, GADD45B, KLF5, TNF, LAMB3, HRH4, TAGAP, and TRIB1) displayed an abnormal expression pattern.⁸⁷ More recently, GEP of CD34+ stem/progenitor cells from ET patients has resulted in data that suggests the JAK2^{V617F} mutation has no influence on the GE profile, and may support the hypothesis that ET may be acquired as a secondary hit, afflicting more mature cells (on the basis of data obtained on PB granulocytes).⁸⁸

Examination of the GE profiles of CD34+ cells in PMF patients has yielded very interesting data not only on aberrant gene expression but also regarding prognostic factors. Through the use of class prediction analysis, Guglielmelli et al⁸⁹ identified that the GE pattern of eight genes (i.e., CD9, GAS2, DLK1, CDH1, WT1, NFE2, HMGA2, and CXCR4) in PB CD34+ cells and granulocytes may discriminate PMF from PV, ET, and normal controls. Additionally, abnormal expression of HMGA2 and CXCR4 was found to be dependent on JAK2^{V617F} mutation status. In regards to prognosis, WT-1 expression levels directly coincided with disease activity.⁹⁰

More recently, microRNA (miRNA) has come into the investigation of the pathogenesis of PV and PMF. MiRNA (as described by Du et al⁹¹) are 18–22ntRNA that regulate GE, either by destabilizing target mRNA or by inhibiting protein translation.⁹¹ Dysregulation of miRNA has been implicated in many physiologic and pathologic processes. A profile of 40 miRNAs from various PB cells (i.e., granulocytes, reticulocytes, mononuclear cells, or platelets) of PV patients has recently been shown to be statistically different from normal individuals.⁹² Some overlap of expression was seen with other hematopoietic disorders, such as chronic lymphocytic leukemia, and some dysregulated miRNA expression was dependent on the JAK2^{V617F} mutation status.⁹² In regards to PMF, Guglielmelli et al⁸⁹ identified an miRNA profile that could distinguish PB granulocytes of PMF patients with those of normal individuals; however, some overlap was seen with PV and ET. Twelve of the 60 miRNAs found to be statistically significant by analysis of variance included upregulation of miR-190, -182, and -183, and downregulation of miR-31, -150, -95, -34a, -342, -326, -105, -149, and -147.⁸⁹

Molecular-Targeted Therapy

A substantial portion of the current literature in pathology revolves around the molecular pathogenesis of disease. This is in part due to the advances in technology over the past decade and also to the success of disease treatment, such as the use of imitinab for CML, other Philadelphia chromosome-positive diseases, and other diseases associated with tyrosine kinase abnormalities, such as PDGFRA or PDGFRB. Therapeutics focused on molecular targets that are more specific and generally have a better side effect profile, as compared to traditional chemotherapy regimens. There is no current FDA-approved therapy for non-CML MPNs.

In regards to non-CML MPNs, there are limited studies focused on kinase inhibitors, especially JAK. Currently, there are four JAK inhibitors in Phase I clinical trials, which include Lestaurtinib (Cephalon), INCB18424 (Incyte), XL-019 (Exelixis),⁹³ and LS104.⁹⁴ Lestaurtinib was initially investigated as an fms-like kinase (FLT-3) inhibitor for use in treatment of AML. Recently, in a study conducted by Hexner et al.,⁹⁵ it was also found to be a JAK2/STAT5 signaling pathway inhibitor and limits proliferation of cells taken from patients with MPNs, with only partial inhibition of normal patient samples. Initial studies of INCB18424 reveal effective responses in clinical and laboratory parameters of patients with a decrease in proinflammatory cytokines. The only negative response seen thus far is cytopenia with higher dosages.⁹³ No clinical data is currently available for XL-019.

LS104 is the first non-ATP competitive inhibitor of JAK2 to enter clinical trials for the treatment of non-CML MPNs. LS104 is not a new drug, and previous in vitro studies have identified it as a novel inhibitor of oncogenic kinases in leukemia.⁹⁶ More recently, Lipka et al.⁹⁴ demonstrated the ability of LS104 not only to induce apoptosis of JAK2 positive cells but also to inhibit autophosphorylation of JAK2 and downstream pathway targets. This molecule is unique in that it appears to work through a non-ATP competitive inhibition, as compared to other JAK inhibitors.

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Molecular Pathology of Myelodysplastic/Myeloproliferative Neoplasms, Myeloid and Lymphoid Neoplasms with Eosinophilia and Abnormalities of PDGFRA, PDGFRB, and FGFR1, and Mastocytosis

Robert P. Hasserjian

Myelodysplastic/Myeloproliferative Neoplasms: General Aspects

Myelodysplastic/myeloproliferative neoplasms (MDS/MPN) are clonal hematopoietic neoplasms that display features of both myeloproliferative neoplasms (MPN) and myelodysplastic syndromes (MDS). They typically display some degree of effective hematopoiesis, manifested by an increase in one or more peripheral counts and/or organomegaly due to extramedullary hematopoiesis. However, they also exhibit aspects of ineffective hematopoiesis with one or more cytopenias, morphologic dysplasia, and/or abnormal effector cell function. Although MDS/MPN entities have in common this combined disease phenotype, within each disease entity there is often a wide spectrum of clinical presentations, in some cases resembling “pure” MDS and in others “pure” MPN entities.^{1,2} Myeloblasts may be increased in MDS/MPN cases, and in some of these entities blast count defines prognostic groups as with MDS; however, the bone marrow (BM) and peripheral blood (PB) blast count is always less than 20%.

Unlike the MPN and MDS groups, there are no entities within the MDS/MPN group of neoplasms that are defined by a particular genetic feature. Nevertheless, recurring genetic abnormalities are found in many MDS/MPN entities, and in some instances provide useful prognostic information. In general, the cytogenetic abnormalities in MDS/MPN cases are more often numerical (i.e., trisomies, monosomies, additions, and deletions), rather than structural (i.e., translocations), and more resemble those seen in MPN rather than MDS or AML. Some genetic tests, such as assessment for BCR–ABL rearrangement, are required to exclude certain entities that may be in the differential diagnosis of MDS/MPN diseases. Moreover, identification of a genetic abnormality may help confirm a diagnosis of a neoplasm, as the clinical presentation and even morphologic features of many

MDS/MPN entities may overlap with a reactive process due to an infection, inflammatory process, metabolic derangement, drug, or toxin. MDS/MPN are clonal disorders originating from a neoplastic hematopoietic precursor. In spite of their frequently mixed clinical picture of effective, overexuberant production of one lineage and ineffective production of other lineage(s), X-inactivation and molecular cell subset analysis in MDS/MPN diseases have confirmed clonality across multiple myeloid and, in some instances, lymphoid lineages.

Chronic Myelomonocytic Leukemia

Chronic myelomonocytic leukemia (CMML) is an MDS/MPN entity characterized by persistent PB monocytosis ($>1 \times 10^9/L$) in the setting of morphologic dysplasia of one or more myeloid lineages. If convincing dysplasia is not present, the diagnosis may be made in the setting of a monocytosis that is persistent (longer than 3 months) and unexplained after rigorous clinical investigation, and/or by demonstrating a clonal molecular and/or cytogenetic abnormality. Chronic myeloid leukemia must always be excluded by showing absence of BCR–ABL fusion and cases with eosinophilia should show absence of a PDGFRA, PDGFRB, or FGFR1 rearrangement. CMML may variably manifest as an elevated white blood count mimicking a myeloproliferative neoplasm, or with a cytopenic picture mimicking a myelodysplastic syndrome.¹

There is no specific genetic lesion associated with CMML, and the disease is heterogeneous in terms of its cytogenetic abnormalities and observed oncogene mutations. Between 24 and 50% of CMML cases exhibit cytogenetic abnormalities^{3–9}; the incidence of cytogenetic abnormalities in CMML may be lower in Asian countries.⁸ Patients with an abnormal karyotype have a significantly shorter survival than patients with a normal karyotype,⁷ although cytogenetics has not been shown to be

significant in multivariate analysis with other features, such as PB counts and blast count, and is not used in proposed prognostic scoring systems for CMML.^{6,7} The most commonly reported cytogenetic abnormalities in CMML are the numerical aberrations -7 and $+8$ ⁷; however, the frequency of -7 may be overstated in earlier studies that included pediatric patients, likely representing juvenile myelomonocytic leukemia cases.^{4,5} Less frequent abnormalities include -5 , $+21$, $\text{del}(12\text{p})$, and $\text{i}(17\text{q})$.⁷ CMML cases presenting with isolated isochromosome 17q characteristically show markedly hypolobated and nonsegmented dysplastic neutrophils, and may have a more aggressive clinical course.¹⁰ While the 17q abnormality results in loss of 17p material, including the p53 gene, p53 mutations have not been identified in the intact p53 gene in these neoplasms.¹¹ Trisomy of chromosome 1 or partial trisomy of chromosome 1q has also been reported to occur in CMML.¹² Complex karyotypes comprise about 18% of cytogenetically abnormal cases.⁷ In cytogenetically abnormal CMML cases, the abnormality is present in all myeloid cell lines, but not in lymphocytes, suggesting that this is a clonal neoplasm deriving from an early myeloid stem cell.¹³

CMML is considered to represent a *de novo* disease; however, monocytosis resembling CMML may develop in some MDS cases and is associated with disease progression, although no concomitant genetic changes have been identified in these cases correlating with the monocytosis.¹⁴ CMML may also occur as a therapy-related MDS/MPN following treatment with topoisomerase II inhibitors. Such cases usually manifest with cytopenias and are associated with a $\text{t}(11;16)(\text{q}23;\text{p}13.3)$ translocation that involves the MLL and CBP genes.¹⁵ A case of CMML bearing a partial tandem duplication of the MLL gene has also been reported.¹⁰

The presence of a $\text{t}(9;22)(\text{q}34;\text{q}11)$ translocation and/or presence of BCR-ABL fusion confirms a diagnosis of CML and excludes a diagnosis of CMML; thus it is critical to exclude the presence of BCR-ABL fusion by cytogenetics, fluorescence in situ hybridization (FISH), and/or reverse transcription polymerase chain reaction (RT-PCR) prior to making a diagnosis of CMML. In particular, the rare cases of CML that bear a minor bcr breakpoint variant translocation resulting in a 190 kDa BCR-ABL fusion protein (p190) often exhibit absolute monocytosis and may have dysplastic features and lack basophilia, mimicking CMML. Although the absolute monocyte count often exceeds $1 \times 10^9/\text{L}$ in these cases, in CML (unlike CMML), the monocyte percentage is usually less than 10%.^{16,17}

The JAK2 V617F mutation appears to be rare in CMML, having been identified in 3–13% of cases.^{18–22} It is unknown if the JAK2 mutation is more common in CMML cases presenting with proliferative features, such as splenomegaly and leukocytosis. FLT3 internal tandem duplications and mutations and KITD816 mutations that characterize some cases of acute myeloid leukemia (AML) have not been reported to occur in CMML.¹⁰

The RAS family genes (HRAS, NRAS, and KRAS) encode small GTPases that regulate transduction pathways

involved in cell proliferation and differentiation. Activating point mutations in the GTP-binding region of RAS proteins are common in human cancers, including AML and MDS, and are present in 10–60% of CMML cases.^{3,10,23} While solid tumors more commonly bear KRAS mutations, NRAS mutations are most common in hematologic malignancies, including CMML.²⁴ HRAS mutations have also been identified in CMML.²⁵ MDS patients with NRAS mutations have shorter survival rates and increased likelihood of progression to AML than patients lacking RAS mutations; the prognostic effect of RAS mutations has not been specifically studied in CMML patients.³ Activated NRAS genes have induced myeloid malignancies resembling CMML in a mouse model, characterized by leukocytosis, monocytosis, anemia, BM granulocytic and monocytic proliferation, and hepatosplenomegaly²⁶ and myeloproliferative diseases have also been induced in mice by introducing mutated KRAS genes.²⁷ These data suggest that the activating RAS point mutations identified in many CMML cases play a critical role in the disease pathogenesis. The CMML-like disease induced by activated NRAS differed from the CML-like disease induced in murine models by BCR-ABL by involvement of the monocytic as well as the granulocytic lineage. Thus, although the BCR-ABL and RAS pathways are interrelated, involvement of different signaling pathways may underlie the distinct clinical and biologic features of CMML and CML.²⁶ Further study is needed to determine what specific effectors downstream of RAS are activated in CMML cases.

There is no targeted molecular therapy available to treat CMML; imatinib is not effective.²⁸ Imatinib is effective in CML and in myeloid neoplasms with eosinophilia and abnormalities of PDGFRB, diseases that may have monocytosis mimicking CMML.

Atypical Chronic Myeloid Leukemia, BCR-ABL1 Negative

Atypical CML, BCR-ABL1 negative (aCML) is a rare MDS/MPN entity characterized by leukocytosis ($\text{WBC} > 13 \times 10^9/\text{L}$) with circulating dysplastic neutrophil precursors and usually dysplasia of erythroid and/or megakaryocytic lineages as well. The disease is distinguished from CMML by its lack of monocytosis and from CML by its lack of BCR-ABL fusion. Although the morphologic dysplasia and common anemia and thrombocytopenia mimic MDS, the persistent leukocytosis excludes “pure” MDS and there is often splenomegaly and hepatomegaly that would be uncommon in MDS cases. Some cases exhibit prominent, abnormally condensed nuclear chromatin in neutrophils, and have been previously termed as “syndrome of abnormal chromatin clumping in granulocytes.”²⁹

As with CMML, there is no specific genetic lesion associated with aCML. Between 20 and 82% of aCML cases are cytogenetically abnormal.^{2,30–32} The most common abnormalities are $+8$ (14/49, 28% of reported karyotypically

abnormal cases) and del(20q)(12/49, 24% of reported abnormal cases). Other reported abnormalities include deletions or additions of chromosomes 5, 7, 11, 12, 13, 14, 17, 21, and X. Some myeloid neoplasms with isochromosome 17q (see CMML section above) may lack monocytosis and are classified as aCML.^{13,30} Translocations are less common than numerical aberrations, being reported in 7/49 (14%) of cytogenetically abnormal aCML cases. A t(8;9)(p22;p24) PCM1–JAK2 translocation has been reported in some cases, but most of these cases have eosinophilia and likely represent examples of chronic eosinophilic leukemia.^{33,34} Cases with translocations involving BCR–ABL1 or the PDGFRA, PDGFRB, and FGFR1 genes should be excluded. Interestingly, rare cases of myeloid neoplasms resembling CML or aCML have been reported that bear a t(9;22)(p24;q11.2) translocation, resulting in BCR–JAK2 fusion, distinct from the t(9;22)(q34;p11.2) BCR–ABL translocation of CML. Although dysplasia was not a prominent feature, such cases are probably best classified as aCML and are not responsive to imatinib mesylate.^{35,36} A complex karyotype is present in less than 10% of aCML cases.^{2,30–32} While there is significant overlap between the cytogenetic abnormalities reported in aCML and CMML, del(20q) is more common in aCML while –7 is less common. Cytogenetic abnormalities have not been correlated with prognosis in aCML, although only a small number of cases have been analyzed.³⁰

A mutation in NRAS or KRAS has been reported in 23% of cases.³⁰ The JAK2 V617F mutation has not been identified in the small number of aCML cases analyzed to date.³⁷ Imatinib does not appear to be effective in treating aCML, although in one series 1/7 patients apparently showed some improvement in anemia following single agent imatinib therapy.²⁸

Juvenile Myelomonocytic Leukemia

Juvenile myelomonocytic leukemia (JMML) is a rare, aggressive MDS/MPN entity of childhood characterized by an abnormal proliferation of monocytic and granulocytic lineages involving the BM, PB, and usually the liver and spleen. There is PB leukocytosis with circulating immature myeloid precursors and monocytosis. Patients characteristically present with lymphadenopathy, hepatosplenomegaly, and skin rash, and there is elevation of Hemoglobin F levels.³⁸ The disease is closely associated with mutations of genes of the RAS/MAP-kinase pathway, and is markedly increased in incidence in children with neurofibromatosis type-1 (NF-1) and Noonan syndrome.

The karyotype of JMML is normal in about 65% of cases. Monosomy 7 is present in about 26% of cases, usually alone, but in combination with other abnormalities in about one-fifth of the cases. Other cytogenetic abnormalities, including +8, +13, +21, and additional material on 7q or 12p are present in 10% of cases.³⁹ Monosomy 7 may occur together with RAS/MAP-kinase genetic lesions (discussed later) and, unlike

its adverse prognostic significance in adult and pediatric MDS, does not appear to stratify JMML patients prognostically or clinically.⁴⁰ For these reasons, cases previously designated as “infant monosomy 7 syndrome” are classified as JMML, provided that they fulfill the diagnostic criteria.³⁸ In one series in which JMML and pediatric MDS patients were combined, complex karyotype conferred an inferior prognosis, similar to adult MDS however, the incidence and potential prognostic impact of complex karyotype in JMML patients is not specifically known.⁴¹

Myeloid progenitors from JMML patients show marked hypersensitivity to granulocyte–monocyte colony-stimulating factor (GM-CSF), due to constitutive activation of the RAS/MAP-kinase signaling cascade that regulates the response of cells to GM-CSF (Figure 32.1) (reviewed in Niemeyer⁴²). For example, an activating point mutation in NRAS or KRAS (similar to that identified in many CMML cases, as described previously) is present in about 25% of JMML cases.⁴³ Other genetic abnormalities in the RAS/MAP-kinase pathway in JMML cases include activating point mutations in PTPN11 (that encodes the tyrosine phosphatase SHP2) and truncating inactivating mutations in NF1 (that encodes the negative regulatory protein neurofibrin).^{44,45} In contrast, no mutations have been found in BRAF, another downstream effector of RAS,⁴⁶ nor have mutations in the GM-CSF receptor gene itself been identified in JMML.⁴⁷ The specific genetic lesions affecting the RAS/MAP-kinase GM-CSF signal transduction pathway appear to be mutually exclusive, and altogether are present in 65–75% of JMML cases.⁴⁸ While JMML bears some clinical similarities to pediatric MDS cases, hypermethylation of the cell cycle regulatory genes p15 and p16, commonly present in pediatric MDS cases, is rare in JMML cases; thus, unlike pediatric MDS, aberrant DNA methylation may not be an important pathogenetic mechanism in JMML.⁴⁹ Activating FLT3 mutations, that characterize many myeloid malignancies, have not been identified in JMML cases.^{50,51}

As expected from the panmyelosis that characterizes JMML, X-chromosome inactivation and RAS mutational analysis have shown clonal involvement of erythroid as well as myeloid lineages. Some JMML cases show involvement of the B-cell lineage, further supported by the evolution of some JMML cases B-acute lymphoblastic leukemia, and even those of T-cell and NK cell lineage.^{43,52,53} The pluripotent nature of the JMML stem cell is further supported by the observation that T and NK-cells derived from JMML stem cells engraft in NOD/SCID mice.⁵⁴

Eleven percent of all JMML patients carry a diagnosis of neurofibromatosis type I, an autosomal dominant condition caused by congenital germ-line mutations of NF1.⁴² Subsequent loss of the normal NF1 allele is thought to underlie the development of tumors in these patients; specifically, loss of the normal NF1 allele with duplication of the abnormal allele by mitotic recombination (uniparental disomy) has been identified in JMML cells from patients with NF1.⁵⁵

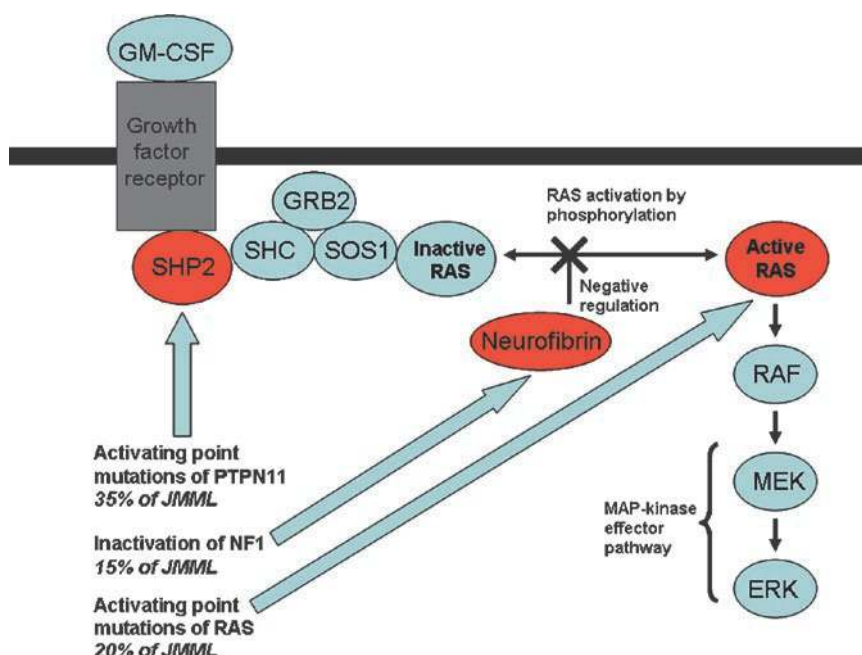


FIG. 32.1. Genetic lesions in JMML and their effects on the RAS pathway. These mutations promote constitutive activation of RAS and underlie the hypersensitivity of JMML cells to GM-CSF stimulation.

The median age of presentation of JMML is under 2 years, often preceding diagnostic clinical manifestations of NF1.³⁹ Thus, JMML may be the presenting feature of neurofibromatosis and this disease may be under-recognized in JMML patients. The latter is supported by the identification of point mutations in the NF1 gene in nonleukemic tissues from JMML patients not known to have NF1.⁴⁵ JMML is also associated with Noonan syndrome, a congenital disorder caused by a germ-line mutation in the PTPN11 gene.⁵⁶

The close association of aberration of a specific signaling pathway (i.e., GM-CSF–RAS–MAP-kinase) in JMML suggests the potential effectiveness of targeted therapy for this often aggressive disease. Such biological approaches have included inhibition of GM-CSF, farnesyltransferase inhibitors that affect posttranslational processing of RAS, the bisphosphonate zoledronic acid that inhibits activation of RAS, enzymatic depletion of downstream kinase Raf-1, and negative regulation of GM-CSF using the SHP1 protein (reviewed in Koike⁴⁸). While these agents have shown variable inhibition of JMML cell growth in vitro, the clinical efficacy of these therapies has yet to be fully evaluated.

Refractory Anemia with Ring Sideroblasts and Marked Thrombocytosis

Refractory anemia with ringed sideroblasts associated with marked thrombocytosis (RARS-T) is a provisional entity manifesting as ineffective erythropoiesis with anemia, erythroid dysplasia, and ring sideroblasts (>15% of

all erythroid precursors) combined with the proliferative features of thrombocytosis ($>450 \times 10^9/L$) and abnormal, enlarged megakaryocytes, resembling those of essential thrombocythemia. Patients may present with clinical sequelae of thrombocytosis, anemia, or both.

Myelodysplastic syndromes with isolated del(5q), inv(3)(q21q26), or t(3;3)(q21;q26) may also have ring sideroblasts and thrombocytosis and must be excluded by cytogenetic and/or FISH analysis before making a diagnosis of RARS-T.

Based on reported series, about 21% of RARS-T cases show karyotypic abnormalities, with isolated +8 characterizing almost half of the cytogenetically abnormal cases; other reported abnormalities reported in single cases include del(11q), del(7q), del(12p), and inv(10). A complex karyotype was noted in 3/12 karyotypically abnormal cases and included del(5q) in two of these three cases, suggesting possible progression from MDS with isolated del(5q).^{57–60}

The JAK2 V617F point mutation has been found in approximately 60% of RARS-T patients.^{57,59,61,62} Unlike cases of essential thrombocythemia, in which the JAK2 mutation is usually present in only a single allele, many cases of RARS-T show an allelic ratio consistent with a homozygous JAK2 mutation.⁶³ As with MPN cases bearing the JAK2 mutation, RARS-T cases show aberrant staining of megakaryocyte nuclei with phospho-STAT5 immunohistochemistry, indicating aberrant STAT5 activation resulting from the activating JAK2 mutation.^{59,64} RARS-T cases with JAK2 mutation have a more favorable prognosis than cases lacking the mutation.⁶³ Mutated cases also have higher platelet, erythrocyte, and white blood counts and megakaryocyte

morphology more resembling MPN megakaryocytes than cases lacking the mutation, suggesting that heterogeneous molecular mechanisms may underlie this disease.^{60,63,65} The combination of both abnormal morphology and phospho-STAT5 staining in megakaryocytes and observed abnormal erythroid colony formation in vitro in RARS-T suggest that this disorder originates from a myeloid stem cell.^{59,61}

Imatinib mesylate has shown some therapeutic benefit in JAK2-mutated MPN,²⁸ and has recently been shown to reduce the platelet count of an RARS-T patient with JAK2 V617F mutation⁶⁶; it is unclear if this activity is related to imatinib inhibition of the mutated JAK2 kinase or effects on another tyrosine kinase.

Other Unclassifiable Myelodysplastic/Myeloproliferative Neoplasms

Occasional myeloid neoplasms manifesting with both dysplastic features (ineffective hemopoiesis and/or dysplasia of one or more lineages) and proliferative features (typically one or more elevated counts) are not classifiable in any of the previously cited entities. Most commonly, these cases present with anemia and thrombocytosis ($>450 \times 10^9/L$) and/or leukocytosis ($>13 \times 10^9/L$).

Aside from failing to fulfill the morphologic criteria for any MDS/MPN, MPN, or MDS entities, cytogenetics, FISH, and/or molecular studies should be performed to exclude the presence of isolated del(5q), inv(3), t(3;3), BCR-ABL, or translocations involving the PDGFRA, PDGFRB, or FGFR1 genes. The incidence of JAK2 mutations or cytogenetic abnormalities in this group of cases is unknown; it is controversial whether myeloid neoplasms with isolated del(5q) and a concomitant JAK2 V617F mutation, which often have more prominent thrombocytosis and higher white blood count than typical MDS with isolated del(5q), should be considered as variants of MDS or unclassifiable myelodysplastic/myeloproliferative neoplasms.⁶⁷

Myeloid and Lymphoid Neoplasms Associated with Eosinophilia and Abnormalities of PDGFRA, PDGFRB, and FGFR1 Rearrangement: General Aspects

The updated 2008 World Health Organization (WHO) Classification created a new category of myeloid neoplasms characterized by eosinophilia and expression of fusion genes involving specific tyrosine kinases and apparently originating from a pluripotent (lymphoid and myeloid) stem cell. Although each entity exhibits a clinical and pathologic spectrum, these entities are defined by their specific genetic

lesion, akin to the use of BCR-ABL to define CML irrespective of its clinical/pathologic presentation. This approach underscores the increasing reliance of classification on genetic (rather than clinical, morphologic, or immunophenotypic) features, particularly when a therapeutic agent targeting the specific genetic lesion is available. Nevertheless, recognition of the presenting clinical and pathologic spectrum of these entities is important, as the diagnostician must be aware of the scenarios in which testing for PDGFRA, PDGFRB, and/or FGFR1 rearrangement is appropriate. The main differential diagnosis with these entities is chronic eosinophilic leukemia (CEL), an MPN characterized by autonomous, clonal proliferation of eosinophil precursors. Although there is no specific cytogenetic or molecular genetic abnormality that has been identified in CEL, the following rearrangements must be absent to establish a diagnosis of CEL: BCR-ABL1, t(5;12)(q31-35p13) or other PDGFRB rearrangement, or a PDGFRA, and FGFR1 rearrangement or the finding of a recurring karyotypic abnormality that is usually observed in myeloid disorders (i.e., trisomy 8 or isochromosome 17q) supports a diagnosis of CEL. It should also be recognized that occasional CEL patients may have a JAK2 mutation (discussed in Chap. 31), and X-linked polymorphism analysis may be used in female patients to establish clonality.

Myeloid and Lymphoid Neoplasms Associated with PDGFRA Rearrangement

Myeloid and lymphoid neoplasms associated with PDGFRA rearrangement most commonly presents as an eosinophilic leukemia (PB eosinophil count $>1.5 \times 10^9/L$), but may occasionally present as an acute myeloid or precursor T-lymphoblastic leukemia.⁶⁸ The BM is usually hypercellular with a prominent proliferation of eosinophils; mast cells are also increased in most cases and usually are scattered, or form loose aggregates rather than the large, cohesive clusters of mast cells that characterize systemic mastocytosis.⁶⁹ Serum tryptase is elevated (>12 ng/ml) in nearly all patients. There is no BCR-ABL fusion and the karyotype is normal in most cases; the PDGFRA gene at 4q12 is fused with another partner gene and.⁷⁰ In the vast majority of cases, the fusion protein FIP1L1-PDGFR results from a cytogenetically cryptic interstitial deletion at chromosome 4q12.⁷⁰ This rearrangement generates a constitutively active tyrosine kinase, that induces IL-3 independent growth in cell lines in vitro and causes a myeloproliferative disease in a murine model (although characterized by a pan-granulocytic hyperplasia as opposed to the striking eosinophilia present in the human disease).⁷¹ Although cytogenetically cryptic, this genetic lesion may be detected by RT-PCR spanning the two genes, or by FISH detecting the commonly deleted CHIC2 site at 4q12 or fusion of the FIP1L1 and PDGFRA genes.⁷⁰ Using purified cells from affected patients, the FIP1L1-PDGFR fusion has been detected in eosinophils, neutrophils, monocytes,

mast cells, T-cells, and B-cells, proving the pluripotent stem cell origin of this disease.⁷² Distinction from systemic mastocytosis may be difficult in some cases, although in the latter disease the mast cells usually form tight and large, rather than loose, smaller aggregates of mast cells. Immunophenotyping of the mast cells is not helpful, as FIP1L1–PDGFRA neoplasms may demonstrate an aberrant CD25+ mast cell phenotype.⁶⁹ However, unlike systemic mastocytosis, FIP1L1–PDGFRA neoplasms do not show a D816V mutation in the C-KIT gene.⁶⁹ Thus, in cases with both BM mast cell infiltrates and eosinophilia, screening for both C-KIT mutation and PDGFRA translocations is recommended to distinguish between the two entities.

Subsequent to the initial report of FIP1L1–PDGFRA fusion in myeloid neoplasms with eosinophilia, a number of other partner genes fusing with and similarly constitutively activating PDGFRA have been recognized. These include the following: STRN–PDGFRA, resulting from a t(2;4)(p24;q12); ETV6–PDGFRA, resulting from a t(4;12)(q24;p13)⁷³; and KIF5B–PDGFRA⁷⁴ and CDK5RAP2–PDGFRA, resulting from an ins(9;4)(q33;q12q25) abnormality.⁷⁵ Rare cases previously reported as aCML bear a t(4;22) translocation, resulting in BCR–PDGFRA fusion.^{76,77} FISH is the most common test used to detect the FIP1L1–PDGFRA fusion. Use of RT-PCR spanning the translocation may be insensitive, due to widespread breakpoints in FIP1L1 and complex alternative splicing of the fusion product. RT-PCR detecting overexpression of PDGFRA mRNA may represent a useful screening test, as this technique will pick up overexpressed PDGFRA, due to variant translocations other than FIP1L1–PDGFRA and will not be affected by the breakpoint loci.⁷⁴

Imatinib is an inhibitor of PDGFRA as well as ABL, and all thus these diseases, responds to targeted therapy with imatinib. Complete molecular remission may be achieved and the use of imatinib and other tyrosine kinase inhibitors has dramatically improved the outcome of this disease.^{68,70} Analogous to point mutations that confer imatinib-resistance in CML, the T674I point mutation in the ATP binding site of the PDGFRA portion of the fusion protein may result in imatinib resistance; however, this may be overcome by newer generation tyrosine-kinase inhibitors, such as sorafenib.⁷⁸ FIP1L1–PDGFRA is inhibited by imatinib as well as newer-generation tyrosine kinase inhibitors at lower doses than is BCR–ABL, allowing the use of lower doses than are used to treat CML.⁷⁸

Myeloid Neoplasms Associated with PDGFRB Rearrangement

Myeloid neoplasms associated with PDGFRB rearrangement usually present with prominent eosinophilia accompanied by neutrophilia, monocytosis, and a hypercellular bone marrow with increased eosinophils and myeloid hyperplasia.

Mast cells may be increased similar to myeloid neoplasms with PDGFRA rearrangement.^{79,80} Due to the monocytosis and neutrophilia, these cases were previously often classified as CMML or aCML or (in children) JMML, in spite of the nearly universal eosinophilia. The defining genetic feature of this disease is fusion of the PDGFRB gene at 5q31–33 with another partner gene. The PDGFRB catalytic tyrosine kinase domain is retained in the resulting fusion protein and is activated constitutively, most likely by dimerization via protein–protein interaction motifs such as coiled-coil or ankyrin domains.⁸⁰ Most cases have ETV6–PDGFRB fusion, due to a t(5;12)(q31–33;p12),⁸¹ that may be detected by conventional cytogenetics or FISH. RT-PCR can also detect most cases, as the vast majority have conserved breakpoints at exon 4 of ETV6 and exon 11 of PDGFRB; however, rare variant fusions may occur outside these breakpoints and may be missed by standard primers directed to these exons.⁷³ Moreover, since the original description, at least 15 other partner fusion genes have been reported (reviewed in Bain⁸²), as well as fusions of PDGFRB with unknown partner genes. Fortunately, conventional cytogenetics may pick up these variant translocations, since cryptic translocations involving PDGFRB have not yet been described. FISH break-apart probe to PDGFRB may be used if conventional cytogenetics fails or is not available.⁸³

Like myeloid neoplasms with PDGFRA rearrangement, myeloid neoplasms with PDGFRB rearrangement are sensitive to imatinib investigation for involvement of the PDGFRB gene should be performed on all myeloid neoplasms bearing a genetic lesion at 5q31–33, particularly if there is monocytosis and/or eosinophilia. One caveat is that some t(5;12)(p31;p13) genetic lesions in MPN do not result in detectable ETV6–PDGFRB fusion, but rather cause upregulation of the IL3 gene located at 5q31 close to ETV6.⁸⁴ These diseases may be distinguished from “true” ETV6–PDGFRB fusion by RT-PCR spanning known breakpoints of these genes.⁸⁵ MPN with t(5;12) cytogenetic abnormalities that lack an ETV6–PDGFRB fusion product do not respond to imatinib and should not be classified with this entity.

Myeloid and Lymphoid Neoplasms Associated with FGFR1 Rearrangement

Myeloid and lymphoid neoplasms associated with FGFR1 rearrangement constitute another group of pluripotent stem cell disorders with particular propensity to manifest as neoplasia in both myeloid and lymphoid lines, and has been referred to as “stem cell leukemia/lymphoma syndrome”. Patients may present with leukocytosis and eosinophilia resembling an eosinophilic MPN, as a T-cell or (less commonly) B-cell lymphoblastic lymphoma/leukemia, or both simultaneously. Patients may also present as (or subsequently transform to) AML.⁸⁶ The disease is defined by a translocation involving the

FGFR1 gene at 8p11. The most common partner is ZNF198, resulting in a translocation t(8;13)(p11;q12) and a ZNF198–FGFR1 fusion product that contains both zinc-finger motifs of ZNF198 and the catalytic kinase domain of FGFR1 and that relocates to the cytoplasm.⁸⁷ Interestingly, the reciprocal FGFR1–ZNF198 fusion transcript is also expressed; however, based on murine models, this product does not appear to contribute to the neoplastic transformation.⁸⁸ Similar to other tyrosine–kinase fusion proteins in myeloid neoplasms, the ZNF198 partner contributes a proline-rich oligomerization domain that activates FGFR1 by self-association of the fusion protein. In contrast, the zinc finger motifs do not appear to be required for neoplastic transformation.^{89,90} Numerous variants have also been described, all of which encode a constitutively activated FGFR1 tyrosine kinase.^{86,91} Particular features of specific translocations include basophilia associated with t(8;22)(p11;q11) and BCR–FGFR1 fusion and polycythemia associated with t(6;8)(p27;p11–12) and FGFR1OP1–FGFR1 fusion.⁹¹ The FGFR1 translocation may be identified in both the BM eosinophilic proliferation as well as the lymphoblastic lymphoma, indicating common derivation of both neoplastic proliferations from a single transformed stem cell (Figure 32.2a–d). Murine models of FGFR1-translocated neoplasms demonstrate both abnormal myeloid proliferations characterized by extramedullary myeloid infiltrates as well as primitive T-cell lymphomas, mimicking the human disease.⁸⁸

Unfortunately, imatinib does not inhibit activated FGFR1 kinase and is not effective in this disease. The small-molecule tyrosine kinase inhibitor PKC412 inhibits the activity of ZNF198–FGFR1 *in vitro*, inhibits the proliferation of ZNF198–FGFR1 transformed cells, and prolongs survival in a murine model of FGFR1 neoplasia. Preliminary data has shown some response in one affected patient treated with PKC412.⁹⁰ Other possible approaches include targeting downstream effectors of FGFR1; ZNF198–FGFR1 activates the AKT and MAP-kinase prosurvival signaling pathways that prevent apoptosis. Competitive inhibition of 14-3-3 phosphoserine/threonine-binding proteins that function in these pathways induced apoptosis in ZNF198–FGFR1 transformed cells *in vitro*.⁹² However, at the current time, unlike myeloid neoplasms with PDGFRB and PDGFRA rearrangement, targeted therapies for myeloid neoplasms with FGFR1 rearrangement have not yet been validated in large numbers of patients, and their potential effectiveness is unknown. Cytotoxic therapy is generally not effective and myeloid neoplasms with FGFR1 rearrangement carry a poor prognosis.

Mastocytosis

Mastocytosis encompasses neoplastic proliferations of mast cells, including cutaneous mastocytosis, extracutaneous mastocytoma, systemic mastocytosis (SM), mast cell

leukemia, and mast cell sarcoma. A somatic mutation in KIT is present in the neoplastic mast cells in about 95% of adult SM cases.^{93,94} The KIT gene encodes a tyrosine kinase receptor for stem cell factor that is expressed on hematopoietic progenitor cells as well as normal mast cells.⁹⁵ The most common mutation is a point mutation at codon 816 in exon 17 (D816V) that results in constitutive ligand-independent activation of the KIT kinase. This mutation also results in resistance to the effects of imatinib, which can inhibit the activity of the wild-type KIT kinase; recent data suggest that the tyrosine kinase inhibitor PKC412 may be effective in treating SM with mutated KIT.⁹⁶ A small percentage of SM may also bear other activating point mutations of exon 17. The D816V mutation is less frequent in cutaneous mastocytosis cases and other exon 17 mutations appear to be more common.⁹⁴ SM may occur in association with hematological clonal non-mast cell disorders (AHNMD), most commonly CMML, AML, and other myeloid neoplasms.⁹⁷ In such cases, genetic abnormalities characteristic of the AHNMD (such as AML-related translocations or a JAK2 mutation) may be present in addition to the D816V.⁹⁸ Interestingly, eosinophils in SM cases lacking AHNMD have been shown to bear the D816V mutation, suggesting that at least some cases of SM may derive from a more undifferentiated hematopoietic precursor.⁹⁹ PB and BM eosinophilia are commonly associated with SM and important entities in the differential diagnosis are the myeloid and lymphoid neoplasms with eosinophilia and tyrosine kinase gene rearrangements, particularly FIP1L1–PDGFRA. As discussed previously, this neoplasm characteristically has elevated serum tryptase and increased BM mast cells, but at lower levels than SM. Moreover, unlike SM, the BM mast cells in the FIP1L1–PDGFRA neoplasm do not form large, compact aggregates and show less prominent cytologic atypia; however, they may aberrantly express CD2 and/or CD25.^{68,100} Distinction between these two diseases is critical, as they have differing clinical courses and, unlike FIP1L1–PDGFRA disease, SM does not respond to imatinib.

Mast cell leukemia is a rare mast cell neoplasm that involves the PB and BM. The mast cells are markedly atypical, with hypogranulation, nuclear folding or bilobation, and prominent nucleoli, resembling blasts and potentially causing confusion with some subtypes of AML.¹⁰¹ The genetics of mast cell leukemia are not well characterized, but at least some cases bear a D816V mutation, similar to SM.¹⁰¹

Summary

The diagnostic genetic features of these previously discussed entities are summarized in Table 32.1.

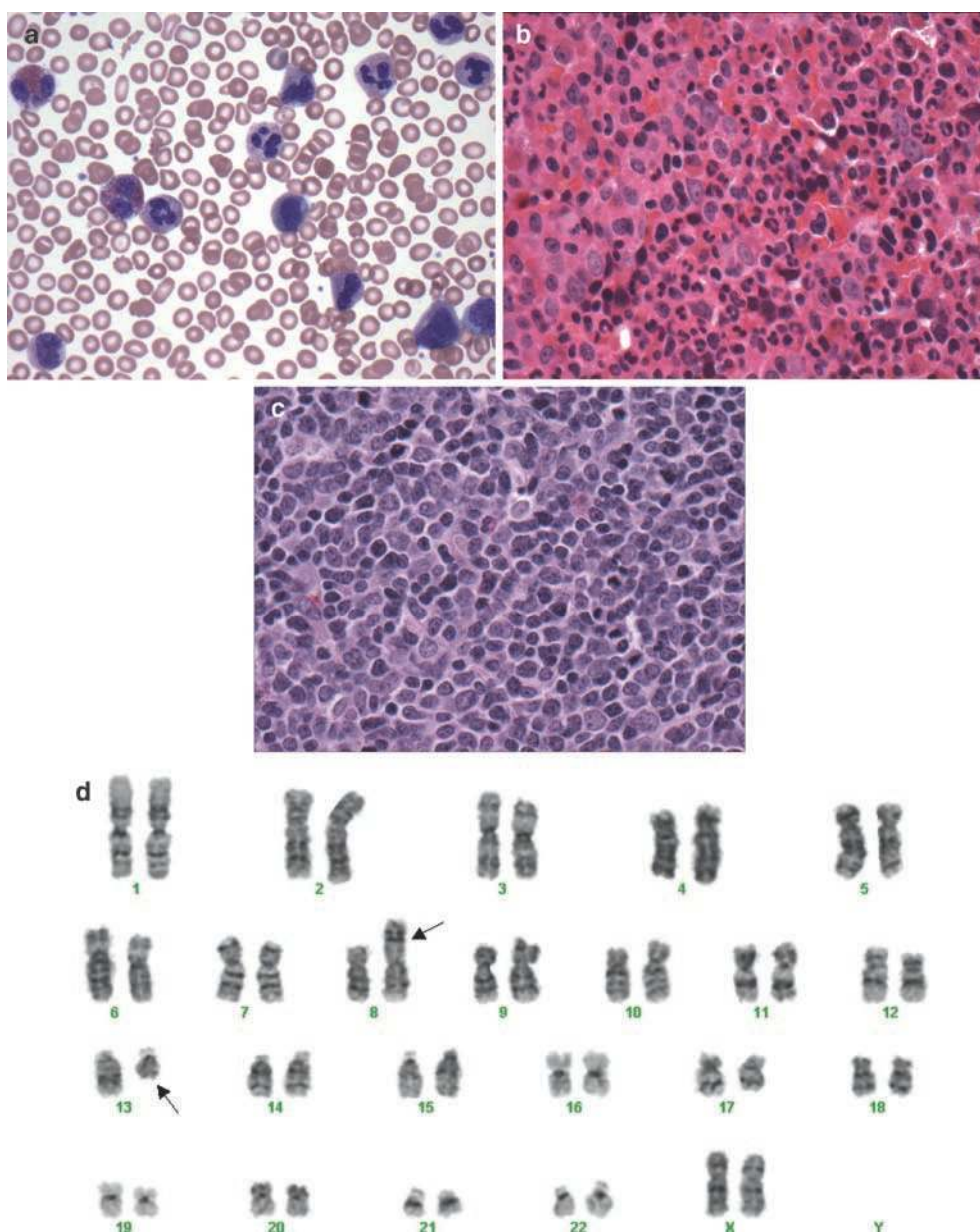


FIG. 32.2. Myeloid and lymphoid neoplasm associated with FGFR1 rearrangement. (a) The peripheral blood smear shows leukocytosis with immature myeloid elements and eosinophilia (Wright-Giemsa stain). (b) The bone marrow biopsy specimen is markedly hypercellular with a marked myeloid hyperplasia and increased eosinophilic forms. There is no increase in myeloblasts and no lymphoblast population was identified by flow cytometry (H&E stain).

(c) Concurrent inguinal lymph node biopsy shows a precursor T-lymphoblastic lymphoma; scattered mature eosinophils are present and can represent a clue to the diagnosis (H&E stain). (d) Karyotype from both the bone marrow and the lymph node reveals an identical 48, XX, t(8;13)(p12;q12) karyotype (ZNF198–FGFR1 fusion), indicating the clonal relationship of the chronic myeloproliferative process and the T-cell lymphoma.

TABLE 32.1. Diagnostic Genetic Features of Myelodysplastic/Myeloproliferative Neoplasms and Related Entities.

Disease	Important genetic features
Chronic myelomonocytic leukemia	Absence of BCR–ABL1 rearrangement Absence of PDGFRB rearrangement (especially in cases with eosinophilia)
Atypical chronic myeloid leukemia, BCR–ABL1 negative	Absence of BCR–ABL1 rearrangement
Juvenile myelomonocytic leukemia	Absence of BCR–ABL1 rearrangement Monosomy 7 and/or mutations in RAS–MAP-kinase pathway common
Refractory anemia with ring sideroblasts and marked thrombocytosis (provisional entity)	Absence of isolated del(5q), inv(3)(q21q26) or t(3;3)(q21;q26) cytogenetic abnormalities JAK2 V617F mutation in ~60% of cases
Myelodysplastic/myeloproliferative neoplasm, unclassifiable	Absence of BCR–ABL1, FGFR1, PDGFRA, and PDGFRB rearrangements. Absence of isolated del(5q), inv(3)(q21q26), and t(3;3)(q21;q26) cytogenetic abnormalities
Chronic eosinophilic leukemia	Absence of BCR–ABL1, FGFR1, PDGFRA, and PDGFRB rearrangements Trisomy 8 and/or iso(17q) may be encountered Occasional presence of JAK2 ^{V617F} mutation X-linked polymorphism analysis supports clonality in female patients
Myeloid and lymphoid neoplasms with eosinophilia associated with PDGFRA rearrangement	Absence of BCR–ABL1 rearrangement Rearrangement of PDGFRA, usually with FIP1L1 gene partner (cytogenetically cryptic).
Myeloid neoplasms with eosinophilia associated with PDGFRB rearrangement	Absence of BCR–ABL1 rearrangement Rearrangement of PDGFRB, usually with ETV6 gene partner resulting in t(5;12) translocation
Myeloid and lymphoid neoplasms with eosinophilia associated with FGFR1 rearrangement	Absence of BCR–ABL1 rearrangement Rearrangement of FGFR1, usually with ZNF198 gene partner resulting in t(8;13) translocation
Systemic mastocytosis	C-KIT D816V point mutation

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Molecular Pathogenesis of Myelodysplastic Syndromes

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Introduction

Myelodysplastic syndromes (MDSs) are a collection of clonal stem cell hematopoietic disorders that are characterized by ineffective hematopoiesis, multilineage dysplasia, peripheral cytopenias, and susceptibility to leukemic transformation. The complexity of the biologic, clinical, morphologic, and genetic features of MDSs has led to evolving classification systems, including the French–American–British (FAB) classification, which was introduced in 1982, and the subsequent World Health Organization (WHO) classification (1999), which was most recently updated in 2008. Although some of the revisions to the FAB classification remain controversial, the WHO recommendations for MDS classification continue to gain increasing acceptance.¹ Table 33.1 presents the current 2008 WHO classification and criteria for MDSs² along with the corresponding FAB designations. Common presenting symptoms include fatigue, infection, pallor, bruising, and/or bleeding; however, patients may also be asymptomatic at diagnosis. Adverse outcomes arising from MDSs include bleeding, anemia, infection, and progression to acute myeloid leukemia (AML), which is often refractory to standard treatments.

The onset of MDSs may be primary/de novo or therapy-related. De novo or primary MDSs predominantly affect older patients, with 60–70 years being the median age of onset. Although the precise epidemiologic statistics of de novo MDSs are not known, it is estimated that its annual incidence is approximately 20 per 100,000 in individuals over 70 years of age, and approximately 3 per 100,000 in the general population.³ MDSs have also been documented in younger patients at a lower incidence.^{4–6} Possible predisposing factors for de novo MDSs include viruses, occupational exposure to benzene, pesticides, and other cytotoxic agents or environmental carcinogens. Therapy-related MDSs arise in patients with a known exposure to radiation therapy and/or chemotherapeutic agents. Although the incidence of therapy-related MDSs remains unknown, it may represent as many as 10–15% of all the AML and MDS cases diagnosed each year.³

Currently, the diagnosis and classification of MDSs is primarily based on a combination of clinical findings, peripheral blood (PB) cell counts, and morphologic examination of PB and bone marrow (BM). Additional studies including cytogenetic analysis, immunophenotyping, molecular genetic tests, and in vitro colony growth assays have also been applied to aid in the diagnosis of MDS.¹

For prognostic stratification of MDS, two systems have been proposed and commonly used: the International Prognostic Scoring System (IPSS) and the WHO Classification-Based Prognostic Scoring System (WPSS) (Table 33.2). The IPSS defines 4 distinctive risk groups with varying probability for transformation to acute leukemia and survival based on the percentage of BM blasts, degree of cytopenia, and chromosomal pattern. Based on the prognostic model proposed by Greenberg et al.,⁷ the median overall survival times for patients with MDS based on IPSS risk categories are as follows: low risk, approximately 5.7 years; intermediate 1 risk, approximately 3.5 years; intermediate 2 risk, approximately 1.2 years; and high risk, approximately 0.4 years. A WHO classification-based prognostic scoring system (WPSS) has also been described recently and validated in untreated patients.⁸ This scoring system is based on the WHO subgroups (i.e., RA/RARS/5q–, RCMD/RCMD-RS, RAEB-1, and RAEB-2), karyotypic abnormalities categorized according to IPSS, and red blood cell transfusion requirements.⁸ It identifies five risk groups of MDS patients with differences in risks of leukemic progression and survival (median survival from 12 to 103 months, depending on risk groups) (Table 33.2).^{8,9}

It is postulated that the development of MDSs occurs through multiple evolutionary stages. The initial genetic insult to hematopoietic stem cells initiates an emergence of an aberrant clone that exhibits morphologic dysplasia, disparate proliferative advantage, and cellular dysfunction. The promotion of genomic instability and heightened susceptibility to acquisition of additional genetic lesions is thought to be a result of the initiating clonal defect. Ensuing evolution of the mutant clone is associated with progressive cellular dysfunction, as well as ineffective hematopoiesis characterized by excessive apoptosis.

TABLE 33.1. WHO classification for MDS.

MDS subtype (WHO)	Peripheral blood	Bone marrow	FAB subtype
Refractory cytopenias with unilineage dysplasia (RCUD), Refractory anemia (RA); Refractory neutropenia (RN); Refractory thrombocytopenia (RT)	Unicytopenia or bicytopenia No or rare blasts (<1%)	Unilineage dysplasia: ≥10% of the cells in one myeloid lineage <5% blasts <15% ringed sideroblasts	RA
Refractory anemia with ring sideroblasts (RARS)	Anemia No blasts	≥15% ringed sideroblasts Isolated erythroid dysplasia <5% blasts	RARS
Refractory cytopenia with multilineage dysplasia (RCMD)	Cytopenia(s) No or rare blasts (<1%) No Auer rods <1 × 10 ⁹ /L monocytes	Dysplasia in ≥10% of the cells in ≥2 myeloid lines <5% blasts No Auer rods ±15% ring sideroblasts	RA
Refractory anemia with excess blasts-1 (RAEB-1)	Cytopenia(s) <5% blasts No Auer rods <1 × 10 ⁹ /L monocytes	Unilineage or multilineage dysplasia No Auer rods 5–9% blasts	RAEB
Refractory anemia with excess blasts-2 (RAEB-2)	Cytopenia(s) 5–19% blasts Auer rods ±<1 × 10 ⁹ /L monocytes	Unilineage or multilineage dysplasia Auer rods ±10–19% blasts	RAEB
Myelodysplastic syndrome-unclassified (MDS-U)	Cytopenias ≤1% blasts	Unequivocal dysplasia in less than 10% of cells in one or more myeloid cell lines when accompanied by a cytogenetic abnormality considered as presumptive evidence for a diagnosis of MDS <5% blasts	–
MDS associated with isolated del(5q)	Anemia Usually normal or increased platelet count No or rare blasts (<1%)	Normal to increased megakaryocytes with hypoblasted nuclei <5% blasts Isolated del(5q) No Auer rods	RA

TABLE 33.2. International prognostic scoring system (IPSS) and WHO classification-based prognostic scoring system (WPSS) FOR MDS.

Scoring system		Score variable					
IPSS	Prognostic variable	0	0.5	1.0	1.5	2.0	3.0
	Marrow blast %	<5	5–10	–	11–20	21–30	–
	Karyotype ^a	Good	Intermediate	Poor	–	–	–
	Cytopenia (s) ^b	0–1	2–3	–	–	–	–
WPSS	WHO category	RA, RARS, 5q-	–	RCMD, RCMD-RS	–	RAEB-1	RAEB-2
	Karyotype	Good	–	Intermediate	–	Poor	–1
	Transfusion requirement	No	–	Regular	–	–	–
Risk category	Very low	Low	Intermediate 1	Intermediate	Intermediate 2	High	Very high
IPSS score	–	0	0.5–1.0	–	1.5–2.0	≥2.5	–
WPSS score	0	1	–	2	–	3–4	5–6
Median survival years							
IPSS	–	5.7	3.5	–	1.2	0.4	–
WPSS	8.5	6	–	3.3	–	1.75	1

MDS myelodysplastic syndrome, RA refractory anemia, RARS refractory anemia with ringed sideroblasts, 5q- myelodysplastic syndrome with isolated del(5q) and marrow blasts less than 5%, RCMD refractory cytopenia with multilineage dysplasia, RCMD-RS refractory cytopenia with multilineage dysplasia and ringed sideroblasts, RAEB-1 refractory anemia with excess of blasts-1, RAEB-2 refractory anemia with excess of blasts-2.

^aKaryotype was as follows: good: normal, -Y, del(5q), del(20q); poor: complex (≥3 abnormalities), chromosome 7 anomalies; and intermediate: other abnormalities.

^bNeutropenia indicates neutrophil level less than 1,800/μL; anemia, hemoglobin level less than 10 g/dL (<6.21 mm/L); and thrombocytopenia; platelet level less than 100,000/μL.

RBC transfusion dependency was defined as having at least one RBC transfusion every 8 weeks over a period of 4 months.

Adapted from ref. ^{7,8}.

The assemblage of multiple alterations that affect cell cycle regulation, transcription factors, tumor suppressors, and growth factor receptors allow these clonal cells to further expand with abnormal maturation and increased apoptotic rates, leading to ineffective marrow hematopoiesis and peripheral cytopenias.

The specific pathogenesis of MDSs continues to be investigated as its complex biologic features are further understood.

Morphologic dysplasia is not completely specific for MDS, particularly for low-grade MDSs, such as refractory anemia and refractory anemia with ringed sideroblasts. The dysplastic

morphology may be seen in other conditions, including megaloblastic anemia, congenital dyserythropoietic anemia, exposure to toxins (such as arsenic), as well as other conditions.¹⁰ As a result, causes of secondary dysplasia must be considered and excluded before making a diagnosis of MDS. In addition, a small number of dysplastic erythroid, granulocytic, or megakaryocytic cells may be seen in the BM of normal individuals.¹¹ Thus, a minimum of 10% of the cells in a lineage should be dysplastic in order to consider the lineage as dysplastic and evidence for MDS.¹⁰ Overall, an accurate diagnosis of low-grade MDS requires integration of clinicopathologic findings.

MDS Associated with Recurrent Cytogenetic Abnormalities and Molecular Genetic Lesions

Chromosomal abnormalities may be identified in approximately 30–50% of primary MDSs and in over 80% of secondary/therapy-related MDSs.¹² Common recurrent chromosomal lesions associated with MDSs include del(5q), –7/del(7q), trisomy 8, del(20q) and –Y. Complete or partial deletion of chromosome 5 is reported in 10–15% of primary MDSs and 50% of secondary MDSs.^{13,14}

The best characterized chromosomal abnormality is the isolated del(5q) chromosome abnormality, which is commonly referred to as 5q-syndrome. The cytogenetic abnormality involved in this syndrome is an interstitial deletion within chromosome 5 [5q31–5q33].¹ The most common clinical features are associated with refractory macrocytic anemia and normal to mildly increased platelet counts, occurring most commonly in middle-aged to older women. The number of blasts in the BM is usually less than 5%. Atypical megakaryocytes with mono/hypolobated nuclei, as well as erythroid hypoplasia are commonly observed.^{3,15} Recently, it was found that partial loss of function of the ribosomal subunit protein RPS14 phenocopies (i.e., mimics as a photocopy) 5q- disease in normal hematopoietic progenitor cells and that forced expression of RPS14 rescues the disease phenotype in patient-derived BM cells.¹⁶ These findings suggest that 5q- syndrome is caused by a defect in ribosomal protein function.¹⁶ Furthermore, this group identified a block in the processing of pre-ribosomal RNA in RPS14-deficient cells that is functionally equal to the defect in Diamond-Blackfan anemia, linking the molecular pathophysiology of 5q- syndrome to a congenital syndrome that leads to BM failure.¹⁶ The isolated interstitial deletion of 5q has been categorized as “good risk” according to the IPSS and WPSS.

Lenalidomide, a nonteratogenic analog of thalidomide has recently been approved by the US Food and Drug Administration (FDA) for treatment of patients with 5q- syndrome.^{17,18} The first clinical trial reporting on the safety and efficacy of lenalidomide in MDS included 43 patients with symptomatic anemia who were poor candidates for benefit from erythropoietin

(EPO) therapy, or who had failed treatment with EPO.¹⁹ Using the International Working Group (IWG) criteria, 56% of patients experienced an erythroid response and 20 of 32 patients who previously required RBC support became transfusion-independent.²⁰ Ten of 12 (83%) patients with an isolated interstitial deletion of chromosome 5(5q31.1) experienced an erythroid response, as compared to 57% who had a normal karyotype and 12% who had another chromosomal abnormality.²⁰ In general, patients with 5q31.1 also were found to have longer duration of transfusion independence and 75% of patients with 5q31.1 were found to experience a complete cytogenetic remission.²⁰

Abnormalities involving chromosome 20q interstitial deletions and clonal loss of the Y chromosome are also classified as “good risk,” while abnormalities of chromosome 7 are classified as “poor risk” according to the IPSS and WPSS. Abnormalities involving chromosome 7 have been seen in increased frequency in the sixth decade. Localized to 7q22 and 7q32–34, the critical deletions involving the long arm of chromosome 7 have been associated with tumor suppressor genes.^{21,22} MDS with monosomy 7 has been described as having a median survival of 9 months with the rate of evolution to leukemia being 33% of cases (if 7q- is the only anomaly) and 71% (if other chromosomal abnormalities are also present).²³ In 10% of primary MDS cases, 7q- has been reported,²³ while in secondary MDS, structural/numerical deletions of chromosome 7 have been reported in 40% of cases.¹⁵ Del(20q) occurs less frequently, and is characterized by low progression to AML and a long survival median of 42 months.²³ The most common deleted region is located between 20q1.2 and 20q13.2, with the critical deleted region being reported to extend between D20S174 and D20S75 in MDS.^{24,25} Loss of the Y chromosome has been observed in 7.7% of healthy elderly men, 10.7% of MDS cases, and 3.7% of AML cases.²⁶

Other recurrent, but less frequent, cytogenetic abnormalities observed in MDSs include trisomy 8, isochromosome 17q, del(12q), and abnormalities of chromosome 1q, 3q21, and 11q23. Trisomy 8 been associated with an increased risk of transformation to AML, and is predominantly seen in males. Isolated isochromosome (17q) has also been associated with rapid transformation to leukemia.¹⁵ Del(17p) is associated with TP53 mutation and unfavorable clinical outcome.³ Del(12q) when occurring as the sole lesion has been affiliated with an indolent disease course. The ETV6 gene located at 12p13 is often involved in translocations in this region. Abnormalities of chromosome 1q, 3q21, and 11q23 are associated with an overall inferior survival.¹⁵ Overexpression of the MDS1/EVI1 gene has been associated with 3q21 translocations, while 11q23 translocations are involved in the rearrangement of the MLL gene.¹⁵ Isodicentric X is another chromosomal abnormality reported in low frequency in MDS. It is seen exclusively in elderly females and characterized by BM iron accumulation.^{15,27}

Point mutation of RAS family proto-oncogenes represent one of the most frequently reported molecular abnormalities in MDS (see Molecular Pathways Involved in MDS section).

Other reported molecular genetic changes include p15 promoter hypermethylation, p53 mutations, and FLT3 duplications, which are all less frequent.²⁸ All of these abnormalities have been associated with progression of MDS to AML, but are nonspecific for MDS.^{15,28} Point mutations involving RUNX1/AML have been linked with more advanced forms of MDS, as well as increased progression to overt leukemia.^{29,30} Furthermore, some specific gene mutations have been associated with specific MDS phenotypes. An example is the mutation affecting the ATRX gene at Xq13.1, which is associated with acquired α -thalassemia arising in the setting of MDS.^{31,32}

Gene Expression Profiling Findings in MDS

The application of cDNA microarray technology to evaluate RNA expression profiles has facilitated the identification of several previously unidentified genes associated with MDS.¹ These genes include PF4, COX2, BIRC5, NAIP, IEX1, PLAB, BRCA2, P21, SPHAR, RAD17, CDC25, CDK2, VCAM1, AXL, PRAME, BMI1, RAB20, FOS, CCNE1, IGFR1, V-ABL, RAD51C, ZNF261, ZNF26, TPO, GATA2, CD14, ACPL, ZNF183, ILFR, HOXA9, GSTT1, DLK1, ZNF183, ARG1, DEFA 4, CD 18, as well as others.^{1,28,33–36} It was also found that a subset of genes are more commonly associated with 5q syndrome, including the upregulation of CREEM, CYLD, and RB1CC1, and the downregulation of ATOX1 and CRSP9.²⁸ Additional gene abnormalities identified in the context of specific cytogenetic abnormalities are discussed in the previous section (MDS Associated with Recurrent Cytogenetic Abnormalities).

MicroRNAs (miRNAs) are nonprotein-coding RNAs composed of 20–24 nucleotides that regulate posttranscriptional gene expression through the destabilization of target transcripts or the inhibition of protein translation.³⁷ More than 230 miRNAs have been identified in mammals with approximately 10% having identified functional attributes, which include signal pathway (i.e., ras signaling) and potent antioncogenic effects.³⁸ In a recent study, 33 miRNAs were expressed in CD34+ hematopoietic stem-progenitor cells (HSPCs) from normal human BM and mobilized human PB stem cell harvests.³⁹ The findings in the study showed that miRNA-17, -24, -146, -155, -128, and -181 may block the differentiation of early hematopoietic cells to more mature cells, that miRNA-155 is a potential potent inhibitor of HSPC differentiation, and that miRNA-221, and -222 may have critical roles in erythroid development, while miRNA-223 may be involved in granulocytic development.³⁹ To the best of our knowledge, no specific miRNA expression profile for MDS has been reported.

In a study conducted by Aivado et al, surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) was used to establish a serum proteome

profile for patients with MDS and patients with conditions resembling MDS (non-MDS cytopenia).^{40,41} A profile that predicts MDS with an accuracy of 80% was identified and validated.⁴⁰ In addition, two separate chemokine (CXC) ligands, CXCL4 and CXCL7, were identified using tandem MS as two potential proteins that may serve as biomarkers to distinguish not only between MDS and non-MDS anemias, but also to differentiate among the different MDS subtypes, being that their levels were significantly decreased in the serum of MDS patients with advanced disease.^{40–42}

Single Nucleotide Polymorphisms Profiling Findings in MDS

Recent advances in microarray technology have led to the study of single nucleotide polymorphisms (SNPs) for high-resolution genome-wide genotyping. Loss of heterozygosity (LOH) may result from copy number (CN) imbalances or uniparental disomy (UPD), which pertains to the acquisition of a duplicated copy of an entire or partial chromosome through mitotic recombination without net loss or gain of genetic material. Segmental acquired UPD has been found to be present in 20% of MDS cases. While metaphase cytogenetics (MC) may detect chromosomal aberrations in approximately 50% of MDS patients, the higher resolution of SNP analysis allows for the detection of smaller, previously cryptic deletions and duplications that may not be detected by MC.⁴³

In a recent study, SNP analysis not only confirmed the lesions identified by MC, but also identified new previously cryptic defects, including defects in 3p14.2, 8q24.23, and 14q11.2.⁴⁴ In this study conducted by Gondek et al,⁴⁴ the most frequent lesions detected by SNP analysis involved chromosomes 8, 7, 5, and 11, with 16, 9, 8, and 6 lesions identified, respectively. Segmental LOH (due to acquired UPD) was present in 33% (24 of 72 cases) of MDS patients.⁴⁴ In addition, LOH was distributed across all WHO subtypes with UPD being identified in 47% of patients with RA/RARS (7 of 15) versus 26% (5 of 19) with RCMD/RCMD-RS.⁴⁴ The chromosomes most frequently associated with gains included chromosome 8, while the chromosomes most frequently showing losses included chromosomes 1, 5, 7, and 11.⁴⁴ Defects identified that were not previously described in association with MDSs included alterations on chromosome 1p31.1, add(2)(q37), and del(14)(q11).⁴⁴ In a study conducted by Mohamedali et al,⁴⁵ the most prominent region for UPD was chromosome 4q in patients with RCMD and normal cytogenetics (i.e., RCMDnc), RARS, and RAEB. In a study by Huang et al,⁴⁶ flow cytometry sorting was performed to sort MDS BM samples into blastic, erythroid, immature myeloid, and lymphoid fractions, and genotyping was performed using 250 K NspI SNP-microarray chips. In this study, the authors concluded that their utilization of a lineage-based analysis showed that different chromosomal regions of UPD, deletions and/or gains are present in different lineages in MDS.⁴⁶

These findings suggested that multiple distinct clones may coexist in different lineages in MDS, and may contribute to cytopenias in specific lineages and the heterogeneous clinical manifestations seen in MDS patients.⁴⁶

It is postulated that SNPs array results play a significant role in the prognosis stratification of MDSs. Studies have shown that 68% of patients with normal MC were found to have new chromosomal lesions by SNP analysis.⁴⁴ According to the study by Mohamedali et al,⁴⁵ SNP microarray results were shown to correlate with IPSS score in cytogenetically normal cases. As a result of this, it is hypothesized that the use of molecular methods for the detection of genomic abnormalities may serve as a reliable addendum to the prognosticators in MDS.

Cytokine Profiling Findings in MDS

Immune dysregulation involving proinflammatory or immunoregulatory molecules has been suggested as having a pathophysiologic role in the hematopoietic insufficiency and BM failure characteristic of MDSs. Multiple cytokines with proapoptotic properties, including interleukin (IL) 1 β , tumor necrosis factor (TNF)- α , p 38 mitogen activated protein kinase (MAPK), Fas ligand (L), and tumor growth factor (TGF)- β 1, have been shown to be upregulated in patients with MDSs.⁴⁷ In addition, cytokine-related signaling molecules are also reportedly involved in the processes leading to ineffective hematopoiesis in MDS and include TNF-related signaling molecules, such as TNF-Related Apoptosis Inducing Ligand (TRAIL).⁴⁷

TNF- α is one of the most well-characterized immunomodulatory cytokines shown to express strong inhibitory activity in hematopoiesis.^{48–50} TNF- α has been implicated in altering the expression of related negative regulatory cytokines, such as interferon (INF)- γ , INF- γ receptor, and Fas in hematopoietic cells.⁵¹ Furthermore, elevated TNF- α levels have been associated with apoptotic death in MDSs.^{51–55} As the disease progresses, the malignant clones in some patients acquire resistance to the proapoptotic effects exerted by TNF and other signals leading to unregulated proliferation, and transformation into the more aggressive forms of MDS.⁵⁶ Several studies report upregulation of TNF- α in the stroma and in BM cells of MDS patients,^{53,57–61} with the highest TNF- α positivity being linked with RA⁵⁸ and advanced MDS subtypes (i.e., RAEB-2) and in AML transformed from MDS (i.e., RAEB-in transformation: RAEB-IT, FAB subtype).⁶² In a recent study, TNF was shown to decrease the mRNA expression of antiapoptotic genes (i.e., BCL2L1), to increase the mRNA expression of pro-apoptotic genes (i.e., BID), and to increase the mRNA expression of pro-inflammatory cytokines (i.e., IL6, IL8, and IL32), which in combination suggest that TNF is involved in the regulation of pro-inflammatory and pro-apoptotic signals in stromal cells that promote apoptosis in malignant myeloid clones.⁵⁶ Of interest, Powers et al were able to demonstrate

an association between single nucleotide polymorphisms of TNF- α and TGF- β and the MDS phenotype in a recent study.⁶³ It was reported that the -308A and -238A polymorphism in TNF- α and the polymorphisms that encode a leucine at amino acid 10 and an arginine at amino acid 25 in TGF β are associated with increased expression and a possible susceptibility to MDS.⁶³

It has been reported that anti-TNF therapies produce improvement in selected patients with MDS.⁵⁶ However, numerous signals induced by TNF may be responsible for its role in the pathogenesis of MDS, and many of these pathways may also be activated by a number of other signals. As a result, a single therapy targeted against TNF may not properly counteract the numerous signals involved in this process.

P 38 mitogen activated kinase (p38 MAPK) is a member of the MAPK family that is involved in the activation of transcription factors, regulation of cytokine production, cell differentiation, apoptosis, and cell death.^{64–67} In normal human hematopoiesis, its primary function is the mediation of proapoptotic and growth inhibitory signals. In a study conducted by Munir et al, phosphorylated p38 MAPK expression was found to be significantly increased in MDS, suggesting that the p38 MAPK signaling pathway may have an important role in the pathogenesis of MDS.⁶⁸

TRAIL is a member of the TNF superfamily of cytokines that has been shown to selectively affect erythroid development by specifically targeting immature erythroblasts,^{69–72} in part by exerting an antidifferentiative activity on erythroid maturation.⁷¹ In a recent study, TRAIL expression was shown to be upregulated in myeloid precursors of granulocytic lineage and in a subset of monocytes and pro-erythroblasts in MDS patients.⁴⁷ In addition, upregulation and increased release of the TRAIL protein at the BM level was found to correlate positively with the degree of anemia and thus hypothesized to be involved in the impairment of erythropoiesis.⁴⁷ Of interest is that endogenous TRAIL was predominantly observed in early disease (RA and RARS), which are characterized by a lower blast count while the RAEB-IT (FAB subtype) group as well as the AML-transformed MDS were negative for TRAIL release.⁴⁷ These findings are compatible with the postulated protective role of TRAIL against blast cell population expansion.⁷³

Molecular Pathways Involved in MDS

Oncogenes

The *RAS* gene superfamily is associated with the regulation of signal transduction, cellular proliferation, and maintenance of the malignant phenotype. When activated by ligands, such as tyrosine kinases, *RAS* proteins function in intracellular signaling pathways to promote cellular proliferation and differentiation.¹ *Ras* oncogene mutations in initial or advanced stages of MDSs may contribute to the pathogenesis of the disease by stimulating early progressive clonal expansion

or late leukemic transformation.^{74–76} N-ras mutations have been linked with decreased survival and higher incidences of leukemic progression.^{74,77} In addition to RAS, the FMS gene region has also been shown to be associated with the pathogenesis of hematopoietic diseases, including MDSs.^{21,78–80} The FMS gene promotes the proliferation and differentiation of hematopoietic cells of the monocyte–macrophage series.^{81,82} It has been observed that point mutation at codon 969 occurs with increased incidence in more advanced MDS subtypes.^{78,83} In addition, overexpression of the proto-oncogene EVI-1 has been documented in AML transformed from MDSs.^{84–86}

Cell Cycle Regulatory Genes

Dysregulation through the cell cycle may contribute to the pathogenesis of MDSs. Inactivation of gene p15 (INK4B) by methylation has been observed in the hematopoietic progenitor cells in MDSs and may be associated with disease progression and leukemic transformation.^{87–89} Additional mutations, including CHK2, p53, and MLL, have also been implicated in cell cycle abnormalities that may contribute to the pathogenetic processes in MDSs.^{90–93}

Apoptotic Genes

Gene products for bcl-2, c-myc, and p53 have been implicated as key regulators in the apoptotic pathway. Increased bcl-2 expression has been associated with MDS progression, with the highest bcl-2 over-expression occurring in more advanced subtypes (RAEB and RAEB-IT by FAB classification).^{94–96} C-myc and p53 also show abnormal expression in hematopoietic cells of MDSs. Increased levels of c-myc have been reported in MDS subtypes RA and RARS,⁹⁷ while enhanced p53 expression has been associated with MDS evolution to leukemia.^{95,98}

Growth Factor and Angiogenesis Genes

Reported regulators of angiogenesis include vascular endothelial growth factor (VEGF), blastic fibroblast growth factor, angiogenin, angiotropin, angiopoietin-1, platelet-derived growth factor, hepatocyte growth factor, EGF, TNF- α , TGF- α , TNF- β , IL-1, IL-6, IL-8, and granulocyte-macrophage CSF.^{99–103} Increased angiogenesis has been observed in MDSs,^{104–106} and found to be associated with elevated levels of basic fibroblast growth factor, VEGF, and hepatocyte growth factor.¹⁰⁷ Advantageous influence of the tumor microenvironment is supported by the overexpression of specific angiogenic mediators in hematopoietic cells.^{106,108}

Receptor Tyrosine Kinase Genes

The FLT3 gene encodes for a class III receptor tyrosine kinase that is preferentially expressed on hematopoietic progenitor

cells. An internal tandem duplication of this gene (FLT-ITD) has been observed in approximately 3% of patients with MDS and 20% of patients with AML, and it is thought that this mutation is associated with poor prognosis or increased incidence of leukemic transformation in MDSs.^{109,110} Another mutation within the activation loop of FLT3 has been identified and is referred to as D835. This D835 mutation has been reported in about 3% of patients with MDSs.¹¹¹

Epigenomic Changes in MDS

Epigenetics is defined as heritable biochemical modifications in chromatin that do not alter the primary sequence of DNA. Epigenetic regulation of gene promoter regions is critical for gene expression control and may involve DNA methylation or posttranslational modifications of histone proteins. Epigenomic processes (involving the transcriptional silencing of genes necessary for the proliferation and differentiation of hematopoietic cells) are thought to contribute to the leukemogenic event underlying MDSs.¹¹²

Methylation

The process of DNA methylation is catalyzed by DNA methyltransferases (DNMTs), and involves the addition of a methyl group to the 5' carbon position of the cytosine ring in the palindrome CpG dinucleotide rich areas, known as CpG islands. These heavily enriched CpG dinucleotide areas are predominantly located in the promoter regions, and the methylation of cytosine within these regions is a major epigenetic mechanism of transcriptional control that is dysregulated in human cancer.¹¹² The role of methylation in relation to cancer has been linked to an aberrant promoter hypermethylation, which leads to transcriptional silencing of known or candidate tumor suppressor genes.¹¹³

Genes vital for carcinogenic pathways are often hypermethylated in MDS. Of the well studied tumor suppressor genes involved in MDSs, p15 (CDKN2B), a cyclin-dependent kinase inhibitor, has been found to be hypermethylated in 40–80% of MDSs.¹¹⁴ In addition, hypermethylation of the suppressor gene E-cadherin (CDH1) has been found in approximately one-third of MDS samples. Furthermore, DNMTs that mediate DNA methylation have been shown to be overexpressed in MDS samples, when compared to controls.¹¹⁵

Histone Deacetylation

In addition to methylation, histone deacetylation has also been found to play a vital role in the epigenomic changes associated with MDSs. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) regulate histone acetylation, which facilitates active gene transcription. HATs are involved in the activation of transcription through their ability to mediate the acetylation of lysine residues on the N-terminus of histones.

In contrast to HATs, HDACs facilitate deacetylation of lysine residues on histones. This deacetylation is associated with the repressive state of chromatin, which is characterized by gene silencing.¹¹² Studies have shown that leukemia- or lymphoma-associated oncogenic proteins, such as AML1-ETO, PML-RAR α , and BCL-6 have been found to recruit HDACs to regulatory DNA sequences and inhibit DNA access to activation transcription factors.¹⁴ Defects in HDAC or HAT that affect normal acetylation have been identified in MDS. An example of the importance of this in regards to the epigenomics of MDSs may be appreciated by noting that the suppression of α catenin (CTNNA 1) expression by histone deacetylation contributes to the growth advantage in human MDS or AML with del (5p).¹¹⁶

Targeted Therapies

The epigenomic changes associated with MDS are of particular interest, because increased understanding of them has led to the development of new targeted therapies that may reactivate tumor suppressor genes that have been epigenetically silenced. Although several epigenetic alterations, including histone methylation, have been considered as targets for therapy, DNA methyltransferases and histone deacetylases are the two most studied drug targets for epigenetic inhibition to date.¹¹⁷

At present, two DNA hypomethylating agents have been approved by the US Food and Drug Administration (FDA) for use in patients with MDSs: 5-azacitidine (azacitidine) and 5-aza-2'-deoxycytidine (decitabine). Azacitidine is FDA approved for use in patients with the following forms of MDS: RA or RARS (if accompanied by neutropenia or thrombocytopenia, or requiring transfusions), RAEB, RAEB-IT (FAB subtype), and chronic myelomonocytic leukemia (CMML).^{118,119} Decitabine is FDA approved for the treatment of patients with previously treated, untreated, de novo, and secondary MDS of all FAB subtypes, including chronic myelomonocytic leukemia, and patients with intermediate-1 or higher risk as assessed by the International Prognostic Score.¹²⁰ Azacitidine and decitabine are categorized as pyrimidine nucleoside analogs of cytidine that are phosphorylated upon uptake into the cell and incorporated into DNA, where they irreversibly bind to DNMTs, inhibit their function, and lead to the progressive loss of methylation, which restores the function of the tumor suppressor genes.¹⁴ Of note is that decitabine is incorporated into replicating DNA only, while azacitidine is incorporated into RNA as well as DNA by the action of ribonucleotide reductase. These therapeutic agents have been shown to produce a clinical response in AML and MDS patients in vivo, and to cause growth inhibition and differentiation of transformed myeloid cell lines and primary leukemic blasts in vitro.¹²¹

HDAC inhibition has been hypothesized to restore normal acetylation of histone proteins and to be of benefit in the treatment of MDSs. Studies have shown that HDAC inhibition results in hyperacetylation of the histone tail

lysine residues, which allow the chromatin to convert to a transcriptionally active state and thus the reexpression of silenced genes. Various compounds from both synthetic and natural sources have been found to have an inhibitory effect on HDACs. Histone deacetylase inhibitors (HDACIs) may be divided into four chemical classes: (1) short chain fatty acids, which include valproic acid; (2) hydroxamic acid derivatives, which include suberoylanilide hydroxamic acid (SAHA) and LBH589; (3) benzamides, which include MS-275 and MGCD0103; and (4) cyclic peptides, which include desipeptide.¹¹² Several of these HDACIs have been tested in clinical trials as single agents in patients with MDS or AML, including phenylbutyrate, valproic acid, desipeptide, MS-275, and MGCD0103, some of which resulted in complete remission.¹⁴ Of these, valproic acid, a fatty acid chain derivative with HDAC inhibitory activity, has been most studied.¹²⁰ While most HDACIs have equal inhibitory effects on all HDACs, some have been found to work preferentially on class I versus class II HDACs. Despite this, there is no definitive evidence that distinct HDACs have a defined role in cancer, and it is thought that all HDACIs have similar patterns of cellular response.¹¹²

Combination strategies associated with the use of a hypomethylating agent and an HDAC inhibitor have been found to synergize effectively in the reactivation of epigenetically silenced genes.¹²⁰ Although it is unclear whether HDAC inhibitors contribute to the efficacy of DNA hypomethylating agents, the concept seems promising, and combination studies with both agent types are ongoing.

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Acute Myeloid Leukemias with Recurrent Cytogenetic Abnormalities

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Introduction

Acute leukemias are clonal malignant disorders resulting from genetic alterations in hematopoietic stem cells that limit the ability of stem cells to differentiate into red cells, granulocytes, and platelets, and lead to the proliferation of abnormal leukemic cells or “blasts.”¹ Acute myeloid leukemias (AML), also referred to as acute nonlymphocytic leukemias, are heterogeneous disorders.^{2,3} The current World Health Organization (WHO) classification scheme of acute myeloid leukemia and myelodysplastic syndrome (MDS) has evolved away from the French–American–British (FAB) classification scheme, which only uses morphologic features for classifying those neoplasms. The current WHO classification scheme includes not only morphologic features, but also clinical, immunophenotypic, and cytogenetic features.^{2–4} The current 2008 WHO classification includes four main categories of AML: AML with recurrent genetic abnormalities, AML with myelodysplasia-related changes, therapy-related myeloid neoplasms, and AML not otherwise specified (Table 34.1). The pathogenesis and underlying molecular processes substantially differ between each of these AML groups: AMLs with recurrent cytogenetic abnormalities are discussed in this chapter after a general introduction to the topic. However, AMLs with mutated *NPM1* and AMLs with mutated *CEBPA* will be discussed in Chap. 35, as will AMLs with normal cytogenetics. AMLs with myelodysplasia-related changes and therapy-related AMLs are discussed in Chap. 36.

Epidemiology

Approximately 11,000 new cases of AML are diagnosed annually in the United States, with an overall annual incidence of 3.4 new cases per 100,000 people.^{5,6} Although leukemia is the most common malignancy in children (≤ 15 years of age), most cases of leukemia occur in older adults, with a median age at presentation of 68 years.⁵ The incidence of AML is less than 1 per 100,000 for patients younger than

30 years and is 16–17 per 100,000 for patients aged 75 years or older.^{5,6} AML accounts for less than 15% of leukemia cases in children younger than 10 years of age and 25–30% of leukemia cases in children between 10 and 15 years of age^{5,7}; in contrast, AML accounts for 80–90% of acute leukemia cases in adults.⁵ It would appear that the incidence of leukemia, along with its precursor, myelodysplasia, is rising, particularly in patients older than 60 years.⁸ Several factors, including improved diagnosis capabilities and longer life expectancies, which increase a person’s exposure to environmental factors that may increase the risk of AML, are probably responsible for this increase.⁸

In adults, AML is by far the most common type of acute leukemia. The incidence of AML is higher in males than in females; in addition, the incidence of AML is higher in Caucasians^{5,9} and in populations of European descent than in African-Americans.⁹ Furthermore, acute promyelocytic leukemia (APL), a distinct subtype of AML, is more common among certain populations with a Hispanic background.¹⁰ An increased incidence of AML has also been found in patients with certain disorders with excessive chromatin fragility, such as Bloom syndrome, Fanconi anemia, and Kostmann syndrome, and in individuals with Wiskott–Aldrich or ataxia-telangiectasia syndrome. Other syndromes, such as Down (chromosome 21 trisomy), Klinefelter (XXY and variants), and Patau (chromosome 13 trisomy) have also been associated with AML.^{11,12} Survivors of the atomic bombs in Japan had an increased incidence of AML that peaked 5–7 years following the radiation exposure.^{13,14} Therapeutic radiation also appears to impart a minimal risk of developing AML, and this risk may increase, if the therapeutic radiation was administered concurrently with chemotherapy with an alkylating agent.

Clinical Features

The signs and symptoms seen in AML are a result of the proliferation of abnormal leukemic cells and their impact on normal hematopoiesis. For example, fatigue, bruising or

TABLE 34.1. 2008 WHO classification of acute myeloid leukemias.

AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); <i>RUNX1-RUNX1T1</i>
AML with inv(16)(p13.1;q22) or t(16;16)(p13.1;q22); <i>CBFB-MYH11</i>
Acute promyelocytic leukemia with t(15;17)(q22;q21); <i>PML-RARA</i>
AML with t(9;11)(p22;q23); <i>MLLT3-MLL</i>
AML with t(6;9)(p23;q34); <i>DEK-NUP214</i>
AML with inv(3)(q21;q26.2) or t(3;3)(q21;q26.2); <i>RPN1-EVII</i>
AML (megakaryoblastic) with t(1;22)(p13;q13); <i>RBM15-MKL1</i>
AML with mutated <i>NPM1</i>
AML with mutated <i>CEBPA</i>
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukemia, NOS
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic and monocytic leukemia
Acute erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis
Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm

WHO World Health Organization.

bleeding, fever, and infection are an indication of bone marrow (BM) failure. Although the term “leukemia” suggests marked elevations in white blood cell counts, pancytopenia is more common. More than 95% of patients in whom AML is diagnosed have hemoglobin levels less than 12 g/dl. Only 10% of patients with newly diagnosed AML present with a leukocyte count greater than 100,000/ μ L and are, therefore, at higher risk of tumor lysis syndrome and leukostasis.¹⁵ Tumor lysis syndrome may result from spontaneous or treatment-related cell destruction, and is characterized by hyperuricemia, renal failure, acidosis, hypocalcemia, and hyperphosphatemia.^{16,17} Leukostasis may manifest as dyspnea, chest pain, headaches, altered mental status, cranial nerve palsies, or priapism.^{18,19} Leukostasis and tumor lysis syndrome are both oncologic emergencies and require prompt recognition and management. Disseminated intravascular coagulation is another oncologic emergency and is most common in cases of AML with the translocation t(15;17).²⁰ Disseminated intravascular coagulation is typically seen at AML presentation or during induction chemotherapy^{21,22} and is manifested by diffuse oozing of blood with thrombocytopenia, hypofibrinogenemia, elevated fibrin split products, and deficiency in coagulation factors.²³ Other physical findings may include organomegaly, sternal tenderness, retinal hemorrhages; and infiltration of the gingivae, skin, soft tissues, or meninges (more common in AML with a monocytic component (i.e., AML, M4 or AML, M5)).²⁴

Myeloid sarcomas (MSs) occur in 2–14% of cases of AML, occurring more frequently in children than in adults.^{25–33} MS most often involves the soft tissues, skin, bone (including periosteum), lymph nodes, and/or the orbit and paranasal sinuses, but MS has been reported in virtually all anatomic locations.^{25–33} Testicular involvement is less common in AML than in acute lymphoblastic leukemia (ALL), occurring in 1–8% of cases.³³ The BM may be hypocellular and often, but not invariably, reveals increased blasts. MS may be present at diagnosis of AML, or may even precede the diagnosis. In children, the presence of MS involving sites other than the skin was an independent prognostic factor for AML, when compared to children with MS involving the skin or children without MS.²⁶ In adults, those with MS appeared to have worse outcomes than those without MS.³⁴

Examination of the Peripheral Blood and Bone Marrow

Morphologic Features

According to the FAB criteria, acute leukemia is diagnosed when a 200-cell differential reveals the presence of >30% blasts in a BM aspirate. The WHO classification criterion, assuming that any such criterion is arbitrary, changed this minimum criterion to 20%. In addition, patients with the clonal, recurring cytogenetic t(8;21)(q22;q22), inv(16)(p13;q22) or t(16;16)(p13;q22), and t(15;17)(q22;q21) abnormalities should be considered to have AML, regardless of the blast percentage. If the BM contains >50% normoblasts and pronormoblasts, the blast percent is based only on the nonerythroid cells. In these cases, the diagnosis is typically acute erythroid leukemia, which may be confirmed, if glycophorin A is expressed on the blasts' surface.

In AML, blasts are classified as myeloblasts, monoblasts, erythroblasts, or megakaryoblasts. Three major types of myeloid blasts have been reported in the literature. Type I myeloblasts have fine nuclear chromatin, 2–4 distinct nucleoli, and a moderate rim of pale to basophilic cytoplasm without azurophilic granules. Type II myeloblasts have nuclear and cytoplasmic features similar to type I myeloblasts, with the addition of up to 20 delicate azurophilic granules in the cytoplasm. Type III myeloblasts have numerous azurophilic granules in the cytoplasm. However, distinguishing between type III myeloblasts and promyelocytes is somewhat arbitrary and may even be impossible. One characteristic feature of myeloblasts in AML is the presence of Auer rods, which are seen in 60–70% of all cases and represent abnormal azurophilic granules. In some cases, leukemic cells contain numerous Auer rods and are referred to as “faggot cells.” The faggot cells are typical, but not unique, for cases of AML with the t(15;17)(q22;q21) abnormality. Long, slender Auer rods with tapered ends are typical for cases with the t(8;21)(q22;q22) abnormality.

Monoblasts have a round, sometimes folded nucleus, finely dispersed nuclear chromatin, variably prominent nucleoli, abundant, slightly basophilic cytoplasm with fine granulation, and occasional vacuoles. Erythroblasts have a round nucleus, slightly condensed nuclear chromatin, variably prominent nucleoli, and a moderate amount of deeply basophilic cytoplasm. Megakaryoblasts vary in their appearance from one case to another, ranging from undifferentiated blasts to mature-appearing megakaryocytes. In some cases of AML, the full range of megakaryoblasts is present, while in others there is a predominance of a particular type of megakaryoblast. Cells are moderately sized with dispersed nuclear chromatin, distinct nucleoli, and a moderate amount of light basophilic cytoplasm with very fine azurophilic granules frequently located in the perinuclear zone. The cytoplasm may be irregular and show pseudopod formation.

In some cases of AML, blasts exhibit any of several unusual morphological features. For example, marked nuclear lobulation is seen in cases with the t(15;17)(q22;q21) abnormality. Multinucleation is seen in many types of blasts, especially erythroblasts and megakaryoblasts. Pseudo-Chédiak-Higashi-type giant granules result from fusion of primary granules and are occasionally seen in leukemic blasts and granulocytic precursors, especially in cases with the t(8;21) abnormality. Erythrophagocytosis is seen most commonly in cases of AML with monocytic differentiation, especially in cases with the translocation t(8;16). Some myeloblasts have prominent vacuolization, with an appearance similar to the L3 blasts of ALL.

To fulfill the diagnostic criterion of 20% blasts established for AML, in some cases it is necessary to combine “real” blasts with so-called “blast equivalents”. The term “blast equivalent” is specific to a particular type of AML. For example, the neoplastic promyelocyte is considered a blast equivalent in cases with the t(15;17)(q22;q21) abnormality, or FAB M3 AML, and has an eccentric, often folded and lobulated nucleus with slightly condensed nuclear chromatin, intense cytoplasmic granularity, and an apparent Golgi zone. The promonocyte is considered a blast equivalent in cases of AML with monocytic differentiation.

Cytochemical Features

An important aspect in classifying AML is the cytochemical reactivity pattern of the leukemic blasts. Cytochemical stains include myeloperoxidase (MPO; or, to a lesser extent, Sudan black B [SBB]), nonspecific esterase (alpha-naphthyl butyrate or alpha-naphthyl acetate), and *p*-aminosalicylic acid (PAS). Some laboratories also use naphthol ASD chloroacetate esterase. If $\geq 3\%$ of the blasts stain positive for MPO or SBB, the diagnosis is AML. If the blasts are negative for MPO but positive for butyrate or nonspecific esterase, the diagnosis is acute monocytic leukemia (a variant of AML). A minority of MPO-negative and

SBB-negative cases are AML and may include minimally differentiated leukemia (M0), acute monocytic leukemias (M5), and megakaryocytic leukemias (M7), which require flow cytometry for characterization.³⁵

Immunophenotyping Using Flow Cytometry

Immunophenotyping using flow cytometry has dramatically improved the accuracy in diagnosing AML. It is strongly recommended, however, that the immunologic marker study results be interpreted in conjunction with the morphologic studies. Only the typical immunophenotypic features of AML and some of the pertinent information related to the differential diagnosis of AML are presented here.

Various T-cell, B-cell, and myeloid-associated markers are available for flow cytometric analysis. Table 34.2 summarizes the immunophenotypic profile of immature myeloid cells. The European Group for the Immunological Classification of Leukemias proposed a scoring system of lineage specificity for different markers in 1995 that was later revised in 1998.^{36,37} For instance, two markers, CD3 and cytoplasmic MPO, are highly specific for T-cell and myeloid cell lineages, respectively. The myeloid antigens are CD13, CD33, CD117, CD14, CD64 (the latter two are monocytic markers), glycophorin A (an erythroid marker), and CD41 (a megakaryocytic marker). Nevertheless, the specificity of these markers is not absolute.

TABLE 34.2. Immunophenotypic profiles of immature myeloid elements.

Cell type	Characteristic immunophenotypic features/ comments
Myeloblast	wCD45, CD34, CD33 ^a , CD13 ^a , vCD11c, CD4, HLA-DR, MPO
Promyelocyte	CD11c, CD13, CD15, CD33, CD45, MPO, loss of HLA-DR and acquisition of strong CD15 and CD11c associated with maturation. Gradual loss of CD33 also characterizes successive maturation stages.
Monoblast	wCD45, vCD34, CD33, CD15, CD13, CD11c, vCD4, HLA-DR
Promonocyte	HLA-DR, CD13, CD33, CD14, CD4, CD15, CD11c, CD45
Erythroblast	Glycophorin A, hemoglobin A, CD45
Megakaryoblast	HLA-DR, vCD34, CD41 ^b , CD13, vCD33, CD61, CD31. Progressive maturation characterized by loss of CD34 and acquisition of CD42 and von Willebrand factor.

MPO myeloperoxidase, v variable antigen expression, w weak antigen expression.

^aRare AML lacks surface CD13 and CD33.

^bFalse-positive CD41 expression by flow cytometry may be secondary to platelet adherence to the blasts.

For more details on flow cytometric immunophenotyping in leukemia, please refer to the appropriate review articles in the reference list.^{38–40}

Immunohistochemical Analyses

The antigen retrieval technique allows routinely fixed, paraffin-embedded BM biopsy sections to be used for antigenic characterization. When fresh BM aspirate and peripheral blood samples are not available, antigen retrieval is useful in classifying acute leukemias. The following markers are often included in the antigen retrieval panel:

- Progenitor-associated markers: CD34 and TdT;
- Myeloid-associated markers: MPO, lysozyme, CD117, CD68, CD15, and CD33;
- Erythroid-associated markers: hemoglobin A and glycophorin A;
- Megakaryocyte-associated markers: factor VIII, CD41, and CD61;
- T-cell-associated markers: CD3, CD5, CD4, and CD8; and
- B-cell-associated markers: CD20, CD22, CD79a, Pax-5, immunoglobulin kappa and lambda, and CD10.

In patients with MS without previous or concurrent AML, CD45 (LCA) antibodies are useful for establishing the hematopoietic origin of the tumor.

Electron Microscopy

With improvements in laboratory investigations, the role of electron microscopy has diminished. Demonstration of platelet peroxidase is useful in documenting the diagnosis of acute megakaryocytic leukemia (M7). Characteristically, the platelet peroxidase reaction product is deposited in the perinuclear envelope and endoplasmic reticulum, but not in the Golgi zone.

Cytogenetic and Molecular Markers

Table 34.3 summarizes the frequency of recurrent chromosomal abnormalities in pediatric and adult patients with AML.^{41–44} Molecular studies of many recurring cytogenetic

abnormalities have revealed genes that may be involved in leukemogenesis, which will be discussed in more detail below. Recently, new approach consolidating multiple assays and/or technologies (Cytogenetics, Fluorescence in-situ hybridization (FISH) reverse transcriptase polymerase chain reaction (RT-PCR), etc.) into a single test was developed by Asuragen, a molecular diagnostics company (Austin, TX, USA). Signature® LTx Leukemia Translocation Panel is capable to detect nine Translocations and their subtypes in a single test: t(9;22) (b2a2, b3a2, e1a2), t(1;19), t(12;21), t(4;11) (e10/e4, e9/e5), t(15;17) (long and short forms)m inv(16) (A and D types), and t(8;21).

Gene Expression Profiling

Oligonucleotide or cDNA microarray technology is an alternative to an extension of conventional karyotyping and fluorescence in situ hybridization (FISH) for diagnosing leukemia subtypes. Several groups have demonstrated that the sensitivity and specificity of microarray technology is as high as 100% for AML with recurring cytogenetic abnormalities and more than 90% for other types of AML.⁴⁵ It appears unlikely; however, that microarray technology will completely replace other techniques in the near future.

Prognostic Indicators

The prognosis for patients with AML depends on several parameters, including the patients' age, cytogenetic findings, preceding MDS, and/or exposure to chemotherapeutic agents, and coexisting morbidities. AMLs with the t(8;21), inv(16), or t(15;17) abnormalities are associated with a good prognosis, while AMLs with the 11q23 abnormality, a complex karyotype, preceding MDS, or previous chemotherapy are associated with a worse prognosis. Tools are becoming available to better determine prognosis in these types of AML. It has been established, for example, that patients who have a normal karyotype but duplication of *FLT3*^{46,47} or *MLL*^{48,49} have considerably poorer prognoses than those described previously. See Chap. 11 for a discussion of *FLT3*. The patients who have a normal karyotype, mutation in *CEBPA*, and no duplication of *FLT3* (approximately 10% of all patients with AML have a prognosis similar to that seen

TABLE 34.3. Recurrent cytogenetic abnormalities in children and adults with AML.^{41–44}

Abnormalities	Fusion genes	FAB subtype	Frequency	
			Children	Adults
t(8;21)(q22;q22)	<i>AML1-ETO</i>	M2/M1	10–15%	8–12%
inv(16)(p13;q22)	<i>CBFB-MYH11</i>	M4Eo	6–12%	8–12%
t(15;17)(q22;q21)	<i>PML-RARα</i>	M3/M3v	8–15%	8–10%
t(9;11)(p22;q23)	<i>MLLT3-MLL</i>	M4/M5	9–12%	2%
t(6;9)(p23;q34)	<i>DEK-NUP214</i>	M2–M5	1%	1%
inv(3)(q21;q26.2)	<i>RPN1-EVII</i>	M2–M5	NR	1–2%
t(1;22)(p13;q13)	<i>RBM15-MKLI</i>	M7	1%	NR

NR not reported.

in patients with core binding factor (CBF) AML.⁵⁰ In addition, an increased expression of *BAALC* has been shown to worsen prognosis in patients with a normal karyotype.⁵¹ Further identification of such genes will provide new therapeutic “targets” and refinement of prognoses. Once covariates such as those noted above are specified, neither the presence of dysplasia⁵² nor the morphologic diagnosis (i.e., FAB subtype) affects prognosis.⁵³

Molecular Abnormalities in AML

Approximately 50% of de novo AML have distinctive molecular abnormalities, most frequently chromosomal translocations.⁵⁴ These translocations typically involve genes that are involved in transcription and differentiation.⁵⁵ In most translocations, the genes are disrupted, and the 5' segment of one gene is joined to the 3' end of a second gene to form a novel fusion gene, from which chimeric mRNA is transcribed and protein is translated. Other translocations involve the juxtaposition of intact genes as well as inversions.^{54,55}

Traditionally, genes that are implicated in cancer pathogenesis are divided into tumor suppressor genes and proto-oncogenes. Proto-oncogenes are considered to play an important role in pathogenesis, having acquired constitutive activation through translocation or sporadic mutation, whereas tumor-suppressor genes are involved in pathogenesis through their inactivation of gene/protein function. Point mutations that cause missense or nonsense mutations, resulting in abnormal or truncated protein products, are a common mechanism of tumor-suppressor gene inactivation. Although some genes involved in leukemogenesis follow this paradigm (for example, the typical protooncogenes *HOX* and *EVI-1* and the typical tumor-suppressor genes *PU.1* and *RAR α*), some genes demonstrate overlapping features of both oncogenes and tumor-suppressor genes (such as *CBF*, *CEBPA*, *GATA-1*, and *NPM1*), suggesting that this dichotomy is an oversimplification.^{56–64}

Molecular Mechanisms of AML

A well-accepted concept states that for an AML to develop, at least two broad mutations are necessary, referred to as Class I and Class II mutations. Class I mutations, including *RAS* and *FLT3* mutations, activate signal-transduction pathways and confer a proliferation advantage to hematopoietic cells. Class II mutations complement Class I mutations and affect transcription factors and serve primarily to impair hematopoietic differentiation.⁶⁵

The *inv*(16) and *t*(8;21) abnormalities are Class II mutations and involve subunits of the transcription factor complex CBF. The *inv*(16) abnormality results in the fusion of *CBF β* (*CBFB*) gene on the q arm and the myosin heavy chain (*MYH11*) gene on the p arm. The *t*(8;21) translocation involves the *CBF α* (*CBFA*) gene at chromosome 21, called

the *AML1* (also *RUNX1*) gene, joining the *ETO* (*MTG8*) gene at chromosome 8⁶⁶. Like the *t*(15;17) gene product, the *AML1/ETO* protein acts to block transcription of *CBFA-CBFB*-controlled genes.

Another example of a Class II mutation is involvement of the myeloid-lymphoid (or *MLL*) gene located at chromosome 11q23. *MLL* has two regions that encompass multiple zinc fingers and at least two additional potential DNA-binding motifs. Abnormalities in *MLL* are relatively common in patients with AML who do not have 11q23 rearrangements on cytogenetic analysis. The *MLL* locus at 11q23 is involved in more than 30 leukemia-associated translocations.^{67,68} The most common translocation is *t*(4;11)(q21;q23), which gives rise to a chimeric *MLL/AF4* fusion gene. Patients with AML with 11q23 rearrangements generally have a poor prognosis.

The translocation *t*(15;17) does not fit readily into the Class I–Class II mutation concept, as it has features of both. The translocation *t*(15;17) encodes a chimeric protein, promyelocytic leukemia (PML)/retinoic acid receptor- α (*RAR α*), which is formed by the fusion of the *RAR α* gene from chromosome 17 and the *PML* gene from chromosome 15. The *RAR α* gene then encodes a member of the nuclear hormone receptor family of transcription factors. After binding retinoic acid, *RAR α* can promote the expression of a variety of genes. The translocation *t*(15;17) juxtaposes PML with *RAR α* in a head-to-tail configuration that is under the transcriptional control of PML. The PML-*RAR α* fusion protein tends to suppress gene transcription and blocks differentiation of the cells. However, pharmacologic doses of the *RAR α* ligand all-trans-retinoic acid (ATRA; or tretinoin) relieve this block and promote differentiation.

The presence of the translocation *t*(15;17) may also be detected by a unique immunofluorescence method using antibody specific for the PML protein. Normally, PML is compactly located at multiple nuclear domains, known as PML oncogenic domains (POD), whose structures are dynamically regulated and disrupted in *t*(15;17) cases.

While some hematologic malignancies appear to follow this scheme, there are too many exceptions to believe that this rule is universal for all hematologic malignancies. For example, in a recent study of 140 cases of therapy-related AML, 33 (26%) had evidence of Class I mutations, 47 (34%) had evidence of Class II mutations, and only 18 (13%) demonstrated both Class I and Class II mutations.⁶⁹

Besides conventional cytogenetic studies, FISH may be used to detect these abnormalities, as well as RT-PCR. Both techniques use archival formalin-fixed, paraffin-embedded tissue; however, FISH studies using paraffin-embedded tissue are more technically difficult, and the results are generally not straightforward enough to interpret. Please see the review articles listed in the references for more details.^{70–73}

A new concept of “gatekeeper genes” has been recently introduced in the study of hematologic malignancies. This concept was first established in solid tumors, such as colon

cancer, where inactivation of a particular pathway was the first genetic alteration in pathogenesis.⁷⁴ Several studies have suggested that the genetic alterations affecting hematopoietic transcription factors may be the initiating event resulting in the establishment of a malignant clone, but then secondary events are required for disease penetration. For example, MDS, which is known to precede AML, is often associated with *RUNX1* mutations, which might be an initiating event in AML.⁷⁵ Additionally, cytogenetic and genetic abnormalities identified in therapy-related MDS and AML might be initiating genetic events in leukemogenesis.⁷⁶ The observation of *RUNX1* and *CEBPA* mutations in hereditary AML syndromes and concordant AML induction in identical twin pairs, which share the initiating clone carrying the same translocation affecting *MLL*, also supports the idea of gatekeepers in leukemias.⁷⁷

Other molecular mechanisms have been implicated in leukemogenesis. For example, gene amplification may result in gene overexpression. In addition, numerical gains or losses of chromosomes, such as trisomy or monosomy as a result of nondisjunction, are detected in a large subset of acute and chronic leukemias. Gene deletions, such as those arising from partial chromosomal deletions or unbalanced translocations, may result in tumor-suppressor gene inactivation or loss. Hypermethylation is another mechanism of gene inactivation. Often more than one of these mechanisms is involved in leukemogenesis, leading to the accumulation of genetic lesions that culminates in leukemogenesis or subsequently contributes to disease progression.

AML with Recurrent Genetic Abnormalities

From the molecular mechanism viewpoint, cases of AML with recurrent genetic abnormalities represent biologically unique entities. For example, cases of therapy-related AML and AML with multilineage dysplasia have overtly overlapping biologic and cytogenetic features. The fourth category, which recapitulates the old morphological approach of FAB classification, clearly represents a heterogeneous group driven by a variety of different molecular alterations.

AML with t(8;21)(q22;q22.3); *RUNX1*–*RUNX1T1* (*AML1/ETO* or *RUNX1/MTG8*)

The translocation t(8;21)(q22;q22.3) has been identified in 8–12% of de novo AMLs.⁷⁸ Less commonly, the translocation t(8;21) has been identified in therapy-related AMLs and in MDS.⁷⁹ In adults, de novo AMLs with the translocation t(8;21) respond well to AraC-containing chemotherapy, with high rates of complete remission and relatively long survival times.⁸⁰ However, the presence of the translocation t(8;21) in pediatric patients with AML may be less predictive of a good prognosis.⁸¹

Morphologically, most cases of AML are classified as M2 using the FAB classification, and 30% of all M2 cases carry the translocation t(8;21). The myeloblasts in these leukemias typically have characteristic cytological features characterized by abundant evidence of maturation, including numerous, thin Auer rods and salmon-colored cytoplasmic granules.⁸²

The translocation t(8;21) is a reciprocal chromosomal translocation involving the *ETO* gene at chromosome 8 and the *AML1* (also known as *RUNX*) gene at chromosome 21.^{79,82,83} As a result of this translocation, *ETO* and *AML1* are disrupted and fused in a 5'→3' direction, forming an *AML1*–*ETO* fusion gene located on the derivative chromosome 8. The fusion protein includes the promoter and runt-like domains of the AML1 protein, and the translocation domain of normal *AML1* is replaced by sequences derived from *ETO*. A reciprocal *ETO*–*AML1* fusion gene located on the derivative chromosome 21 has not been identified.

One proposed mechanism for the role of *AML1*–*ETO* in leukemogenesis is competitive inhibition of the normal AML1 protein.⁸² The breakpoints in the *AML1* gene are consistently detected in intron 5. The breakpoints in the gene are also relatively uniform, in the 5' end of the *AML1* gene. *AML1* encodes for one of many members of a family of heterodimeric transcriptional regulatory proteins. *AML1* is highly homologous with the *Drosophila runt* gene, and encodes the α subunit of CBF, the human counterpart of the murine nuclear polyoma enhancer binding protein (PEBP2). The AML1 protein binding to DNA occurs via the runt-like central domain and requires heterodimerization with CBF. The latter protein does not bind to DNA but improves the binding affinity of *AML1*. *AML1* is normally expressed by myeloid and T-cells, which are thought to play an important role in hematopoietic differentiation.⁷⁹ *ETO* (also referred to in some studies as *CDR* or *MTG8*) encodes a transcription factor protein that has two zinc-finger-like motifs at its C-terminus. *ETO* is expressed in the brain, lungs, and gonads, but it is not normally expressed in hematopoietic cells.

A variety of methods are available to detect the translocation t(8;21). This translocation is not difficult to recognize using conventional cytogenetics, which detects the translocation t(8;21) in over 95% of AML cases.⁷⁹ However, rare cases have been reported, in which the translocation was not detected by cytogenetics but by other molecular methods.⁸⁴ For example, FISH may effectively identify the translocation t(8;21). In addition, RT-PCR methods detect almost all AML cases with the translocation t(8;21). Because of their extraordinary sensitivity, RT-PCR methods have also been used to monitor residual disease. However, initial studies with small numbers of patients have demonstrated the presence of *AML1*–*ETO* transcripts in patients who have achieved complete clinical remission after chemotherapy or BM transplantation and who have remained in complete remission with prolonged clinical follow-up.^{85,86} Thus, RT-PCR does not

appear to effectively predict risk of relapse after therapy in patients in clinical remission.^{85,86}

Cases with *AML1-ETO* transcripts appear to follow the "Class I and Class II mutation rule," as *AML1-ETO* fusion genes can be detected in long-term survivors who are presumably cured of their disease.⁶⁵ For example, "knock-in" mice expressing an *AML1-ETO* fusion gene do not develop leukemia until they are exposed to mutagens presumably causing mutations that complement *AML1-ETO*.⁸⁷ These findings support the hypothesis that a single genetic event is not sufficient to cause AML.

AML inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*

The inversion (16)(p13q22) and, more rarely, the translocation t(16;16)(p13;q22) have been identified in 5–12% of de novo AMLs.^{88,89} In most cases, the blasts have monocytic cytological features and are associated with immature eosinophils containing coarse basophilic cytoplasmic granules.^{89,90} The inv(16) has also been identified in rare cases of chronic myeloid leukemia (CML) in blast crisis.^{91,92}

The detection of the inv(16) in de novo AMLs is a favorable prognostic finding. Patients with AML with the inv(16) usually have higher rates of complete response to chemotherapy, longer durations of remission, and prolonged survival times than patients with other forms of AML.⁹³ The inv(16) involves the *CBF* (or *PEBP2*) β subunit gene located at chromosome 16q22 and the *MYH11* gene located at chromosome 16p13. The inversion results in a *CBFB-MYH11* fusion gene, from which chimeric mRNA and a novel protein are generated.⁹⁴ *CBFB* and *MYH11* are fused in a 5' → 3' direction and are transcribed in the centromeric to telomeric direction.⁹⁵ The translocation t(16;16) also involves *CBFB* and *MYH11* and results in an identical *CBFB-MYH11* fusion gene. A reciprocal *MYH11-CBFB* mRNA or protein has not been identified.

The CBF protein has two components, α and β , of which there are three α subunits and one β subunit. The α subunits all share a runt domain sequence, which allows the CBF protein to bind to DNA and to the β subunit. The β subunit binds to the α subunit and stabilizes CBF binding to DNA. Normal *CBFB* spans 50 kb with 6 exons, and the breakpoints in *CBFB* are relatively constant. In most cases, the breakpoint occurs in intron 5 at nucleotide 495 (corresponding to amino acid 165).⁹⁵ However, a small subset of cases with a more proximal breakpoint at nucleotide 399 (amino acid 133) have been reported.⁹⁶ Normal *MYH11* encodes for the smooth-muscle form of the myosin heavy chain, and *MYH11* is a member of the myosin II family.⁹⁴ Although the function of the CBF β -MYH11 protein is not completely known, it is thought to bind to the enhancer or promoters of a number of genes involved in hematopoietic cell differentiation.⁸⁹

The breakpoints in *MYH11* are more variable than in *CBFB*, in that a number of different breakpoint sites have

been reported, although most occur in a small 370-bp intron.^{89,95,96} This common breakpoint corresponds to nucleotide 1921 in *MYH11*. However, at the time of this writing, it is unclear whether different breakpoints correlate with differences in clinical findings or prognosis. Despite the variability in the different fusion genes generated by the inv(16) and t(16;16), one form of the fusion gene is created in approximately 85% of cases and involves nucleotide 495 of *CBFB* and nucleotide 1921 of *MYH11*. This fusion gene results in the generation of the CBF β -MYH11 protein, which includes the first 165 amino acids of the normal CBF β protein and a relatively small tail portion of the normal MYH11 protein.

Conventional cytogenetic methods, Southern blot analysis, RT-PCR, and immunohistochemistry⁹⁴ have been used to detect the inv(16) and t(16;16). One major advantage of conventional cytogenetics is that this method will also identify additional chromosomal abnormalities, which have been found in up to 50% of AML cases.⁹³ However, one potential disadvantage of karyotyping is that the inv(16) may be difficult to recognize or it may be misinterpreted as a del(16).⁹² RT-PCR analysis identifies *CBFB-MYH11* transcripts in most cases of AML with either the inv(16) or the t(16;16), demonstrating that the genetic consequences of both inversion and translocation are identical.^{91,95,96} However, the number of potential transcripts generated by the inv(16) can result in a small (<10%) subset of cases being falsely negative by RT-PCR but detectable by conventional cytogenetics.^{95,96}

Recent gene expression profiling studies of AML with inv(16) have shown distinctive upregulation of the NF- κ B pathway. In addition, genes associated with high cell proliferation are upregulated.⁹⁸

Acute Promyelocytic Leukemia with t(15;17)(q22;q21); *PML/RARA*, and Variants

The t(15;17)(q21;q21) abnormality occurs exclusively in APL, which is classified in the FAB system as AML M3. APLs represent 5–13% of all de novo AMLs,¹⁰ and there appear to be variations in patients' genetic predisposition to developing APL. For example, in Los Angeles County in the United States, the incidence of APL is higher in adult Latino patients (24%) than in non-Latino patients (8%), and similarly high incidences of APL have been reported in children from Central and South America and Italy.¹⁰ The presence of the t(15;17) abnormality consistently predicts responsiveness to with ATRA. Retinoic acid is a ligand for *RAR α* that is involved in the t(15;17) abnormality. ATRA is thought to overcome the block in cell maturation, allowing the neoplastic cells to mature and be eliminated.⁹⁹ Additional genetic abnormalities are rare in APL, suggesting that the t(15;17) abnormality by itself is sufficient for neoplastic transformation. This hypothesis was confirmed in an animal model, in which transgenic mice expressing the PML-RAR α protein in myeloid cells developed APL and responded to ATRA ther-

apy.¹⁰⁰ Of interest, ubiquitous and unrestricted expression of *PML-RAR α* in embryos has proven to be lethal.¹⁰¹

Two morphologic variants of APL have been described, typical and microgranular, with both variants carrying the t(15;17) abnormality.¹⁰² In the typical or hypergranular variant, promyelocytes have numerous azurophilic cytoplasmic granules that often obscure the border between the cell nucleus and cytoplasm. Cells with numerous Auer rods in bundles (or faggot cells) are common. In contrast, in the microgranular variant, promyelocytes contain numerous small cytoplasmic granules that are difficult to discern with the light microscope on routinely stained smears, but which are highlighted by MPO cytochemical staining and which are easily detected by electron microscopy.

The t(15;17) abnormality is a balanced and reciprocal translocation in which *PML* at chromosome 15 and *RAR α* at chromosome 17 are disrupted and fused to form a hybrid gene.^{103,104} The *PML-RAR α* fusion gene encodes a chimeric mRNA and protein. On the derivative chromosome 15, both *PML* and *RAR α* are oriented in a head-to-tail orientation. However, the function of normal *PML* is poorly understood. *PML* is ubiquitously expressed and encodes a protein characterized by an N-terminal region with two zinc-finger-like motifs, known as a ring or a B-box motif, and thought to be involved in DNA binding.^{103,104} A dimerization domain is also present. The normal *PML* protein appears to have an essential role in cell proliferation, and *RAR α* encodes a transcription factor that binds to DNA sequences in *cis*-acting retinoic acid-responsive elements. High-affinity DNA binding also requires heterodimerization with another family of proteins, the retinoic acid X receptors. The *RAR α* protein, from N-terminal to C-terminal, has transactivation, DNA binding, heterodimerization, and ligand-binding domains, with the normal *RAR α* protein playing an important role in myeloid differentiation.

There are three major forms of the *PML-RAR α* fusion gene corresponding to different breakpoints in *PML*.¹⁰⁵ The breakpoint in *RAR α* occurs in the same general vicinity within intron 2 in all cases. Approximately 40–50% of APL cases have a *PML* breakpoint in exon 6 (the so-called long form, or *BCR1*); 40–50% of APL cases have a *PML* breakpoint in exon 3 (the so-called short form, or *BCR3*); and 5–10% of APL cases have a *PML* breakpoint in exon 6 (the so-called variable form, or *BCR2*). In each form of the translocation t(15;17), the *PML-RAR α* fusion protein retains the 5' DNA binding and dimerization domains of *PML* and the 3' DNA binding, heterodimerization, and ligand (retinoic acid) binding domains of *RAR α* . Studies have found that the different forms of the *PML-RAR α* fusion protein's mRNA are associated with different clinical presentations and prognoses in patients with APL. In particular, the *BCR3* type of *PML-RAR α* is associated with higher leukocyte counts at the time of APL presentation and is more commonly present in the microgranular variant of APL.¹⁰⁵ Both higher leukocyte counts and microgranular variant morphology are adverse

prognostic findings in APL. Furthermore, the *PML-RAR α* transcript is not associated with survival.¹⁰⁶

In addition to the *PML-RAR α* protein, the t(15;17) abnormality results in two other abnormal proteins. First, an aberrant *PML* protein is expressed in virtually all cells with the t(15;17) abnormality, as a result of alternative mRNA splicing. This *PML* protein retains its DNA binding capacity, and could possibly play a role in neoplastic transformation. Secondly, a *RAR α -PML* fusion gene is also formed, which is located at the derivative chromosome 17. The *RAR α -PML* protein is expressed in approximately 75% of cases of APL.¹⁰⁷ Because the *RAR α -PML* protein lacks the DNA-binding regions of both normal *PML* and *RAR α* , it is not thought to play a role in leukemogenesis. Furthermore, *RAR α -PML* expression does not correlate with response to ATRA *in vitro* or with clinical outcome *in vivo*.¹⁰⁷

A number of methods may be used to detect the t(15;17) abnormality. Conventional cytogenetic methods detect the t(15;17) abnormality in 80–90% of APL cases at the time of initial diagnosis. Suboptimal clinical specimens and poor-quality metaphases explain a large subset of the negative results. FISH is another useful method for detecting the t(15;17) abnormality in APL.¹⁰⁸ Different FISH methods employ probes specific for either chromosome 15 or chromosome 17 (or both), and commercial kits are available. In the past, Southern blot hybridization was also used to detect gene rearrangements that resulted from the t(15;17) abnormality, but this approach is cumbersome. Additionally, RT-PCR is a convenient and reliable method for detecting the various *PML-RAR α* fusion transcripts.

Polyclonal and monoclonal antibodies that react with the *PML* and *RAR α* proteins have been generated, and immunohistochemical studies assessing the pattern of *PML* and *RAR α* protein staining are useful for diagnosis.¹⁰⁹ In normal cells, the *PML* protein is localized in 5–20 spherical structures per nucleus, known as PODs, which are highlighted in a punctate pattern of staining by the anti-*PML* antibody. The *RAR α* protein is not present in these structures. In contrast, in APL cells, immunostaining for either *PML* or *RAR α* reveals a microgranular pattern. Thus, the *PML-RAR α* fusion protein is present in the microgranules. The *PML-RAR α* fusion protein may prevent *PML* from forming normal PODs because treatment with ATRA allows *PML* reorganization into these domains.

To diagnose residual disease or early relapse after therapy, conventional cytogenetic studies, Southern blot analysis, and immunohistochemical methods are limited by low sensitivity. In contrast, RT-PCR and FISH methods are very useful. The sensitivity of RT-PCR, which may detect one cell with the *PML-RAR α* gene in 1×10^5 benign cells, makes this method most useful for monitoring residual disease after therapy. For the first few months after therapy, RT-PCR results may be positive, with no significant correlation with relapse rate. However, after more than 3 months post-therapy, a positive RT-PCR result is significantly correlated with an increased

rate of relapse.¹¹⁰ FISH methods compare favorably with RT-PCR methods; however, the greater sensitivity of RT-PCR allows the detection of disease in occasional cases that are negative on FISH.¹¹¹ However, FISH methods offer an advantage over RT-PCR in that FISH studies may detect variant forms of the t(15;17) abnormality that appear to be negative on RT-PCR.¹¹¹

The t(11;17)(q23;q21) abnormality has rarely been identified in AML (<1% of all cases). Many of the patients with t(11;17)-positive AML present with a bleeding diathesis, and tumors are often originally classified as APL. Licht et al¹¹¹ suggested that neoplastic cells have cytological features that are intermediate between the blasts of AML, FAB M2, and the promyelocytes of APL. Others have noted that many of these cases have distinctive cup-like nuclei.¹¹² However, unlike APLs, t(11;17)-positive AMLs respond poorly to ATRA.

The t(11;17) abnormality is a reciprocal and balanced translocation involving the *PLZF* (promyelocytic leukemia zinc finger) gene at chromosome 11 and the *RAR* gene at chromosome 17.^{113,114} Two fusion genes are produced via this translocation: *PLZF-RAR α* and *RAR α -PLZF*. It is still uncertain at the time of this writing which chimeric protein is leukemogenic. The structure of both chimeric proteins suggests that either can bind to DNA and influence transcription. Normal *PLZF* is expressed in a tissue-specific manner, particularly in cells of myeloid lineage. Furthermore, *PLZF* is a member of a large family of zinc finger transcription factors and is probably involved in myeloid differentiation. The inability of ATRA to induce differentiation in t(11;17)-positive AMLs suggests that disruption of normal *PLZF*, rather than disruption of *RAR α* (as is the case in APL), is the primary leukemogenic event. Analogous to the t(15;17) abnormality, there is some variability in the breakpoints within *PLZF*. In contrast, the breakpoints in *RAR α* specifically occur in the second intron, similar to those that occur in the t(15;17) abnormality.

Rare cases of APL carry the translocation (5;17)(q32;q21). In a case reported by Redner et al,¹¹⁵ the neoplastic cells were morphologically indistinguishable from APLs with the t(15;17), and the neoplasm responded well to ATRA. The t(5;17) is a balanced and reciprocal translocation involving the *NPM* (nucleophosmin) gene at chromosome 5 and the *RAR α* gene at chromosome 17. *NPM* and *RAR α* are fused in a 5' $\rightarrow$ 3' direction. In addition, the breakpoint region in *RAR α* is identical to the breakpoint region in the t(15;17) abnormality.

AML with t(9;11)(p22;q23), *MLLT3-MLL*

The translocation (9;11)(p22;q23) is the most common translocation involving chromosome 11q23 in AML patients. AMLs with the t(9;11) represent approximately 2% of all adult AMLs and is as often as 9–12% in pediatric patient population.^{41,42} AMLs with the t(9;11) may be either de novo or therapy-related. De novo neoplasms occur in both children

and adults. Patients with de novo AML with the t(9;11) tend to have a more favorable outcome than adult patients with other 11q23 abnormalities.¹¹⁶ In addition, therapy-related cases of AML have been reported in patients previously treated with chemotherapeutic agents that target topoisomerase II.¹¹⁷

The t(9;11) is a balanced and reciprocal translocation that disrupts *MLL* gene at chromosome 11q23 and the *AF9* gene (also known as *LTG9* for the leukemia translocation gene) at chromosome 9p22. The translocation results in the formation of an *MLL-AF9* fusion gene located at the derivative chromosome 11. The function of normal *AF9* is incompletely understood, but it is normally expressed in megakaryocytes and erythroid cells. There are contradictory reports describing the role of the *MLL-AF9* fusion protein on differentiation. For example, while the *MLL-AF9* fusion protein blocked the differentiation of 32D cells,¹¹⁸ it induced the differentiation of U937 cells into macrophages.¹¹⁹ Finally, secondary cytogenetic abnormalities may be detected in t(9;11)-positive AMLs, with trisomy 8 being the most common secondary abnormality.

AML with t(6;9)(p23;q34); *DEK-NUP214*

The t(6;9)(p23;q34) has been identified in approximately 1–2% of cases of AML.¹²⁰ Acute myelogenous leukemia (AML) with chromosomal translocation (6;9)(p23;q34) is a rare disease with poor prognosis and distinct clinical and morphologic features. Patients with AML and the t(6;9) abnormality are typically young (<40 years old) and have a poor prognosis.^{120,121} Distinct morphologic features of this entity include marrow basophilia and myelodysplasia, and immunophenotypically, the blast cells are positive for CD9, CD13, CD33, and HLA-DR; are usually positive for CD45 and CD38; and may be positive for CD15, CD34, and terminal deoxynucleotidyl transferase. Approximately 50% of patients with AML with the t(6;9) achieve clinical remission with chemotherapy, but relapse is common. A subset of patients present with aggressive MDS (rather than AML), and others present with overt AML with multilineage dysplasia.¹²⁰

The t(6;9) disrupts the *DEK* gene at chromosome 6p23 and the *CAN* (*NUP214*) gene at chromosome 9q34, resulting in a *DEK-CAN* fusion gene at the derivative chromosome 6.¹²² The breakpoints in the t(6;9) are clustered, with the breakpoints in *DEK* occurring in one intron and known as *icb-6* (or intron-containing breakpoint on chromosome 6) and the breakpoints in *CAN* clustering in another intron and known as *icb-9*. *DEK* is approximately 40 kb in size and encodes a 43-kDa protein located in the cell cytoplasm and thought to be a transcription factor. *CAN* is relatively larger (over 140 kb) and encodes a 214-kDa protein. The normal *CAN* protein is a component of the nuclear-pore complex (hence the name nucleoporin) involved in the transport of mRNA and proteins between the cytoplasm and the nucleus.¹²² The *DEK-CAN* fusion protein has a nuclear distribution with a predicted size of 165 kDa, suggesting it is a part of the transcription factor system.¹²³

The t(6;9) is effectively detected by conventional cytogenetic analyses, which also allows the detection of additional abnormalities, which may occur with disease progression; trisomy 8 and trisomy 13 are the most common additional abnormalities.¹²⁰ Southern blot analysis has also been used in the past. The clustering of breakpoints in *icb-6* of *DEK* and *icb-9* of *CAN* allows for convenient detection using RT-PCR methods.¹²⁴ *FLT3* gene mutations are common in t(6;9)-positive AML.¹²⁰ Prevalence of FLT3-ITD is as high as 70% among patients with t(6;9) AML, and patients with t(6;9) AML and FLT3-ITD mutations usually have higher white blood cell counts, higher bone marrow blasts, and significantly lower rates of complete remission.

AML with inv(3)(q21q26.2) or t(3;3) (q21;q26.2); *RPN1-EVII*

Most of the patients with inv(3) or t(3;3) described in the literature are adults; men and women are equally affected.^{41,125} Both de novo cases and cases arising from MDS have been described.^{126,127} Several cases of blast crisis of CML with inv(3) or t(3;3) have been also described.¹²⁸ The incidence of inv(3) or t(3;3) in AML is reported to be between 1 and 3%^{41,125}; however, as a sole cytogenetic abnormality, inv(3) and t(3;3) occur infrequently. For example, Cancer and Leukemia group B detected inv(3) or t(3;3) as a sole cytogenetic abnormality in only 2 of 1,213 (0.16%) patients.⁴¹

A small proportion of patients may demonstrate prominent hepatosplenomegaly.¹²⁷ Platelet counts in the peripheral blood at presentation may be increased, normal or decreased,^{129,130} demonstrating wide range of values, from $20 \times 10^9/L$ to $1,731 \times 10^9/L$.¹²⁹ The literature describes an association of inv(3) and t(3;3) with increased number of micromegakaryocytes and monolobated and bilobated megakaryocytes in bone marrow^{128,129}; significant number of cases also demonstrates dysplastic changes in erythroid and/or myeloid lineages.^{127,128}

It has been demonstrated that the chromosomal breakpoints in 3q26 are scattered either in the 5' or the 3' region of the *EVII* gene,¹³¹ while the chromosomal breakpoints in the 3q21 region are restricted to a relatively narrow area. Two different clusters account for around 100 kb that have been defined downstream of the *RPN1* gene.¹³² Alternative splicing of *EVII* results in the *MDS1/EVII* transcript by fusion with *MDS1*. Suzukawa et al suggested that the housekeeping gene *RPN1* acts as an enhancer of *EVII* expression, which results in the leukemogenic effect.¹³³ It has been demonstrated that ectopic expression of *EVII* in immature hematopoietic cells interferes with erythroid and granulocytic development.¹³⁴

While there is a single report in the literature describing good response of two patients with inv(3) to thalidomide and arsenic trioxide,¹³⁵ in general, the prognosis of patients with inv(3) or t(3;3) is poor with short survival.^{128,136,137} However, most reports dealing with results of therapy were published

in the last century; hopefully, new therapeutic modalities may change the outcome of these patients.

AML (Megakaryoblastic) with t(1;22) (p13;q13); *RBM15-MKL1*

Most of the cases of AML with t(1;22) have been reported in infants.^{138,139} The largest series published to date describes 39 cases with a median age of 4 months (range, 1 day to 36 months).¹³⁸ The translocation (1;22) represents a sole cytogenetic abnormality in more than half of the cases; of interest, it represents a sole cytogenetic abnormality much more often in infants younger than 6 months (19 of 23, 83%, cases), than in infants older than 6 months (3 of 16, 19%, cases).¹³⁸ There is a slight female predominance; all patients reported had no evidence of Down syndrome.¹³⁸ In rare cases, *RBM15-MKL1* transcript resulted from 3-way translocations involving 1p13 and 22q13, such as t(1;14;22) or t(1;22;4).¹⁴⁰

Almost all patients present with organomegaly^{138,139}; a subset of patients was initially diagnosed with a solid tumor.¹³⁸ Moderate leukocytosis with circulating blasts, marked anemia, and variable thrombocytopenia are usually observed at presentation.¹³⁸⁻¹⁴⁰ The vast majority (35 of 39–90%) of patients in the largest series¹³⁸ fulfill the diagnostic criteria for AML M7. The majority of patients demonstrate a hypercellular or normocellular BM; however, cases with a hypocellular BM also occur.¹⁴⁰ While in some cases blasts demonstrate typical morphologic features of megakaryocytic differentiation (i.e., cytoplasmic basophilia, blast clumping, cytoplasmic blebs), in others, blasts have a rather undifferentiated appearance.¹³⁹ Blasts usually express immunologic markers of megakaryocytic differentiation (i.e., CD42b, CD41a, Factor VIII).¹³⁹ A subset of blasts also expresses CD34 and HLA-DR; some blasts may express the myelomonocytic marker, CD33.¹³⁹

The translocation (1;22)(p13;q13) results in a fusion of RNA-binding motif protein-15 (*RBM15*) and megakaryocyte leukemia -1 (*MKL1*) genes.¹⁴¹ It has been demonstrated that t(1;22)-positive blasts express both reciprocal fusion transcripts, *RBM15-MKL1* and *MKL1-RBM15*, as detected by RT-PCR.¹⁴¹ However, the predicted *RBM15-MKL1* chimeric protein encompasses all putative functional motifs encoded by each gene, which makes it the candidate oncoprotein of t(1;22). Ma et al suggested that in *RBM15-MKL1*, the *MKL1* SAP domain is expected to aberrantly relocalize the RRM and SPOC motifs of *RBM15* to sites of transcriptionally active chromatin, deregulating RNA processing and/or Hox and Ras/MAP kinase signaling and altering the normal proliferation or differentiation of megakaryoblasts.¹⁴¹

The prognosis of patients with t(1;22) could be called intermediate and appears to improve dramatically with modern therapy. The largest series published in 1999 reported 53% complete remission rate and median survival of 8 months (range, 1 day to 104 months).¹³⁸ The most recent report of

11 pediatric patients published in 2003 describes 6 of 11 patients being alive with more than 2 years follow-up.¹⁴⁰

AML with Gene Mutations

The current 2008 WHO classification includes AML with mutated *NPM1* or *CEBPA* genes into the category of AML with recurrent genetic abnormalities. These entities are discussed in details in Chap. 35.

Translocations in AML Not Included in AML with Recurrent Genetic Abnormality Category of WHO 2008 Classification

In the original 2001 WHO classification, the category of AML with recurrent cytogenetic abnormalities included t(15;17), inv(16), t(8;21), and 11q23 (*MLL*). The current 2008 WHO classification added t(9;11), t(6;9), inv(3), and t(1;22) to this category, but excluded other translocations involving 11q23 (*MLL*) from the category. In addition, there are other well-recognized cytogenetic abnormalities, not yet included in the category of recurrent genetic abnormalities. In our attempt to have our review more structured, we would like to discuss those abnormalities as follows.

AML with 11q23 (MLL) Abnormalities Other than t(9;11)

Translocations involving the 11q23 locus are detected in 3–10% of all patients with de novo AML.^{142,143} In infants (age <1 year old), AMLs with 11q23 translocations typically present with hyperleukocytosis and have a poor prognosis.¹⁴⁴ These translocations involving chromosome 11q23 are found in more than 70% of patients younger than 1 year of age regardless of whether the immunophenotype is designated as AML or ALL.¹⁴⁵ In adults, AMLs with 11q23 translocations most commonly exhibit monocytic maturation and are classified as FAB M4 or M5. These AMLs do not have specific clinical features and may have a poorer prognosis than AMLs without 11q23 translocations. In addition, 80–90% of therapy-related AMLs that occur in patients previously treated with topoisomerase II inhibitors are associated with 11q23 translocations. However, translocations involving the 11q23 locus are not restricted to AML and are associated with different hematologic malignancies, including precursor T- or B-cell lymphoblastic leukemia, MDS, and Burkitt lymphoma.^{142,143,146} In addition, *MLL* has been labeled as a “promiscuous” oncogene, since translocations involving over 60 partner genes or regions have been identified.¹⁴²

All 11q23 translocations disrupt the *MLL* gene, also known as *ALL1*, *HRX*, or *HRTX*.¹⁴⁷ *MLL* is a relatively large gene, which is homologous to the *Drosophila trithorax* gene.¹⁴⁸ *MLL* consists of 36 exons distributed over 100 kb and produces a 12 mRNA that encodes a 3,968 amino acid protein, with an estimated molecular weight of 430 kD.¹⁴² Recent

experiments have indicated that *MLL* is normally processed via a cytoplasmic cleavage event into a 320-kD amino terminus (*MLL-N*), and a 180-kD carboxy terminus fragment.¹⁴² A number of protein motifs/domains have been identified in the primary structure of *MLL*, including amino-terminal hooks, a DNA methyltransferase domain, PHD domains, a transactivation domain, and a SET domain.¹⁴⁸ An important function of *MLL* is maintaining *HOX* gene expression during embryonic development. For example, it was demonstrated that loss of *MLL* function in flies and mice results in embryonic lethality and homeotic transformation.¹⁴² The ability of *MLL* fusion proteins to regulate *HOX* gene expression might be partially responsible for immortalization of myeloid progenitor cells.¹⁴⁹ It was demonstrated that *MLL* affects *HOX* gene expression via direct binding to promoter sequences¹⁵⁰ and that *HOXA9* is highly expressed in leukemias with *MLL* expression.¹⁵¹ The *MLL-ENL* fusion protein was shown to immortalize immature myelomonocytic cells in vitro. After transplanting these cells in mice, the mice developed myeloid leukemia.¹⁵² By fusing a truncated *MLL* protein to an inducible dimerization domain, Martin et al found that *MLL* fusion proteins must dimerize to immortalize hematopoietic cells and inhibit their differentiation.¹⁵³ Biochemical analysis of *MLL* suggests that it normally functions as a transcriptional regulator, and expression of *MLL* fusion proteins has been shown to be leukemogenic in mice.¹⁴⁸

Despite the large size of *MLL*, most (but not all) *MLL* translocations associated with hematologic malignancies can be mapped to an 8.3-kb breakpoint cluster region (bcr).¹⁵⁴ This region is bounded by *Bam*HI restriction sites and encompasses *MLL* exons, originally designated as exons 5–11. Subsequent studies revealed that the *MLL* exon 4 actually consisted of three discrete exons (now designated as exons 4A, 4B, and 4C).

There is a correlation between the location of the breakpoints in de novo when compared to therapy-related AML. Using the *Xba*I restriction enzyme, the 8.3-kb fragment of DNA can be divided into a 5' 4.6-kb region I and a 3' 3.9-kb region II.¹⁵⁵ De novo AMLs more commonly have breakpoints in region I. In contrast, most cases of therapy-related AML have region II breakpoints.¹⁵⁵ The presence of scaffold attachment regions and possible topoisomerase II consensus binding sites in region II may explain the increased likelihood of region II breakpoints in therapy-related AMLs.¹⁵⁵

Conventional cytogenetic analysis is an excellent method to detect 11q23 abnormalities.¹⁵⁶ This technique allows the detection of 11q23 translocations and also identifies all possible partner chromosomes. However, a subset of AMLs with 11q23 translocations may have a normal karyotype.¹⁵⁷ FISH techniques are also useful and may detect a small subset of cases not recognized by conventional cytogenetics. The clustering of breakpoints within *MLL* is well suited to their detection by Southern blot analysis.¹⁴⁶ Using *Bam*HI-digested DNA, a single *MLL* cDNA probe spanning an 8.3-kb genomic fragment detects most of the common and

uncommon 11q23 translocations as gene rearrangements. The introns between exon 5 and exon 11 are very large; thus, standard PCR methods cannot be used for detecting 11q23 translocations. However, the cDNA corresponding to exons 5 through 11 is <700 bp, which allows for RT-PCR analysis. A panel of primers needs to be used to detect the 11q23 translocations because of the number of possible partner chromosomes. Therefore, multiplex RT-PCR approaches are most convenient.¹⁵⁸ RT-PCR methods are probably more useful for monitoring residual disease, once the partner chromosome involved is known, rather than in establishing an initial diagnosis (or for screening).

One of the most unusual features of *MLL* fusion genes is that they have a very brief latent period. Leukemias with *MLL* fusion genes have been detected in newborns¹⁵⁹ and even in aborted fetuses.¹⁶⁰ The concordance rate of leukemia in monozygotic twins who have *MLL* fusion genes is almost 100%.¹⁶¹ It has been hypothesized that additional mutations occur very rapidly in cells that acquire *MLL* fusion genes.¹⁶² One possible mechanism is that *MLL* fusion genes lead to a "mutator phenotype."¹⁶² *MLL* fusion genes have been shown to impair DNA double-strand break recognition¹⁶³ and cell cycle checkpoints,¹⁶⁴ both of which can lead to increased accumulation of mutations. It is also possible that the mechanism that caused the *MLL* translocation may cause additional mutations, especially if the individual had an inherited deficiency in DNA repair or toxin-metabolizing enzymes. This possibility is supported by studies showing that certain isoforms of *NQO1* or *CYP3A4* confer an increased risk of infant leukemia¹⁶⁵ or therapy-related leukemia.¹⁶⁶

AML with the Translocation t(11;16)(q23;p13.3)

The translocation t(11;16) is a rare, recurrent translocation that has been identified in therapy-related AML and MDS and occurring almost exclusively in patients previously treated with agents that inhibit topoisomerase II.¹⁶⁷ The translocation t(11;16) is a reciprocal translocation that disrupts the *MLL* gene at chromosome 11q23 and the *CBP* gene (for cAMP response element or CREB-binding protein) at chromosome 16p13.3, resulting in the formation of an *MLL-CBP* fusion gene located on the derivative chromosome 11.¹⁶⁷ A *CBP-MLL* fusion gene is also produced in most, but not all cases of AML and, therefore, is not thought to be leukemogenic. The normal CBP protein is a transcriptional adaptor/coactivator protein.

AML with Other Translocations Involving 11q23

Other 11q23 translocations less commonly identified in AMLs include the t(6;11)(q27;q23), t(11;19)(q23;p13.3), t(11;19)(q23;p13.1), t(1;11)(p32;q23), t(1;11)(q21;q23), t(11;17)(q23;q21), and t(10;11)(p11;q23) translocations. In each of these translocations, the *MLL* gene is disrupted and its 5' end is fused with the 3' end of the partner gene: A-6

in t(6;11), *ENL* in t(11;19)(q23;19p13.3), *ELL* in t(11;19)(11q23;19p13.1), *AFIp* in t(1;11)(p32;q23), *AFIq* in t(1;11)(q21;q23), *AF17* in t(11;17), and *AF10* in t(10;11).^{156,168-172} The formation of these fusion genes, all located at the derivative chromosome 11, results in the generation of a novel chimeric protein.

AML with 11q23 Rearrangements and Normal Conventional Cytogenetics or Trisomy 11

Caligiuri and colleagues¹⁵⁷ have screened a number of cases of AML with normal conventional cytogenetics by Southern blot analysis and have identified *MLL* gene rearrangements in approximately 10% of the cases. All were shown to have partial tandem duplications (PTDs) of the 5' part of *MLL*.¹⁵⁷ These mutations occur in 3-10% of adult AML cases, but are uncommon in pediatric AML. PTDs of *MLL* are extremely frequent in conjunction with trisomy 11^{157,173,174} and are detected in approximately 10% of AML cases without cytogenetic aberrations. PTDs of *MLL* have also been associated with an unfavorable outcome in patients with AML.^{49,157} Another type of intrachromosomal rearrangement of *MLL*, intrachromosomal amplification, also occurs, which most likely leads to increased *MLL* expression in AML and MDS.¹⁷⁵

AML with inv(11)(p15q22)

The inv(11)(p15q22) has been identified in a very small number of de novo and therapy-related cases of AML.¹⁷⁶ This inversion disrupts the *DDX10* gene at chromosome 11q22 and the *NUP98* gene at chromosome 11p15, creating the *NUP98-DDX10* fusion gene. A reciprocal *DDX10-NUP98* fusion gene is also created and may be expressed, but it is not thought to be involved in neoplastic transformation.¹⁷⁶ Normal *DDX10* is large, spanning 200 kb, and is composed of at least 12 exons. *DDX10* is a DEAD-box putative RNA helicase gene that encodes a protein that may be involved in ribosomal assembly.¹⁷⁷ *DDX10* is ubiquitously expressed in normal tissues. In addition, normal *NUP98* encodes a nuclear-pore complex protein that is also ubiquitously expressed.

AML with t(16;21)(p11;q22)

Most neoplasms with the translocation t(16;21) are AMLs, but rare cases of CML in blast crisis and MDS with the translocation t(16;21) have been described.^{178,179} Patients with AML associated with the translocation t(16;21) are generally young and have a poor prognosis. The translocation t(16;21) is a reciprocal and balanced translocation that disrupts the *TLS/FUS* gene at chromosome 16p11 and the *ERG* (*ETS*-related gene) gene at chromosome 21q22.¹⁸⁰ As a result, two fusion genes are created, *TLS/FUS-ERG* on the derivative chromosome 21 and *ERG-TLS/FUS* on the derivative chromosome 16. Although both fusion genes may be expressed, only the *TLS/FUS-ERG* fusion gene is consistently expressed

in all cases, suggesting that this chimeric protein is involved in leukemogenesis.¹⁸⁰ Both *TLS/FUS* and *ERG* are fused in a 5'→3' direction. Normal *TLS/FUS* encodes an RNA-binding protein that is highly homologous to the *EWS* gene involved in Ewing sarcoma. *TLS/FUS* protein plays a role in activating transcription. Normal *ERG*, a member of the *ETS* proto-oncogene superfamily, also encodes an RNA-binding protein and is a potent transcriptional activator.¹⁸¹ At least four different transcripts of *TLS/FUS-ERG* have been identified. These transcripts result from variability in the breakpoints and alternative RNA splicing. In most cases, the breakpoint in *ERG* is tightly clustered in one intron, and the breakpoints in *TLS/FUS* are more variable.¹⁷⁸

AML with t(12;22)(p13;q11)

The translocation t(12;22)(p13;q11) is found in AMLs and rare cases of MDS and CML.¹⁸² The translocation t(12;22) is a reciprocal translocation involving the *TEL* gene at chromosome 12p13 and the *MNI* (meningioma) gene at chromosome 22q11.¹⁸³ The translocation disrupts *TEL* and *MNI*, resulting in the formation of the *MNI-TEL* and *TEL-MNI* fusion genes. The *MNI-TEL* fusion gene is likely to encode the leukemogenic protein based on the predicted structure of the fusion protein, which is consistent with an altered transcription factor. The *MNI-TEL* fusion gene has also been constantly expressed in the small number of neoplasms analyzed, unlike the *TEL-MNI* fusion gene.¹⁸³ Normal *TEL* is very large, exceeding 150 kb, and is a member of the *ETS* gene family of transcription factors. In addition, *TEL* is ubiquitously expressed in tissues. However, normal *MNI* is less well known. *MNI* was originally cloned from the translocation t(4;22)(p16;q11) and was identified in a case of sporadic meningioma.¹⁸⁴ *MNI* spans 70 kb, has at least two exons separated by a large intron, and encodes a protein predicted to have 1,319 amino acids. The breakpoints in *MNI* appear to be clustered in the 5' region of the intron.¹⁸⁴

AML with t(8;16)(p11;p13)

The translocation t(8;16)(p11;p13) has been identified in <1% of all cases of AML. Most AMLs with the translocation t(8;16) exhibit monocytic differentiation and are morphologically classified as FAB M4 or M5,¹⁸⁵ and the blasts often exhibit evidence of erythrophagocytosis.¹⁸⁵ Both de novo and therapy-related AMLs with the translocation t(8;16) have been reported, with de novo cases commonly occurring in children and adolescents (<18 years old).^{185,186} The translocation t(8;16) is a reciprocal translocation involving the *MOZ* (monocytic leukemia zinc finger protein) gene located at chromosome 8p11 and the *CBP* gene located at chromosome 16p13.¹⁸⁷ This translocation disrupts *MOZ* and *CBP*, resulting in the formation of a *MOZ-CBP* fusion gene on the derivative chromosome 8. A *CBP-MOZ* fusion gene is also created, but it is thought to be nonfunctional. *MOZ* encodes a 225-kDa protein that is widely expressed in tissues.

The *MOZ* protein has both zinc finger and acetyltransferase domains and may mediate leukemogenesis by aberrant acetylation of chromatin.¹⁸⁷ *CBP* spans 190 kb and encodes a protein involved in transcriptional activation. *CBP* mutations have been identified in a rare genetic disease, the Rubinstein-Taybi syndrome, characterized by mental retardation, dysmorphic cranial features, and digital abnormalities.¹⁸⁸

AML with t(3;21)(q26;q22)

The translocation t(3;21)(q26;q22) has been reported in cases of CML in blast crisis, in MDS, and in AMLs following treatment with topoisomerase II inhibitors, such as etoposide.¹⁸⁹ The breakpoint in *AML1* on chromosome 21 may be identical to that in the translocation t(8;21), but it occurs more often approximately 60 kb downstream. The chromosome 3q26 locus is the site of three different genes that are involved in the translocation t(3;21): from telomere to centromere, *EAP*, *MDS1*, and *EVIL*. These genes are located within 200 kb of each other. In each of these translocations, the 5' end of *AML1*, including sequences that encode the runt-like domain, is fused to the 3' end of one of these partner genes.

In the *AML1-EAP* fusion gene, the 3' *EAP* sequence is very small and is not fused to *AML1* in the reading frame. Thus, the *AML1-EAP* chimeric protein lacks transactivation activity and may exert its effect by inhibiting the normal *AML1* protein. In contrast, the *AML1-MDS1* and *AML1-EVIL* fusion genes contain significant 3' sequences of *MDS1* and *EVIL*, suggesting that their proteins possibly have unique functions in addition to inhibiting the normal *AML1* protein. One potential explanation for the occurrence of these different translocations, which has not yet been proven, is that the entire 3q26 region is transcribed as a result of the translocation t(3;21), and different fusion genes result from alternative splicing of mRNA.^{79,190} The normal *EAP*, *MDS1*, and *EVIL* proteins are not normally expressed in hematopoietic cells, but are expressed as a result of the translocation t(3;21).

AML with t(9;22)(q34;q11)

In some cases, distinguishing AML from an initial presentation of CML in blast phase can be difficult. The presence of the Philadelphia chromosome and/or *BCR/ABL* fusion gene transcripts are not reliable criteria for diagnosing CML as some cases of AML with these features have been reported,¹⁹¹ including cases in which *BCR/ABL* fusion gene transcripts were acquired in MDS. To complicate the issue, the blast karyotype is often very complex. Some authors believe that the only reliable way to distinguish these entities is by assessing therapeutic response and by testing maturing hematopoietic elements for the *BCR/ABL* fusion gene transcript present in CML only.

The translocation t(9;22)(q34;q11) is rarely identified in apparently de novo cases of AML (1–2% of cases).^{191,192}

The translocation t(9;22) involves the *ABL1* gene at chromosome 9q34 and the *BCR* gene at chromosome 22q11. This translocation results in a *BCR-ABL* fusion transcript. Two different forms of the translocation t(9;22) have been detected in AML that result in either a 190-kDa or 210-kDa fusion protein.¹⁹² In both translocations, the breakpoint in *ABL1* occurs in the same general area in the proximal portion of the gene. In the p190^{bc_r-abl} form, the breakpoint in *BCR* occurs in the first exon. In contrast, in the p210^{bc_r-abl} form, the breakpoint occurs in the 5.8-kb major breakpoint region of *BCR*. The detection of the p190^{bc_r-abl} form of the t(9;22) abnormality in a case of AML supported the diagnosis of de novo AML.

AML with t(7;11)(p15;p15)

The translocation t(7;11)(p15;p15) is a rare translocation in AML. Morphologically, most AMLs with the translocation t(7;11) exhibit granulocytic maturation and are classified as FAB M2, and coexistent myelodysplastic features are common.^{193,194} Most patients with t(7;11)-positive AML have been Japanese adults, suggesting a possible genetic predisposition for this type of AML.¹⁹⁴

The translocation t(7;11) disrupts the *HOXA9* gene at chromosome 7p15 and the *NUP98* gene at chromosome 11p15, resulting in the formation of the *NUP98-HOXA9* and *HOXA9-NUP98* fusion genes.^{187,195} The *NUP98-HOXA9* fusion gene, located on the derivative chromosome 11, is thought to be involved in leukemogenesis, based on the predicted structure of the chimeric protein.^{187,195} The reciprocal *HOXA9-NUP98* fusion gene is probably nonfunctional and was not amplifiable by RT-PCR in a subset of cases tested.¹⁸⁷ Normal *HOXA9* is a member of the homeobox gene family and is involved in development and differentiation. *HOXA9* encodes a class I homeodomain protein that is expressed in hematopoietic cells and the kidneys.^{187,195} Normal *NUP98* is relatively small, with five exons, and encodes a component of the nuclear-pore complex that is 98 kDa.

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Acute Myeloid Leukemias with Normal Cytogenetics

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Introduction

In this chapter, we discuss acute myeloid leukemia with normal cytogenetics (CN AML). In 40–50% of patients with AML, no chromosomal abnormalities are detected by conventional karyotyping, suggesting that other mechanisms are responsible for leukemogenesis in these cases.¹ Attempts to stratify such cases on the basis of complementary DNA microarrays demonstrated gene expression patterns with differences in responses to treatment; however, no specific genetic subgroups have emerged from these studies.^{2,3} Abnormalities detected in patients with AML with a normal karyotype could be arbitrarily divided into two major groups:

- Abnormalities directly affecting proliferation/apoptosis of leukemia cells, and
- Abnormalities affecting proliferation/apoptosis of leukemia cells through interaction with bone marrow stroma.

Clinical relevance of genetic abnormalities is summarized in Table 35.1. In this chapter, we first describe the molecular abnormalities directly affecting leukemic cells, and then we describe the interaction of leukemic cells with bone marrow (BM) stroma. Although this separation is quite arbitrary, it allows us to describe the different aspects of leukemic cell biology. However, it is important to realize that alterations in the leukemic cells themselves as well as alterations in the BM microenvironment play a critical role in leukemogenesis. It also needs to be stressed that molecular abnormalities directly affecting leukemic cells are not limited to patients with AML with a normal karyotype, as molecular abnormalities also play an important role in other types of AML, including in patients with recurrent cytogenetic abnormalities.

Abnormalities Affecting Proliferation/ Apoptosis of Leukemia Cells Directly

FLT3 Gene Mutations (Also See Chapter 11)

The FMS-like receptor tyrosine kinase 3 (*FLT3*), also known as fetal liver kinase 2 (*FLK-2*) and stem cell tyrosine kinase 1 (*STK-1*), belongs to a class III receptor tyrosine kinase (TK) family.⁴ The human *FLT3* gene is located at chromosome 13q12 and contains 24 exons⁵ *FLT3* gene encodes a protein, which exists in two forms: a 158- to 160-kd membrane-bound protein glycosylated at *N*-linked glycosylation sites in the extracellular domain, and a 130- to 143-kd nonmembrane-bound unglycosylated protein.⁶ *FLT3* is preferentially expressed by hematopoietic stem cells and is also expressed in the brain, placenta, and liver.⁷ The *FLT3* ligand, which is expressed as a membrane-bound or soluble form by BM stromal cells, stimulates stem cells by itself or in cooperation with other cytokines.^{6,8–11} *FLT3* is also expressed on the surface of a high proportion of AML cells.^{12–14}

Two unique forms of the *FLT3* gene mutation have been described:

- An internal tandem duplication in the juxtamembrane domain-coding sequence (*FLT3/ITD*)¹⁵
- A missense point mutation at the D835 residue and point mutations, deletions, and insertions in the codons surrounding D835 within the *FLT3* TK domain (*FLT3/KDM*)^{16–22}

FLT3/ITD and *FLT3/KDM* mutations occur in 15–35% and 5–10% of adults with AML and are associated with a poor prognosis.^{23–28} In addition, extremely high levels of *FLT3* transcripts have been demonstrated in a proportion of patients with AML without *FLT3* mutations, which was also associated with a poor prognosis.²⁹ Rarely, both types

TABLE 35.1. Summary of clinical relevance of genetic abnormalities.

Abnormalities associated with favorable clinical course
<i>NPM1</i> gene mutations (in the absence of <i>FLT3/ITD</i>)
<i>CEBPA</i> gene mutations
Abnormalities associated with dismal clinical course
<i>FLT3</i> gene mutations
<i>RAS</i> gene mutations
<i>KIT</i> gene mutations
<i>p53</i> gene mutations
<i>ERG</i> gene mutations
<i>MN1</i> gene mutations
CXCR4 overexpression
BAALC overexpression

of mutations occur simultaneously in AML, which may be related to clonal progression.³⁰

FLT3 mutations lead to constitutive activation of *FLT3*, resulting in increased receptor signaling, which in turn is responsible for tumorigenesis. *FLT3/ITD* is constitutively phosphorylated on tyrosine residues and forms homodimers and, if the ITD-containing *FLT3* is cotransfected with wild-type *FLT3*, a heterodimer with wild type *FLT3*.^{31,32} *FLT3/ITD* or *FLT3/KDM* result in autonomous proliferation of murine interleukin 3-dependent cell lines, such as 32D, and induce constitutive activation of downstream signaling molecules, such as signal transducer and activation of transcription 5 (STAT5), MAP kinase, SHC, AKT, and BAD.^{32–36} 32D cells are known to differentiate into mature neutrophils in response to granulocyte colony-stimulating factor (G-CSF). However, both *FLT3/ITD*-expressing and *FLT3/KDM*-expressing 32D cells do not differentiate into mature neutrophils when they are treated with G-CSF.³⁷ These results indicate that *FLT3* mutations are involved in cascades for autonomous cell proliferation and differentiation block. In addition, mutant *FLT3* has antiapoptotic effects on leukemia cells.³⁵

FLT3 mutations are mainly found in de novo AML and are less frequent in AMLs that have developed from myelodysplastic syndrome (MDS) or in response to therapy.²⁸ *FLT3/ITD* or *FLT3/KDM* occurs in approximately 3% of patients with low-grade MDS.³⁸ However, *FLT3/ITD* or *FLT3/KDM* can occur in up to 15% of patients with AML that has developed from MDS.^{39–41} The frequency of *FLT3* mutations in patients with AML has been associated with patient age. For example, *FLT3/ITD* has been found in about 25% of all adult patients, but was more prevalent in patients older than 55 years (31.4%).^{18,39} In contrast, *FLT3/ITD* has been found in approximately 10% of pediatric patients^{42–45} and has been rare in infants with AML.⁴⁶ *FLT3* mutations are frequently found in patients with AML with normal or intermediate-risk cytogenetic characteristics and patients with the t(15;17) translocation, but these mutations are rare in patients with core binding factor translocations, such as t(8;21) and inv(16). Recently, it was demonstrated that the *NPM1* gene (discussed below)

was mutated in more than 60% of adult patients with AML with *FLT3* mutations.^{47–49}

Kelly et al found that *FLT3/ITD* induced an oligoclonal myeloproliferative disorder, but not AML, in mice, suggesting that additional mutations impairing hematopoietic differentiation and/or proliferation may be necessary for AML development.⁵⁰ Similarly, transplanting *FLT3/ITD*-transfected bone marrow cells into PML/RAR α transgenic mice shortened the latency period and increased penetrance for developing an APL-like disease.⁵¹

FLT3/ITD is strongly associated with leukocytosis and an increased percentage of blast cells in the peripheral blood and bone marrow of patients with AML.^{40,41} However, the relationship between *FLT3/KDM* and leukocytosis is still controversial. Only one study has demonstrated significant leukocytosis in adult patients with AML with *FLT3/KDM*.¹⁹

Several large-scale studies have demonstrated the impact of *FLT3* mutations on patients' clinical outcome.²⁸ *FLT3/ITD* has been found to be a strong adverse predictive factor for overall survival, disease-free survival, and event-free survival within the intermediate-risk cytogenetic category, but the clinical impact of *FLT3/KDM* on long-term outcome is unclear. A recent meta-analysis of four studies with a total of 1,160 adult patients with AML suggested an adverse effect of *FLT3/KDM* on long-term outcome.

RAS Gene Mutations

RAS is a GTP-dependent, 21-kD protein that is localized at the inner side of the cell membrane and is encoded by the *RAS* gene family (*K-RAS* is located at 12p12.1, *H-RAS* is located at 11p15.5, and *N-RAS* is located at 1p13.2). RAS binds guanine nucleotides, displays intrinsic GTPase activity, and transduces signals from growth factor receptors to downstream effectors. The GTPase-mediated cycling between RAS-bound GTP (active conformation) and GDP (inactive conformation) serves as a regulatory switch for signal transduction.

Activating somatic mutations in the first two exons of *RAS* have been described at codons 12, 13, and 61 of *N-RAS*,^{52,53} sporadically in *K-RAS*, and rarely in *H-RAS*.⁵⁴ These point mutations disrupt *RAS* function by diminishing GTPase activity. In several large studies, the reported frequency of *N-RAS* mutations was 10–27% in patients with AML.^{55–58} *K-RAS* mutations were found in approximately 5–10% of pediatric and adult patients with AML,^{57–59} whereas *H-RAS* mutations were rare.⁵⁶ In two studies including age-adjusted analyses of patients with AML, the presence of *RAS* mutations correlated with lower blast counts in the bone marrow and longer survival times.^{58,60} In other reports, however, patients with *RAS* mutations showed a trend toward worse outcomes when compared to patients without *RAS* mutations.⁵⁹

Patients with MDS and *N-RAS* mutations have a high risk of progression to AML,⁶¹ suggesting that *N-RAS* mutations play a role in progression. On the other hand, analyses of

RAS mutation status in patients with AML at the time of first diagnosis and at the time of relapse revealed a marked instability of *RAS* mutations, with loss of mutation in some cases and replacement by mutations in different codons in others.⁶² These analyses indicate that *RAS* mutations may not be leukemia-initiating events.⁶² *RAS* has been shown to be an important mediator of G-CSF – and thrombopoietin-induced differentiation and to enhance the function of c/EBP α in promoting myeloid differentiation.^{63,64}

Kit Gene Mutations

Activating mutations of *KIT*, which are well known in mast cell disorders and in gastrointestinal stromal tumors, also occur in AML. *KIT* mutations usually occur in the juxtamembrane region, which functions as a negative regulatory region,⁶⁵ and in residues in the activation loop.⁶⁶ *KIT* mutations are infrequent in AML in general,⁶⁷ but these mutations are more common in patients with core binding factor leukemias, with an incidence rate between 2 and 20%.^{68,69}

G-CSF Receptor Gene Mutations

Approximately 20% of patients with severe congenital neutropenia have G-CSF receptor point mutations.⁷⁰ These mutations result in truncation of the C-terminal region, which is crucial for G-CSF signaling.⁷¹ The mutated G-CSF receptor interrupts signals required for normal myeloid cells. Thus, patients with these mutations have an increased risk of progression to AML.^{71,72} In 32D cells, the expression of the truncated G-CSF receptor resulted in increased proliferation without neutrophilic differentiation.⁷³ Herman et al found that transgenic mice expressing the mutant G-CSF receptor (truncated at amino acid 715) developed a form of neutropenia, in which the myeloid progenitors reacted to G-CSF with hyperproliferation and neutrophilia.⁷⁴ Transgenic mice overexpressing a form of the G-CSF receptor truncated at amino acid 718 or 731 had low neutrophil counts when compared to the wild-type controls and increased numbers of immature myeloid cells in the bone marrow.⁷⁵ These effects have been attributed to ligand-induced internalization of the receptor,^{74,76} reduced ability of the mutant G-CSF receptor to activate STAT3,⁷⁷ and reduced STAT5 inhibition by SOCS3.⁷⁸ All these features may be responsible for the increased risk of AML in patients with severe congenital neutropenia.

NPM1 Gene Mutations

The *NPM1* gene is located at chromosome 5q35 and contains 12 exons.^{79,80} *NPM1* encodes for three alternatively spliced nucleophosmin (NPM) isoforms: B23.1, B23.2, and B23.3. NPM is a highly conserved phosphoprotein that is ubiquitously expressed in tissues.^{79,80} While the bulk of NPM resides in the granular region of the nucleolus, it shuttles continuously between the nucleus and the cytoplasm.^{79,80}

NPM plays a central role in cell growth and proliferation through its involvement in ribosome biogenesis.

Except for two cases involving the splicing donor site of *NPM1* in exon 9 or exon 11,⁸¹ all reported *NPM1* mutations occur in exon 12 and typically result in an elongated NPM protein remaining in the cytoplasm; this change is relatively specific for AML, whereas almost all other human neoplasms consistently show nucleus-restricted NPM expression.¹

NPM1 mutations are characteristically heterozygous and retain a wild-type allele. Although *NPM1* mutations have been described in a few patients with chronic myelomonocytic leukemias and MDS, many patients with *NPM1* mutations rapidly progress to overt AML.^{74–76} *NPM1* mutations are common in de novo AML; but AML secondary to myeloproliferative disorders/MDS and therapy-related AML rarely show cytoplasmic NPM.⁷¹

In children with AML, the incidence of *NPM1* mutations ranges from 2.1% in Taiwan to 6.5% in Western countries, accounting for 9.0–26.9% of all children with AML with a normal karyotype. In adult patients with AML, the incidence of *NPM1* mutations ranges from 25.0 to 35.0%, accounting for 45.7–63.8% of all adult patients with AML with a normal karyotype.^{47–49,76–80} *NPM1* mutations are restricted to exon 12. Loss of *NPM1* mutations at the time of AML relapse is rare and may be a result of either the emergence of a different leukemic clone or the inability to detect mutations because few leukemic blasts infiltrate the bone marrow.⁷⁷ In some cases, loss of *NPM1* mutations was associated with a change from a normal to an abnormal karyotype. Patients with AML with wild-type *NPM1* at diagnosis rarely acquire *NPM1* mutations during the course of disease, suggesting that mutations are unlikely to play a role in disease progression.

NPM1 mutations are associated with a normal karyotype and do not occur in core binding factor leukemias or APL.¹ The chromosomal abnormalities observed in 14% of patients with *NPM1* mutations are probably secondary events, because cells with an abnormal karyotype often represent subclones within the leukemic population with a normal karyotype. As noted earlier, several studies have shown a strong correlation between *NPM1* mutations and *FLT3-ITD*.^{47,48}

NPM1 functions both as an oncogene and a tumor-suppressor gene depending on the gene dosage, expression levels, interacting partners, and compartmentalization.⁸² *NPM1* has been implicated in promoting cell growth, as its expression increases in response to mitogenic stimuli and above-normal amounts are detected in highly proliferating and malignant cells. On the other hand, *NPM1* contributes to growth-suppressing pathways through its interaction with ARF.

NPM1-mutated AML cases show a wide morphologic spectrum, but *NPM1* mutations are more frequent in the French–American–British (FAB) M4 and M5 AML subtypes^{48,73,76} and in AMLs with prominent nuclear invaginations (“cuplike” nuclei).⁸³ More than 95% of *NPM1*-mutated AML cases are negative for CD34.¹ *NPM1* mutations were

demonstrated in several cell lineages (myeloid, monocytic, erythroid, and megakaryocytic but not lymphoid).⁸⁴

Patients with *NPM1*-mutated AML with a normal karyotype often have high blast counts high *FLT3/ITD* and LDH serum levels, extramedullary involvement (mainly gingival hyperplasia and lymphadenopathy), and elevated platelet counts.^{47,48} Bone marrow biopsy specimens from *NPM1*-mutated AML cases frequently show increased numbers of megakaryocytes exhibiting dysplastic features.⁸⁵ After induction therapy, patients with AML with a normal karyotype carrying *NPM1* mutations achieve higher complete remission rates than similar patients who do not carry *NPM1* mutations.^{42,43,80,86} However, *NPM1* mutation status was not found to be an independent predictor for responsiveness to chemotherapy.⁴³

NPM1 mutations can be detected using several techniques. Conventional screening of *NPM1* mutations is usually performed by polymerase chain reaction (PCR) using a fluorescence-conjugated primer prior to fragment analysis by capillary electrophoresis or by immunohistochemical analysis to detect abnormal cytoplasmic localization of mutated *NPM1* protein.⁸⁷ Alternative methods include denaturing high-performance liquid chromatography and the fluorescence resonance energy transfer technique.⁸⁸

Rb1 Gene Mutations

The *Rb1* tumor-suppressor gene, located at chromosome 13q14, encodes a 110-kDa nuclear phosphoprotein that can bind to DNA and form complexes with other proteins. The Rb protein is integrally involved in cell cycle transition from the G₁ phase to the S phase. Abnormalities in the *Rb1* gene have been identified in a subset of AMLs. In a study by Ahuja et al⁸⁹ using Southern blot analysis, 5/54 (9.3%) cases of AML had structural abnormalities in the *Rb1* locus, including 4/15 (26.7%) cases of AML with monocytic differentiation (FAB subtypes M4 and M5). In most of these neoplasms, the frequencies of *Rb1* gene abnormalities were associated with the absence of protein expression. *Rb1* gene abnormalities were also identified in 6/42 (14.3%) cases of chronic myelogenous leukemia (CML) in myeloid blast crisis and 2/18 (11.1%) cases of MDS.⁸⁹

P53 Gene Mutations

The *p53* tumor-suppressor gene, located at chromosome 17p13, is often mutated in a wide variety of human cancers, including AMLs. The normal *p53* gene encodes for a nuclear phosphoprotein that binds to DNA and that can influence the expression of other genes. In addition, the *p53* protein appears to be involved in cell proliferation and apoptosis. In a review of the literature, Prokocimer and Rotter,⁹⁰ identified over 200 cases of AML, in which the status of the *p53* gene was assessed; point mutations were identified in approximately 6% of the cases. *p53* gene mutations have

been identified in all morphologic types of AML, except FAB subtype M3. The frequency of *p53* gene mutations has been associated with older patient age, presence of myelodysplasia, and poor prognosis.^{91,92}

STAT Activation

Several studies have revealed constitutive phosphorylation of STAT3 and STAT5 in AML blasts ex vivo, and constitutive DNA binding of STAT proteins or induction of their target genes has also been shown.^{93,94} Constitutive STAT3 activation has been associated with an unfavorable prognosis.⁹⁵ In addition, STAT3 and STAT5 may be activated by the mutationally activated isoforms of KIT and FLT3 in AML blasts.^{96,97} For full transcriptional activity, STAT proteins also have to be phosphorylated on serine residues, which is often accomplished by MAP kinases; this pattern of activation has been observed in AML blasts.⁹⁸ Aberrant regulation of gene expression contributes to the constitutive activation of STAT proteins.

SOCS proteins are potent repressors of STAT activation, and SOCS protein transcriptional repression through aberrant DNA methylation of regulatory regions upstream of the SOCS proteins' transcriptional start site has been shown in AML.⁹⁹

Constitutive activation of STAT3 and STAT5 appears to work as a signal integration site for several pathways on the way to leukemic transformation. The transforming ability of constitutively activated STAT5 proteins in mouse bone marrow was demonstrated in a retroviral transduction/transplantation study.¹⁰⁰ It was shown that the presence of STAT3 or STAT5 was necessary for the TK-mediated transformation of myeloid cells.^{97,100}

CXCR4 Overexpression

CXC chemokine receptor 4 (CXCR4) is one of a number of chemokine receptors with the ability to induce cell migration toward a chemotactic cytokine gradient (chemotaxis). CXCR4 has received much attention in the literature because it is the receptor for stromal-derived factor (SDF)-1 α , also known as CXCL12, and the CXCR4–CXCL12 axis is essential for the migration of normal cells to the bone marrow microenvironment.¹⁰¹ CXCR4–CXCL12 also appears to play a role in the metastatic spread of both hematopoietic and solid tumors.^{102–104}

CXCR4 has been shown to be expressed and functional in a subset of patients with AML and is associated with a poor prognosis,^{102,103} suggesting that the CXCR4–CXCL12 axis is a potential therapeutic target. In addition, CXCR4 expression was reported to be expressed more frequently and at a higher level in patients with AML with *FLT3/ITD* mutations when compared to patient with wild type *FLT3*.⁹⁴ Based on this last observation, a link between CXCR4 expression and the *FLT3/ITD* mutation has been proposed. However, it has also been

demonstrated that CXCR4 expression is associated with poor prognosis in patients with AML with normal cytogenetics and a wild type *FLT3* gene.¹⁰⁵

Antigen Receptor Gene Rearrangement

Immunoglobulin heavy chain (IgH) and/or *T-cell receptor β - and γ -chain* gene rearrangements were detected in less than 5% of patients with AML by investigators using Southern blot analysis.^{106,107} However, other investigators using PCR-based methods found a substantially higher number of patients with AML with IgH gene rearrangements.¹⁰⁸ Many of the cases with antigen receptor gene rearrangements were undifferentiated neoplasms, often TdT-positive and expressing one or more lymphoid-associated antigens, suggesting that these leukemias arose from an early bone marrow precursor cell with multilineage potential.¹⁰⁷ Patients with AMLs with IgH gene rearrangements have been reported to have a poorer prognosis than those without IgH gene rearrangements.¹⁰⁸

BAALC Gene

The brain and acute leukemia cytoplasmic (*BAALC*) gene is located at chromosome band 8q22.3 and encodes a protein that has no homology to any known proteins or functional domains.¹⁰⁹ The *BAALC* gene is expressed in neuroectoderm-derived tissues and hematopoietic precursors. In patients with de novo AML with a normal karyotype, high *BAALC* expression was associated with worse DFS and OS in patients with wild-type *FLT3* or with *FLT3/ITD* who also concurrently expressed the wild-type *FLT3* allele.^{110,111} When the analysis was restricted to patients with normal karyotype without *FLT3/ITD* or *CEBPA* mutations, high *BAALC* expression was still associated with worse DFS and OS.¹¹⁰ On multivariable analysis, patients with high *BAALC* expression were 2.7 times more likely to die and 2.2 times more likely to relapse than patients with low *BAALC* expression.¹¹¹ Based on these results, it was concluded that *BAALC* expression was particularly useful as a prognostic marker in patients with AML with normal karyotype and no *FLT3/ITD* or *CEBPA* mutations.¹¹⁰

In the large study of more than three hundred patients with normal karyotype, high expression of *BAALC* was an independent adverse prognostic factor for resistance to initial induction chemotherapy, CIR, and OS.¹¹² On multivariable analysis, high *BAALC* expression and a high *FLT3/ITD*/wild-type *FLT3* ratio were the only factors predicting a high CIR and shorter OS. Notably, preliminary data from the same study also suggested that allogeneic stem cell transplantation (SCT) in first CR might overcome the higher relapse rate associated with high *BAALC* expression.¹¹² These results suggest that patients with high *BAALC* expression might benefit from allogeneic SCT. Patients with high *BAALC* expression who underwent allogeneic SCT had a significantly lower

cumulative incidence of relapse (CIR) than patients with high *BAALC* expression who underwent autologous SCT. Moreover, among all patients undergoing allogeneic SCT, there was no difference in CIR between patients with high or low *BAALC* expression.¹¹²

CEBPA Gene

The CCAAT/enhancer-binding protein α (*CEBPA*) gene encodes a protein member of the basic region leucine zipper (bZIP) transcription factor family, playing a crucial role in granulopoiesis.¹¹³ In AML, *CEBPA* gene mutations are associated with a normal karyotype at diagnosis.¹¹³ There are 2 main categories of *CEBPA* mutations: C-terminal mutations that occur in the bZIP domain, which are usually in-frame and predict mutant proteins lacking DNA binding and/or homodimerization activities; and N-terminal nonsense mutations that prevent expression of the full-length protein and result in truncated isoforms with dominant-negative activity. Some patients present with biallelic mutations at the C-terminus, whereas others are heterozygous for separate mutations or found to have a C-terminal mutation coexisting with a mutation in the N-terminus.^{114,115}

CEBPA mutations occur in 15–19% in patients with normal cytogenetics and are associated with a favorable prognosis^{49,110,114} and significantly better EFS,^{115,116} DFS,¹¹⁶ and OS.^{115,116} At diagnosis, patients with *CEBPA* mutations had higher percentages of blood blasts, lower platelet counts, less lymphadenopathy and extramedullary involvement, less frequent *FLT3/ITD* and *FLT3/TKD* mutations, and no *MLL/PTD* mutations than patients without *CEBPA* mutations.¹¹⁴ CR rates did not differ between patients with and without *CEBPA* mutations,^{49,110,114} whereas CRD,¹¹⁴ DFS,¹¹⁰ EFS,⁴⁹ and OS^{110,114} were significantly better for patients with *CEBPA* mutations. On multivariable analysis, *CEBPA* mutational status has added prognostic information to that provided by *MLL/PTD* and *FLT3/ITD* status, age, and resistant disease after the first course of induction therapy with regard to CRD and by *FLT3/ITD* status, age, and WBC for OS.¹¹⁴ Similar results were obtained in another study, where *CEBPA* mutations, *BAALC* expression, and *FLT3/ITD* status were prognostic for DFS, and *CEBPA* mutations, age, *BAALC* expression, and *FLT3/ITD* status were prognostic for OS.¹¹⁰

Fröhling et al¹¹⁴ analyzed clinical outcomes in patients with N-terminal nonsense *CEBPA* mutations, patients with other *CEBPA* mutation types, and patients with wild-type *CEBPA* and found that the CRD was the longest in patients with N-terminal mutations, followed by the CRD in patients with other mutations and in those without *CEBPA* mutations. However, in pairwise comparisons, there were no significant differences in the CRD between patients with N-terminal mutations and those with other mutations or between patients with other mutations and patients with wild-type *CEBPA*.¹¹⁴

ERG Gene

The v-ets erythroblastosis virus E26 oncogene homolog (avian; *ERG*) gene is located at the long arm of chromosome 21 (band 21q22)¹¹⁷ and is a member of the *ETS* gene family, which encodes downstream nuclear targets of the signal transduction pathways regulating and promoting cell differentiation, proliferation, and tissue invasion.^{118,119} The *ERG* gene was discovered in patients with AML with prognostically unfavorable complex karyotypes that contained cryptic amplification of chromosome 21, which was first uncovered by spectral karyotyping.^{117,120} In AMLs carrying the t(16;21)(p11;q22) abnormality, *ERG* was found to be rearranged with *FUS*, linking *ERG* with myeloid leukaemogenesis.¹²¹ However, *ERG* overexpression was not always associated with genomic amplification and was also detected in CN AML,¹¹⁷ suggesting that *ERG* overexpression might bestow an adverse prognosis in patients with CN AML. In one study of patients with AML patients normal cytogenetics, patients with the highest *ERG* expression levels (4th quartile) had a worse CIR than those with low expression (1st–3rd quartiles; $P < 0.001$); the estimated 5-year relapse rate for patients in the 4th quartile was 81% versus 33% for patients in the 1st–3rd quartiles.¹²² Patients in the 4th quartile had a median survival of 1–2 years and an estimated 5-year survival rate of 19%, while median survival for patients in the 1st–3rd quartiles had not been reached and the estimated 5-year survival rate was 51%.¹²² On multivariate analyses, high *ERG* expression and *FLT3/ITD* status independently predicted worse CIR and OS. Analyses restricted to the more favorable subset of patients lacking *FLT3/ITD* or expressing both *FLT3/ITD* and the wild-type *FLT3* allele revealed that high *ERG* expression and *MLL/PTD* both impacted remission duration. With respect to OS, there was an interaction between *ERG* and *BAALC* expression, with *ERG* overexpression predicting shorter OS times only in patients with low *BAALC* expression.¹²²

MNI Gene

The meningioma 1 (*MNI*) gene is located at the long arm of chromosome 22 (22q11) and was initially isolated from a meningioma with the t(4;22)(p16;q11) abnormality.¹²³ Later, *MNI* was found to be fused with *ETV6* as a result of the t(12;22)(p13;q11–12) abnormality, a recurrent translocation in AML.¹²⁴ In one cDNA microarray study, *MNI* overexpression was associated with poor response to induction chemotherapy.¹²⁵ In another study of patients with AML with a normal karyotype, high *MNI* expression measured by real-time reverse transcriptase polymerase chain reaction (RT-PCR) was associated with a lower response rate on day 15 after the first course of induction therapy than the response rate seen in patients with low *MNI* expression.¹²⁶ Interestingly, in this study, the presenting clinical features did not differ between patients with low and high *MNI* expression; however, high *MNI* expression was associated with high CD34 expression. There was no significant difference in CR rates between the groups. Furthermore, high *MNI* expression

bestowed a higher relapse rate and worse RFS and OS rates. Thus, *MNI* expression was found to be an independent unfavorable prognostic factor for OS and RFS.¹²⁶

Other Prognostic Factors in Karyotypically Normal AML

Although most patients with CN AML harbored at least one of the aforementioned genetic alterations, in a study by Döhner et al⁴⁸ almost a quarter of these patients did not carry *FLT3/ITD*, *FLT3/TKD*, or *MLL/PTD* or *CEBPA* or *NPM1* mutations. Thus, it is likely that novel gene mutations and/or abnormal gene expression with prognostic significance will be discovered in the future.

A recent study found a significantly shortened complete remission duration (CRD) in patients with CN AML that was associated with overexpression of the breast cancer resistance protein (BCRP) encoded by the *ABCG2* gene.¹²⁷ Interestingly, in another study that used gene-expression profiling to classify adult AML, overexpression of the *ABCG2* gene was a consistent finding in a cluster of patients with worse disease-free survival (DFS) rates and a high induction failure rate, 42% of whom had a normal karyotype.¹²⁸ It will be of interest to determine if high expression of the *EVII* gene, hitherto reported as bestowing an adverse prognosis in patients classified in the intermediate-risk cytogenetic group,¹²⁹ or overexpression of the *WT1* gene, shown to unfavorably impact clinical outcomes in patients with cytogenetically heterogeneous AML in some¹³⁰ but not all¹³¹ studies, also confer an adverse prognosis in patients with CN AML. Likewise, although a recent study revealed no differences in outcomes in patients with CN AML patients either with or without *NRAS* mutations,¹³² further studies of *NRAS*, *KRAS*, and *KIT* mutations seem warranted given the prospect of developing therapies with tyrosine kinase inhibitors targeting these mutations. Finally, future analyses should address potential prognostic relevance in CN AML with epigenetic changes, which have already been correlated with clinical outcome in adult AML in some studies.^{133–136}

Abnormalities Affecting Proliferation/Apoptosis of Leukemia Cells Directly

Similar to normal hematopoiesis, leukemic hematopoiesis occurs in a unique bone marrow (BM) microenvironment that is integral to the production and differentiation of both normal and leukemic hematopoietic progenitor cells. Such hematopoietic cell development is regulated by BM stromal cells (BMSC) through the production of cytokines and intracellular signals.^{137,138} It is still debated, however, whether certain mesenchymal elements are altered in specific hematologic disorders, in terms of either their levels and/or their functions, and to what extent such alterations may contribute

to the progression of the disease.^{139–141} In a recent study, karyotypic abnormalities were found in a pure population of mesenchymal stem cells (MSC) in a significant proportion of patients with MDS.¹⁴² Interestingly, in those cases with chromosomal alterations, the karyotypes showed complex abnormalities that, in some cases, were totally different from those observed in their hematopoietic counterparts. The same phenomenon has been noted in cultures of stromal cells showing trisomy 8 and monosomy 7 from patients with AML.¹⁴³

Osteoblasts

Osteoblasts play a central role in skeletal development. Derived from pluripotent MSC, osteoblasts mature along a specific lineage to become highly specialized cells. Primary human osteoblasts produce growth factors, including G-CSF, that are likely essential for the survival of CD34+ BM cells^{144–148} and support the growth of CD34+ human BM progenitor cells in vitro.¹⁴⁹ While osteoblasts play a pivotal role in support of normal hematopoiesis, the possible role of these cells in leukemic hematopoiesis has also been recently suggested. In two published studies, the culture of AML blasts with osteoblastic sarcoma cells (Ca172, SJSA-1), as well as with normal osteoblasts, was shown to increase AML blast proliferation and to increase the levels of the proangiogenic mediators IL-8¹⁵⁰ and vascular endothelial growth factor (VEGF).¹⁵¹

Leptin

Leptin, a 16-kDa protein produced in abundance in BM adipocytes,¹⁵² was originally identified as a cytokine that regulates fat metabolism.¹⁵³ Leptin's role in the interaction between BM and leukemic cells was recently identified when it was discovered that leptin significantly reduced ATRA- and doxorubicin-induced apoptosis in PML-RAR α cells expressing high levels of the leptin receptor.¹⁰⁰ This antiapoptotic effect required direct cell-cell interactions, was associated with the phosphorylation of STAT3 and Ras/mitogen-activated protein kinase (MAPK), and was reduced when the leptin receptor (OB-R) was blocked. This finding provides a mechanistic basis for the BM adipocyte-leukemic cell interaction and indicates that leptin secreted by BM adipocytes in the vicinity of leukemic cells could play a role in the proliferation and survival of APL cells through paracrine interactions in the BM microenvironment.

Leptin is known to induce the proliferation and differentiation of normal hematopoietic cells¹⁵⁴ and the proliferation of AML cells.¹⁵⁵ It has recently been shown that primary APL cells express high levels of the OB-R long isoform and that the induction of PML-RAR α increased the levels of OB-R mRNA.¹⁰⁰ The expected apoptosis in PML-RAR α -expressing APL cells induced by ATRA and doxorubicin was blocked by direct contact between a layer of cultured mesenchymal stem cell-derived adipocytes and the APL cells.¹⁰⁰

It was also demonstrated that the cells in the NB4 cell line spontaneously migrated beneath the MSC-derived adipocytes layer^{156,157} (a behavior known as pseudoemperipolesis)¹⁵⁸, which is responsible for the engulfment of leukemic cells by adherent stromal cells. NB4 cells migrated below the adipocytes after 3–96 h of coculture, which suggests that intimate cell-to-cell contact through the membrane starts early and continues thereafter. In addition, NB4 cells below the adipocytes were protected from apoptosis.¹⁵⁷ Pseudoemperipolesis has also been observed between BM stromal cells and lymphoid or myeloid leukemic cells, and such direct contact between the cancer cells and the stromal cells prevented the apoptosis of leukemic cells in vitro.¹⁵⁹

Microvasculature

The microvasculature is an active component of the stroma and is responsible for supplying the appropriate oxygen and nutrients. The microvasculature provides a route for homing and metastasis as well, and BM endothelial cells are also involved in autocrine and paracrine interactions with leukemic cells. Angiogenesis is defined as the formation of new capillaries from preexisting blood vessels, and it plays an important role in the progression of solid tumors. Recently, a similar relationship has been noted in several hematologic malignancies. For example, the development of clinical disease that involves the infiltration of leukemic cells throughout the BM compartment seems to require the interaction of leukemic blasts and neighboring nonleukemic cells in the context of local angiogenesis and increased BM vessel density.^{160,161} Endothelial cells are also morphologically abnormal in hematologic malignancies, in that they form branching, smaller-caliber microvessels^{162–164} that are functionally abnormal. Increased angiogenesis has been observed in MDS^{162,165} and AML.^{160,166} In addition, the synthesis of VEGF and other angiogenic factors, such as bFGF and hepatocyte growth factor, has been observed in leukemic cells.^{167,168} Furthermore, increased plasma as well as intracellular AML blast levels of proangiogenic VEGF are associated with an increased risk of relapse after intensive chemotherapy,¹⁶⁹ and low systemic levels of the antiangiogenic cytokine endostatin are associated with reduced survival.¹⁷⁰ These findings suggest that angiogenesis induction is involved in the pathogenesis of hematologic disease in a similar way as the angiogenic switch that has been proposed to be responsible for the formation of solid tumors. In line with these results, recent studies have indicated that VEGF not only plays a role in angiogenesis, but also promotes hematopoietic cell survival.¹⁷¹ In particular, VEGF was found to inhibit apoptosis in leukemic cells after exposure to etoposide and doxorubicin by inducing MCL1, a member of the BCL2 family.

While the mechanisms of increased angiogenesis are likely to be multifarious, one of the major determinants of angiogenesis (via VEGF expression) and glycolysis, two leading characteristics of tumor invasion and metastasis, is HIF-1.

The fact that increased cellular hypoxia occurs during the progression of AML in rats and, unlike normal cells, some of the leukemic cells proliferate even during hypoxia points to the potential relevance of this transcription factor.^{172,173} It is, therefore, conceivable that the expression of HIF target genes, such as VEGF, plays an important role in leukemia progression, therapy response, and outcome. Of interest, HIF-1 was recently found to regulate SDF-1 gene expression in endothelial cells, resulting in the selective *in vivo* expression of SDF-1 in ischemic tissue in direct proportion to reduced oxygen tension.¹⁷⁴ Such HIF-1-induced SDF-1 expression increased the adhesion, migration, and homing of circulating CXCR4-positive progenitor cells to ischemic tissue. Together with the finding that HIF-1 regulates CXCR4,¹⁷⁵ these data suggest that a hypoxic BM microenvironment represents a conditional stem and progenitor cell niche, in which the HIF-1-induced stabilization and activation of both the trafficking stimulus (SDF-1) and receptor (CXCR4) facilitate the recruitment and retention of leukemic progenitor cells. In this context, since HIF-1 is expressed in hypoxic areas containing infiltrating stromal cells and endothelial cells, it may represent the molecular target of the tumor microenvironment.

Integrins are heterodimers that mediate the adhesion of cells to ECM proteins and in some cases to counterreceptors on other cells.^{176,177} One protein that plays a central role in integrin activation and signaling is integrin-linked kinase (ILK)¹⁷⁸; ILK connects integrins to the actin cytoskeleton and transduces signals through integrins to the ECM and from integrins to various subcellular compartments. In addition, ILK activates Akt either directly or indirectly and has recently been biochemically confirmed to be an *in vivo* activator of Akt.¹⁷⁹ ILK is involved in cell survival, cell cycle progression, cell adhesion, cell spreading, and ECM modification.¹⁸⁰ ILK is also involved in regulating changes in cell shape and cell migration through a PINCH/ILK interaction¹⁸¹ and through Rac1 activation.¹⁸² Recent studies have demonstrated that ILK is constitutively activated in AML, which provides survival signals to leukemic cells.¹⁸³ Conversely, the inhibition of ILK signaling by small-molecule ILK inhibitors induced apoptosis in leukemic cells cocultured with BMSC. Moreover, the blockade of ILK/Akt signaling inhibited other signaling pathways as well (i.e., the MAPK and STAT-3 pathways), demonstrating a proximal role for ILK/Akt in stromal-leukemic cell interactions. These results have established ILK as an attractive target for therapy in leukemias.

The BM microenvironment is presumed to be the primary site for minimal residual disease after chemotherapy, as leukemic cells may acquire resistance to chemotherapy by interacting with BMSC components. It was demonstrated that BMSCs protect AML cells from cytosine arabinoside (AraC)-induced apoptosis and that this is mediated through the induction of BCL-2 and BCL-X_L in leukemic cells.¹⁸⁴ Evidence supporting this concept includes the finding that VLA-5, a β_1 integrin on AML cells, interacts with fibronectin on BMSCs, thereby triggering antiapoptotic and proliferative signals.¹⁸⁵

In addition, AML cells bind to BMSCs through combined β_1 and β_2 integrin mechanisms.¹⁸⁶ Recent data suggest that the interaction between VLA-4 on leukemic blasts and fibronectin on BMSC activates PI3K/AKT/Bcl-2 signaling, which is a critical determinant of the chemosensitivity of AML cells, the level of minimal residual disease in patients with AML, and disease-free survival in patients with AML.¹⁸⁷ In a mouse model of minimal residual disease, the combination of VLA-4-specific antibodies and AraC resulted in a 100% survival rate, whereas AraC alone prolonged survival only slightly. Thus, the interaction between VLA-4 on leukemic cells and fibronectin on stromal cells may be crucial in determining the extent of minimal residual disease in the BM and the prognosis in patients with AML. The CD44 adhesion molecule, a receptor for hyaluronan, is strongly expressed on AML blasts, and CD44 ligation was shown to decrease drug-induced apoptosis of AML blasts.¹⁸⁸ However, the CD44 ligand, hyaluronan, is expressed on endothelial cells as well as on BMSCs, and AML cells have a much lower affinity for hyaluronan than for other stromal cell components in the BM, making the CD44-hyaluronan interaction unlikely to be the main cause of minimal residual disease in BM.

Conclusions

Both molecular abnormalities directly affecting leukemic cells and abnormalities affecting the interaction of leukemic cells with BMSCs are crucial for leukemic cell biology. Although many issues remain unresolved, dramatic progress in understanding leukemic cell biology and the interaction of leukemic cells with BM stroma has been made. Further success will not only enrich our knowledge, but will also dramatically improve patient care.

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Acute Myeloid Leukemia with Myelodysplasia-Related Changes and Therapy-Related Acute Myeloid Leukemia

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous clonal malignant disorder resulting from genetic alterations in multipotent hematopoietic stem cells.^{1,2} These alterations limit the ability of stem cells to differentiate into erythrocytes, granulocytes, and platelets and lead to the proliferation of leukemic cells or blasts.^{3,4}

Approximately 11,000 cases of AML are diagnosed annually in the United States, where the overall annual incidence rate is 3.4 cases per 100,000, rising to 17.9 cases per 100,000 in adults 65 years old and older.⁵ The median age at presentation is 68 years,⁵ and it occurs more frequently in males than in females.^{5,6} AML accounts for less than 15% of leukemia cases in children younger than 10 years and 25–30% of cases in children aged 10–15 years.^{5,7} Among adults, AML accounts for 80–90% of acute leukemia cases.⁵ The AML in people older than 60 years may result from several factors, including improved diagnosis and longer life expectancy, resulting in increased environmental exposures that lead to genetic alterations.⁸ Many cancers are dependent on subpopulations of the so-called cancer stem or initiating cells. Human leukemia stem cells (LSC) are not functionally homogeneous, but like normal hematopoietic stem cells, fall into a distinct hierarchy.² The heterogeneity in the self-renewal potential of all leukemia stem cells supports the hypothesis that they derive from normal hematopoietic stem cells.¹ Warner et al⁹ showed that coordinating genetic events enabled the leukemic transformation of normal hematopoietic stem cells. They developed a system whereby the first step in the leukemogenic processes was an engineered disruption of differentiation and self-renewal resulting from the expression of the *TLS-ERG* oncogene, followed in some cases by the overexpression of *hTERT*. The transduced cells then underwent step-wise transformation and immortalization through the spontaneous acquisition of additional changes including karyotypic modifications. The acquired karyotypic abnormalities and alterations, including the upregulation of the *Bmi-1* oncogene and telomerase, all occur in this system. When exposed to

cytokines, the undifferentiated blasts exhibited cytoplasmic myeloid differentiation as shown by the expression for myeloperoxidase and butyrate esterase.⁹

Knowledge of the genetic mutations and deregulated expression of particular genes or sets of genes will allow us to dissect the diversity present in cases of AML defined by cytogenetic subsets, which will lead to the development of leukemia stem cell – selective or “targeted” therapies. Such genetic alterations appear to fall into different classes of mutations.¹⁰ One group comprises mutations that activate signal transduction pathways. These are class I mutations that are frequently represented by mutations of receptor tyrosine kinases, which result in enhanced cell proliferation and/or survival. Examples include mutations in genes such as *FLT3*, *RAS*, *KIT*, *PIK3C2B*, *BCL-2*, *MYC*, *CCNA2*, *CCNE2*, and *CDC2*. Class II mutations affect transcription factors (i.e., *RUNX1*, *RARA*, *EVII*, *NPM1*, *TAL1*, *GATA1*, *EKLF*, and *WT1*) or components of the transcriptional co-activation complex and result in impaired differentiation. Recurring gene fusions resulting from balanced translocations, or mutations in *CEBPA* or other genes, fall into this category.

A new dimension of complexity has been added by the analysis of alterations in the epigenome. It has become clear that changes in epigenetic signatures comprising aberrant methylation and histone deacetylation are common modifications in various types of leukemia. Because both DNA methylation and histone deacetylation are reversible processes, they have become attractive targets for epigenetic therapy.

Classification of AML

The current classification of acute leukemias and myelodysplastic syndrome (MDS) by the World Health Organization (WHO) includes morphologic, clinical, immunophenotypic, and cytogenetic features.^{3,4,11–14} The WHO classification for AML includes four categories: AML with recurrent cytogenetic abnormalities, AML with myelodysplasia-related changes,

therapy-related myeloid neoplasms, and AML not otherwise specified (Table 36.1). Thus, to diagnose AML completely, one must evaluate the percentage of immature cells and their morphologic, cytochemical, and immunophenotypic characteristics; evaluate residual hematopoiesis for the presence and degree of dysplasia, obtain the patient's history of chemotherapy, and learn from cytogenetic and/or molecular studies whether a patient has a recurrent cytogenetic abnormality.

AML with myelodysplasia-related changes is diagnosed when 20% or more of the blasts are present in peripheral blood or bone marrow (BM) and there are morphologic features of myelodysplasia or the patient has a prior history of MDS or myelodysplastic/myeloproliferative neoplasm, or MDS-related cytogenetic abnormalities.¹³ Patients should not have a history of prior cytotoxic or radiation therapy for an unrelated disease. Morphologic multilineage dysplasia is significantly correlated with secondary AML and unfavorable cytogenetics. In a multivariate analysis, no adverse prognostic impact of multilineage dysplasia and prior chemo/radiotherapy was detected; whereas, cytogenetic risk and patient age maintained their prognostic value.^{15,16} Multilineage dysplasia is prevalent in *FLT3-ITD*-negative patients and equally distributed between patients with and without *NPM1* mutation.¹⁵ Chromosomal abnormalities are similar to those found in MDS and often involve the gain or loss of major segments of certain chromosomes with complex karyotypes, with $-7/\text{del}(7q)$ and

$-5/\text{del}(5q)$ being the most common (76% of all cases with abnormal karyotype).¹³ Additional abnormalities including balanced translocations that are sufficient for inclusion in this category are listed in Table 36.2. One of the balanced translocation involves 5q32-33. The $t(3;5)$ is associated with multilineage dysplasia and a younger age at presentation than most other cases in this disease group.¹⁷ Cases with multilineage dysplasia may carry *NPM1* and/or *FLT3* mutations.¹⁸ These mutations are discussed in Chap. 35. Most *NPM1* mutated cases would be expected to have normal karyotype, CD34 negative blasts, and no history of prior MDS. Cases of adult acute megakaryoblastic leukemia frequently have multilineage dysplasia, and a history of an antecedent hematologic disorder or MDS or both, and 19% of patients have previously received chemotherapy for other malignancies.¹⁹ De novo adult AML with multilineage-related changes and 20% megakaryoblasts are considered AML with myelodysplasia-related changes (megakaryoblastic type).²⁰

The category of therapy-related myeloid neoplasms includes therapy-related AML (t-AML), therapy-related MDS (t-MDS), and myelodysplastic/myeloproliferative neoplasms occurring as late complications of chemotherapy or radiation therapy administered for prior neoplastic or non-neoplastic disorders.^{21,22} Survivors of the atomic bombs in Japan had an increased incidence of myeloid leukemias that peaked 5–7 years following their exposure to radiation.^{23,24}

TABLE 36.1. World Health Organization classification of AML.

Acute myeloid leukemia (AML) with recurrent genetic abnormalities
• AML with $t(8;21)(q22;q22)$, <i>RUNX1-RUNX1T1</i>
• AML with $\text{inv}(16)(p13.1q22)$ or $t(16;16)(p13.1;q22)$, <i>CBFβ-MYH11</i>
• Acute promyelocytic leukemia (APL) with $t(15;17)(q22;q21)$, <i>PML-RARA</i>
• AML with $t(9;11)(p22;q23)$; <i>MLLT3-MLL</i>
• AML with $t(6;9)(p23;q34)$; <i>DEK-NUP214</i>
• AML with $\text{inv}(3)(q21q26.2)$ or $t(3;3)(q21;q26.2)$; <i>RPN1-EVII</i>
• AML (megakaryoblastic) with $t(1;22)(p13;q13)$; <i>RBM15-MKLI</i>
• Provisional entity: AML with mutated <i>NPM1</i>
• Provisional entity: AML with mutated <i>CEBPA</i>
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, not otherwise specified
• AML with minimal differentiation
• AML without maturation
• AML with maturation
• Acute myelomonocytic leukemia
• Acute monoblastic/monocytic leukemia
• Acute erythroid leukemias
– Pure erythroid leukemia
– Erythroleukemia, erythroid/myeloid
• Acute megakaryoblastic leukemia
• Acute basophilic leukemia
• Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
• Transient abnormal myelopoiesis
• Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasms

From ref.¹²

TABLE 36.2. Cytogenetic abnormalities sufficient to diagnose AML with myelodysplasia-related features when 20% PB or BM blasts are present.

Complex karyotype ^a
Unbalanced abnormalities
$-7/\text{del}(7q)$
$-5/\text{del}(5q)$
$i(17q)/t(17p)$
$-13/\text{del}(13q)$
$\text{del}(11q)$
$\text{del}(12p)/t(12p)$
$\text{del}(9q)$
$\text{idic}(X)(q13)$
Balanced abnormalities
$t(11;16)(q23;p13.3)^b$
$t(3;21)(q26.2;q22.1)^b$
$t(1;3)(p36.3;q21.1)$
$t(2;11)(p21;q23)^b$
$t(5;12)(q33;p12)$
$t(5;7)(q33;q11.2)$
$t(5;10)(q33;q21)$
$t(3;5)(q25;q34)$

^aMore than three unrelated abnormalities, none of which are included in the subgroup for AML with recurrent genetic abnormalities. Such cases should be classified in the appropriate cytogenetic group.

^bThese abnormalities most commonly occur in therapy-related AML, which should be excluded before using these abnormalities as evidence for a diagnosis of AML with myelodysplasia-related features.

PB peripheral blood, BM bone marrow, AML acute myeloid leukemia.

From ref.²⁰

TABLE 36.3. Frequency of cytogenetic abnormalities in de novo and therapy-related MDS and AML.

Type of disease	Cytogenetics		
	Unbalanced 5q-/-5, 7q-/-7, +8 (%)	Balanced 11q23, 21q22, 17q21, 16q22 (%)	Normal karyotype (%)
De novo MDS	15–25	Rare	50–60
t-MDS	50–70	Rare	5–10
De novo AML	15–25	15–20	40–50
t-AML	40–50	15–20	10–15

AML acute myeloid leukemia, MDS myelodysplasia syndrome.

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Therapeutic radiation levels appear to impart minimal risk, but the risk may be significantly increased if radiation was administered concurrently with chemotherapy that included an alkylating agent.

There are two types of chemotherapy-related AML.²⁵ The “classic” type caused by exposure to alkylating agents (e.g., cyclophosphamide, melphalan, or nitrogen mustard) has a latency period of 5–10 years and often is associated with t-MDS, evidence of bone marrow failure, and abnormalities of chromosome 5 and/or chromosome 7.^{26,27} The second type is associated with exposure to agents that inhibit the DNA repair enzyme topoisomerase II (e.g., etoposide, teniposide). This latter type has a shorter latency period, usually 1–5 years, and is not associated with a MDS phase but is associated with a balanced chromosomal translocation (Table 36.3).²⁸

Drugs such as chloramphenicol, phenylbutazone, chloroquine, and methoxypsoralen may result in BM damage, that may later evolve into AML.¹⁴ Exposure to benzene, a ubiquitous solvent used in several industries and products such as cigarettes, dyes, herbicides, and pesticides has been implicated as another potential risk factor (see Chap. 6).^{29,30}

Dysplasia in Myeloid Cells

Differential diagnosis among AML categories requires evaluation not only of blasts and blasts equivalents, but also cells appearing as mature or maturing because these cells may be part of a neoplastic clone and demonstrate unusual morphological features, such as giant neutrophils or giant platelets, or contain Auer rods. Marked dysplasia may be a hint of a history of previous chemotherapy. In the absence of such a history, marked dysplasia warrants classification as AML with myelodysplasia-related changes. Dysplastic features are also seen in cases of t-AML. By definition, the dysplasia in AML with multilineage dysplasia must be severe, that is, present in at least 50% of cells of at least two lines. The criteria for dysplasia are as follows:

- Granulocytic dysplasia: agranular or hypogranular polymorphonuclear cells, hyposegmented nuclei (pseudo Pelger-Huët anomaly), or pseudo Chédiak-Higashi-type giant granules.

- Megakaryocytic dysplasia: micromegakaryocytes, multiple separated nuclei, or large mononuclear forms.
- Erythroid dysplasia: karyorrhexis, megaloblastoid aspect, multinuclearity, nuclear fragments, nuclear budding, internuclear bridging, cytoplasmic vacuolization, or aberrant positivity for periodic acid-Schiff.³¹

Detection of Dysplasia Using Flow Cytometric Immunophenotyping

Dysplastic features may be assessed by using flow cytometric immunophenotyping.³² The proposed criteria for myeloid cells are abnormal granularity, abnormal decrease in CD45 expression, presence of HLA-DR or lack of CD11b, abnormal relationship between CD13 and CD16, expression of CD56 on myeloid cells, lack of CD33 expression, asynchronous shift to the left, presence of lymphoid antigens, or expression of CD34 on myeloid cells.³² For monocytic cells, the findings include abnormal granularity, abnormal CD11b or HLA-DR expression, loss of CD13 or CD16 expression, lack of CD33 or CD14 expression, and expression of CD56 and/or CD34, and the lymphoid antigen.³² Dyspoiesis that is detectable by flow cytometric analysis results in increased risk of relapse after allogeneic hematopoietic cell transplantation.^{33,34}

Cytochemical Features of AML

An important aspect of the classification of AML is the cytochemical reactivity pattern of the blasts. Cytochemical stains used to demonstrate the reactivity pattern of leukemia blasts include myeloperoxidase (or to a lesser extent, Sudan Black B), nonspecific esterase (alpha-naphthyl butyrate or alpha-naphthyl acetate), naphthol ASD chloroacetate esterase, and occasionally periodic acid-Schiff. If 3% or more of the blasts stain positive for myeloperoxidase or Sudan Black B, the diagnosis is AML. If the blasts are negative for either or both of those stains, but positive for butyrate or nonspecific esterase, acute monocytic leukemia is diagnosed.³¹

Immunophenotype

There are no specific immunophenotypic findings in AML with myelodysplasia-related changes or in t-AML. Blasts generally express CD34 and express panmyeloid markers (CD13, CD33). Aberrant expressions of CD56 and/or CD7 are frequent.

Genetics and Molecular Markers

MDS and AML are heterogeneous, closely associated diseases arising de novo or following irradiation or chemotherapy with alkylating agents or topoisomerase II inhibitors. Whereas

de novo MDS and AML are almost always subclassified according to cytogenetic characteristics, t-MDS and t-AML are often considered as separate entities and are not subdivided. Alternative genetic pathways were previously proposed in t-MDS and t-AML on the basis of cytogenetic characteristics.³⁵⁻³⁸ An increasing number of gene mutations are now observed to cluster differently in these pathways, with identical patterns seen in de novo and in therapy-related forms of both diseases (Figures 36.1 and Tables 36.4 and 36.5).^{38,39} An association is observed between activating mutations of genes in the tyrosine kinase *RAS-BRAF* signal transduction pathway (class I mutations) and inactivating mutations of genes encoding hematopoietic transcription factors (class II mutations). Point mutations of *AML1* and *RAS* seem to cooperate and predispose hematopoietic stem cells to progression from t-MDS to t-AML. Recently, critical genetic effects underlying chromosomal abnormalities at 5q-/5 and 7q-/7 have been proposed.³⁸ Their association and cooperation with point

mutations of *p53* and *AML1*, respectively, extend the scenario of cooperating genetic abnormalities in t-MDS and t-AML.³⁸ The *p53* tumor suppressor directs the cellular response to DNA-damaging agents and is selected during the pathogenesis of t-AML. Recent data indicate that MDM2 and *p53* variants interact to modulate responses to genotoxic therapy and are risk determinants for t-AML.^{40,41}

The mechanisms responsible for the evolution of early MDS to advanced MDS and AML consist of an equilibrium switch in favor of signals that prevent apoptosis and cause increased cell proliferation. The transcription factor *RELA/NFKB1* and the *FLT3/PI3KC2A/AKT1* pathways seem to play key roles in such a process (Figure 36.1).^{18,42-45} Inhibition of the *FLT3* receptor reduces constitutive *RELA/NFKB1* activation in patients with high-risk MDS and AML.⁴⁶ Interestingly, bortezomib induces DNA hypomethylation and silences gene transcription by interfering with *Sp1/NFKB1*-dependent DNA methyltransferase activity in AML.⁴⁷

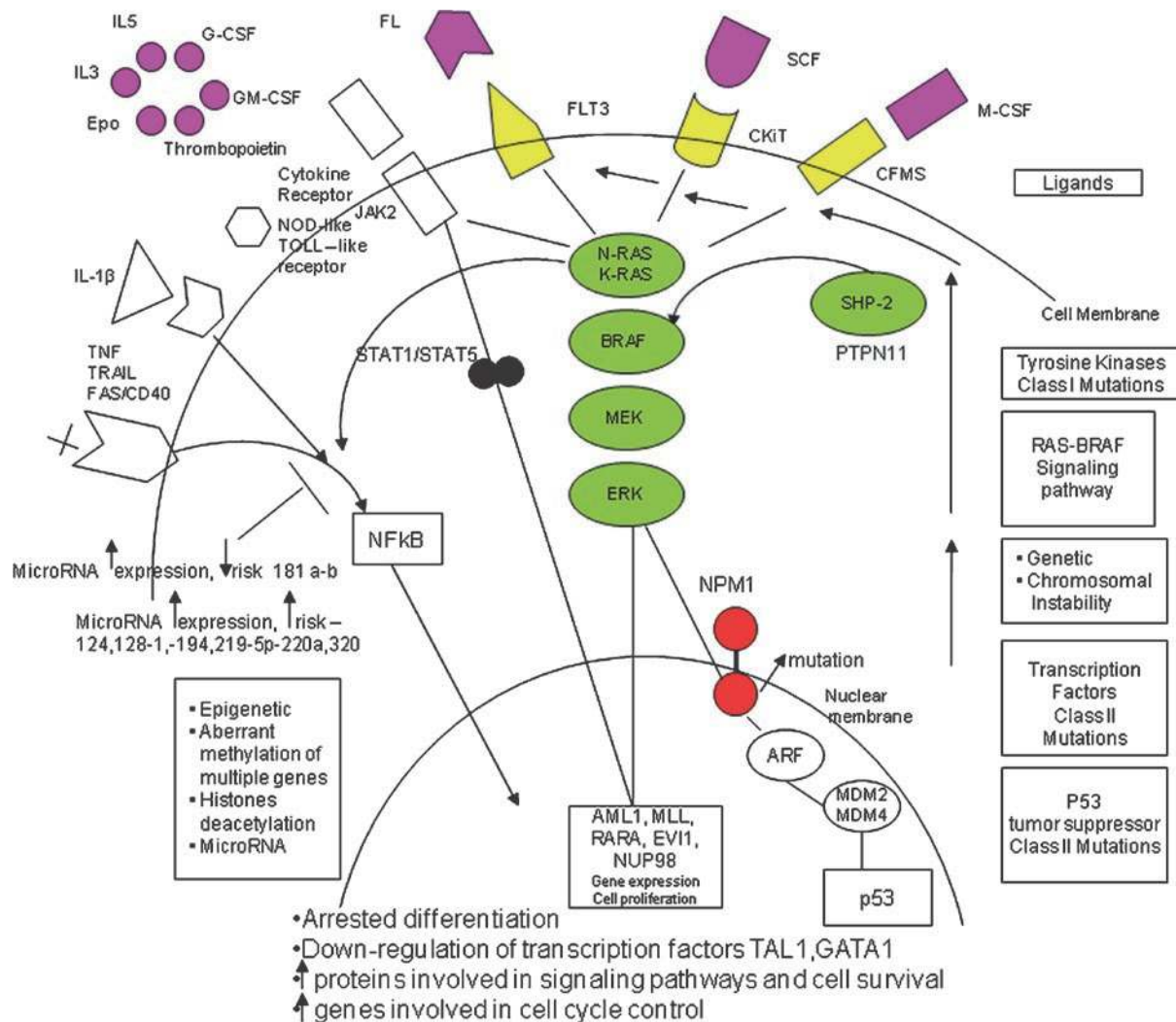


FIG. 36.1. Diagram of signal transduction pathways and classification of mutations in MDS and AML. AML acute myeloid leukemia, MDS myelodysplasia syndrome. Adapted by permission from Macmillan Publishers Ltd, ref.³⁸

TABLE 36.4. The eight alternative genetic pathways in secondary MDS/AML (according to ³⁸ with modifications).

Inducing agent	Chromosomal defect	Additional chrom. defect	Principal molecular lesion	Other molecular lesion	Last genetic defect
Alkylating agents	-7/7q	None	RUNX1 mutation	RAS mutations, TP53 mutations	Frequent CDKN2B
Deletions	-5/5q-	-7/7q- 17p- complex karyotype	TP53 mutations	MLL and RUNX1 amplification/duplication	promoter methylation P15INK4B
Topoisomerase II Inhibitors	11q23rearr.		MLL rearrangement	RAS mutations BRAF mutations; ↓ miR-29b	
Gene Fusions	21q22/16q22 rearr.	-7/7q-	RUNX1 or CBFB rearrangement	KIT RUNX3 Downregulation	Frequent CDKN2B promoter methylation
	t(15;17) 11p15 defects	None None	RARA NUP98	FLT3 ITD ??	
De novo diseases	Normal karyotype	None	FLT3 mutations, RAS mutations, MLL PTD	RUNX1 mutations, ↓ miR-181a in poor prognosis AML with trilineage dysplasia	Less common CDKN2B promoter methylation
	+8, other defects	None	NPM1, ↓ miR-191, miR199a	↓ miR-204 in NP Mmutated target HOXA10 and MEIS1	NF-kB activation

TABLE 36.5. Frequency of gene mutations in de novo and therapy-related MDS and therapy-related AML.³⁷

Type of gene	Name of gene	Frequency de novo MDS (%)	t-MDS (%)	De novo AML (%)	t-AML (%)
Tumor suppressor	p53 p.m.	5–10	25–30	10–15	20–25
Tyrosine kinase	FLT3 ITD	Rare	Rare	35–50	10
	JAK2 p.m.	2–5	2–5	Rare	Rare
RAS/BRAF pathway	KRAS/NRAS p.m.	10 ^a	10	10	10
	PTPN11 p.m.	3–5 ^b	3–5	3–5	3–5
Transcription factor	AML1 c.r.	Rare	2	7–10	5–7
	CBFB c.r.	Rare	Rare	5–8	2–3
	MLL c.r.	Rare	Rare	5–7	5–7
	RARA c.r.	Rare	Rare	5–10	2–3
	EVII c.r.	Rare	Rare	2–3	2–3
	AML1 p.m.	10–15	15–30	5–10	2–3
	NPM1 p.m.	Rare	4–5	40–50	15
	CEBPA p.m.	Rare	Rare	15–20	Rare

p.m. point mutation, ITD internal tandem duplication, c.r. chimeric rearrangement, AML acute myeloid leukemia, MDS myelodysplasia syndrome.

^a15–20% in juvenile myelomonocytic leukemia.

^b30–40% in juvenile myelomonocytic leukemia.

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Gene Expression Profiling

Oligonucleotide or cDNA microarray technology is being established as an alternative to and extension of conventional karyotyping and fluorescence in situ hybridization in the diagnosis of leukemia subtypes. Several groups have demonstrated that the sensitivity and specificity of microarray technology is higher than 90% for some types of de novo AML and t-AML.^{48–50} Common to each of the subgroups are gene expression patterns typical of those marking arrested differentiation in early progenitor cells.⁴⁹ Relative to other leukemias, those with a –5/del(5q) and complex karyotype have high expression of genes involved in cell cycle control (*CCNA2*, *CCNE2*, *CDC2*), checkpoints (*BUB1*), or growth (*MYC*), and a loss of expression of the gene encoding the interferon–consensus sequence binding protein. A second subgroup of t-AML (occurring in patients with a –7

chromosomal abnormality but no abnormality of chromosome 5, patients with a normal karyotype, or patients with other cytogenetic abnormalities), is characterized by the downregulation of transcription factors involved in early hematopoiesis (*TAL1*, *GATA1*, and *EKLF*) and the overexpression of proteins involved in the signaling pathways of myeloid cells (*FLT3*) and those for cell survival (*BCL2*).⁴⁹

Pedersen-Bjergaard et al.³⁵ have proposed that t-AML can be subdivided into at least eight genetic pathways with different etiologies and biologic characteristics (Table 36.4). The first genetic pathway is characterized by abnormalities of chromosome 7, with *NRAS* mutations and *CDKN2B/p15* methylation may also occur as cooperating mutations. The second genetic pathway is characterized by abnormalities of chromosome 5, complex karyotypes [often with –7/del(7q)], and *p53* mutations. Please refer to (Table 36.4) for characteristics of the additional pathways.

Recently the discovery of a novel class of gene regulators, named microRNAs, has changed the landscape of human genetics. MicroRNAs are non-coding RNA of approximately 22 nucleotides that regulate gene expression by binding to the mRNA 3' untranslated regions. Should a perfect complementarity occur, the microRNA is cleaved and degraded, whereas a translational silencing is the main microRNA regulatory mechanism in the case of imperfect base pairing.⁵¹ MicroRNA's regulation of gene expression is complex. Of the 500 or so known microRNAs, some bind to many genes; similarly, the 3' untranslated region of a single messenger RNA can bind multiple microRNAs.⁵² Extensive deregulation of microRNAs has been observed in cases of leukemia and mechanistic studies support a role for microRNAs in the pathogenesis of AML (Table 36.4).^{51,53–55} In this context, Nervi et al⁵⁶ recently reported a link between the differentiation block of leukemia and the epigenetic silencing of the microRNA-223 gene by the AML1/ETO oncoprotein.

DNA Methylation

It is now recognized that not only genetic but also epigenetic alterations are important in the pathogenesis of AML. DNA methylation and modifications of histone tails are key cooperating mechanisms in maintaining epigenetic memory in mammalian cells. Along with genetic alterations, epigenetic abnormalities play an important role in the deregulation of genes in cancer.⁵⁷ Aberrant hypermethylation in cancer or leukemia cells may affect hundreds of promoter-associated CpG islands and cause stable epigenetic silencing of methylated genes. While many genes that are methylated in tumors are not expressed in relevant normal tissues, silencing of genes that are critically important to controlling cell proliferation contributes to the development of a malignant phenotype in the same manner as inactivating mutations of tumor suppressor genes.⁵⁸ In leukemia, epigenetic silencing by DNA methylation of cyclin-dependent kinase inhibitors,^{59–62} DNA repair genes,⁶³ apoptosis mediators,^{64,65} nuclear receptors,^{66–68} transcription factors,⁶⁹ cell adhesion molecules,⁷⁰ and many other genes has been reported.^{71–73}

Because these alterations do not change the DNA sequences and are pharmacologically reversible, they have been regarded as optimal targets for what is known as epigenetic therapy.⁷⁴ Kroeger et al⁷⁵ investigated DNA methylation of 16 promoter-associated CpG islands in 21 leukemia cell lines using bisulfite pyrosequencing. They further evaluated nine CpG islands in primary leukemia cells and documented their methylation in 23–83% of AML patients at diagnosis. They found that the frequency and density of methylation in studied genes was further increased at relapse.⁶¹ Whether gene methylation affects the decision-making functions of hematopoietic stem cells or is just a signature of epigenetic instability is unknown. The *p15* cell-cycle inhibitor is expressed at low levels in the bone marrow and peripheral blood, and its expression has been shown

to decrease with CpG hypermethylation in leukemia patients.⁷⁶ Methylation of *p15*, *PGR*, and *CDH13* has been described in leukemia.^{59,67,70} Although *PGR* and *CDH13* genes are not significantly expressed in mature blood cells, their expression or silencing may be important in the balance of stem cell self-renewal and differentiation. In most patients, methylation of multiple genes has been observed, suggesting a general disturbance of epigenetic memory in AML and confirming the findings of a CpG island methylator phenotype⁷⁷ in leukemia.^{71,78,79} The mechanism of concordant methylation of multiple genes is unknown. Hypermethylation of multiple genes is associated with a poor prognosis in older patients at high risk for t-MDS and t-AML following MDS.⁸⁰ Kroeger et al⁷⁵ found that levels of DNA methylation increased in most patients with relapsed AML, particularly those with a stable karyotype, which suggests that an epigenetic instability process is involved in AML progression. Conversely, chromosomal instability may be the major driving force behind the development of additional cytogenetic changes at relapse. Clinical research demonstrated differences in the degree of methylation levels in patients with or without chromosomal instability. This supports the hypothesis of Kroeger et al⁶¹ that epigenetic and chromosomal instability may be inversely correlated in AML (Figure 36.1). Understanding the nature and extent of DNA methylation and other epigenetic alterations in AML will help to develop strategies for the use of DNA-demethylating and histone-modifying agents in the treatment of AML.

Summary

With our present knowledge, the optimal management of individual cases of AML requires that all cases be studied by morphologic, cytochemical, cytogenetic, immunologic, and molecular techniques. The outcome of patients with t-AML has been poor compared with people in whom AML develops de novo. The identical pattern of cytogenetic abnormalities and gene mutations in de novo AML, t-MDS, and t-AML supports the hypothesis that these are biologically similar diseases that share some signaling pathways, among which RAS-BRAF, and RELA and PI3KCA2A/AKT1 are the most relevant. It is now recognized that not only genetic but also epigenetic alterations are equally important in t-MDS and t-AML. Because these alterations do not change the DNA sequences and are pharmacologically reversible, they are regarded as optimal targets for what is now known as epigenetic therapy. It is also anticipated that genotype-specific therapies will be introduced and patients will be entered into different treatment protocols on the basis of their individual genetic profiles. Future studies on the clinical importance of the expression of various markers will aid clinicians in treating AML and in preventing refractory disease.

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Molecular Pathology of Hemoglobin and Erythrocyte Membrane Disorders

Murat O. Arcasoy and Patrick G. Gallagher

Introduction

Inherited abnormalities of the erythrocyte comprise an important group of hemolytic disorders. These defects include abnormal hemoglobins and abnormal proteins of the erythrocyte membrane. Because of the easy accessibility of the erythrocyte, these disorders represent some of the best understood disorders at the molecular level. A logical approach to the evaluation and diagnosis of these disorders allows implementation of rational management strategies and the avoidance of ineffective or deleterious therapies.

Molecular Pathology of Hemoglobin Disorders

Hemoglobin molecules – the principal oxygen-carrying pigments in the body – are packaged into red blood cells and consist of tetramers containing two pairs of dissimilar globin polypeptide chains, each with an identical heme group. Abnormalities in the globin proteins due to mutations that change the amino acid composition of the respective globin chain give rise to the hemoglobinopathies, a heterogeneous group of disorders characterized by alteration of the normal biosynthesis, structure, and function of the hemoglobin molecule. Inherited hemoglobin disorders, prevalent in many parts of the world, including Southeast Asia, the Middle East, the Mediterranean, the Indian subcontinent, Africa and the Caribbean constitute the most common human single gene disorders, the severe forms of which represent a major global public health issue. An estimated 300 million people, nearly 5% of the world population, carry a potentially pathological hemoglobinopathy trait.^{1,2} Approximately 300,000 infants are born annually with major hemoglobin disorders, the most common being thalassemias and sickle cell disease.³ Increasing global migration in the last several decades has introduced the major hemoglobinopathies into many parts of the world where they were not originally endemic.^{4–9}

The major hemoglobin in adult red blood cells (RBCs) is Hb A and consists of two α and two β globin chains ($\alpha_2\beta_2$). Adult red blood cells (RBCs) also contain small amounts (<3.5%) of the minor adult hemoglobin Hb A₂ ($\alpha_2\delta_2$) and 0.5–1% fetal hemoglobin Hb F ($\alpha_2\gamma_2$). The α - and β -like globin genes are localized in two separate gene clusters on chromosomes 16 and 11, respectively, as illustrated in Figure 37.1. There are two copies of α globin genes ($\alpha 2$ and $\alpha 1$) on each chromosome 16 and a single β globin gene on each chromosome 11. Two hemoglobin switches occur during development involving both globin gene clusters. These switches include the embryonic to fetal (ζ to α and ϵ to γ globin switches) and fetal to adult (γ to β) switch, controlled at the transcriptional level.¹⁰ The balanced expression of the genes encoding α - and β -globin chains produced in coordination with heme synthesis maintains the solubility of the hemoglobin tetramer in the red blood cell. The tightly regulated expression of the individual genes encoding for each globin polypeptide chain determines the type and amount of hemoglobin produced in different developmental periods.¹¹

The study of the normal structure and function of the hemoglobin molecule and the investigation of the genetic and physiologic mechanisms of the hemoglobinopathies have contributed to the development of molecular medicine in the last five–six decades.¹² There are more than 1,300 naturally occurring hemoglobin mutations described to date and cataloged in the Globin Gene Server (<globin.cse.psu.edu>, accessed February 16, 2009).^{13,14} Many of these mutations do not affect globin gene expression or the structure and function of the hemoglobin molecule to cause clinical disease. The most important globin gene mutations and hemoglobinopathies that lead to clinical symptoms, morbidity, and mortality are encountered in the thalassemia syndromes and sickle cell disease (Table 37.1). Hemoglobin disorders are typically detected during the evaluation of hematologic abnormalities, such as anemia and microcytosis, during neonatal screening, genetic counseling of prospective parents at risk for a child with a major hemoglobinopathy, prenatal diagnosis, and

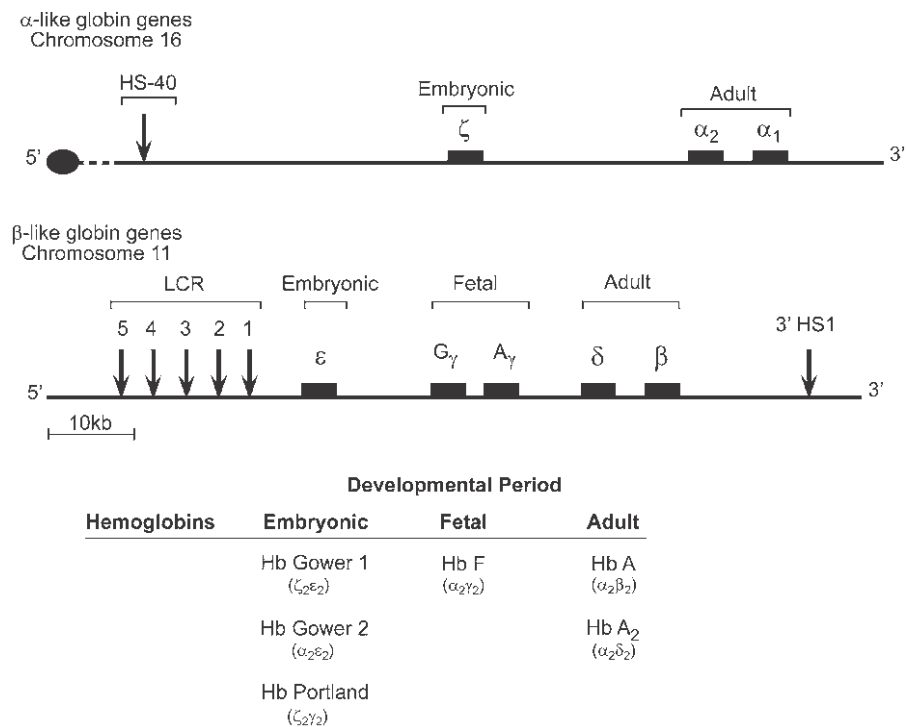


FIG. 37.1. *The globin genes and hemoglobins.* α -like globin genes are located on chromosome 16. The ζ -globin gene is transcriptionally active during the embryonic period. The HS-40 regulatory element for the α -globin genes and the upstream telomeric region involved in large deletions detected in α thalassemia mental retardation (ATR) syndromes

are shown. β -like globin genes are located on chromosome 11. The upstream locus control region (LCR) and the 3' HS1 site consisting of specific sites hypersensitive to DNase I digestion are illustrated. The LCR is a key element regulating the expression of the β -like globin genes. Hemoglobins produced during each developmental period are shown.

TABLE 37.1. Classification of hemoglobin disorders.

- Thalassemia syndromes and defective production of globin chains
 - α thalassemia
 - β thalassemia
 - Other thalassemias and structural hemoglobin variants
- $\delta\beta$ thalassemia
- Hb E
- Hb Constant Spring
- Hb Lepore
- Sickle cell disease and abnormal hemoglobin polymerization
 - Homozygous Hb SS (sickle cell anemia)
- Hb SC disease
 - Sickle- β thalassemia
 - Sickle cell trait (Hb S)
 - Other Hb variants interacting with Hb S
- Hereditary persistence of fetal hemoglobin syndromes
 - Deletion forms
 - Nondeletion forms
- Unstable hemoglobin variants
- Hemoglobin variants with altered oxygen affinity
 - Hb variants with increased oxygen affinity
 - Hb variants with reduced oxygen affinity
- Methemoglobinemias
 - Congenital disorders
- M hemoglobins
- Cytochrome b_5 reductase deficiency
 - Acquired methemoglobinemia

preoperative screening for the presence of sickle hemoglobin (Hb S). Rare hemoglobin variants associated with familial cyanosis, or polycythemia have been described.

The Thalassemia Syndromes

Introduction

The thalassemias are genetic disorders of globin chain production. The α - and β -thalassemia syndromes, characterized by decreased or absent synthesis of α - and β -globin chains, respectively, represent the most common monogenic disorders in the world. α thalassemia is prevalent in the Mediterranean region, tropical Africa, the Middle East, and Southeast Asia, where the carrier frequency is between 5 and 10%. The high gene frequency of α thalassemia carriers in these populations has been associated with the protection of heterozygotes against severe forms of *Plasmodium falciparum* malaria.^{15–19} The potential mechanisms of the protective effect are interesting. Increased susceptibility to infection with the nonlethal *P. vivax* in younger children is thought to induce cross-species immunity against subsequent *P. falciparum* infection, a more severe form of malaria.^{20,21} Other studies have reported reduced parasite multiplication in thalassemic red cells.²²

The geographic distribution of β thalassemia indicates high frequency in the Mediterranean region, Africa and the Middle East, as well as India and Southeast Asia. Approximately 3% of the world population carries genes for β -thalassemia, and an estimated 2,000 individuals in the United States have β -thalassemia. An interaction between β thalassemia trait and malaria protection has been suggested.²³ Global migration and changing population demographics have been associated with

increased numbers of children with thalassemia syndromes in the United States and the developed world.^{4,9,24–27} Following the introduction of preventive programs, the birth rate of thalassemic children has declined significantly in high frequency populations.^{28–30}

Although both α - and β -thalassemia syndromes result in partially reduced or absent synthesis of adult Hb A ($\alpha_2\beta_2$), it is important to note that severe forms of α and β thalassemias have quite different clinical implications for the developing fetus, the neonate, and the pregnant mother. The distinctive clinical manifestations are due, in part, to differences in the developmental stage-specific regulation of the α - and β -globin genes. The severe form of homozygous α -thalassemia, due to deletion of all four functional α -globin genes, is incompatible with life, and manifests itself during early development. This syndrome is associated with severe in utero anemia, fetal hydrops, and significant risk of maternal morbidity. In contrast, clinical manifestations of severe β -thalassemia major generally do not occur until after the first 6 months of life, as the γ - to β -globin switch is completed, resulting in a chronic illness, requiring intensive treatment, with significant associated morbidity as well as great psycho-social and financial burden, due to the dependence of affected children on red blood cell transfusions and iron chelation therapy to sustain life.

β Thalassemia Syndromes

Introduction

β thalassemias are characterized by the deficiency or absence of β globin chain biosynthesis leading to an imbalance of α and β globin chain amounts in the RBC. Mutations that affect β globin gene synthesis and that give rise to β thalassemia syndromes have been described sporadically in all ethnic groups, but are especially common in the Mediterranean, Southeast Asia, and Africa. Despite marked heterogeneity at the molecular level, a unique spectrum of mutations is prevalent in a given population at risk for β -thalassemia.³¹ The clinical severity is heterogenous, and genetic factors may play a role.^{32,33} Patients who are heterozygous for a β thalassemia mutation have β thalassemia trait and are asymptomatic. Individuals who are homozygous for β thalassemia mutations, resulting from the inheritance of two β thalassemia alleles, may develop the severe form (i.e., β thalassemia major), or in some cases, a milder form of the disease, termed β thalassemia intermedia.³⁴

Molecular Pathology and Pathophysiology

Most β -globin gene defects that result in β -thalassemia syndromes are due to point mutations, short deletions, or insertions within the β -globin gene, or its immediate flanking regions.³⁵ Homozygous or compound heterozygous inheritance of β -thalassemia alleles associated with severely reduced or absent synthesis of β -globin chains, or the interaction of β -thalassemia alleles with Hb E, usually lead to transfusion-dependent β -thalassemia major, also known as Cooley's anemia. More than 200 different defects in the β -globin gene that result

in β -thalassemia have been described, but approximately 15 mutations account for the vast majority of the patients.³⁶ In a specific ethnic group or geographic area, such as in blacks or those individuals of Mediterranean, Southeast Asian or Asian Indian origin, only five or six mutations are responsible for >90% of the cases.³⁵ The mutations that give rise to β thalassemia syndromes involve nearly every pathway in β -globin gene expression, including transcriptional regulation due to promoter mutations, mRNA processing defects due to splicing abnormalities, or mutations that abrogate the translation of β globin mRNA due to premature termination.³⁶ The clinical severity of the disease may exhibit variability between different ethnic groups harboring the identical mutation. The level of γ globin gene expression is a modulating factor as higher Hb F levels are associated with reduced disease severity in hemoglobinopathies involving the β globin gene.³⁷

The reduction or absence of β globin chain synthesis in β thalassemia prevents adequate accumulation of Hb A which consists of $\alpha_2\beta_2$ tetramers. Homozygosity for β^0 thalassemia alleles, encountered in approximately one third of patients with β thalassemia major, is associated with total absence of β globin chain synthesis. The remaining patients are either compound heterozygotes for β^0/β^+ thalassemia mutations or homozygotes for β^+ thalassemia mutations and exhibit variable, reduced amounts of β globin chain production and Hb A levels ranging from 5 to 30% in the red blood cells. As the Hb F levels decrease in the first 6 months following birth, the residual γ globin gene transcription is not sufficient to replace β globin chain production. Hb A levels are severely reduced or absent, and severe anemia, hypochromia, and microcytosis occur. The unpaired, excess α globin chains aggregate, precipitate, and form inclusion bodies in the RBCs, leading to severe ineffective erythropoiesis due to membrane damage. The hemolytic anemia and its consequences as well as the potential complications of chronic transfusion therapy lead to the clinical manifestations of severe forms of β thalassemia major as illustrated in Figure 37.2. The co-inheritance of α thalassemia may reduce the severity of the disease, by partially ameliorating the imbalance of α and β globin chain synthesis and the accumulation of free α globin chains.³⁸ Similarly, increased levels of Hb F also reduce the severity of the disease by providing an alternative source of non- α globin chains.^{32,37}

Clinical Features and Therapy

β Thalassemia Major

The clinical picture of β thalassemia major was first described by Thomas Cooley in 1925. Infants affected with this disorder are well at birth, but develop progressively severe anemia during the first several months of life. Failure to thrive is typical with feeding problems and recurrent fevers. Transfusion-dependent homozygous β thalassemia patients will present in the first year of life. Those patients with a later onset are likely to have a milder form, termed β thalassemia intermedia, and do not require regular transfusions to sustain life. Adequate transfusion of β thalassemia

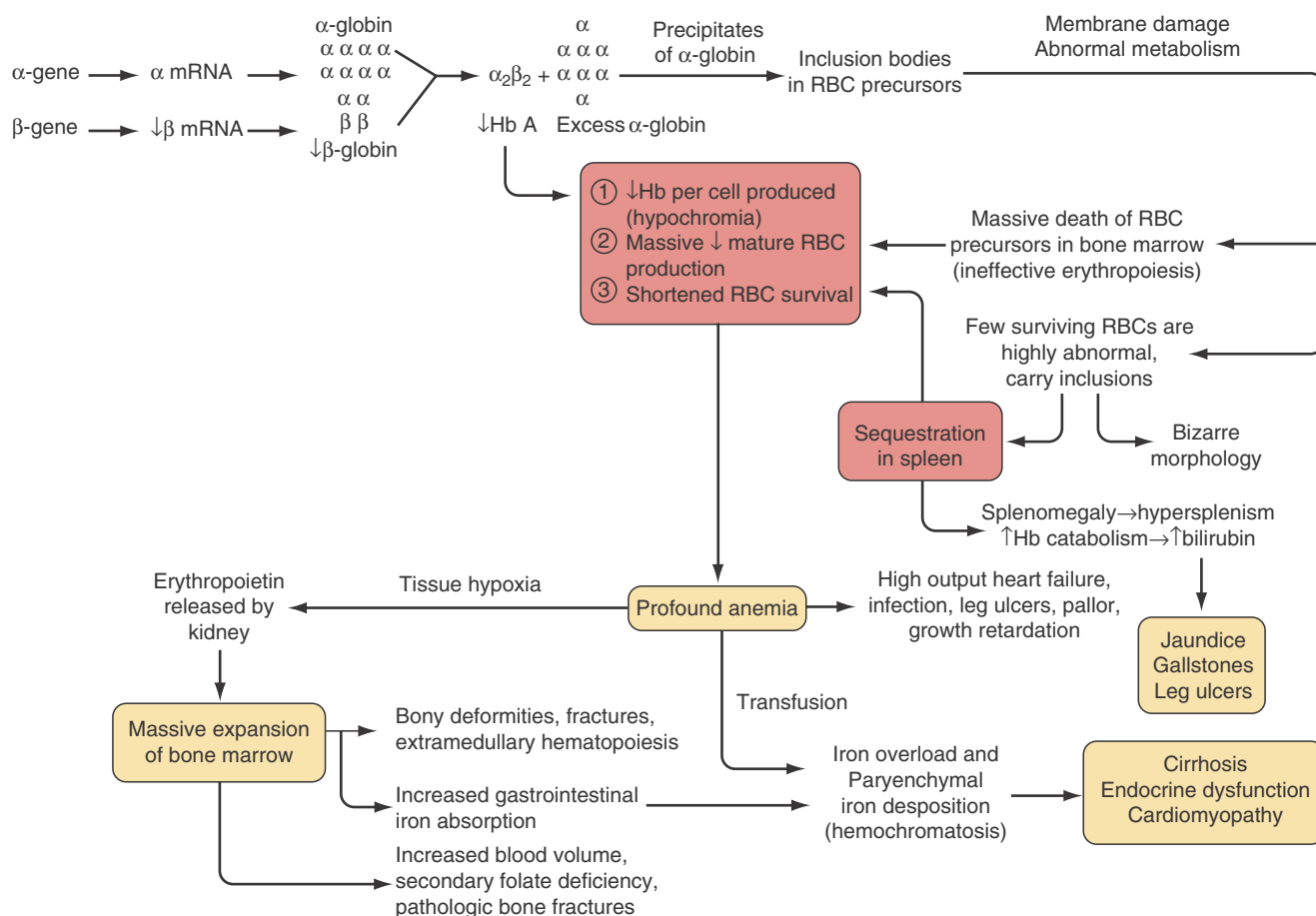


FIG. 37.2. Pathophysiology of β thalassemia syndromes. The primary defect in β globin chain synthesis and relative excess of α globin chains give rise to transfusion-dependent anemia and other

clinical manifestations of severe β thalassemia major (reprinted with permission from Giardina and Forget 36).

major patients is essential for the normal growth and development of the child. Inadequately transfused children will develop the typical features of Cooley's anemia, including delayed growth, bossing of the skull with a prominent maxillary region giving rise to a characteristic facial appearance, hepatosplenomegaly, increased pigmentation of the skin, and skeletal abnormalities involving the skull, long bones, and hands. Infection risk is increased and is a frequent cause of death. Hemoglobin levels at the time of presentation may be as low as 2–3 g/dL. RBC morphology is markedly abnormal with severe hypochromia, microcytosis, polychromasia, basophilic stippling, and target cells. Many nucleated RBCs are present in the circulation, but reticulocytosis is relatively low in proportion to the severity of anemia due to ineffective erythropoiesis in the bone marrow (BM).

The mainstay of therapy for β thalassemia major is regular red cell transfusions, which are required to maintain hemoglobin levels above 9–10 g/dL in affected children. The major therapeutic challenges include the requirement for life-long red blood cell transfusions, availability of adequate blood supply, risk of transfusion-transmitted illness, transfusional iron overload and its fatal complications, development

of hypersplenism and potential need for splenectomy, and control of osteoporosis. Complete red cell antigen phenotyping and optimal matching of donor red cell units are important to minimize the risk of alloimmunization. If BM transplantation is a consideration, the use of cytomegalovirus-negative blood products is appropriate. Adequately transfused children usually require monthly transfusions, exhibit less prominent splenomegaly, and do not develop the typical features of Cooley's anemia described earlier. It is imperative, however, to administer regular chelation therapy to remove excess iron accumulation derived primarily from the blood transfusions as well as from the increased absorption of iron in the gastrointestinal tract. Until recently, the only available chelation therapy agent was deferoxamine, typically given as an overnight subcutaneous infusion using an infusion pump for at least 5–6 days per week.³⁹ Potential adverse effects include ototoxicity, retinal changes, and bone dysplasia with truncal shortening. The number of hours, typically 10–12 h per night, is more important than the dose of the drug for optimal chelation of excess iron. Compliance with this treatment regimen has been difficult for many children, leading to irreversible organ damage and dysfunction, due to the severe,

progressive iron overload. Cardiac iron overload leads to the development of potentially lethal complications, including arrhythmias and congestive heart failure. Endocrine abnormalities due to iron deposition occur, including diabetes mellitus, hypothyroidism, and gonadal failure. The oral iron-chelating agent (i.e., deferasirox) has become available and appears to be as effective as deferoxamine.^{40,41}

Despite adequate transfusion therapy, many patients will develop massive splenomegaly associated with hypersplenism and worsening anemia with increased transfusion requirements. Splenectomy may be beneficial in these patients, but should generally be avoided in children younger than 4 or 5 years of age, due to the increased risk of fatal sepsis in splenectomized children. BM transplantation is potentially curative in approximately 80% of children with β thalassemia major who have HLA-identical siblings.⁴² Most successful outcome has been achieved in younger children without excessive iron overload and hepatic damage.^{43,44} The detection of heterozygous adults of reproductive age and genetic counseling have been successful in the prevention of severe forms of β thalassemia major using prenatal diagnosis.^{28,30,45,46} Fetal DNA obtained by chorionic villus sampling or amniocentesis is analyzed using molecular techniques.^{47,48} Noninvasive methods of fetal DNA isolation from maternal blood samples have been applied to the prenatal diagnosis of β thalassemias.⁴⁹

β Thalassemia Intermedia

Unlike β thalassemia major patients, β thalassemia intermedia patients do not develop anemia that is severe enough to become dependent on regular blood transfusions to sustain life. This clinical picture is associated with different genetic interactions, including inheritance of mild β^+ thalassemia mutations, coinheritance of α thalassemia, or genetic defects associated with high Hb F levels, such as hereditary persistence of fetal hemoglobin (HPFH). The degree of anemia is highly variable. In some patients, the presentation may occur during childhood in a manner similar to β thalassemia major, but late in the first year of life with hemoglobin levels ranging from 5 to 7 g/dL. In other patients, symptoms may not occur until they reach adult life and present with hemoglobin levels ranging from 8 to 12 g/dL. Potential complications may include progressive splenomegaly and hypersplenism, arthritis, cholelithiasis, iron overload due to increased absorption, leg ulcers, thromboembolic complications, extramedullary hematopoiesis in unusual sites such as the vertebral canal, and increased susceptibility to infections. Chelation therapy may be indicated in some patients to prevent or treat iron overload.

β Thalassemia Trait

These individuals are heterozygous for a β thalassemia allele and are asymptomatic. Anemia is either absent or mild. Significant hypochromia and microcytosis is present so the major differential diagnosis is iron deficiency. Ferritin, serum iron, and total iron binding capacity are all normal. Hemoglobin levels are typically higher than 10 g/dL; mean corpuscular

volume (MCV) is <75 fL; and mean corpuscular hemoglobin (MCH) is <26 pg. The RBC count may be elevated. Reticulocyte count is normal or mildly increased. Blood film examination shows microcytosis, target cells, basophilic stippling, and ovalocytes. Diagnosis is established by hemoglobin electrophoresis and quantitation of the Hb A₂ level, which is typically >4%. Hb F levels may be slightly increased in 50% of cases. A normal Hb A₂ level does not rule out β thalassemia trait in the presence of iron deficiency. An accurate diagnosis of β thalassemia trait is important for the identification and counseling of couples at risk for having a child with β thalassemia major.

$\delta\beta$ Thalassemia

This is a heterogeneous group of disorders encountered much less commonly than β thalassemias, with a mild clinical phenotype due to the relatively high levels of γ globin chain production. The hematologic picture is similar to β thalassemia trait. The deletion forms of $\delta\beta$ thalassemia remove both the δ - and β globin genes in the β globin gene cluster, leaving one or both γ globin genes intact (Figure 37.1). In heterozygous individuals, the hematologic picture resembles β thalassemia trait, but Hb A₂ levels may be low or normal, and Hb F levels are elevated ranging from 5 to 20%. Homozygous individuals are anemic with hemoglobin levels ranging from 9 to 10 g/dL, consisting of 100% Hb F and no detectable Hb A and Hb A₂; the clinical picture is similar to β thalassemia intermedia.

Hb E- β Thalassemia

The Hb E structural variant is very common in Southeast Asian populations with a gene frequency of 10–20%. Hb E trait is protective against severe malaria, as RBCs containing Hb AE are resistant to invasion by *P. falciparum*.⁵⁰ Hb E is synthesized in reduced amounts because of abnormal metabolism and instability of the β globin mRNA precursor due to the $\beta^{26\text{Glu}\rightarrow\text{Lys}}$ mutation. Hb E trait is associated with 30–45% Hb E in red cells and microcytosis. Individuals with homozygous Hb E are asymptomatic with mild anemia, microcytosis, and hypochromia. Splenomegaly is uncommon. Compound heterozygosity for Hb E and a β thalassemia mutation (Hb E- β thal) is common because of the high frequency of β thalassemia mutations in the same populations where Hb E is commonly encountered. The coinheritance of Hb E and β^0 thalassemia or severe forms of β^+ thalassemia is associated with a phenotype that is similar to severe β thalassemia major.^{51,52} The severe phenotype of Hb E- β thalassemias is in contrast to the mild clinical features of homozygous Hb E disease (>90% Hb E and no Hb A), which resembles β thalassemia trait. On routine hemoglobin electrophoresis, Hb E migrates like Hb A₂ but is present at higher amounts (30%) compared to the typical amounts of Hb A₂ in RBCs of β thalassemia heterozygotes.

Hemoglobin Lepore

An uneven crossing over and recombination event between adjacent δ and β globin genes gives rise to Hb Lepore, a fused globin chain that is stable and functional and does not

undergo sickling.⁵³ The level of production of the abnormal globin chain is low, ranging from 3 to 20%, due to the low activity of the δ globin gene promoter driving the expression of the $\delta\beta$ fusion gene. Heterozygotes have a clinical picture that is similar to β thalassemia trait. The electrophoretic mobility of Hb Lepore is similar to Hb S at alkaline pH and similar to Hb A at acid pH. Hb A₂ levels are low and Hb F levels are mildly increased around 2–3%. The clinical phenotype of homozygous Hb Lepore is similar to β thalassemia major, or in some cases, β thalassemia intermedia. RBC hemoglobin consists of Hb Lepore and Hb F (75%) without detectable Hb A or Hb A₂.

Thalassemia Syndrome not Associated with a Globin Gene Mutation

Nearly all mutations that give rise to β thalassemia syndromes are located in or around the flanking regions of the β globin locus. One notable exception is the R216Q missense mutation, detected in the erythroid transcription factor GATA-1 that results in the X-linked thrombocytopenia with thalassemia syndrome.^{54,55} Affected males exhibit mild, compensated hemolysis, thrombocytopenia, splenomegaly, elevated Hb A₂ level, and unbalanced globin chain synthesis, leading to an increased $\alpha:\beta$ globin ratio similar to β thalassemia trait.⁵⁶

α Thalassemia Syndromes

α thalassemia is the most prevalent hemoglobin disorder in the world. Normal individuals have four functional α globin genes ($\alpha\alpha/\alpha\alpha$), two on each chromosome 16 (Figure 37.1). The α thalassemias are mainly due to deletions involving the α globin genes ($-\alpha/\alpha\alpha$ or $---/\alpha\alpha$), and less commonly, to point mutations of one or both α globin genes. The severity of the disorder is related to the number of remaining functional α globin genes, with Hb Bart's hydrops fetalis syndrome representing the most severe form of α thalassemia in which all four α globin genes are deleted. Couples at risk for the development of this syndrome during pregnancy have heterozygous α^0 thalassemia ($---/\alpha\alpha$). This trait is particularly prevalent in people of Southeast Asia (where 3.5–14% of the population are heterozygous carriers ($---^{SEA}/\alpha\alpha$)) and the eastern Mediterranean region (where the prevalence is less than 1% ($---^{MED}/\alpha\alpha$)). Thus, carrier detection and prenatal diagnosis are primarily performed in people originating from these parts of the world to prevent the most severe form of α thalassemia.⁵⁷

Molecular Pathology and Pathophysiology

The reduction or absent production of α globin chains leads to the development of various α thalassemia syndromes depending on the number of genes involved (Figure 37.3). There have been at least 18 large deletions that remove both α globin genes.⁵⁸ The chromosomes containing α^0 thalassemia

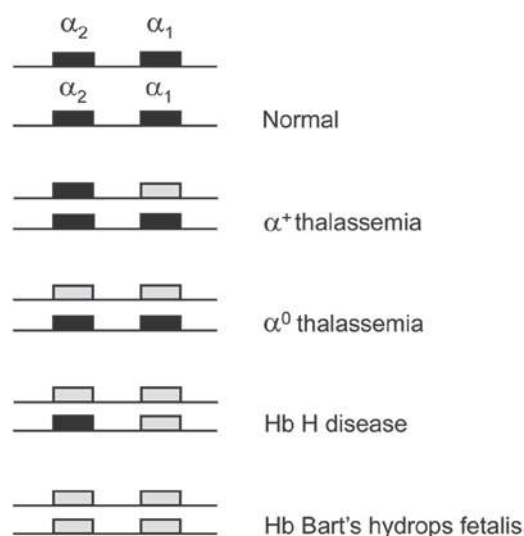


FIG. 37.3. The genotypes of classical α thalassemia. The two α globin genes on each of the homologous pairs of chromosome 16 are illustrated as *black boxes*. The *open boxes* illustrate deleted or otherwise inactivated α genes. Hb H disease occurs due to compound heterozygous state for α^+ and α^0 thalassemia. Hb Bart's and hydrops fetalis results from homozygosity for α^0 thalassemia.

deletions produce no α globin chains at all and exhibit limited geographical distribution primarily in Southeast Asia ($---^{SEA}/\alpha\alpha$) and the Mediterranean ($---^{MED}/\alpha\alpha$). Some deletions that have been described remove the HS-40 locus control region for the α -like globin cluster, leaving the α globin genes intact, but leading to complete inactivation of the α globin genes. The deletion of one or the other of the duplicated α globin genes on the same chromosome gives rise to α^+ thalassemia. Nondeletion types of α^+ thalassemia are much less common than deletion types and typically involve the $\alpha 2$ gene. The expression of the $\alpha 2$ gene predominates over $\alpha 1$ globin by 3:1 during all stages of development. These mutations affect the processing of α globin mRNA transcripts or its translation.

Individuals with loss of one α gene (α^+ thalassemia), two α genes in cis on the same chromosome (α^0 thalassemia), or two genes in trans on each chromosome with homozygous α^+ thalassemia ($-\alpha/-\alpha$) are usually asymptomatic. Hb H disease is due to a compound heterozygosity for α^0 and α^+ thalassemia ($---/-\alpha$), with excess β globin chains forming β_4 tetramers (Hb H). The precipitation of β_4 tetramers in mature RBCs as the cells age results in the formation of inclusions, which are removed by the spleen and lead to RBC damage and hemolytic anemia. Although Hb H is unstable, it does not precipitate readily in early BM erythroid precursors, unlike excess α globin chains produced in severe β thalassemia. Therefore, ineffective erythropoiesis is not a major feature of α thalassemia and Hb H disease. The homozygous state of α^0 thalassemia results in the syndrome of Hb Bart's hydrops fetalis. The total absence of α globin chains leads to the formation of γ_4 tetramers (Hb Bart's). The very high oxygen affinity

of Hb Bart's precludes adequate oxygen delivery to the fetal tissues. Severe hypoxia gives rise to hydropic changes and death in utero.

Clinical Features and Therapy

α^+ Thalassemia

The hematologic changes in carriers are extremely mild with generally normal hemoglobin values and minimal abnormalities in red cell indices. Newborn infants may have 1–2% Hb Bart's detectable, but this typically disappears by the age of 6 months. In adults, hemoglobin electrophoresis is normal, and the condition may be definitively diagnosed by DNA analysis. The MCV and MCH may be mildly low, and no abnormalities are detected on examination of the peripheral blood film.

α^0 Thalassemia

Carriers for α^0 thalassemia are usually asymptomatic and frequently have normal or near-normal hemoglobin levels associated with an increased RBC count. Some individuals may exhibit mild anemia (hemoglobin 10–12 g/dL). Reduced MCV and MCH are typical. At birth there is 5–10% Hb Bart's detectable, but this is not replaced by Hb H in adult life, and Hb Bart's typically disappears by 6 months of age. In adults, no hemoglobin pattern abnormality is noted on electrophoresis. Hb F level is normal and Hb A₂ level is normal or decreased. Diagnosis is clinical, based on the exclusion of iron deficiency and β thalassemia. Definitive diagnosis is established using DNA-based diagnostic techniques that are available in referral laboratories. The detection of small amounts of embryonic ζ -globin chains in adult RBC hemolysates has been used as a detection technique of carriers at potential risk for a baby with homozygous α^0 thalassemia and hydrops fetalis.^{59,60} In the absence of documented iron deficiency, unnecessary therapy with iron supplementation should be avoided.

Hb H Disease

The deletion or inactivation of three α globin genes gives rise to Hb H disease.⁶¹ It has been estimated that in California, where there is a large Asian population, approximately 1 in 15,000 newborns have Hb H disease. Hb H disease also occurs in the Mediterranean, but is rare in black individuals. Diagnosis is established by hemoglobin electrophoresis, demonstrating variable Hb H levels ranging from 5 to 40%. In newborns, 20–40% Hb Bart's is found. The parents should be evaluated to investigate for α thalassemia to obtain further evidence for definitive diagnosis. DNA analysis for α globin gene deletions is indicated. Hb H inclusions may be demonstrated by incubation of the blood with brilliant cresyl blue, resulting in vitro precipitation of Hb H. In splenectomized individuals, large Hb H inclusions have been shown to form in vivo during aging of RBCs and may be visualized on the blood film. Hb H disease is associated with moderate hemolytic anemia with variable hemoglobin levels of 8–10 g/dL, moderate reticulocytosis, jaundice, and mild splenomegaly.

RBC morphology is hypochromic and microcytic with the MCV ranging from 50 to 73 fl. RBC transfusions are frequently not necessary, except in cases with more severe, symptomatic hemolytic anemia. Iron overload may occur and result in morbidity. Complications of Hb H disease include progressive splenomegaly and hypersplenism, gallstones, superimposed folate deficiency, infections, and leg ulcers. The severity of anemia may increase due to the ingestion of certain drugs with oxidant properties or during pregnancy or infections. Occasional patients with more severe anemia and hypersplenism may benefit from splenectomy. Nondeletion forms of Hb H disease are associated with more severe hemolytic anemia and splenomegaly.⁶²

Hb Bart's and Hydrops Fetalis

The homozygous inheritance of two forms of α^0 thalassemia gives rise to Hb Bart's hydrops fetalis syndrome that typically occurs in at risk couples from Southeast Asia and the Mediterranean. No α globin chains are produced in affected fetuses and their red cells contain 80% Hb Bart's and 20% Hb Portland, which persists up to birth in this condition. Profound anemia, hypoxia, and cardiac failure develop during fetal life, and result in hydrops and in utero death between 36 and 40 weeks gestation or soon after birth. The fetuses exhibit anasarca and massive hepatosplenomegaly associated with extramedullary hematopoiesis in response to severe tissue hypoxia. Severe anemia is present with variable hemoglobin values, ranging from 3 to 10 g/dL. Peripheral blood findings include large numbers of nucleated RBCs, target cells, severe hypochromia, and microcytosis. Hemolytic disease due to blood group incompatibility is excluded by the finding of a negative direct Coombs test. Hemoglobin electrophoresis reveals predominantly Hb Bart's with a small amount of Hb H. There is complete absence of Hb A and Hb F. There are reports of salvaged newborns following exchange transfusion immediately after birth or fetuses using in utero blood transfusions. However, the risk of urogenital, neurologic, and limb defects as well as developmental delay exists, and life-long management with blood transfusions analogous to β thalassemia major and iron chelation therapy are required. The increased incidence of maternal complications, such as pregnancy-induced hypertension, hemorrhage, difficult vaginal delivery, and retained placenta underscores the importance of carrier screening, counseling, and prenatal diagnosis using fetal DNA analysis.⁴ Noninvasive methods to diagnose affected fetuses with serial imaging studies or using fetal cells isolated from maternal blood for globin expression analyses have also been utilized in prenatal diagnosis.^{57,63,64}

α Thalassemia Associated with Mental Retardation

There are two variants of the α thalassemia mental retardation (ATR) syndromes. The ATR-16 syndrome is due to large telomeric deletions (1–2 Mb), involving the α globin gene cluster on the short arm of chromosome 16p (Figure 37.1).⁶⁵ The α thalassemia phenotype is associated with mental retardation and skeletal and facial abnormalities. Coinheritance of a

common, single α globin gene deletion from the other parent is associated with the development of Hb H disease in the affected child. The ATR-X syndrome results from a mutation in the ATRX gene located on chromosome Xq13.3. This syndrome is associated with defective α globin synthesis and a mild Hb H disease phenotype in affected males.^{66–68} The mental retardation is severe, and facial, genital, and skeletal abnormalities are characteristically present in the majority of patients.

Acquired Hb H Disease

This syndrome is observed in rare patients with a hematologic malignancy, typically myelodysplastic syndrome (MDS), with a strong male preponderance and a median age at diagnosis of 68 years.⁶⁹ Most thalassemic MDS patients (ATMDS) have been reported to exhibit hypochromia and microcytosis with an average MCV of 75 fl and variable proportions of Hb H-containing RBCs in the periphery. Analysis of chromosome 16 has been found to be normal in the majority of cases, except one patient who was found to have the loss of the telomeric region of one copy of chromosome 16 in a clonal fashion.⁷⁰ In a subset of the 68 patients in the acquired α thalassemia registry, 12 patients were found to have point mutations or splicing abnormalities in the ATRX gene, suggesting that ATRX plays an important role in the regulation of α globin gene expression.^{66,71}

Hb Constant Spring

This unusual nondeletion α thalassemia variant involves a mutation in the termination codon of the dominant $\alpha 2$ globin gene, leading to an elongated α^{CS} globin polypeptide. Two α^{CS} chains pair with two β globin chains to form Hb Constant Spring. The α^{CS} globin chain is unstable, and the output from the affected $\alpha 2$ globin gene is severely reduced by incompletely understood mechanisms. In homozygotes for Hb Constant Spring ($\alpha^{CS}\alpha/\alpha^{CS}\alpha$), the total variant hemoglobin ranges only from 5 to 7%, associated with moderate hemolytic anemia, jaundice, and splenomegaly. Hb F and Hb A₂ levels are normal, and interestingly, 1–2% Hb Bart's may be detected.⁷² Heterozygotes for Hb Constant Spring ($\alpha^{CS}\alpha/\alpha\alpha$) are phenotypically similar to heterozygous α^+ thalassemia. Patients with Hb H disease and Hb Constant Spring ($\alpha^{SEA}/\alpha^{CS}\alpha$) have higher levels of Hb H and Hb Bart's and more severe anemia than patients with ordinary Hb H disease, as a result of compound heterozygosity for α^0 and α^+ thalassemia.⁷³

Sickle Cell Disease

Introduction

Sickle cell disease (SCD) results from homozygosity for the sickle cell mutation giving rise to Hb S (sickle cell anemia), or less commonly, from coinheritance of the sickle cell gene β^S with other β -globin structural variants, such as Hb C, β -thalassemia alleles, Hb D, or Hb O Arab.⁷⁴ In sickle cell anemia, >90% of the hemoglobin in the RBCs consists of Hb S, whereas in other SCD syndromes with compound

heterozygosity for a structural variant, Hb S comprises >50% of all hemoglobin. Hb A is absent unless a β^+ thalassemia allele is co-inherited, and Hb F is usually slightly to moderately elevated. Nearly 70% of SCD occurs in sub-Saharan Africa, where up to 2% of all children are born with SCD. Sickle trait, the heterozygous state for Hb S and Hb A, confers protection against severe malaria in Africa the mechanisms of which have been under investigation.^{75–78} In the United States, neonatal screening programs identify nearly 2,000 infants with SCD each year.⁷⁹ The estimated gene frequency for SCD variants in African-Americans has been reported as 4% for Hb S, 0.4% for β -thalassemia, and 1.5% for Hb C.⁸⁰

Molecular Pathology and Pathophysiology

SCD results from a point mutation in codon 6 of the β -globin gene that leads to a substitution of valine for glutamic acid, giving rise to the β^S globin chain ($\beta^{Glu\rightarrow Val}$). The structurally abnormal β globin chain leads to the formation of a highly viscous gel polymer under low oxygen conditions.⁸¹ The polymerized deoxy Hb S molecules self-associate in the RBC and distort the shape of the cell to assume an elongated, sickle morphology. These cells may be visualized as irreversibly sickled cells on the peripheral blood film. The abnormal RBCs exhibit decreased deformability and may become trapped in small blood vessels giving rise to a wide variety of severe vasoocclusive clinical complications of the disorders and increased mortality. RBC survival is markedly diminished to approximately 20 days, compared to the average survival of normal RBCs of 120 days. The hemolytic anemia is associated with impaired deformability of the cells, increased mechanical fragility, and coating of the RBCs with immunoglobulins, leading to erythrophagocytosis. Hb F in RBCs is a strong inhibitor of Hb S polymerization.³² In patients with co-inheritance of a hereditary persistence of fetal hemoglobin (HPFH) mutation and Hb S, elevated Hb F levels have resulted in a mild clinical phenotype, leading to the investigation of γ globin gene regulation and potential anti-sickling therapeutic strategies to increase Hb F production in RBCs.³⁷ Abnormalities in the sickled RBC membrane and altered endothelial biology, including coagulation activation, adhesion, and vasoregulation, have all been implicated in the pathogenesis of the diverse clinical manifestations of SCD.⁸²

Clinical Features and Therapy

The clinical manifestations of SCD begin to appear during the first several months of life as the Hb F levels decline after birth. The major clinical features include chronic hemolytic anemia, recurrent vaso-occlusive pain episodes, and chronic end-organ damage, typically involving the bones, spleen, lungs, brain, retina, and kidneys. The clinical features of SCD are extremely variable, due to complex interactions with many modulators of disease phenotype, including genetic and environmental factors.⁸³ The diagnosis and management of the acute and

chronic complications of SCD require comprehensive medical care under the direction of a hematologist.⁸⁴ In most children with sickle cell anemia, functional asplenia develops by 5 years of age, resulting in an increased risk for fatal infection and sepsis. Prophylactic oral penicillin administration and vaccination for *Streptococcus pneumoniae* have reduced sepsis risk and mortality in children.⁸⁵ Other complications that occur typically during childhood include dactylitis (hand-foot syndrome) with symmetric swelling of the hands and feet, splenic sequestration crisis associated with engorgement of the spleen and worsening anemia with a propensity for recurrent episodes, and aplastic crisis associated with parvovirus B19 infection and the development of transient red cell aplasia. Vasoocclusive pain episodes in children and adults are frequently managed in a hospital with intravenous fluids, supplemental oxygen, and parenteral narcotic analgesics as appropriate by patient-controlled analgesia.⁸⁶

Neurologic complications, including stroke is a major cause of morbidity and mortality. The approximate risk of stroke in children is 0.5–1% per year. Primary prevention of stroke in high risk children (>10% per year) screened for abnormal transcranial Doppler blood flow velocity measurements is achieved by initiation of a prophylactic chronic transfusion program.^{87,88} Genetic factors associated with increased stroke risk have been investigated in some studies.^{89,90} Chronic transfusion therapy is also indicated in patients who have had a cerebrovascular accident to reduce the 70% risk of a recurrent event and its associated morbidity and mortality.^{91–93} Acute chest syndrome is a frequent potential cause of death in both children and adults with SCD and is characterized by the rapid development of fever, dyspnea, chest pain, hypoxia, and pulmonary infiltrates on chest radiographic studies.^{94,95} Prompt management with intravenous fluids, supplemental oxygen, antibiotics, and transfusion therapy (simple or exchange transfusions) are indicated. Patients with SCD who need to undergo surgery benefit from careful preoperative assessment of their risk and from the decision for simple or exchange transfusion, as appropriate, to reduce postoperative risk of complications, such as acute chest syndrome.^{96,97}

Comprehensive medical care to prevent and treat acute and chronic complications has reduced sickle cell-related mortality in the last three decades.^{98–100} Adult patients with frequent vasoocclusive pain episodes (>3 per year), high white blood cell count, low Hb F levels, and pulmonary hypertension exhibit increased risk for mortality.^{98,101} Death from sickle cell anemia may occur during an acute complication, such as vasoocclusive pain episode, acute chest syndrome, cerebrovascular accident, or organ failure. The use of hydroxyurea therapy has been associated with a significant reduction in mortality from SCD.¹⁰² Long-term follow-up of randomized clinical studies have reported reduced rate of hospitalization, decreased frequency of pain episodes and acute chest syndrome, and reduced requirement for blood transfusions.^{103–106} Iron chelation therapy for patients with transfusional iron overload is essential to prevent end-organ damage. The oral iron-chelating agent

(i.e., deferasirox) appears to be as effective as deferoxamine.¹⁰⁷ BM transplantation has been employed in selected patients as a potentially curative therapeutic modality.^{108,109} The broad variability in the clinical phenotype of SCD, the limited availability of HLA-identical siblings as optimal stem cell donors, and the potential risk of transplant-related morbidity and mortality have presented challenges for the use of this approach.

Sickle Cell Variants

Hb SC Disease

The mutation that gives rise to Hb C ($\beta^{6\text{Glu}\rightarrow\text{Lys}}$) is at the same site as the Hb S mutation but a different amino acid substitution. Individuals with Hb C trait are asymptomatic, and homozygous Hb C disease is associated with mild anemia, splenomegaly, and cholelithiasis. Hb C in RBCs has been reported to be protective against malaria.¹¹⁰ Hb C has a propensity to crystallize and interact with membrane ion transport. In compound heterozygotes for Hb SC with equal amounts of Hb S and Hb C in RBCs, Hb C does not participate in polymerization with deoxy Hb S, but contributes to the dehydration of the cell and induction of sickling. The peripheral blood film shows target cells, folded RBCs and occasional irreversibly sickled cells. Patients with Hb SC disease exhibit clinical manifestations and complications similar to those patients with sickle cell anemia (Hb SS), but with reduced severity and frequency and an increased life expectancy.^{98,111} The hematocrit in patients with Hb SC disease tends to be higher and may account for the increased frequency of proliferative retinopathy. Some adults with Hb SC, who have not developed functional asplenia, are at risk for splenic infarction and sequestration crisis. Chronic organ complication risk is reduced in patients with coinheritance of α thalassemia.

Sickle- β Thalassemia

Compound heterozygosity for Hb S and β^0 thalassemia has a clinical course that is similar to sickle cell anemia (Hb SS), given the absence of any normal β globin chain synthesis and lack of Hb A. In the presence of sickle- β^+ thalassemia, variable levels of Hb A are produced, ranging from 3 to 25% as in many African-American individuals who carry mild forms of β^+ thalassemia mutations. The amount of Hb A correlates with disease severity by mitigating the solubility and polymerization of Hb S. In sickle- β thalassemia, the RBCs are microcytic and hypochromic, and the reduced mean corpuscular hemoglobin concentration (MCHC) modulates the disease phenotype by retardation of Hb S polymerization.

Sickle Cell Trait

Individuals who are heterozygous for the β^S gene have sickle cell trait, detected in up to 8% of African-American individuals. In some regions in Africa where the β^S gene originated in at least three different locations, the frequency approaches

as high as 25%.¹¹² The Hb S amount in RBCs is typically <50% with a Hb A to Hb S ratio of 60:40, due to the greater post-translational affinity of α globin chains for β^A than for β^S globin chains. Thus, hemoglobin electrophoresis typically demonstrates the presence of 40% Hb S and 60% Hb A. The coinheritance of α thalassemia is associated with a reduction in the amount of Hb S, depending on the number of deleted α globin genes.¹¹³ Sickle cell trait is associated with a normal life span and these individuals do not have vasoocclusive complications under physiologic conditions. The complete blood count is normal. The diagnosis and carrier detection for β^S are established frequently during newborn screening, which necessitates genetic counseling for the parents, especially if both parents are carriers for the β^S gene, and are therefore, at risk for a subsequent child with SCD. Children with sickle cell trait do not require any restrictions of activities. Serious complications associated with sickle cell trait are rare and include intermittent hematuria, hyposthenuria, urinary tract infections, splenic infarction at high altitude, rhabdomyolysis, sudden death risk associated with rigorous and prolonged physical exercise or during exercise at high altitude, increased venous thrombosis risk, and medullary renal carcinoma in young adults.¹¹⁴

Other Sickle Cell Variants

These syndromes arise from the coinheritance of the β^S gene with other less common β globin variants. Some examples include compound heterozygous conditions for Hb S and Hb D, Hb O Arab, Hb E, or Hb Lepore. These disorders give rise to hemolytic anemias and vasoocclusive complications of varying severity.^{115–119}

Hereditary Persistence of Fetal Hemoglobin Syndromes

As the physiologic switch from γ - to β globin chain synthesis occurs in the fetus and neonate, adult Hb A replaces Hb F. Mutations that affect the normal developmental regulation of the globin genes and switching that lead to continued synthesis of high levels of fetal hemoglobin in adult life give rise to hereditary persistence of fetal hemoglobin (HPFH) syndromes. Increased amounts of Hb F in affected individuals have no adverse consequences. The major clinical importance of HPFH mutations is the interaction with Hb S or β thalassemia mutations, which results in an ameliorating effect on the phenotype of these disorders and milder clinical syndromes due to elevated Hb F levels. More than 50 mutations that give rise to HPFH syndromes have been discovered.¹²⁰ Some of these mutations involve deletions in the β globin gene cluster and are termed deletional HPFH; Hb F levels typically range from 15 to 30% and are distributed uniformly in nearly all RBCs in a panellular fashion. Heterozygotes are clinically and hematologically normal. Homozygotes produce 100% Hb F and are healthy with only minor hematologic

abnormalities, including slight microcytosis, hypochromia, mild elevation of hemoglobin level, and erythrocytosis due to the high oxygen affinity of Hb F and resulting in a left-shifted oxygen–hemoglobin dissociation curve. Nondeletion HPFH syndromes are associated with point mutations in or around the regulatory elements of the γ globin genes, leading to upregulation of gene expression. Hb F levels do not exceed 20%, and the distribution of Hb F may be either panellular or heterocellular with Hb F detected in some of the RBCs. Certain variants of heterocellular HPFH are not linked to mutations in the β globin gene cluster and exhibit mildly increased Hb F levels. Such genetic determinants that are distant from the β globin gene cluster have been reported in chromosome 6 and the X chromosome.^{121–123}

Unstable Hemoglobin Variants

The unstable hemoglobins are typically associated with an autosomal dominant inheritance pattern; heterozygous individuals harbor unstable hemoglobin concentrations ranging from 10 to 30% of the total with the remaining hemoglobin predominantly consisting of Hb A. More than 130 unstable hemoglobin variants have been reported to date, with some due to spontaneous mutations without affected parents.¹⁴ Unstable hemoglobins exhibit reduced solubility and a propensity to denature and precipitate as insoluble globins that often attach to the RBC membrane and are recognized as Heinz bodies, hence the term “congenital Heinz body hemolytic anemia.” The deformability of the RBC membrane is impaired and hemolysis occurs in the splenic sinuses and microcirculation. Hemolytic anemia may be worsened by the ingestion of “oxidant drugs” or an infection. The mutations may affect either the α or the β globin (75% of cases) chains and lead to amino acid substitutions or deletions in the vicinity of the heme pocket, typically weakening the binding of heme to globin and disrupting the forces that maintain the normal structure of the hemoglobin molecule. Many unstable hemoglobins exhibit an increased susceptibility to oxidation to methemoglobin containing ferric (Fe^{3+}) iron.

Confirmation of the diagnosis requires demonstration of an unstable hemoglobin variant. Incubation of a whole blood sample with brilliant cresyl blue leads to oxidative denaturation and precipitation of unstable hemoglobins. This may allow the visualization of precipitated hemoglobin as Heinz bodies on the peripheral blood film. The isopropanol solubility test may establish the presence of unstable hemoglobins that exhibit increased propensity to precipitate compared to normal hemoglobin. Hemoglobin electrophoresis may aid in diagnosis, only if the unstable hemoglobin mutation is associated with an electrical charge change that alters mobility during electrophoresis. Thus, a normal hemoglobin electrophoresis does not exclude the presence of an unstable hemoglobin, if the mutation is electrophoretically silent or neutral, such as hemoglobin Köln ($\beta^{98\text{Val}\rightarrow\text{Met}}$).¹²⁴ Isoelectric focusing and sequencing of PCR-amplified α and β globin

genes may be performed in cases where further evaluation and characterization are indicated. Because the oxygen affinity of unstable hemoglobins may be altered, determination of the P_{50} by co-oximetry is indicated in all suspected cases. The clinical presentation may be associated with jaundice and splenomegaly, due to a nonspherocytic hemolytic anemia with reticulocytosis, although the severity, manifestations, and age at presentation are quite variable. Most patients exhibit a relatively benign course. As in other chronic hemolytic anemias, splenomegaly, cholelithiasis, and leg ulcers may complicate the course. Hemolytic episodes associated with infections or drugs may necessitate the use of blood transfusions.

Hemoglobin Variants with Altered Oxygen Affinity

Mutations in the hemoglobin molecule that affect the ability of hemoglobin to release oxygen in the tissues may give rise to increased or reduced oxygen affinity variants that impair normal oxygen transport. The kinetics of the sequential oxygenation and deoxygenation of the four separate heme pockets in each hemoglobin molecule is reflected in the oxygen-hemoglobin dissociation curve which relates partial oxygen pressure (P_{aO_2}) to oxygen saturation of hemoglobin. The overall oxygen affinity of hemoglobin is expressed as the P_{50} , the partial oxygen pressure at which 50% of the hemoglobin is saturated with oxygen. The transition of hemoglobin from the deoxygenated, low affinity state (T for tense) to the oxygenated, high affinity state (R for relaxed) is a process that involves the movements of many amino acids and the breakage and formation of many intramolecular bonds. Mutations that affect the interaction of heme with oxygen, the contact between the α and β globin chains, the regions affecting the hydrogen bonds, or the binding of 2,3-diphosphoglycerate (2,3-DPG) may favor the high affinity "R state," leading to decreased tissue oxygen delivery at normal capillary oxygen pressure. One example is Hb Chesapeake ($\alpha^{92\text{Arg}\rightarrow\text{Leu}}$), identified in a family with autosomal dominantly inherited erythrocytosis.¹²⁵ Reduced tissue oxygen delivery triggers erythropoietin production and stimulates erythropoiesis. Individuals with high affinity hemoglobins are generally asymptomatic and do not require therapy. In some patients, elevated RBC mass may be associated with mild symptoms, including occasional headaches, facial fullness, and plethora. The P_{50} determined by cooximetry is decreased. For diagnosis, hemoglobin electrophoresis may be helpful, but electrophoretically silent variants need to be investigated further by other detection methods. The differential diagnosis of decreased P_{50} includes elevated carbon monoxide levels, which stabilize hemoglobin in the high affinity "R state" without the need for oxygen binding. This may occur as a result of acute or chronic carbon monoxide exposure and in some heavy cigarette smokers who may develop smoker's polycythemia.

Hemoglobin variants that cause cyanosis – bluish discoloration of the skin and mucous membranes are associated either

with increased methemoglobin formation (M hemoglobins) or with reduced oxygen affinity variants, such as Hb Kansas ($\beta^{102\text{Asn}\rightarrow\text{Thr}}$), which favors the formation of the deoxy (T state) conformation of hemoglobin.¹²⁶ Hemoglobin variants with decreased oxygen affinity readily release oxygen in the capillaries, such that oxygen transport may occur efficiently with lower than normal hematocrit values, giving rise to a clinical picture termed "pseudoanemia."¹²⁷ These individuals are asymptomatic and do not require transfusions. No intervention is necessary for the treatment of those individuals who have cyanosis. Cooximetry reveals an increased P_{50} value, consistent with the shift of the hemoglobin-oxygen dissociation curve to the right.

Methemoglobinemias

Methemoglobin is an oxidized form of hemoglobin that is incapable of transporting oxygen and in which the ferrous (Fe^{2+}) iron molecules of heme are oxidized to the ferric (Fe^{3+}) state. Methemoglobinemia is a situation where the level of methemoglobin exceeds 1%, leading to the development of cyanosis. Methemoglobinemia occurs either due to increased production or to decreased methemoglobin reduction, and either represents a congenital or an acquired disorder. Deficiency of the enzyme cytochrome b_5 reductase is a rare cause of congenital methemoglobinemia, inherited in an autosomal recessive fashion.¹²⁸ These individuals have the deficiency of methemoglobin reductase activity in the RBCs, and develop cyanosis. Treatment is not required. A more severe phenotype associated with mental retardation may occur. Cyanosis, which may present a cosmetic problem, may be improved by the treatment with oral methylene blue or ascorbic acid.

Globin gene mutations that are associated with stabilization of heme iron in the oxidized ferric state (Fe^{3+}) give rise to M hemoglobin variants and congenital methemoglobinemia.¹²⁷ The inheritance is autosomal dominant, but de novo mutations also occur. Affected individuals present with cyanosis. There have been 9 M hemoglobin variants described to date, involving the α -, β -, or γ -globin chains. The cyanosis in γ chain variants resolves, as the switch from Hb F to Hb A is completed after birth. In contrast to normal Hb A, which binds four oxygen molecules, M hemoglobins bind only two. For instance, in the α -globin chain variant Hb M-Boston ($\alpha^{58\text{His}\rightarrow\text{Tyr}}$), only the β chains react with oxygen.¹²⁹ Similarly, β -globin chain variants, such as Hb M-Hyde Park ($\beta^{92\text{His}\rightarrow\text{Tyr}}$), carry oxygen only on the α -globin chains.¹³⁰ The oxygen affinity at P_{50} may be normal or decreased. M hemoglobins exhibit abnormal spectrophotometric absorbance, which may be utilized in the diagnosis of methemoglobinemia. Analysis by hemoglobin electrophoresis at pH 7.1 reveals an abnormal band after chemical conversion of hemoglobin M containing samples to methemoglobin prior to electrophoresis. These disorders are benign and no therapy is indicated. Cyanosis associated with M hemoglobins does not respond to methylene blue or ascorbic acid treatment.

M hemoglobins should be suspected in all patients with chronic cyanosis, as misdiagnosis may be associated with unnecessary laboratory testing and investigations for cardiopulmonary causes of cyanosis.

Acquired methemoglobinemias are much more common, and occur when normal hemoglobin is oxidized into methemoglobin, following exposure to exogenous agents, such as nitrites, dapsone, local anesthetics, primaquine, or environmental chemicals.¹³¹ Toxic methemoglobinemia results in decreased oxygen carrying capacity of blood in proportion to the amount of methemoglobin because hemoglobin subunits that have been oxidized are no longer capable of binding oxygen. Furthermore, the partial oxidation of hemoglobin subunits in a tetramer is associated with a marked increase in the oxygen affinity of the remaining ferrous (Fe^{2+}) heme groups in the tetramer. The diagnosis is suspected in the presence of cyanosis associated with “chocolate-brown” discoloration of blood in the presence of normal arterial PaO_2 measurements on arterial blood gas analysis, but with a low and discordant arterial oxygen saturation reading determined by pulse oximetry. In the presence of mildly elevated methemoglobin levels measured by cooximetry, discontinuation of the offending agent is frequently associated with gradual improvement over several days. In symptomatic individuals with high methemoglobin levels, tissue oxygen deprivation may be life-threatening, leading to coma and possibly requiring emergent management by intravenous methylene blue administration and occasionally requiring exchange transfusion.

Laboratory Evaluation and Diagnosis of Hemoglobinopathies

Clinically significant disorders of globin chain synthesis require comprehensive clinical and laboratory evaluation to accurately establish the diagnosis. Practice guidelines, protocols for the systematic laboratory evaluation of hemoglobinopathies, and screening recommendations have been published.^{7,82,132–134}

Complete Blood Count and Examination of a Blood Film

A hemoglobin disorder may be suspected based on clinical presentation with anemia, erythrocytosis, or cyanosis and the presence of abnormal laboratory findings in RBC indices, such as reduced MCV and MCH values in cases where iron deficiency anemia has been excluded. Blood film examination may demonstrate the presence of microcytosis, hypochromia, basophilic stippling, target cells, RBC inclusions, nucleated RBCs, or irreversibly sickled cells. Further evaluation for a hemoglobinopathy is indicated, using hemoglobin separation studies and quantification of Hb A_2 and Hb F as appropriate.

Sickle Solubility Test

This is a rapid test that provides the information whether Hb S is present or not. It is positive in both SCD and SC trait.

Using a reducing agent, such as sodium dithionite, deoxygenated Hb S is insoluble in RBCs and is detected by increased turbidity. This test is not quantitative and does not identify the specific abnormal hemoglobin variant, which should be verified by hemoglobin separation studies, such as electrophoresis and chromatography. The sickle solubility test is not used for newborn screening, due to the large amount of Hb F in the RBCs and the possibility that a small amount of Hb S may not be detected.

Hemoglobin Electrophoresis at Alkaline pH

Hemoglobin is a charged molecule and some, but not all, hemoglobin variants exhibit charge differences from normal hemoglobin and migrate differently in the presence of an electrical field. Hemoglobin electrophoresis on cellulose acetate membranes at pH 8.6 is a relatively inexpensive and simple technique that may be prepared quickly, provides sharp resolution of the hemoglobin bands, and allows quantification by densitometry. When used for screening in newborns where the hemolysate contains about 80% Hb F, the suboptimal resolution of Hb F from Hbs S and A necessitates the use of citrate agar electrophoresis at acid pH as a complementary method. This method is not accurate for Hb A_2 and Hb F quantitations.

Hemoglobin Electrophoresis at Acid pH

This is a confirmatory test for abnormal hemoglobins that exhibit a similar migration pattern in alkaline electrophoresis. Agar gel electrophoresis in a citrate buffer at acid pH 6.2 may differentiate Hb C from Hbs E and O and Hb S from Hbs D, G, and Lepore. This procedure is used as a confirmatory test for Hb S and Hb C and is appropriate for newborn screening, since Hb F separates distinctly from Hb S and Hb A, allowing the detection of small amounts. This test is not used for quantitative measurements.

Isoelectric Focusing

This technique involves the use of a pH gradient in a polyacrylamide gel and allows the separation of hemoglobin fractions into sharp and distinct bands with excellent resolving power compared to other electrophoresis techniques. Isoelectric focusing is an excellent screening method of choice for cord as well as adult blood, but cannot be used for quantitation of variant hemoglobins. Abnormalities detected by IEF may be investigated by a second step with citrate agar electrophoresis, and if available, high-performance liquid chromatography (HPLC).

Capillary Electrophoresis

Automated capillary zone electrophoresis is an efficient technique that has become available for routine detection of variant hemoglobins in clinical laboratories. Advantages include the requirement for only a small amount of sample and the ability to carry out rapid testing with good resolution.

This method allows accurate measurement of Hb A₂ in the presence of Hb E or Hb S, but it cannot separate Hb A₂ from Hb C.

High-Performance Liquid Chromatography

This procedure efficiently separates proteins with high resolution on the basis of charge differences and has become a reference method for the characterization of hemoglobin abnormalities in recent years. Using a cation exchange column, the retention time of unknown hemoglobins is compared to that of a calibrated standard containing Hbs F, A, S, and C. In many clinical laboratories, ion-exchange HPLC is used as the initial test for the evaluation of suspected hemoglobinopathies. HPLC allows rapid, automated quantitation of abnormal and minor hemoglobins with high sensitivity. It is also useful as the primary screening method in some newborn screening programs and provides precise measurements of Hb A₂ (except in the presence of Hb E and Hb Lepore).

Hb A₂ Quantitation by Column Chromatography

This test is very important for the accurate detection of β thalassemia carriers. In an individual with mild hypochromic (MCH <26 pg), microcytic anemia not due to iron deficiency, an elevated Hb A₂ level of >3.5% is consistent with heterozygosity for a β thalassemia mutation. Chromatography on microcolumns followed by spectrophotometric analysis is a commonly used method and allows quantitation of the percentage of Hb A₂. HPLC or capillary electrophoresis is an alternative method to reliably quantitate Hb A₂ levels.

Hb F Quantitation

There are several methods for Hb F measurement, which is indicated in the diagnosis of thalassemia and HPFH syndromes as well as in the surveillance of patients with SCD patients, who are treated with hydroxyurea. Reliable techniques include HPLC and capillary electrophoresis, as well as immunologic methods using specific antibodies against Hb F.

ζ Globin Detection in α^0 Thalassemia Carriers

This procedure utilizes an ELISA assay and has been reported to reliably detect carriers of α^0 thalassemia (—^{SEA}) with high sensitivity and specificity by analyzing the presence of ζ -globin protein in adult RBC hemolysates.

DNA-Based Tests

Determination of the underlying specific genotype using molecular diagnostic techniques is important for epidemiologic studies of carrier identification, to determine the prevalence and types of globin gene defects in a given population, for genetic counseling, and for an accurate prenatal diagnosis in couples at risk for having a child with a major hemoglobinopathy. Using genomic DNA, Southern blotting has been useful in detecting large deletions, such as those involving

the α globin genes. Polymerase chain reaction (PCR)-based techniques have been typically used to detect commonly encountered point mutations, as well as nucleotide sequencing of PCR-amplified globin genes to detect unknown or less frequent mutations. The DNA-based techniques and procedures used in the molecular diagnosis of hemoglobinopathies have been detailed in comprehensive reviews.^{7,47,134,135}

Molecular Pathology of Erythrocyte Membrane Disorders

Disorders of erythrocyte shape caused by defects in the erythrocyte membrane comprise a significant group of hereditary hemolytic anemias. These include the hereditary spherocytosis, the hereditary elliptocytosis, and the hereditary stomatocytosis syndromes. There is significant heterogeneity in the genetic, biochemical, and clinical manifestations of these disorders.

Hereditary Spherocytosis

The hereditary spherocytosis (HS) syndromes are a group of heterogeneous disorders with the common finding of spherocytes on the peripheral blood smear.^{136,137} The HS syndromes occur in all races worldwide, but they are particularly common in people of northern European ancestry. HS has been estimated to occur at an incidence of ~1:2,500 individuals. Inheritance is autosomal dominant in ~two thirds of cases. The remainder is due to autosomal recessive inheritance or occurrence of a de novo mutation.

Pathophysiology

The primary cellular defect in HS is the loss of membrane surface area relative to intracellular volume, leading to the spherocytic shape and decreased deformability of HS RBCs.^{137,138} The loss of membrane surface area in HS RBCs is a consequence of several molecular mechanisms, the common denominator of which is weakening of protein-protein interactions that link the underlying membrane skeleton to the plasma membrane, lipid bilayer leading to microvesiculation, loss of membrane surface area, decreased surface/volume ratio, and spherocytosis.

Qualitative and quantitative defects of several membrane proteins (Figure 37.4, Table 37.2) have been associated with HS. Defects of the membrane proteins α - or β -spectrin, ankyrin, or protein 4.2 lead to decreased membrane skeleton density causing destabilization of the lipid bilayer, resulting in the loss of band 3-containing membrane microvesicles. Abnormalities of band 3, the anion exchanger, lead to the loss of the lipid-stabilizing effect of band 3, resulting in the release of band 3-free membrane microvesicles. Both pathways lead to the loss of membrane material with a reduction in membrane surface area (Figure 37.5). The ensuing decrease in membrane

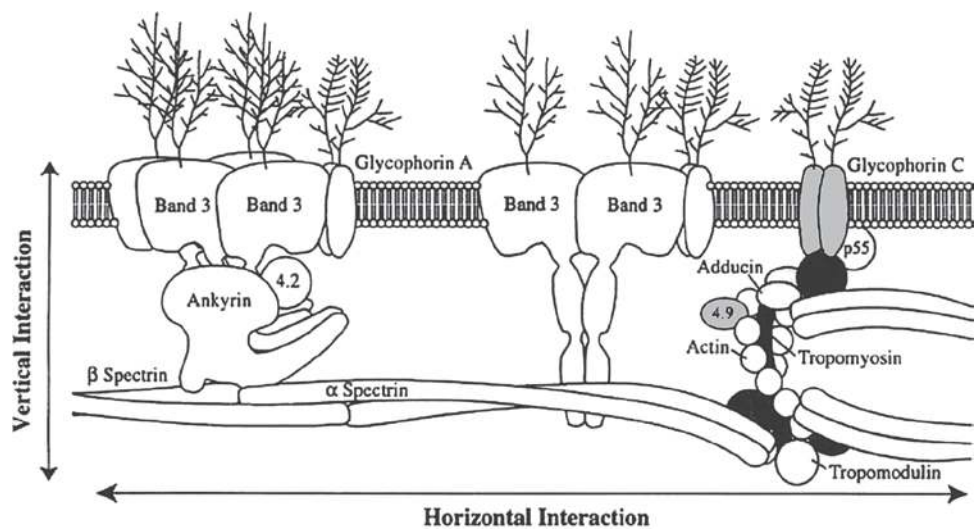


FIG. 37.4. *The erythrocyte membrane.* A model of the major proteins of the erythrocyte membrane is shown: α and β spectrin, ankyrin, band 3 (the anion exchanger), 4.1 (protein 4.1R) and 4.2 (protein 4.2), actin and glycophorin. Membrane–protein and membrane–lipid interactions can be divided into two categories: (1) vertical interactions, which are perpendicular to the plane of the membrane

and involve spectrin–ankyrin–band 3 interactions, spectrin–protein 4.1–glycophorin C interactions, and weak interactions between spectrin and the lipid bilayer, and (2) horizontal interactions, which are parallel to the plane of the membrane (reprinted with permission from Tse WT, Lux SE. Red blood cell membrane disorders. *Br J Haematol.* 1999;104:2).

TABLE 37.2. Erythrocyte membrane gene defects in inherited disorders of red cell shape.

Disorder	Genes	Comment
Hereditary spherocytosis	Ankyrin	Most common cause of typical dominant HS
	Band 3	“Pincered” spherocytes seen presplenectomy
	β -spectrin	“Acanthocytic” spherocytes presplenectomy
	α -spectrin	Typically severe, autosomal recessive
	Protein 4.2	Common in Japan, autosomal recessive
Hereditary elliptocytosis	α -spectrin	Location of mutation in determines phenotype
Hereditary pyropoikilocytosis	β -spectrin	Location of mutation in determines phenotype
	Protein 4.1R	
	Glycophorin C	Concomitant protein 4.1R deficiency
Southeast Asian ovalocytosis	Band 3	In-frame 9 amino acid deletion
Hereditary stomatocytosis	Band 3	Mutations in anion exchange encoding region
	Stomatatin	Protein deficient or absent
Abetalipoproteinemia		Microsomal triglyceride transfer protein
	Apolipoprotein B	
Neuroacanthocytosis	XK	McLeod phenotype, X-linked
	Chorein	Chorea-acanthocytosis
	Junctophilin 3	Huntington’s disease like 2
	PANK2	Pantothenate kinase-associated neurodegeneration

surface area with the formation of spherocytes is paralleled by a decrease in RBC deformability that predisposes the cells to splenic entrapment and conditioning.

Splenic entrapment and destruction of poorly deformable RBCs following phagocytic ingestion is the primary cause of hemolysis experienced by HS patients.¹³⁹ Splenic conditioning, repeated courses of travel through the splenic cords, promotes membrane loss leading to a reduction in cell surface/cell volume ratio and a progressively more spheroid red cell. While detained in the splenic sinuses, the RBC is subjected to an acidotic, metabolically unfavorable environment, where oxidants are prevalent and phagocytic reticuloendothelial cells are ubiquitous. Secondary defects, such as 2,3-BPG depletion and increased cation permeability, are acquired. The more spherocytic the RBC, the greater the likelihood that its lack of deformability will prevent its passage through the narrow fenestrations of the splenic cords. Nondeformable spherocytes accumulate in the red pulp, which becomes grossly enlarged and leads to splenomegaly. Some of these conditioned RBCs re-enter the systemic circulation, as revealed by the “tail” of the osmotic fragility curve, indicating the presence of a subpopulation of cells with a markedly reduced surface area. After splenectomy, this RBC population disappears. Splenectomy interrupts this process of RBC destruction leading to its use in the treatment of HS (see below).

History and Physical

Clinical manifestations of the HS syndromes vary widely.^{137,140} A history of anemia, fatigue, jaundice, gallstones, or splenectomy

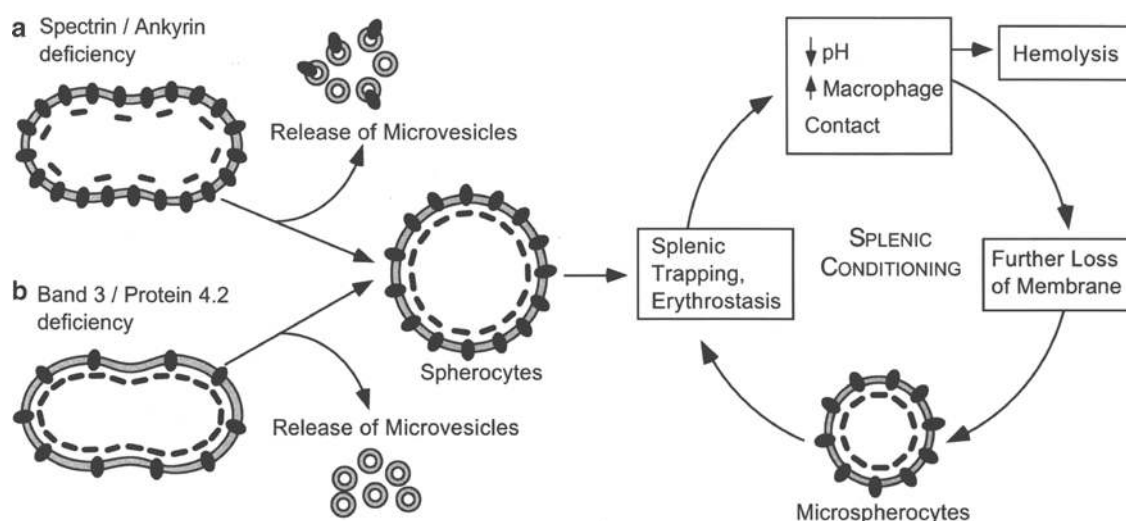


FIG. 37.5. *Pathophysiology of hereditary spherocytosis.* The primary defect in hereditary spherocytosis is a deficiency of membrane surface area. Decreased surface area may be produced by two different mechanisms: (1) Defects of spectrin, ankyrin, or protein 4.2 lead to reduced density of the membrane skeleton, destabilizing the overlying lipid bilayer and releasing band 3-containing microvesicles. (2) Defects of band 3 lead to band 3 deficiency and loss of its lipid-stabilizing effect. This results in the loss of band 3-free microvesicles. Both pathways result in membrane loss,

decreased surface area, and formation of spherocytes with decreased deformability. These deformed erythrocytes become trapped in the hostile environment of the spleen where splenic conditioning inflicts further membrane damage, amplifying the cycle of red cell membrane injury (reprinted with permission from Gallagher PG, Jarolim P. Red cell membrane disorders. In: Hoffman R, Benz EJ Jr, Shattil SJ, Furie B, Cohen HJ, Silverstein LE, McGlave P, eds. *Hematology: Basis Principles and Practice*. 4th ed. Philadelphia: WB Saunders; 2005).

in a patient with suspected HS or their family members is frequently elucidated. Other historical features may include neonatal jaundice and history of red cell transfusion. Physical findings may include pallor, jaundice, and splenomegaly with the spleen occasionally reaching massive proportions. Uncommon findings include leg ulcers and extramedullary tumors.

HS is typically present in childhood, but may be present at any age. Anemia is the most frequent presenting complaint, followed by splenomegaly, jaundice, or a positive family history. Two-thirds to three-quarters of HS patients have incompletely compensated hemolysis and mild to moderate anemia. The anemia is often asymptomatic, except for fatigue and mild pallor. Jaundice is seen some time in about half of patients, usually in association with viral infections. When present, it is acholuric (i.e., with unconjugated hyperbilirubinemia without detectable bilirubinuria).

Laboratory Testing

Typical laboratory findings in HS include spherocytes on the peripheral blood smear, anemia, reticulocytosis, increased mean corpuscular hemoglobin concentration (MCHC), hyperbilirubinemia, and an abnormal osmotic fragility test.

Peripheral Blood Smear

Peripheral blood smears of HS patients exhibit varying numbers of spherocytes (i.e., RBCs lacking a central pallor). Identification

TABLE 37.3. Disorders with spherocytes on peripheral blood smear.

Hereditary spherocytosis
Autoimmune hemolytic anemias
Acute oxidant injury, Heinz body anemias
Liver disease
Thermal injuries
Microangiopathic and macroangiopathic hemolytic anemias
Clostridial sepsis
Transfusion reactions with hemolysis
Poisoning with certain snake, spider, and Hymenoptera venoms
Severe hypophosphatemia
Zinc toxicity
ABO incompatibility (in neonates)

of spherocytes alone is inadequate to make the diagnosis of HS, as spherocytes are seen in various conditions (Table 37.3). RBC morphology less frequently seen in HS includes microspherocytosis, anisocytosis, and poikilocytosis. Detailed genetic studies have suggested that specific RBC morphology may be associated with specific membrane protein defects, such as pincered RBCs (band 3), spherocytic acanthocytes (β spectrin), or spherostomatocytes (band 3 or protein 4.2).

Blood Count and RBC Indices

Blood counts in HS demonstrate variable degrees of anemia (i.e., absent, mild, moderate, or severe) and reticulocytosis, with most patients exhibiting mild to moderate anemia. However, the anemia may be transfusion-dependent and life-threatening.

Despite increased numbers of reticulocytes, which have larger volume than mature RBCs, the mean corpuscular volume (MCV) is normal or slightly low, reflecting membrane loss and dehydration. The mean corpuscular hemoglobin concentration (MCHC) is usually elevated (>35 g/dL) reflecting RBC dehydration. Data obtained by automated cell counters has been examined as a strategy to screen for HS.^{141,142} The combination of a RBC distribution width >14 with an MCHC >35.4 g/dL suggests the diagnosis of HS at a sensitivity of 63% and a specificity of 100%. Analysis of laser-based cell counter-generated histograms of hyperdense erythrocytes with MCHC >40 g/dL has been described as another screening test for HS.

Osmotic Fragility

Osmotic fragility (OF) testing, which measures the lysis of RBCs suspended in solutions of decreasing osmolarity, is frequently utilized in the diagnosis of HS. Membrane redundancy provides the normal RBC with its distinctive discoid shape and its abundant surface area. In HS, RBC membrane loss leads to decreased surface area relative to cell volume, resulting in spherocytic shape (Figure 37.5). This decreased membrane surface area:volume ratio makes HS RBCs susceptible to osmotic lysis in hypotonic solutions, the basis of the osmotic fragility test. This test is performed by adding increasingly hypotonic concentrations of saline to RBCs. Normal RBCs are able to increase cellular volume by swelling, but spherocytes, which are already at maximum volume for surface area, hemolyze at higher saline concentrations than normal. Hemolysis is determined by measuring the amount of hemoglobin released from RBCs into the extracellular fluid.

Incubation at 37°C for 24 h prior to OF testing accentuates the surface area:volume defect, as HS erythrocytes lose membrane surface area more readily than normal. This distinguishing factor is helpful, as ~25% of HS patients exhibit normal OF on freshly drawn cells. The OF correlates well with the degree of spherocytosis detected on the peripheral smear, but does not correlate with hemoglobin concentration. Incubated osmotic fragility testing suffers from decreased sensitivity, as spherocytosis due to any cause leads to increased OF. Moreover, in mild cases of HS, there is poor sensitivity, where up to 10–15% of cases are not detected, even after incubation, likely due to the small numbers of spherocytes present.

In HS patients who have not been splenectomized, a “tail” of the OF curve may be present, representing a subpopulation of very fragile RBCs conditioned by splenic stasis. This subpopulation disappears after splenectomy.

Other Tests

Other tests have been utilized in the diagnosis of HS. These include the autohemolysis test (which examines the spontaneous hemolysis of RBCs incubated under sterile conditions without glucose), the glycerol lysis test and the pink test (which employ glycerol to retard osmotic swelling of RBCs), the cryohemolysis test, and the skeleton gelation test. Like OF testing, these tests lack sensitivity and/or specificity.

Eosin-5-maleimide (EMA) binding to the RBC membrane, an indication of the relative amounts of the band 3 and Rh-related integral membrane proteins, determined by fluorescence activated cell sorting has been utilized as a screening test for HS.^{143,144} Although not widely used, it is a technique that is relatively simple and quickly performed. Like OF and other tests, EMA-binding also detects non-HS spherocytosis, as well as other abnormalities of the RBC, some membrane variants, hydration defects, and dyserythropoietic disorders.

Osmotic gradient ektacytometry can accurately determine the relative contributions of cell dehydration and surface area deficiency in cases of HS. This test is only available in specialized laboratories.

Molecular Studies

Qualitative and quantitative analyses of RBC membrane proteins via sodium dodecyl sulfate–polyacrylamide gel electrophoresis may provide informative data in up to two thirds of HS cases. However, it is time consuming and cumbersome to perform and is not currently commercially available.¹⁴⁵

Mutation detection, either utilizing mutation screening tools or direct DNA sequence analyses, is cumbersome, expensive, and not readily available. The “HS genes,” ankyrin, α - and β -spectrin, band 3, and protein 4.2 are very large. Clinical and laboratory studies provide little guidance as to where to search in the gene for HS-associated mutations, and because there is no “founder” effect in HS (i.e., individual HS kindreds have private mutations spread throughout the genes), mutation detection is difficult. These studies may be performed in specialized laboratories, and are likely to be beneficial only in unusual or diagnostically challenging cases.^{136,146}

Markers of Hemolysis

Other laboratory manifestations commonly observed (i.e., reticulocytosis, hyperbilirubinemia, increased lactate dehydrogenase, decreased haptoglobin, increased urinary, and fecal urobilinogen) are not specific for HS, but are markers of ongoing hemolysis, reflecting increased RBC production or destruction.

Pathologic Findings

Examination of bone marrow samples from HS patients demonstrate erythroid hyperplasia and are not diagnostic. In HS patients with acute parvovirus B19-associated aplasia, giant pronormoblasts are found followed by hyperplastic normoblasts during the recovery phase.

Examination of the spleen demonstrates marked splenic congestion, filled with poorly deformable RBCs.

Differential Diagnosis

Anemia and spherocytosis are found in other inherited and acquired disorders of the RBC, such as immune-mediated hemolysis, G6PD deficiency, Heinz body anemia, unstable hemoglobinopathy, microangiopathic hemolytic anemia,

clostridial sepsis, and thermal burns. These other disorders should be viewed in the appropriate clinical context allowing differentiation from HS by differences in clinical course, peripheral blood smear, and laboratory tests specific for each disease. Additional historical data, such as age of onset, recent prescription of various medications, symptoms attributable to malignancy, or connective tissue disease, may be found. With the exception of autoimmune hemolytic anemia, spherocytes are rarely the sole or dominant morphologic abnormality of the RBC found on the peripheral blood smear. Other laboratory features, such as a positive antiglobulin reaction in immune hemolytic disease, may also be present.

Complications

Complications of HS include the formation of bilirubinate gallstones, found in at least half of adult HS patients, frequently associated with coinheritance of Gilbert syndrome.¹⁴⁷ HS patients may suffer from various “crises,” including aplastic and hemolytic crises. Aplastic crisis occurs due to transient BM suppression by parvovirus B19 or other viral infections.¹⁴⁸ The associated anemia may be severe and require hospitalization and transfusion therapy. Interestingly, aplastic anemia due to parvovirus infection may be the initial manifestation of HS in some patients and their families. Hemolytic crisis with increased anemia may occur with many viral illnesses, likely due to increased splenomegaly and splenic destruction of RBCs. Megaloblastic crisis may occur due to folate deficiency, typically occurring in HS patients with increased folate demands, such as those recovering from an aplastic crisis, pregnant women, and the elderly.

Other rare complications include leg ulcers and/or dermatitis that heal after splenectomy, gout, extramedullary hematopoietic tumors, spinocerebellar degenerative syndromes, hypertrophic cardiomyopathy, and myopathy. Cases of hematologic malignancy, including myeloproliferative disorders and leukemia, have been reported in HS patients. The persistent hematopoietic stress of HS has been suggested to predispose to the development of these disorders, but this is unclear.

Treatment

Specific Therapy

HS is unique among the inherited hemolytic anemias, in which splenectomy permanently cures or markedly improves the anemia in most HS patients. Even patients with severe HS exhibit significant clinical improvement. Postsplenectomy, erythrocyte lifespan normalizes, hemoglobin concentration increases, reticulocyte counts normalize, and transfusion requirements are decreased or eliminated. Jaundice fades and the risk of cholelithiasis is reduced or eliminated. Spherocytosis on the peripheral blood smear and altered osmotic fragility persist, but the “tail” of the fresh osmotic fragility curve, created by splenic conditioning, disappears.

Laparoscopic splenectomy is now the surgical method of choice, resulting in less postoperative pain, shorter hospitalization, and decreased costs. Even huge spleens may be removed laparoscopically. Operative complications include local infection, bleeding, and pancreatitis. Partial splenectomy has been advocated for infants and very young children with severe HS, in part to preserve splenic immunologic function when the risk of overwhelming sepsis is highest, while decreasing the destruction of spherocytes in the spleen and improving the anemia.¹⁴⁹ Although there is rapid regrowth of the spleen in many cases, a second, complete splenectomy is likely to be required, hopefully at a time when the patient is older and the risk of overwhelming postsplenectomy infection (OPSI) is less. Others feel that this is an unnecessary operation and that severely affected patients may be successfully managed with transfusion therapy and close follow-up, avoiding a second operative procedure with general anesthesia.

Splenectomy was once considered routine therapy for the treatment of HS, eliminating both the need for transfusion and the risk of aplastic crises, and decreasing the risk of cholelithiasis and symptomatic gall bladder disease. Over the past decade, the risk of OPSI, particularly with encapsulated bacteria such as *Streptococcus pneumoniae*, the emergence of penicillin-resistant pneumococci, increasing realization of the protective role of the spleen from parasitic diseases, such as malaria or babesiosis, and growing recognition of the increased risk of postsplenectomy cardiovascular disease, particularly thrombosis and pulmonary hypertension, have led to reconsideration of the role of splenectomy in the treatment of HS.^{150–152} This shift in opinion is clearly delineated in recent HS management guidelines, where considerations of these risks be discussed between the patient, the family, and health care providers when considering splenectomy. It has been suggested that HS patients with severe hemolytic anemia and those suffering from significant signs or symptoms of anemia, including growth failure, skeletal changes, leg ulcers, and extramedullary hematopoietic tumors, undergo splenectomy.¹⁵³ HS patients with mild HS and well compensated hemolysis may be followed carefully and referred for splenectomy, if clinically indicated. Splenectomy for patients with moderate HS and compensated, asymptomatic anemia is controversial and should be evaluated on a case-by-case basis. Because the risk of OPSI is highest in young children, splenectomy is typically deferred until at least 6 years of age. Prior to splenectomy, patients should receive immunization with pneumococcal, *Hemophilus influenzae*, and meningococcal vaccines.

Supportive Care

Folic acid is recommended to sustain erythropoiesis and prevent megaloblastic changes in patients with moderate to severe HS and those with inadequate dietary folate intake. Serial ultrasonography to detect cholelithiasis is recommended, beginning in childhood, permitting diagnosis and treatment and preventing biliary tract disease.

Care of patients who have undergone splenectomy includes counseling to seek prompt medical attention during febrile

illness.¹⁵⁴ The use of prophylactic antibiotics postsplenectomy is controversial and data are lacking to make specific recommendations. To illustrate the variations in practice, some caregivers always prescribe prophylactic antibiotics postsplenectomy to adults and children; some prescribe them only to children up to 6 years of age; and some, citing both the lack of data and concerns about the emergence of penicillin-resistant pneumococci, never prescribe them.¹⁵⁵

Several reports have described catastrophic thrombotic events after splenectomy for HS. Some of these patients have actually suffered from stomatocytosis, a condition where postsplenectomy thrombosis risk is well known. There are no studies evaluating prophylactic anticoagulant use in splenectomized HS patients.

Hereditary Elliptocytosis, Hereditary Pyropoikilocytosis, and Related Disorders

The hereditary elliptocytosis (HE) syndromes are a group of heterogeneous disorders with the common finding of elliptical or cigar-shaped erythrocytes on the peripheral blood smear.^{156,157} The HE syndromes occur in all races worldwide, but they are particularly common in people of African and Mediterranean ancestry, presumably because elliptocytes confer some resistance to malaria.¹⁵⁸ World-wide, HE is estimated to occur in 1:2,000–4,000 individuals. The true incidence of HE is unknown because most patients are asymptomatic. In parts of Africa, the incidence of HE approaches 1 in 100. Inheritance is autosomal dominant. Rare cases of nondominant inheritance associated with de novo mutation have been described.

Clinical Syndromes

The elliptocytosis syndromes have been classified into several subtypes.

Silent carriers are clinically and hematologically normal. Their erythrocytes demonstrate subtle defects of the membrane skeleton.

Common hereditary elliptocytosis is (by nomenclature) the most frequent type of HE. Most patients are asymptomatic, coming to attention only because of elliptocytosis. Transient hemolytic anemia may be seen in infancy, but it evolves into typical, asymptomatic HE throughout childhood.

Hemolytic hereditary elliptocytosis is an uncommon manifestation of HE, associated with life-long hemolysis. This usually implies the inheritance of an unusually severe spectrin mutation or coinheritance of a modifier allele that alters membrane protein expression. Homozygous or compound heterozygous HE are also often associated with hemolytic HE.

Hereditary pyropoikilocytosis (HPP), the most severe of the elliptocytosis syndromes, is a rare cause of severe hemolytic anemia with RBC morphology reminiscent of that seen in patients with extensive thermal burns. This morphology leads to studies of the RBCs from these patients, who

have demonstrated abnormal thermal sensitivity compared to normal RBCs.¹⁵⁹ Many HPP patients experience severe hemolysis and anemia in infancy that gradually improves and evolves toward hemolytic or typical HE later in life. HE and HPP often coexist in the same family. Identical molecular defects are often found in HE/HPP kindreds. Unlike HE patients, HPP RBCs are also partially deficient in spectrin.

Spherocytic elliptocytosis is typically only in Caucasians. This dominantly inherited disorder is associated with mild-to-moderate hemolysis, splenomegaly, and RBCs that range in shape from spherocytes to elliptocytes.

Southeast Asian ovalocytosis (SAO) is characterized by the presence of oval erythrocytes with a central longitudinal slit (or transverse bar) on peripheral blood smears of affected individuals. Patients are asymptomatic, with little or no hemolysis. SAO is common in parts of the Philippines, Indonesia, Malaysia, and New Guinea. Remarkably rigid, SAO erythrocytes are resistant to invasion by malarial parasites. The underlying defect is a mutation in a critical region of band 3.

Pathophysiology

Weakening of membrane skeleton protein interactions by mutations altering protein structure, function, or amount leads to diminished membrane mechanical stability and ultimately to hemolysis in severe cases. Mutant RBC membrane proteins associated with HE/HPP include α spectrin, β spectrin, protein 4.1, and glycophorin C (Table 37.2). α - and β -Spectrin mutations near the $\alpha\beta$ spectrin heterodimer self-association region impair spectrin tetramer formation, while protein 4.1 or glycophorin deficiency affect spectrin-actin-protein 4.1 junctional complex formation and membrane attachment. The majority of defects occur in spectrin, the principal structural protein of the membrane skeleton, which provides strength and deformability to the RBC membrane. Numerous mutations in the genes encoding these proteins have been reported, with several mutations found in a number of individuals with the same genetic background, suggesting a “founder” effect for these mutations.¹⁴⁶

Elliptocytes form as the RBC ages, as immature HE erythrocytes do not exhibit any morphologic abnormalities. Acquisition of the elliptocytic shape is thought to be the result of the repeated episodes of elliptocytic deformation that all RBCs undergo during each circulatory cycle. Normal RBCs regain a discocytic configuration by a process of elastic recoil; whereas, HE RBCs become locked into an elliptocytic configuration by disruption of the normally abundant interconnections between the membrane proteins. This elliptocytic shape, per se, does not shorten the life-span of the RBC.

History and Physical

Clinical findings in HE are heterogeneous, ranging from asymptomatic carriers to patients with severe, transfusion-dependent hemolytic anemia. However, the vast majority of HE

patients are asymptomatic and are diagnosed incidentally during testing for unrelated conditions.

Approximately ~10% of patients with HE experience hemolytic anemia, splenomegaly, and intermittent jaundice. Many of these symptomatic patients have parents with typical, asymptomatic HE, and thus are homozygotes or compound heterozygotes for defects inherited from each of the parents. Interestingly, symptomatology may vary between members of the same family; indeed, it may vary in the same individual at different times. To partially explain these findings, modifier alleles of spectrin have been hypothesized to influence spectrin protein expression and clinical severity. One such common modifier allele is the α^{LELY} (low expression Lyon) allele, which has been identified and extensively characterized.¹⁶⁰

Laboratory Testing

Typical laboratory findings in HE include elliptocytes on the peripheral blood smear without anemia or reticulocytosis.

Peripheral Blood Smear

Elliptocytes of varying number, from a few to 100%, are seen on peripheral blood smears of HE patients. In cases of hemolytic HE and HPP, RBC morphology may include anisocytosis, poikilocytosis, and spherocytosis. Microspherocytosis is particularly prominent on smears from HPP patients.

Blood Count and Erythrocyte Indices

Blood counts and RBC indices in typical HE are normal. In the hemolytic HE and HPP syndromes, there are variable degrees of anemia and reticulocytosis, with most patients exhibiting mild to moderate anemia. The MCHC may also be increased. In HPP, the striking microspherocytosis is manifest in a low MCV, as low as 50 fl in some cases.

Osmotic Fragility

RBC osmotic fragility is normal in patients with typical HE. It is increased in samples from patients with hemolytic HE and HPP.

Molecular Studies

Qualitative and quantitative analyses of RBC membrane proteins via sodium dodecyl sulfate–polyacrylamide gel electrophoresis, tryptic digestion of spectrin followed by gel electrophoresis, and spectrin self-association studies may be highly informative in cases of HE and HPP. However, these tests are expensive and time consuming to perform and are not currently commercially available.

Similarly, detection of the causative mutation, either utilizing mutation screening tools or direct DNA sequence analyses, is cumbersome, expensive, and not readily available. These studies may be performed in specialized laboratories, and are likely to be beneficial only in unusual or diagnostically challenging cases. Prenatal diagnosis has been performed by

direct examination of the membrane proteins of fetal RBCs. If the specific mutation(s) present in the family are known, prenatal diagnosis by analysis of fetal DNA is also feasible.

Markers of Hemolysis

In hemolytic HE and HPP, laboratory manifestations of ongoing hemolysis, reticulocytosis, hyperbilirubinemia, increased lactate dehydrogenase, decreased haptoglobin, and increased urinary and fecal urobilinogen may be found.

Differential Diagnosis

Elliptocytes are also seen on the peripheral blood smear in cases of megaloblastic anemia, thalassemia, iron deficiency anemia, myelodysplastic syndromes, and myelofibrosis. Elliptocytes typically make up less than a quarter of RBCs in these conditions. History and additional laboratory studies help differentiate and clarify the diagnosis of these disorders.

Treatment and Outcome

Specific Therapy

Splenectomy has been curative in cases of symptomatic hemolytic HE and HPP. Indications for splenectomy are unclear, but consideration of the same factors involved in the decision for splenectomy in cases of HS seems reasonable.

Supportive Care

Folic acid is recommended to sustain erythropoiesis and prevent megaloblastic changes in patients with severe hemolytic HE and HPP. Serial ultrasonography to detect cholelithiasis is recommended.

Complications and Outcome

Complications are rarely associated with typical HE. Hemolytic forms, like HS, may be complicated by cholelithiasis, gallbladder disease, and the occurrence of viral-related aplastic and hemolytic crises.

Hereditary Stomatocytosis Syndromes

The hereditary stomatocytosis (HSt) syndromes are a heterogeneous group of inherited disorders characterized by RBCs with a mouth-shaped (stoma) area of central pallor on peripheral blood smear.^{161,162} RBC membranes of HSt patients usually exhibit abnormal permeability to the univalent cations sodium and potassium, with resultant modification of intracellular water content and volume, which may be either increased (hydrocytosis) or decreased (xerocytosis), or in some cases, near normal. The pathobiology of the stomatocytic shape is poorly understood and the molecular basis(es) of this group of disorders, with exception of a few cases linked to band 3 (Table 37.2), is unknown.¹⁴⁶

Xerocytosis

The dehydrated stomatocytosis syndromes, also known as xerocytosis or dessiccation, are characterized by contracted and spiculated RBCs, variable numbers of stomatocytes, and target cells on the peripheral blood smear. Anemia is minimal or absent. Splenomegaly, cholelithiasis, and other markers of chronic hemolysis are rare. Inheritance is autosomal dominant.

Laboratory studies reveal RBC dehydration, as evidenced by high MCHC or *decreased* osmotic fragility. The molecular basis of xerocytosis is unknown, with one locus mapped to 16q23–q24.¹⁶³ Isolated pseudohyperkalemia is allelic to xerocytosis and also shows linkage to this region.¹⁶⁴ Treatment is rarely required. Splenectomy is of little or no benefit, and in some cases may be deleterious, predisposing to life-threatening thrombotic episodes.

Hydrocytosis (Stomatocytosis)

The overhydrated stomatocytosis syndromes, also known as hydrocytosis, are marked by the presence of swollen, stomatocytic RBCs on the peripheral blood smear. Patients suffer from mild-to-moderate hemolytic anemia, often accompanied by splenomegaly, jaundice, and other complications of hemolysis. Some affected individuals develop evidence of iron overload, even in the absence of blood transfusions. Inheritance is autosomal dominant.

Laboratory studies reveal RBC overhydration, as evidenced by low MCHC, very high MCV (often >120 fl), and increased osmotic fragility. Hydrocytes are low in K⁺ and high in Na⁺, with total monovalent cation content (Na⁺ and K⁺) greater than that of normal RBCs. Like xerocytosis, the molecular basis of hydrocytosis is unknown. RBCs from many patients lack stomatin, an integral red cell membrane protein also present in lipid rafts, although mutations have not been found in stomatin cDNA from hydrocytosis patients. Splenectomy may lessen hemolysis and ameliorate anemia; however, it has been associated unusually with a high risk of catastrophic thrombotic complications and chronic pulmonary hypertension.

Intermediate Syndromes

Hydrocytosis and xerocytosis represent the extremes of a spectrum of RBC permeability defects. Patients with features of both conditions have been reported with variable severity of permeability defects, stomatin deficiency, hemolysis, anemia, and numbers of stomatocytes. These patients have been categorized as “intermediate” syndromes. These observations suggest that hereditary stomatocytosis is a complex collection of syndromes caused by various molecular defects.

Other disorders associated with stomatocytosis include Rh deficiency syndrome, familial deficiency of high-density lipoproteins, sitosterolemia, and several acquired conditions, including alcoholism, cancer, cardiovascular and hepatobiliary disease, and therapy with drugs.

Acanthocytosis

Acanthocytes are dense, contracted erythrocytes with numerous irregular “thorny” projections. Acanthocytes differ from echinocytes, in which there are fewer projections, and the width and length of these projections vary considerably. No central pallor is evident. By contrast, echinocytic spicules are similar in dimension and evenly distributed around the cell periphery.

Acanthocytes on the peripheral blood smear are found in several inherited disorders, including abetalipoproteinemia, the McLeod phenotype, and neuroacanthocytosis. They are also found in acquired disorders, including spur cell anemia of liver disease, starvation, anorexia nervosa, and hypothyroidism, and postsplenectomy.

Abetalipoproteinemia

Abetalipoproteinemia is a rare, autosomal recessive disorder characterized by malabsorption of fat, hypolipidemia, retinitis pigmentosa, acanthocytosis, and progressive ataxia due to the inability to produce or secrete the B apoproteins (i.e., B100 and B48), or defects in the microsomal triglyceride transfer protein (MTTP), required for the production of apoprotein B-containing beta-lipoproteins (Table 37.2).^{165,166} Intestinal absorption of lipids is abnormal; cholesterol levels are extremely low; and serum beta lipoprotein is absent. The serum appears transparent. Acanthocyte formation likely reflects an increase in the surface area of the outer lipid bilayer relative to the inner bilayer, due to increased sphingomyelin in the outer bilayer, mirroring the altered plasma lipid profile ascribed to lipid exchange.

Clinical manifestations are variable, reflecting the degree of perturbation of apolipoprotein B-mediated metabolic processes. Symptoms of steatorrhea and failure to thrive develop in infancy, with retinitis pigmentosa and progressive ataxia appearing between 5 and 10 years of age. Neurologic symptoms progress without treatment, leading to death during the second or third decade of life. Heart failure and arrhythmias may precede death. Many of the clinical abnormalities have been attributed to the deficiency of fat-soluble vitamins, especially vitamin E.

McLeod Phenotype

The McLeod phenotype, named after the first patient described, is an X-linked abnormality of the Kell blood group system.¹⁶⁷ Affected patient's RBCs, leukocytes, or both react poorly with Kell antisera, due to a mutation in XK, a membrane protein linked to the Kell protein and required for Kell antigen expression. Thus, McLeod erythrocytes lack both XK and Kell proteins. Contiguous gene deletions of the X chromosome, including the XK gene, have led to patients with coinherited McLeod phenotype, chronic granulomatous disease, Duchenne's muscular dystrophy, and/or retinitis pigmentosa.¹⁶⁸

Findings in males include acanthocytosis on the peripheral blood smear with a well-compensated, mild hemolytic anemia and susceptibility to alloimmunization by Kell antigens. Because of the susceptibility to alloimmunization, it is important to diagnose affected patients, since they are transfused, they may develop antibodies compatible only with McLeod RBCs. Elevated serum creatine phosphokinase levels are found often associated with symptoms of myopathy and peripheral neuropathy. Central nervous system abnormalities develop after the fourth decade of life. Females have only occasional acanthocytes on the peripheral blood smear and minimal to no hemolysis.

Neuroacanthocytosis Syndromes

The neuroacanthocytoses are a heterogeneous group of degenerative neurologic disorders with variable numbers of acanthocytes on the peripheral blood smear.¹⁶⁹ These disorders include the McLeod syndrome (described earlier), the chorea-acanthocytosis (ChAc) syndrome, and other neurodegenerative disorders, including a Huntington's disease variant (i.e., Huntington's Disease Like 2-HDL2) and pantothenate kinase-associated neurodegeneration (also known as Hallervorden-Spatz disease) as well as its allelic variant HARP (Hypobetalipoproteinemia, Acanthocytosis, Retinitis pigmentosa, Pallidal degeneration) syndrome. ChAc has been associated with mutations in the chorein gene (also known as CHAC or VPS13A-vacuolar protein sorting 13 homolog A), a gene of unknown function in man. HDL2 has been associated with mutations in the junctophilin-3 gene. Pantothenate kinase-associated neurodegeneration has been associated with mutations in the pantothenate kinase 2 (PANK2) gene. The etiology of acanthocytosis in these disorders is unknown. Investigation of the abnormalities in acanthocytic RBCs may provide valuable insights into the associated neurodegenerative processes.

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White Blood Cell and Immunodeficiency Disorders

John F. Bastian and Michelle Hernandez

Introduction

The human immune system comprises cellular and humoral elements that sense danger, and respond to threat in both non-specific and antigen-specific ways that eliminate the offending agents and restore homeostasis. The innate immune system refers to those elements that recognize danger and activate the immune response. The cellular components of innate immunity include monocytes, dendritic cells, macrophages, mast cells, neutrophils, natural killer (NK) cells, and eosinophils. The humoral components include the complement proteins, C-reactive protein, and mannose lectin binding protein. In addition to activation of the immune system via cytokines and elaboration of effector molecules (such as interferons), some of the cells of the innate system process foreign antigen and present it to the adaptive immune system. The adaptive immune system comprises the T and B cell compartments. Cytotoxic T cells kill infected cells; helper T cells provide support for the production of antibody by B cells; and regulatory T cells moderate the adaptive responses and prevent the emergence of autoimmunity. Primary immunodeficiencies derive from mutations in the genes involved in this elaborate host response. These mutations may occur at any phase of the immune response (i.e., from danger recognition to synthesis of high affinity antibody). The molecular consequences of these mutations lead to undue susceptibility to infection, autoimmunity, and, in some instances, malignancy.

The pattern of inheritance of primary immunodeficiencies may be autosomal recessive, autosomal dominant, and X-linked. The X-linked inheritance predominates in several of the immunodeficiencies, and thus males are more often affected than females. In some disorders, heterozygosity with one affected and one normal gene results in the immunodeficiency. This defect may result from a dominant negative effect, as has been documented in transmembrane activator and CAML interactor (TACI) deficiency, that results in common variable immunodeficiency.¹ Haploinsufficiency may also result in immunodeficiency, as is seen in the 22q11

deletion syndrome, where a deletion on one chromosome of a pair results in the DiGeorge Syndrome.²

For most primary immune disorders, there are several genotypes that lead to the same clinical phenotype. For example, in chronic granulomatous disease, there are three autosomal recessive genes and one X-linked gene that result in the phenotype.³ Severe combined immunodeficiency (SCID) has at least 10 genes, in which mutations may lead to the same clinical presentation.⁴ Conversely, there are some disorders in which mutations in the same gene have different forms of clinical expression. In the recombinase Rag1 and Rag2 deficiency, there is loss of B and T cells due to the inability to recombine the T cell receptor and B cell antibody genes. With some mutations, this disorder presents as SCID, but in others it presents as Omenn's syndrome.⁵ Thus, the molecular basis of these disorders have not only scientific significance, but also clinical and genetic relevance for patients and their families. While a review of more than 150 primary immune disorders are beyond the scope of this chapter, the disorders reviewed here will illustrate the molecular mechanisms inherent to these maladies.⁶ The discussed disorders are summarized in Table 38.1.

Severe Combined Immunodeficiency (Also See Chap. 2)

SCID denotes those disorders where there exists a profound deficiency of B, T and NK cells. These patients have essentially no adaptive immune function and succumb early in life to infection. SCID phenotype may be classified by the presence or absence of T cells, B cells, and NK cells (see Table 38.2). There are several molecular means whereby SCID occurs (Figure 38.1). In adenosine deaminase deficiency, absence of this purine catabolic pathway protein leads to profound lymphopenia. The mechanism appears to be toxicity of the ADA substrate 2,deoxyadenosine. Accumulation of this substrate results in the accumulation of deoxyATP which is toxic for

TABLE 38.1. Summary of immunodeficiency disorders: cellular/molecular mechanism, laboratory findings, and clinical features.

Disorder	Cellular/molecular mechanism	Laboratory findings	Clinical features
Agammaglobulinemia	Arrested B cell development	Very decreased immunoglobulins; absent B cells	SBI; malignancy
Hyper IgM syndrome	Failure of immunoglobulin class switch	Elevated or normal IgM decreased IgG and IgA	SBI; OI
Common variable immunodeficiency	Defects in B cell maturation and isotype switching	Decreased immunoglobulin levels; T Cell dysfunction	SBI; OI; autoimmunity; malignancy
Undue susceptibility to mycobacteria	Defects in the cytokines and receptors of the IL-12/gamma interferon axis	Greatly reduced in vitro production of TNF alpha in response to LPS and γ -IFN	Mycobacterial infections; infections with intracellular organisms; severe/recurrent viral infections
Autoimmune polyglandular syndrome type 1	Failed expression of tissue specific self proteins during thymic development	Presence of multiple autoantibodies	Vitiligo, type I diabetes, alopecia, hypothyroidism, and premature ovarian failure
Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance syndrome	Defect in the gene for FOXP3, transcription factor that functions as a transcriptional suppressor	Lack of CD4+CD25+ regulatory T Cells	Type I diabetes, autoimmune enteropathy
Neonatal onset multi-system inflammatory disease	Increased release of IL-1 beta due to increased inflammasome activity	Mutations in the gene CIAS1 encoding the protein cryopyrin	Neonatal autoimmune type I diabetes, autoimmune enteropathy, eczema, food allergy, autoimmune hemolytic anemia, and severe infections
X-linked lymphoproliferative disease	Impaired development of NKT cells	Overwhelming EBV infection	Fatal EBV infection; hypogammaglobulinemia; lymphoma
Chronic granulomatous disease	Defects in NADPH oxidase with failure to generate superoxide	Absent or greatly reduced oxidation of rhodamine by activated neutrophils	SBI; colitis
Leukocyte adhesion defect-type I	Lack of adhesion molecules on leukocytes prevents binding to endothelium and diapedesis	Absence of CD18 on leukocytes	Recurrent bacterial infections
Primary herpes encephalitis	Failure to produce type I interferons in the central nervous system	Failure of toll-like receptors ligands to induce Type I interferon	Primary herpes encephalitis

SBI serious bacterial infections, OI opportunistic infections, LPS lipopolysacchride, γ -IFN gamma interferon, EBV Epstein–Barr virus.

TABLE 38.2. Summary of genes involved in immunodeficiency disorders, inheritance pattern, and resulting absence (–) or presence (+) of lymphoid cells.

Gene	Inheritance	T cells	B cells	NK cells
Adenosine deaminase	AR	–	–	+
Common γ chain	X-linked	–	+	–
Jak 3 kinase	AR	–	+	–
CD 45	AR	–	+	–
IL-7 receptor α -chain	AR	–	+	+
CD3 δ chain	AR	–	+	+
CD3 ζ chain	AR	–	+	+
CD3 ϵ chain	AR	–	+	+
RAG-1	AR	–	–	+
RAG-2	AR	–	–	+
Artemis	AR	–	–	+

B, T, and NK cells (B–, T– NK– SCID). In thymocytes, there is evidence that apoptosis mediates this toxicity.⁷ In X-linked SCID, there is absence of a protein common to several interleukin receptors that are involved in transmembrane signaling [the common gamma (γ) chain].⁸ In this form of SCID, the deficiency is likely due to loss of function of the IL-7 receptor. IL-7 is an interleukin expressed by thymus epithelial cells and is essential for thymocyte development. Unlike ADA deficiency, this defect affects T cells and NK cells, so B cells are present in these patients (i.e., T–, B+, NK– SCID). The lack of NK cells is likely due to loss of function of

the IL-15 receptor.^{9,10} Jak3 is a kinase associated with the common γ chain and, as expected, its deficiency results in the same phenotype. In recombinae deficiency, as noted earlier, the recombination of variable region genes responsible for the generation of antibody and T cell receptors fails to occur. This failure arrests B and T cell development and results in T–, B–, and NK+ SCID. NK cells do not possess antigen receptors with variable regions, and thus their development is not affected. CD 3 is an essential protein component of the T cell receptor complex. Deficiencies of its component chains result in T cell maturation arrests (i.e., T–, B+, NK+ SCID).¹¹

Antibody Deficiency

The Agammaglobulinemias

Individuals with defects in their humoral immune system commonly present with recurrent and sometimes severe bacterial infections. Genetic mutations affecting antibody production have been identified in various stages of B cell development and maturation. During their development in the bone marrow (BM), cells that are destined to become B lymphocytes (pro-B cells) undergo discrete stages of gene expression of various surface proteins. Once pro-B cells have completed recombination of the immunoglobulin mu (μ) heavy chain locus, some of these μ heavy chains associate

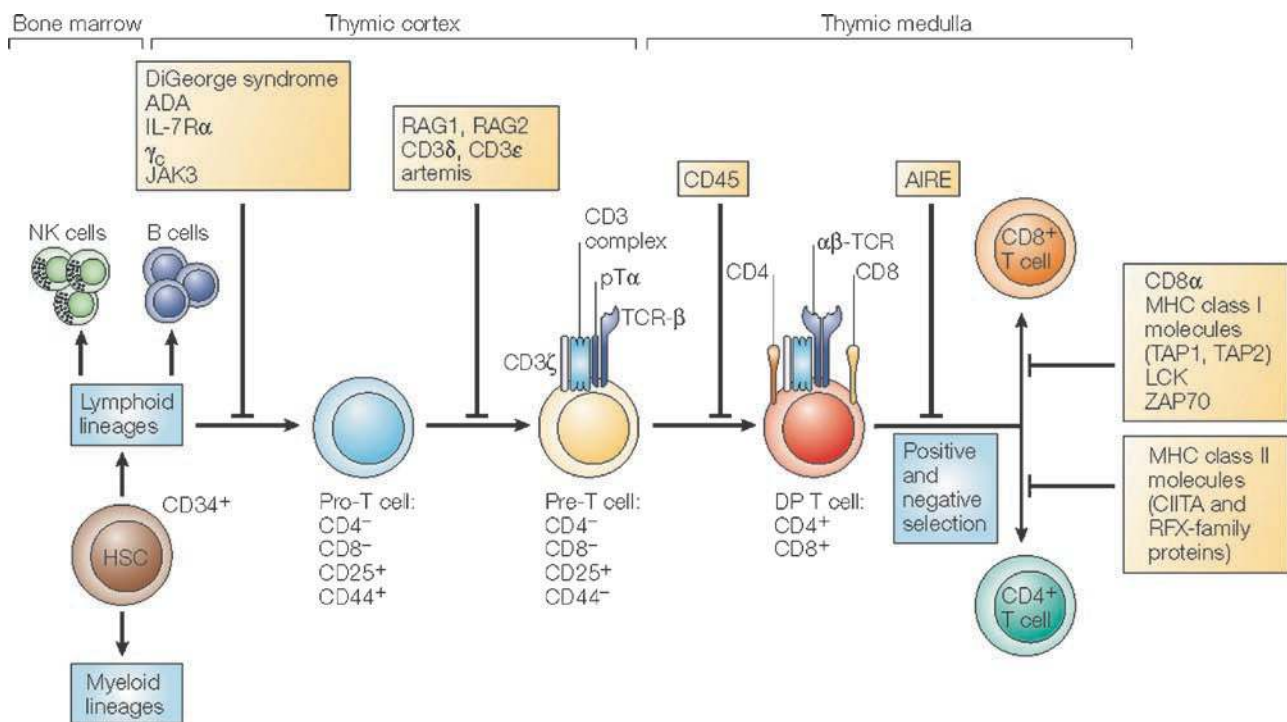


FIG. 38.1. Protein and gene defects in T-cell development. Haematopoietic stem cell (HSC)-derived lymphoid progenitor cells migrate from the bone marrow to the thymus and develop into progenitor (pro)-T cells, which then rearrange their T-cell receptor (TCR) genes and differentiate into either $\gamma\delta$ or $\alpha\beta$ T cells in the cortex. The latter initially co-express CD8 and CD4, which interact with MHC class I and class II molecules, respectively, at the surface of medullary thymic stromal cells. This interaction allows T cells to be “educated” regarding self-antigens and non-self-antigens

(enabling the positive or negative selection of T cells in the thymus) before their migration to the periphery, where they exclusively express CD4 or CD8. Numerous defects in maturation have been elucidated. Defects in the genes encoding the molecules listed in the *yellow boxes* (and the primary immunodeficiency diseases listed) are known to affect the developmental steps indicated. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology, November 2005 (doi: 10.1038/sj.Nat Rev Immunol).

with proteins, called surrogate light chains. Complexes of these μ heavy chains and surrogate light chains are called pre-B cell receptors, and are expressed on the cell surface (Figure 38.2). The pre-B cell receptor associates with surface proteins, called Ig α (Ig alpha) and Ig β (Ig beta), that transduce signals from the receptor. Although it is unknown exactly what stimulates the pre-B cell receptor or what precise signal is transduced, activation of the pre-B cell receptor is required to stimulate the proliferation and continued maturation of developing B cells. Once the pre-B cell progresses into the immature-B cell stage of its development, it expresses low levels of membrane IgM (μ , mu heavy chain and a κ , kappa or λ , lambda light chain). After the immature B cell exits hematopoietic tissues and reaches the periphery, it has undergone alternative splicing to express both IgM and IgD on the cell surface.¹²

The agammaglobulinemias are caused by a variety of genetic mutations that result in the arrest of B cells at the pre-B cell stage and, in essence, produce identical phenotypes (Figure 38.2). Patients with agammaglobulinemias present in infancy or in childhood with recurrent sinopulmonary pyogenic infections, enteroviral meningoencephalitis, and/or

mycoplasma, or ureaplasma, arthritis.¹³ These patients have reduced or absent B cells in lymphoid tissues and peripheral blood, no germinal centers in lymph nodes, and consequently low or absent serum immunoglobulins.

X-linked agammaglobulinemia (XLA) accounts for 85% of all agammaglobulinemias. Also known as Bruton's agammaglobulinemia, it is a disease characterized by failure of B cell maturation.¹⁴ Boys or lyonized females with this condition have a defect in a kinase, called B cell tyrosine kinase (BTK).¹⁵ BTK is associated with the pre-B cell receptor and is required for transducing signals from the pre-B cell receptor downstream, which stimulates B cell maturation. Autosomal recessive mutations that block B cell development at the precell stage have been identified as well. The most common autosomal recessive form of agammaglobulinemia is due to IgM heavy chain mutations, which prevent surface expression of IgM.¹⁶ Mutations in the surrogate late chain ($\lambda 5/14.1$)¹⁷ and in Ig α have also been identified.¹⁸ More recently, mutations in more downstream signaling components of the pre-B cell receptor have also been discovered. B cell linker protein (BLNK) is an adaptor protein that connects several cytoplasmic molecules, and is critical for

mobilization of intracellular calcium stores.¹⁹ In its absence, signaling through the pre-B cell receptor cannot occur. One female with a heterozygous mutation in Leucine-rich repeat containing 8 (LRRC8) has been identified with an XLA phenotype.²⁰ The function of this protein in pre-B cell signaling is still unknown.

Hyper-IGM Syndrome (Elevated IGM, Decreased IGG, A, and E) (Also See Chap. 2)

Once Naïve B cells expressing mature IgM and IgD recognize antigens in the peripheral lymphoid tissues, antigen binding to the membrane IgM and IgD receptors activates these B cells and leads to B cell proliferation. During this activation process, B cells undergo antibody class switching (to make IgG, IgE, and IgA)²¹ and somatic hypermutation (Figure 38.2).²² Heavy chain isotype switching and somatic hypermutation are stimulated by helper T cell signals. After a B cell recognizes its specific antigen through the IgM or IgD receptor, it endocytoses the antigen–antibody complex and processes and presents it as a peptide fragment on MHC class II molecules. It presents this antigen to a helper T cell, which along with the expression of

costimulators (such as the CD80/86-CD28 complex) induce the T cell to express CD40 ligand and secrete cytokines. The CD40 ligand on T cells then binds to CD40 on the B cell surface and initiates B cell proliferation and differentiation.

Defects in B cell isotype switching collectively lead to a group of disorders called the Hyper-IgM (HIGM) syndromes. These patients have normal numbers of B cells, but express elevated levels of IgM with low IgG, IgE, and IgA levels. HIGM I is caused by a defect in CD40 ligand on T cells, is X-linked, and is the most common defect.²³ HIGM 3 is due to autosomal recessive mutations in CD40 on B cells.²⁴ Autosomal recessive mutations have also been identified in the DNA modifying enzymes, Activation Induced Cytidine Deaminase (AID) and Uracil Nucleotide Glycosylase,^{25,26} both of which are required for normal immunoglobulin class switching and somatic hypermutation.

Common Variable Immunodeficiency (Also See Chap. 2)

The above-mentioned antibody deficiencies typically present in childhood because of their severe phenotypes.

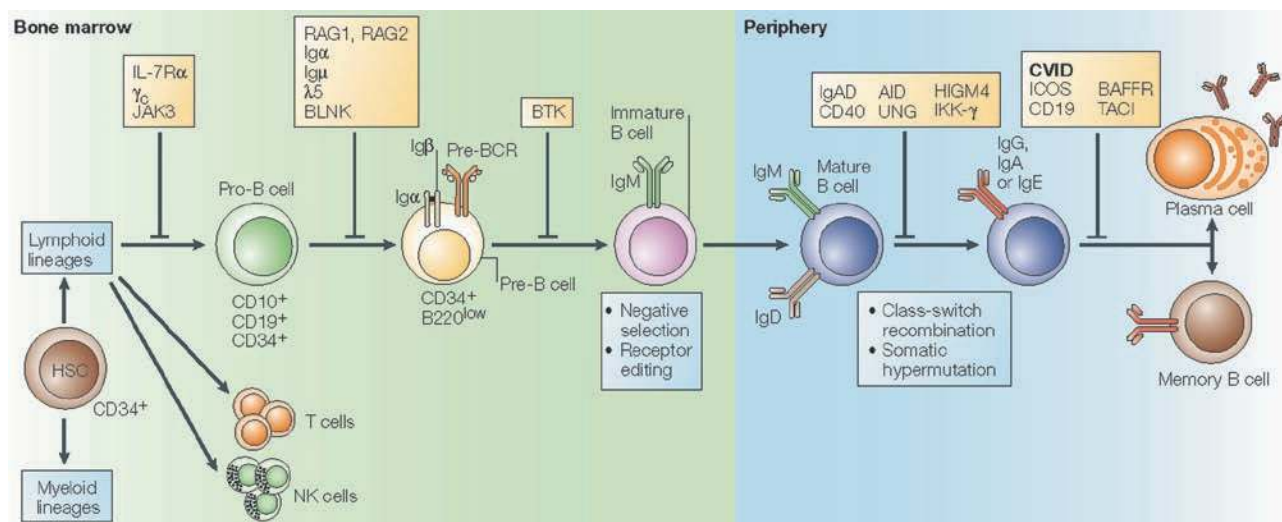


FIG. 38.2. Protein and gene defects in B-cell development and function. Haematopoietic stem cells (HSCs) give rise to progenitor (pro)-B cells, which then rearrange their immunoglobulin heavy-chain gene segments to generate precursor (pre)-B cells. Pre-B cells subsequently rearrange their immunoglobulin light-chain gene segments to produce a functional cell-surface receptor (IgM). This protein is composed of heavy and light chains that are derived from these gene rearrangements, and it functions as a receptor for responding to stimulation with antigen, resulting in the induction of proliferation and differentiation of the B cell. In the periphery, after stimulation with antigen, mature B cells further develop following class-switch recombination and somatic hypermutation and, ultimately, differentiate into memory B cells or plasma cells. Developmental blocks throughout B-cell maturation and differentiation occur as a result of defects in genes encoding the molecules listed in the yellow boxes.

Blocks in the function of mature B cells can also occur. Primary immunodeficiency syndromes that cause these blocks are also listed. *AID* activation-induced cytidine deaminase, *BAFFR* B-cell-activating-factor receptor, *BCR* B-cell receptor, *BLNK* B-cell linker, *BTK* Bruton's tyrosine kinase, γ c common cytokine-receptor γ -chain, *CVID* common variable immunodeficiency, *HIGM4* hyper-IgM syndrome 4, *ICOS* inducible T-cell co-stimulator, *IgAD* selective IgA deficiency, *Igμ* μ immunoglobulin heavy chain, *IKK-γ* inhibitor-of-nuclear-factor- κ B kinase- γ , *IL-7Rα* interleukin-7 receptor α -chain, *JAK3* Janus kinase 3, *NK cell* natural killer cell, *RAG* recombination-activating gene, *TACI* transmembrane activator and calcium-modulating cyclophilin-ligand interactor, *UNG* uracil-DNA glycosylase. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology, November 2005 (doi: 10.1038/sj.Nat Rev Immunol).

Common variable immunodeficiency (CVID), the second most frequent primary immunodeficiency in humans after selective IgA deficiency, is the most prevalent primary immunodeficiency in adults. The phenotype of CVID is heterogeneous, as it denotes a syndrome which comprises a number of different diseases. Unlike the agammaglobulinemias, the causes of CVID have remained elusive, with the genetic basis identified in less than a quarter of cases. Affected individuals have a history of recurrent infections or a life threatening infection, due to hypogammaglobulinemia. They may also suffer from granulomatous diseases, autoimmune disorders, splenomegaly, and certain malignancies.²⁷ In addition to reduced serum immunoglobulin levels of IgG, IgA and/or IgM, patients also have absent or impaired specific antibody responses to previous infection or vaccination.

While the vast majority of CVID cases are sporadic, kindreds do account for approximately 10% of patients. These kindreds usually demonstrate an autosomal dominant pattern of inheritance.²⁸ However, autosomal recessive inheritance of CVID has been noted in families with a homozygous deletion of the inducible costimulator (ICOS), normally present on activated T-cells (Figure 38.2). The interaction of the ICOS molecule with its ligand appears to promote the differentiation of naïve B cells into memory B cells and plasma cells. Thus far, the incidence of ICOS deficiency among patients with CVID is less than 5%, with a total of nine patients identified worldwide.

Mutations of the transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI, TNFRSF13B), a member of the tumor necrosis factor (TNF)-like receptor family, have recently been identified in some CVID patients. TACI is expressed on peripheral B cells. Its ligands are BAFF (B-cell activating factor) and APRIL (a proliferation-inducing ligand), primarily expressed on monocytes and dendritic cells.²⁹ These interactions and key cytokine signaling have important functions in immunoglobulin isotype switching. Four mutations of TNFRSF13B have been identified in CVID patients.^{30,31} Unlike ICOS, both heterozygous and homozygous patients have been identified, with a predominance of TACI heterozygotes. Phenotype did not correlate with genotype (i.e., heterozygote phenotypes range from unaffected to severe CVID). Based on this recent evidence, it appears that 5–10% of CVID patients carry at least one germ line mutation in TNFRSF13B.

Most recently, van Zelm et al have discovered mutations in the CD19 gene, which lead to CVID in the presence of mature (but dysfunctional) B cells.³² Although polymorphisms have been identified in the BAFF-R of CVID patients, these variants did not affect BAFF-R expression and do not appear to be disease-causing mutations.³³ Patients may also have a phenotype consistent with CVID, but have mutations in genes associated with distinct primary immunodeficiencies. Patients with a clinical CVID phenotype have been reported to actually have mutations in the *btk*, *CD40-L*, and *SH2D1A* genes.^{34–37}

Cellular Deficiencies

Undue Susceptibility to Mycobacteria: IFNGAMMA/IL-12 AXIS

Mononuclear phagocytes are critical in defending the host against infections with both pathogenic and nonpathogenic strains of mycobacteria. After a macrophage is infected with mycobacteria, it produces IL-12, which in turn stimulates NK cells and T cells via the IL-12 receptor on their cell surface. These activated NK and T cells produce the cytokine Interferon- γ (Interferon gamma). After Interferon- γ binds to its receptor on nucleated cells (such as macrophages), a signaling cascade is initiated, resulting in the translocation of the transcription factor, signal transducer and activator of transcription 1 (STAT1), to the nucleus. STAT1 is responsible for transcription of the cytokines TNF- α (TNF alpha) and IL-12, both of which are important in mycobacterial killing. Patients with genetic defects in any component of this communication axis between macrophages and NK or T cells typically present with severe disseminated mycobacterial disease during their childhood years.³⁸

Both autosomal recessive mutations in the IFN-gamma receptor (i.e., reduced receptor expression on the cell surface) and autosomal dominant³⁹ mutations (i.e., accumulation of nonfunctional IFN- γ R1 proteins on the cell surface) lead to various degrees of impaired signal transduction capabilities.⁴⁰ Autosomal recessive mutations in IL-12p40 ligand secreted by macrophages, or in the beta chain of the IL-12 receptor on T cells and NK cells also produce a similar phenotype.^{41,42} Dominant negative mutations in STAT1, which impair the phosphorylation of STAT1 and, in turn, leads to reduced translocation of STAT1 to the nucleus, have also been identified.⁴³

Autoimmunity

The autoimmune monogenic diseases highlight the importance of maintaining tolerance to self-antigens. The three syndromes discussed in this book chapter point to the fact that immune tolerance occurs at multiple levels and at multiple sites.

During their maturation in the thymus, T cells undergo the process of negative selection and positive selection. Less than 5% of cells that entered the thymus actually survive these processes. During negative selection, T cells that react too strongly to self-antigen presented by medullary thymic epithelial cells are eliminated through the induction of apoptosis.¹²

Autoimmune polyglandular syndrome type 1 (APS1) is an autosomal recessive disease characterized by autoimmunity that may classically involve hypoparathyroidism, adrenal insufficiency, and chronic mucocutaneous candidal infections. Affected patients may also exhibit other aspects of organ-specific autoimmunity, such as vitiligo, type I diabetes, alopecia, hypothyroidism, and premature ovarian failure.⁴⁴ Through a

series of elegant mouse experiments, the gene responsible for APS1, called the autoimmune regulator (AIRE), has been found to regulate the transcription of many tissue-specific antigens (TSAs)⁴⁵ that are expressed on medullary thymic epithelial cells. Although the exact mechanism is unknown to date, it appears that AIRE drives the production of many TSAs that are to be presented by medullary thymic epithelial cells, so that autoreactive T cells with affinities for those TSAs may be eliminated (Figure 38.1).^{46,47}

Once T cells have successfully exited the thymus, they may still develop autoreactivity to self-antigens. Regulatory T cells are responsible for maintaining homeostasis and tolerance to self-antigens in the periphery.

The immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance syndrome (IPEX; OMIM 304930) is a lethal disorder that afflicts males in early childhood. Children with IPEX develop neonatal autoimmune type I diabetes, autoimmune enteropathy, eczema, food allergy, autoimmune hemolytic anemia, and severe infections.⁴⁸ The gene responsible for IPEX, FOXP3, encodes a transcription factor that functions as a transcriptional suppressor; it inhibits endogenous cytokine expression promoted by nuclear factor of activated T cells (NFAT) and nuclear factor κ B (nuclear factor kappa B) response elements.⁴⁹ Analyses of the subpopulations of purified lymphocytes have shown that FOXP3 is predominantly expressed in CD4+CD25+ regulatory T lymphocytes.⁵⁰ FOXP3 deficiency leads to a failure of CD4+CD25+ regulatory T cell development (Figure 38.3), resulting in unregulated T-cell activation with consequent TH1 and TH2 cytokine dysregulation.⁵¹ However, as individuals have been identified

with IPEX phenotypes, in which mutations of the coding exons of FOXP3 were not detected,⁵² intense research is focused on pathways that modulate regulatory T cell development and function.

A newly identified group of autoinflammatory syndromes, characterized by recurrent episodes of unprovoked inflammation, appear to lack the participation of autoreactive T lymphocytes or immunoglobulins to self antigens. Instead, these disorders result from aberrant regulation of cytokine signaling pathways, leading to persistent or uncurbed inflammation. They also appear to be driven by phagocytes, such as PMNs and monocytes, that share mutations in a group of proteins which regulate apoptosis, inflammation, and cytokine processing. One of the most extreme autoinflammatory syndromes, called chronic infantile neurologic cutaneous and articular syndrome (CINCA) or neonatal onset multisystem inflammatory disease (NOMID), presents in infancy with nonpruritic, urticarial rashes, sensorineural hearing loss, chronic aseptic meningitis, and epiphyseal long bone ossification with resultant osseous overgrowth.⁵³ Patients with NOMID/CINCA have autosomal dominant mutations in the gene *CIAS1*,⁵⁴ encoding the protein cryopyrin,⁵⁵ a component of the inflammasome complex responsible for processing pro-interleukin 1 to its mature form, IL-1 beta. Cryopyrin plays a role in mediating caspase-1 activation, and is thought to upregulate interleukin-1beta and other cytokines that contribute to the profound inflammation seen in this disorder.⁵⁶ Defects in the regulation of the inflammasome complex are currently being investigated in a variety of inflammatory disorders.

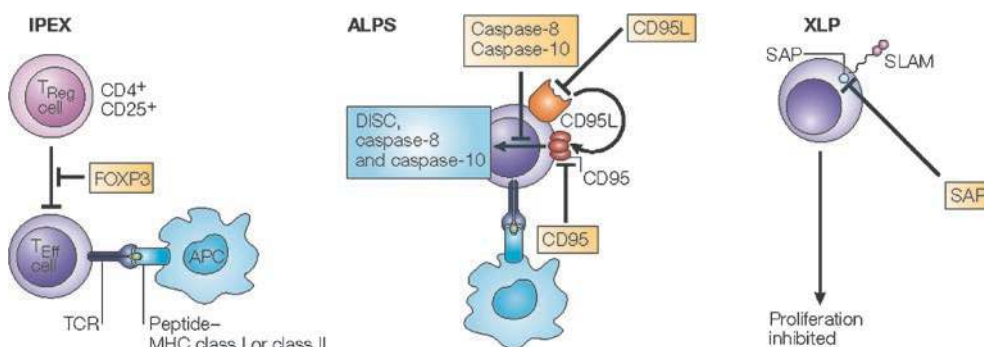


FIG. 38.3. Protein and gene defects in T-cell function after maturation is complete. In patients with IPEX (immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome), self-reactive effector T (Teff) cells are not inhibited, because mutations in the forkhead box P3 gene (FOXP3) result in a loss of CD4+CD25+ regulatory T (TReg)-cell activity. In patients with autoimmune lymphoproliferative syndrome (ALPS), defects in the CD95, CD95 ligand (CD95L), caspase-8 or caspase-10 genes abrogate formation of the death inducing signalling complex (DISC), thereby interfering with apoptosis of Teff cells. In patients with X-linked lymphoproliferative syndrome (XLP), uncontrolled proliferation of T cells occurs as a result of a mutation in SH2D1A,

which encodes SAP (signaling lymphocytic activation molecule (SLAM)-associated protein). ADA adenosine deaminase, AIRE autoimmune regulator, APC antigen-presenting cell, γ common cytokine-receptor γ -chain, *CIITA* MHC class II transactivator, DP double positive, *IL-7R α* interleukin-7 receptor α -chain, *JAK3* Janus kinase 3, *NK cell* natural killer cell, *pre-T cell* precursor-T cell, *pT α* pre-TCR α -chain, *RAG* recombination-activating gene, *RFX* regulatory factor X, *TAP* transporter associated with antigen processing, *ZAP70* ζ -chain-associated protein kinase of 70 kDa. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology, November 2005 (doi: 10.1038/sj.Nat Rev Immunol).

Lymphoproliferative Disorders

Regulation of the immune response is not only necessary in avoiding excessive inflammatory states, like those seen in CINCA, but also in preventing malignant transformation in chronically activated lymphocytes undergoing high rates of proliferation. Activated lymphocytes are susceptible to apoptosis (programmed cell death) via death receptor signaling through the interaction of the surface receptors FAS and FAS-Ligand. Once ligated, these death receptors activate the caspase cascade, causing apoptosis. This is a potent mechanism of cytotoxicity for T cells and NK cells, important in killing virally infected or -transformed cells.

The X-linked lymphoproliferative syndrome (XLP) is a human immunodeficiency, characterized by a dysregulated immune response to infection with Epstein-Barr Virus (EBV). Also refer Chap. 2. Approximately 58% of boys who suffer from this condition present with severe and mostly fatal infectious mononucleosis.⁵⁷ More than half of males who survive into adulthood suffer malignant lymphoma, mostly of B cell origin (the EBV receptor, CD21 is highly expressed in B cells) and about a third from dysgammaglobulinemia. The signaling lymphocytic activation molecule (SLAM) receptor family, which modulates immune cell development, actively couples with the cytoplasmic adaptors SLAM-associated proteins (SAPs) to transduce intracellular signals (Figure 38.3).⁵⁸ Absent SAP function, either through mutations in the gene SH2D1A resulting in absent protein or in functionally inert protein, has been shown to impair the function of various immune cells. As NK cells are particularly important in antiviral and antitumor responses, it is felt that impaired NK cell cytotoxicity is primarily responsible for the compromised ability of patients with SAP mutations to clear EBV infection, and in their propensity to develop B-cell lymphomas after EBV stimulation.^{59–62}

The uncontrolled expansion of potentially harmful self-reactive lymphocytes produces the clinical phenotype in patients with autoimmune lymphoproliferative syndrome (ALPS), who present with chronic nonmalignant lymphadenopathy ± splenomegaly. Also refer Chap. 2. Increased susceptibility to hematological malignancy, particularly lymphoma in approximately 10% of patients, and the development of autoimmune disease are the most worrying clinical phenomena.⁶³ ALPS patients classically suffer from defective antigen-induced apoptosis in cultured activated lymphocytes in vitro. Most have autosomal dominant mutations producing truncated protein or dysfunctional FAS (also known as CD95) on the cell surface (Figure 38.3).^{64–66} However, even kindreds with identical FAS mutations may have varying clinical phenotypes, including asymptomatic states. Others with ALPS have mutations in FAS-ligand (CD95 Ligand) and caspase 8 or 10, producing similar clinical phenotypes.^{67–69} To date, it is unclear whether a second signal, such as another genetic or possibly environmental stimulus, impairs apoptosis signaling in an FAS-independent manner, accounting for the variable penetrance of the FAS mutation.

Phagocyte and Innate Defects

Chronic Granulomatous Disease

In addition to disorders with inadequate production of phagocytes, which will not be discussed in this chapter, various disorders affecting phagocyte function have been identified. The primary function of phagocytes is to eradicate microbes that have been endocytosed in phagocytic vacuoles. When phagocytes are appropriately stimulated, they activate an NADPH oxidase-dependent respiratory burst, resulting in the production of superoxide. It is now thought that production of reactive oxygen species, such as superoxide and hydrogen peroxide, stimulate potassium influx into phagocytic vacuoles, which then facilitates activation of neutrophil elastase and cathepsin G, which are then responsible for eradicating the ingested organism.⁷⁰ The NADPH oxidase complex is composed of the two transmembrane proteins [cytochrome b558 alpha and beta chains (p22phox and gp91phox, respectively)] and the three polypeptides [nuclear cytosolic factor 1 (p47phox), nuclear cytosolic factor 2 (p67phox), and p40phox], and is regulated by two GTPases, Rac2 and Rap1 3.

Defects in any component of the NADPH oxidase complex may produce the phenotype seen in patients with chronic granulomatous disease (CGD), the best understood phagocyte disorder. Patients with this disease suffer from recurrent life-threatening infections with catalase-positive bacteria and fungi (commonly including *S. aureus*, *Nocardia* species, *S. marcescens*, *B. cepacia*, and *Aspergillus* species), and experience tissue granuloma formation, due to failure to degrade inflammatory mediators.⁷¹ Mutations in genes encoding any of the components of the NADPH complex or its regulators render the phagocyte unable to produce superoxide. Approximately 70% of patients with CGD have an x-linked mutation in the gp91phox gene 3. The remaining 30% of patients with CGD have autosomal recessive mutations, most commonly in p47phox; although mutations have been identified in p22phox, p67phox, and Rac2.

Leukocyte Adhesion Deficiency

Of course, before a neutrophil or monocyte can fulfill its destiny of eradicating ingested intracellular organisms, it must first be able to immigrate to the site of infection. As leukocytes travel through blood vessels, they roll along the vessel surface by forming loose associations, via sialo-Lewisx ligands on the neutrophil surface interacting with selectins on the vessel surface. After the appropriate danger signals at a site of trauma or infection are sensed, leukocytes form tighter adhesions with the vessel surface by upregulating the integrins, LFA-1 (CD11a/CD18), complement receptor 3 (CD11b/CD18), and complement receptor 4 (CD11c/CD18), that then interact with intercellular adhesion molecules (ICAMs) on the vessel surface. These tight adhesions allow leukocytes to exit the vessel and enter the tissues.

Leukocyte adhesion defect 1 results from an autosomal recessive mutation in CD18 (HGB2 gene), which is common to the three leukocyte integrin heterodimers.⁷² Patients with this defect generally present with delayed separation of the umbilical cord, as neutrophils are required for this process to occur. These patients have a baseline leukocytosis with a further white blood cell response to infection. However, a biopsy at the site of infection will show few to no neutrophils and rare monocytes. Unfortunately, patients suffer from recurrent necrotizing infections of the skin, perineum, lung, and gut with enteric Gram-negative rods and Gram-positive cocci. If they survive to adolescence, few retain their teeth past this age, because of severe gingivitis, leading to tooth loss and alveolar ridge resorption.

Leukocyte adhesion defect 2 is produced by autosomal recessive mutations in the GDP-fucose transporter, impairing neutrophil rolling via E-selectins. These patients also present with delayed umbilical cord separation, although their global phenotype is slightly different due to the function of fucose in other tissues.^{73,74} More recently, dominant negative mutations in RAC-2, a GTPase that regulates the NADPH oxidase complex, have been identified in two male infants with multiple life threatening infections. In addition to expected deficiencies in the respiratory burst,⁷⁵ it has also been noted that peripheral blood neutrophils are defective in adhesion via impaired capture and rolling via the L-selectin ligand, GlyCAM-1.⁷⁶ This mutation also produces defects in neutrophil primary granules, responsible for microbial killing.⁷⁷

Herpes Virus Encephalitis

Although herpes virus encephalitis is a rare complication of herpes simplex virus type 1 (HSV-1) infection, it is the most common type of sporadic viral encephalitis in western countries (about 1 patient per 250,000 person-years). In the past, the pathogenesis was unclear, as it appeared to affect otherwise healthy patients with no apparent immunodeficiency, or with no increased susceptibility to other forms of HSV-1 infections. However, recently, a group from France noted a high frequency of herpes virus encephalitis (13%) among affected consanguineous families, and hypothesized that it may be inherited as a monogenic trait. This was a novel idea, as susceptibility to specific organisms is being studied in the context of defects in the Toll-like receptor family. As patients with STAT-1 deficiency^{78–81} and with NEMO mutations^{82,83} were found to have predispositions to HSE, due to defective production of type I interferons after viral stimulation, it was felt that other defects impairing type I interferon production would explain the genetic basis for HSE susceptibility. Indeed, through the aid of mouse models, two unrelated patients with HSE from consanguineous families were found to have different autosomal recessive mutations in the same gene, UNC93B1.⁸⁴ As suspected, these patients failed to produce type I interferons after stimulation with stimuli, such as TLR3, TLR7, TLR8, and TLR9 agonists. UNC93b is a transmembrane protein resident

in the endoplasmic reticulum whose function in humans is still unknown. It was recently shown that UNC93b interacts with TLR3, TLR7, and TLR9, and is essential for signaling downstream of these TLRs in mice.⁸⁵

All of the TLRs, except for TLR3, signal through the adaptor protein IL-1R associated kinase 4 (IRAK-4). It had been previously demonstrated that patients with IRAK-4 mutations suffered from recurrent pyogenic infections, yet did not have severe disease with viruses, fungi, or parasites.⁸⁶ This finding suggested that signaling through IRAK-4 independent mechanisms provided adequate antiviral immunity. In light of this, TLR3, which signals through the adaptor TRIF, was found to be deficient in two unrelated patients with HSE.⁸⁷ These patients carried identical heterozygous, dominant negative mutations. Their fibroblasts had diminished interferon production and had increased viral replication after stimulation with a TLR3 agonist or to vesicular stomatitis virus. The fact that patients with TLR3 or UNC93b mutations have not been found to have severe HSV-1 infections in other parts of the body suggests that the importance of this signaling pathway in antiviral immunity is tissue-specific. In any case, these discoveries pave the way for future studies, focusing on upstream defects in TLR signaling and susceptibility to specific organisms.

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Molecular Basis of Disorders of Hemostasis and Thrombosis

Alice Ma

Introduction and Overview of Coagulation

A comprehensive recital of the molecular basis of all of the defects leading to disorders of hemostasis and thrombosis is well beyond the scope of this book. This chapter instead presents broad themes, focusing on defects in the soluble coagulation factors, defects in platelet number and function, other defects leading to hemorrhage, and defects predisposing to thrombosis.

Coagulation may be broken into a series of steps occurring in overlapping sequence. Primary hemostasis refers to the interactions between the platelet and the injured vessel wall, culminating in the formation of a platelet plug. The humoral phase of clotting encompasses a series of enzymatic reactions, resulting in a hemostatic fibrin plug. Finally, fibrinolysis and wound repair occur, to return normal blood flow and vessel integrity. Each of these steps is carefully regulated, and defects in any of the main components or regulatory mechanisms may predispose to either hemorrhage or thrombosis. Depending on the nature of the defect, the hemorrhagic or thrombotic tendency may be either profound or subtle.

Primary hemostasis begins at the site of vascular injury, with platelets adhering to the subendothelium, utilizing interactions between molecules, such as collagen and von Willebrand factor (VWF) in the vessel wall with glycoprotein receptors on the platelet surface. Upon exposure to the cocktail of agonists exposed at a wounded vessel, signal transduction processes lead to platelet activation. Via a process known as inside-out signaling, the integrin $\alpha_{IIb}\beta_3$ (also known as GP IIb/IIIa) undergoes a conformational change to be able to bind fibrinogen, which crosslinks adjacent platelets and leads to platelet aggregation. Secretion of granular contents is also triggered by outside signals, potentiating further platelet activation. Lastly, the surface of the platelet changes to serve as an adequate scaffold for the series of biochemical reactions resulting in thrombin generation.

Following platelet activation, a series of enzymatic reactions take place on phospholipid surfaces, culminating in

the formation of a stable fibrin clot. Several models have attempted to make sense of these reactions. The waterfall model was developed by two groups nearly simultaneously^{1,2} and explained the extrinsic, intrinsic, and common pathways leading to fibrin formation. In this model, the intrinsic pathway is initiated by contact factors (prekallikrein (PK), high molecular weight kininogen (HMWK), and factor XII) which activate factor XI. Factor XIa activates factor IX to IXa, which cooperates with factor VIIIa to form the tenase complex, which then forms factor Xa. Factor Xa, along with its cofactor Va forms the prothrombinase complex. The thrombin formed by this complex cleaves fibrinogen to allow it to polymerize into fibrin strands. The extrinsic pathway requires tissue factor (TF) in complex with factor VIIa to form the tenase complex. Additionally, TF/VIIa may activate factor IX to IXa, serving as an alternate method of activating the intrinsic pathway (Figure 39.1).

While the cascade model explains the prothrombin time (PT) and the partial thromboplastin time (PTT), it fails to explain the bleeding diathesis seen in individuals deficient in factors XI, IX, and VIII, as well as the lack of bleeding in those deficient in factor XII, HMWK, or PK. A cell-based model of hemostasis has been developed to address these deficiencies. In this model, a tissue factor-bearing cell such as an activated monocyte or fibroblast serves as the site for generation of a small amount of thrombin and factor IXa. The small amount of thrombin generated by this initiation step is insufficient to cleave fibrinogen, but serves to activate platelets and cleave factor VIII from VWF, allowing the formation of VIIIa. The IXa formed on the TF-bearing cell acts with VIIIa to form the tenase complex on the surface of the activated platelet. The Xa thus formed interacts with the Va generated on the platelet surface to form the prothrombinase complex. This complex generates a large burst of thrombin which is sufficient to cleave fibrinogen, activate factor XIII and activate the thrombin activatable fibrinolysis inhibitor (TAFI), thus allowing the formation of a stable fibrin clot (Figure 39.2). In this model, XIa binds to the surface of activated platelets and boosts the activity of the tenase complex, but is not necessary for thrombin generation. The proteins

controlling these series of reactions will be discussed later in this chapter.

Fibrinolysis leads to clot dissolution once wound healing has occurred, in order to restore normal blood flow. Plasminogen is activated to plasmin by the action of either tissue plasminogen activator (t-PA) or urokinase (u-PA). Plasminogen degrades fibrin and fibrinogen and may thus dissolve both formed clot as well as its soluble precursor. Plasmin

is inhibited by a number of inhibitors, of which α_2 -plasmin inhibitor (α_2 -PI) is the most significant. Plasminogen activation is also inhibited by a number of molecules; chief among them is plasminogen activator inhibitor-1 (PAI-1). Lastly, cellular receptors act to localize and potentiate or clear plasmin and plasminogen activators.

Disorders of Soluble Clotting Factors

While classic hemophilia (factor VIII deficiency) and hemophilia B (factor IX deficiency) are the best known examples of soluble clotting factor deficiencies, the following overview will discuss each deficiency in the numerical order ascribed by the Roman numeral classification system (Table 39.1)

Fibrinogen (Factor I) Abnormalities

Fibrinogen abnormalities are inherited in an autosomal pattern and occur in two main forms: afibrinogenemia and dysfibrinogenemia.³ Afibrinogenemia is a very rare disorder that occurs when any one of the three genes coding for the alpha, beta, or gamma chains that make up the fibrinogen dimer is mutated. If the mutation is sufficient to disrupt formation or secretion of any of the three chains, afibrinogenemia results.⁴ Afibrinogenemic patients have a severe bleeding disorder manifest by bleeding after trauma into subcutaneous and deeper tissues that may result in dissection. Bleeding from the umbilical stump occurs frequently. Though hemarthroses occur in these patients, they occur less frequently than in severe forms of hemophilia A and B. Diagnosis is usually apparent with lack of clot formation in

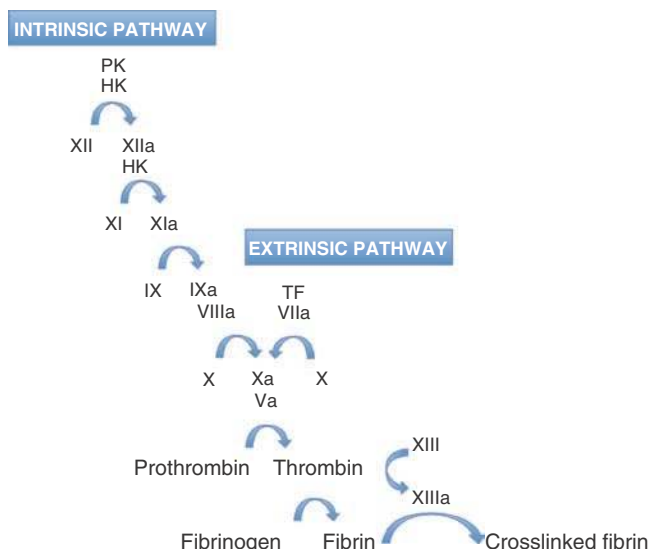


FIG. 39.1. Waterfall model of coagulation. The model shows sequential activation of clotting factors proceeding from the top of the schematic down toward thrombin generation and fibrin formation at the bottom of the schematic. The intrinsic and extrinsic pathways are noted.

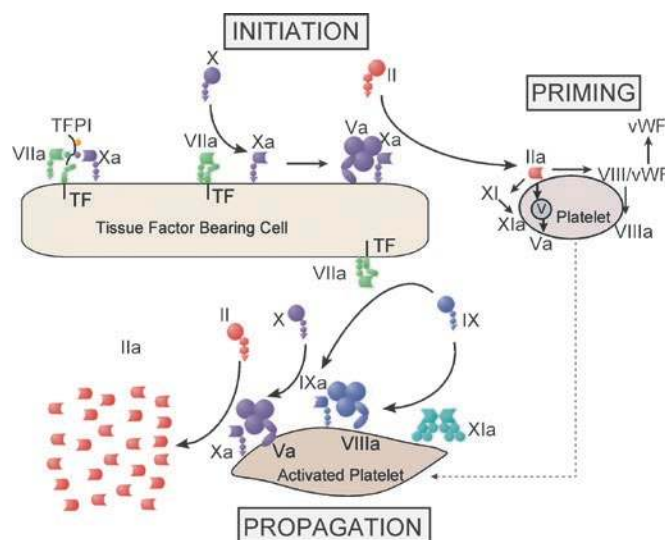


FIG. 39.2. Cell based model of hemostasis. The initiation steps of coagulation take place on the surface of a tissue-factor-bearing cell, such as an activated endothelial cell or macrophage. TF and a small amount of VIIa generate Xa, which joins with Va to form a small amount of thrombin. In the priming step, the small amount

of thrombin generated on the TF bearing cell goes on to activate platelets and activate VIII, which joins with IX to generate Xa. On the platelet surface, the prothrombinase complex can generate a large thrombin burst in the propagation step, allowing for cleavage of fibrinogen into fibrin (used with permission of D. Monroe).

TABLE 39.1. Disorders of soluble clotting factors.

Defect	Inheritance pattern	Bleeding manifestations	Diagnostic testing				Treatment
			PT	PTT	TCT	BT	
Fibrinogen abnormalities	Afibrinogenemia	Severe, but less so than severe Hemophilia A and B	Infinitive	Infinitive	Infinitive	Prolonged	Cryoprecipitate
Prothrombin deficiency	Dysfibrinogenemia	Variable bleeding and/or clotting	Prolonged	Prolonged	Prolonged or shortened	Normal	Cryoprecipitate
Factor V deficiency	Autosomal	Varies with prothrombin levels	Prolonged	Prolonged	Normal	Normal	PCCs
Factor VII deficiency	Autosomal	Mild-moderate	Prolonged	Prolonged	Normal	Prolonged	FFP, potential need for exchange transfusion
Hemophilia A	Autosomal	Moderate-severe	Prolonged	Normal	Normal	Normal	Recombinant activated factor VII
Hemophilia B	X-linked recessive	Variable, depending on Factor VIII level	Normal	Prolonged	Normal	Normal	Factor VIII concentrates, DDAVP in mild cases
Factor X deficiency	X-linked recessive	Variable, depending on Factor IX level	Normal	Prolonged	Normal	Normal	Factor IX concentrates
Factor XI deficiency	Autosomal	Variable, depending on Factor X level	Prolonged	Prolonged	Normal	Normal	Plasma or PCCs
Deficiency of Factor XII, prekallikrein, or high molecular weight kininogen	Autosomal	Variable, but NOT dependent on Factor XI levels	Normal	Prolonged	Normal	Normal	Plasma or Recombinant activated factor VII
Factor XIII deficiency	Autosomal	None	Normal	Prolonged	Normal	Normal	None needed
Deficiency of alpha 2 plasmin inhibitor or plasminogen activator inhibitor-1	Autosomal	Severe	Normal	Normal	Normal	Normal	Cryoprecipitate
		Severe	Normal	Normal	Normal	Normal	Antifibrinolytic agents (epsilon aminocaproic acid or tranexamic acid)

screening clotting tests such as the prothrombin time (PT), partial thromboplastin time (PTT), or the thrombin clotting time (TCT). The bleeding time (BT) is also prolonged because of the absence of fibrinogen in the platelet alpha granule. Treatment consists of transfusing cryoprecipitate to raise the fibrinogen level to the range of around 100 mg/dL. There are a few reports of anti-fibrinogen antibodies occurring in patients with afibrinogenemia who have been repeatedly exposed to fibrinogen replacement.^{5,6}

Dysfibrinogenemia is also rare, but is more common than afibrinogenemia, with the majority of patients being heterozygous for the disorder.⁷ The dysfibrinogens are the result of missense, nonsense, or splice junction mutations. Several hundred different mutations have been recorded, many of which result in neither a hemorrhagic nor thrombotic state. Other dysfibrinogens, however, are associated with bleeding episodes, while a few may be associated with venous or arterial thrombosis. Diagnosis is usually suspected by the observation of abnormalities of the PT, PTT, and TCT, which are usually prolonged. In those dysfibrinogens associated with thrombosis, the TCT may be shortened. The reptilase time may be more sensitive than the TCT to the presence of a dysfibrinogen. Specific diagnosis requires DNA sequencing of the fibrinogen gene. Bleeding patients should be treated with infusions of cryoprecipitate or the newly approved fibrinogen concentrate.

Prothrombin (Factor II) Deficiency

Inherited prothrombin deficiency is rare, with fewer than 50 distinct mutations being reported.⁸ It is an autosomal recessive disorder, and heterozygotes have no bleeding symptoms. Symptomatic patients may be homozygous or double heterozygotes. Bleeding in affected patients varies from mild to severe, depending upon the prothrombin level. The complete absence of prothrombin probably leads to embryonic lethality. Diagnosis depends upon a high index of suspicion in patients with a prolonged PT and PTT who do not have other known clotting factor defects. Specific diagnosis requires an assay for factor II activity.

Factor V Deficiency

Factor V deficiency is an autosomal recessive disorder due to defects in the factor V gene.⁸ Heterozygotes are asymptomatic, while homozygotes or combined heterozygotes may have mild to moderately severe bleeding symptoms. While about 40 mutations in the factor V gene have been reported, they seem to occur less frequently than in genes for other clotting factors.⁸

Bleeding manifestations are similar to those seen in classic hemophilia, except that they tend to be milder, and hemarthroses are less common. The PT and PTT are prolonged in this disease, but the TCT is normal. Factor V deficiency results in a long bleeding time, presumably because of the lack of platelet factor V. It has been reported that 20% of the circulating factor V mass resides in the platelet alpha granule.⁹

Treatment consists of replacing factor V with fresh frozen plasma. It can be difficult to raise the factor V level higher than 15–20% of normal using plasma transfusions alone because of danger of volume overload. Exchange transfusion using fresh frozen plasma may thus be needed when factor V levels above 15 or 20% of normal are required. Inhibitor antibodies against factor V in congenital deficiency are rare.

Factor VII Deficiency

Factor VII deficiency is an autosomal recessive bleeding disorder that occurs in mild, moderate, and severe forms.¹⁰ More than 100 mutations in the gene for factor VII have been reported.¹¹ Bleeding manifestations vary, but in severely affected patients, bleeding can be as severe as that seen in severe classic hemophilia including the occurrence of crippling hemarthroses. Factor VII levels of 10% of normal may be sufficient to control most bleeding episodes, but in some cases, higher levels may be required for hemostasis. There are a few patients with almost no measurable factor VII, who express very few hemorrhagic manifestations. The reason for this is not clear.

Patients with factor VII deficiency have prolongation of the PT while the PTT, TCT and BT are normal. Recombinant human tissue factor should be used in the PT assay, since tissue factor from other species may give spurious results. The best replacement therapy is to use factor VII concentrates. Inhibitor antibodies against factor VII occur, usually in those patients whose genetic mutation results in virtual absence of factor VII protein.

Hemophilia A and Hemophilia B (Classic Hemophilia and Christmas Disease)

Hemophilia A and B result from abnormalities in factor VIII and IX respectively. Factor VIII and IX are necessary for the normal rapid conversion of prothrombin to thrombin. Hemophilia A and B are the only two soluble clotting factor deficiencies that are inherited as X-linked recessive disorders. Several hundred distinct mutations in each gene have been reported.^{8,12,13} These mutations result in mild, moderate, and severe forms of hemophilia, and the clinical manifestations of hemophilia A and B are, for all practical purposes, indistinguishable. In the severe form, both disorders are characterized by recurrent hemarthroses that result in chronic crippling arthropathy unless treated by replacing the deficient factor on a prophylactic basis. Central nervous system hemorrhage is especially hazardous and remains one of the leading causes of death. Retroperitoneal hemorrhage and bleeding into the pharynx may also be life-threatening.

The diagnosis of hemophilia A or B should be suspected in any male patient with hemarthroses, severe bleeding, or excessive bleeding after trauma or surgery. The PTT is prolonged, and the PT, TCT, and BT are normal. Specific diagnosis requires assays for factors VIII and IX.

Safe and effective replacement therapy is available for both hemophilia A and B in the form of highly purified factor VIII and IX concentrates, prepared either from large pools of human plasma or by recombinant DNA technology. Nonplasma products, such as desmopressin (1-desamino 8D-arginine vasopressin; DDAVP), may be used for mild or moderate hemophilia A, but some physicians still prefer specific replacement therapy, even in mildly affected patients.

The main complication of therapy at the present time is the development of inhibitors to factor VIII, which occurs in 10% to greater than 30% of treated patients. Factor IX antibody inhibitors occur in about 3% of severely affected patients, usually in those patients whose mutation results in undetectable factor IX antigen. Some patients with factor IX inhibitors develop anaphylaxis and/or the nephrotic syndrome when exposed to factor IX. Hemophilic patients with inhibitors to factor VIII or IX are resistant to treatment, and hence subject to more complications with subsequent increased morbidity and mortality.

Factor X Deficiency

Factor X deficiency was initially confused with factor VII deficiency, until it was found that the PTT was prolonged in the initial factor X-deficient patient. Like factor VII deficiency, the disorder is inherited in an autosomal recessive fashion and can be mild, moderate or severe. Numerous mutations have been recorded.⁸ Severely affected patients have symptoms similar to severe classic hemophilia, including hemarthroses and chronic crippling hemarthropathy. The PT and the PTT are both prolonged, and the BT and the TCT are normal. Treatment consists of replacement therapy, using either plasma or prothrombin complex concentrates (PCCs) that contain all the vitamin K-dependent factors. The biologic half-life of the factor is about 40 h, so plasma therapy is reasonable, although overload of the circulation may be a problem. Inhibitor antibodies to factor X occur, but are not common.

Factor XI Deficiency

Factor XI deficiency is an autosomal recessive disorder that commonly occurs in patients of Ashkenazi Jewish descent. The deficiency generally produces a mild bleeding tendency. The lack of clinical severity may be explained by the fact that factor XI-deficient patients have normal levels of factor VIII and IX (to form the tenase complex) and normal levels of factors V and X (to form the prothrombinase complex)¹⁴ Whether or not factor XI deficient patients bleed may depend upon differences in their ability to generate thrombin, the ability to activate the thrombin activatable fibrinolytic inhibitor (TAFI) and/or the activity of the fibrinolytic system. In this disorder, the PT and TCT are normal, while the PTT is prolonged. Specific diagnosis depends upon an assay for factor XI.

Deficiencies of Factor XII, Prekallikrein, and High Molecular Weight Kininogen

Deficiencies of factors XII, PK, and HK (the so-called "contact factors") cause a marked prolongation of the PTT, but other screening tests of coagulation are normal. These defects are inherited in an autosomal recessive fashion. They are not associated with bleeding, even after trauma or surgery, although the prolonged PTT may cause a great deal of consternation amongst those not familiar with these defects. A good history revealing the absence of bleeding in these patients and their family members despite a long PTT is the best indication that one is dealing with one of these defects. A specific assay for each is needed for the exact diagnosis. Factor replacement therapy is not needed. The precise role of these factors in hemostasis, if any, is not clear. Furthermore these patients do not appear to have any other disease associated with defects in these factors.

Factor XIII Deficiency

Factor XIII deficiency is caused by a defect in plasma transglutaminase, that covalently cross links the fibrin alpha and gamma chains to form an impermeable fibrin clot. Although a clot may form in the absence of factor XIII and be held together by hydrogen bonds, this clot is permeable to blood and is easily dissolved by the fibrinolytic system. The clot formed in the absence of factor XIII does not form a normal framework for wound healing, and abnormal scar formation may occur.

Factor XIII consists of two A chains and two B chains. The complete molecule is an A₂B₂ tetramer with the A chains containing the active site and the B chains acting as a carrier for the A subunits. Platelet alpha granules contain A chains, but no B chains. Factor XIII deficiency may result from mutations in the genes coding for either the A or B chains, with A chain mutations being more common.¹⁵ Autosomal genes govern hepatic synthesis of the factor, and the disease is expressed as a recessive disorder.

Bleeding manifestations are generally severe, and hemorrhage may occur into any tissue. Umbilical stump bleeding is common in factor XIII deficiency. All the screening tests of clotting function are normal in this disorder, so the diagnosis requires a high index of suspicion, especially in patients with a striking lifelong history of excessive bleeding and in whom the PT, PTT, and TCT are normal. Screening for the diagnosis is done by taking the clot from one of the above tests, placing it in a 5 M urea solution or a dilute solution of trichloroacetic acid, and measuring the time required for its dissolution. If the clot dissolves in less than 24 h, then Factor XIII deficiency should be suspected. However, these screening tests are not very sensitive, and specific diagnosis rests on an assay for plasma transglutaminase, such as measuring incorporation of putrescine into casein.

The plasma half-life of factor XIII is several days, so weekly prophylactic treatment with cryoprecipitate is practical.

Cryoprecipitate is the replacement therapy of choice in the United States, although factor XIII concentrates are available in Europe and are in clinical trials in the United States.

Multiple Clotting Factor Deficiencies

The two most common multiple clotting factor deficiencies are a combined deficiency of factors V and VIII and a combined deficiency of the vitamin K-dependent factors (i.e., factors II, VII, IX, and X, and Protein C and S).^{16,17}

A combined deficiency of factors V and VIII is inherited in an autosomal recessive fashion and may be distinguished from a combined inheritance of mild classic hemophilia and mild factor V deficiency by family studies or by genetic analysis. The disorder is due to defects in one of two genes: the LMAN1 gene and a newly discovered gene called the “multiple clotting factor deficiency 2 (MCFD2) gene.”¹⁷ The products of both genes play a critical role in the transport of factors V and VIII from the endoplasmic reticulum to the Golgi apparatus and are necessary for normal secretion of these factors. The disorder results in a mild to moderate bleeding tendency, with factor V and VIII levels ranging from 5% to 30% of normal. When both the PT and PTT are prolonged and levels of either factor V or VIII are found to be decreased, the combined deficiency should be suspected. Factor VIII is easily replaced using factor VIII concentrates, but the only readily available factor V replacement is fresh frozen plasma, which is limited in its ability to normalize the factor V level. In some cases, plasma exchange is necessary to raise the factor V to hemostatic levels.

Combined deficiencies of the vitamin K-dependent factors may be due to defects in either the gene for vitamin K dependent carboxylase or the gene for vitamin K epoxide reductase.¹⁸ This is an autosomal recessive disorder that may be associated with severe deficiency of prothrombin, factor VII, IX, and X as well as Protein C and S.¹⁷ In this syndrome, both the PT and PTT are prolonged, and assays for the individual factors that influence these tests are necessary. The diagnosis must be distinguished from surreptitious ingestion of

coumarin drugs, which is an acquired disorder with bleeding manifestations of recent onset. Large doses of vitamin K may partially correct the hereditary defect in some cases, but not in all. Some bleeding episodes will require replacement with prothrombin complex concentrates.

Von Willebrand Disease

The most common hereditary bleeding disorder arises from abnormalities in von Willebrand factor (VWF). VWF occurs in plasma as multimers of a 240,000 Dalton subunit, with molecular weights ranging from 1 to 20 million Daltons. The main functions of VWF are to act as a carrier for clotting factor VIII and to mediate platelet adhesion to the injured vessel wall. VWF binds to glycoprotein Ib on the platelet surface and also to collagen in the vessel wall. VWF also crosslinks platelets via binding to glycoprotein IIb–IIIa.

There are three main types of von Willebrand disease (VWD): type 1, 2, and 3¹⁹ (Table 39.2). Type 1 is autosomal dominant and represents a quantitative deficiency of VWF. Type 3 is autosomal recessive, with patients having a near absence of VWF. Type 2 VWD usually occurs as an autosomal dominant disorder with abnormalities in VWF function. Type 2 occurs in four major forms: 2A, 2B, 2N, and 2M. Types 2A and 2B are characterized by the absence of higher molecular weight multimers of VWF. Type 2B is also associated with thrombocytopenia, as a result of a gain of function mutation, resulting in a VWF molecule with higher affinity for the GP Ib receptor, thus enhancing platelet agglutination. Type 2M patients show reduced binding of their VWF to GPIb.

Type 2N VWD is a rare autosomal recessive disorder arising from a mutation in the factor VIII binding site on the VWF molecule. Without the protection provided by VWF binding, factor VIII levels fall because of a markedly decreased half life. VWF multimers and antigen and activity levels may be normal, while the factor VIII levels are low enough, so that this type of VWD may be confused with classic hemophilia. Clinically affected patients are either homozygous for the

TABLE 39.2. Von Willebrand disease subtypes.

			Diagnostic testing					
	Inheritance pattern	Bleeding manifestations	VWF Ag	VWF Activity	Factor VIII activity	VWF multimers	Treatment	
Type 1	Autosomal dominant	Generally mild	Low	Low	Low	Normal	DDAVP, Factor VIII concentrates rich in VWF	
Type 2	2A Autosomal dominant	Mild–moderate	Low	Lower than antigen	Variable	Absent high molecular weight forms	Factor VIII concentrates rich in VWF	
	2B Autosomal dominant	Mild–moderate	Low	Lower than antigen	Variable	Absent high molecular weight forms	Factor VIII concentrates rich in VWF	
	2N Autosomal recessive	Mild	Normal	Normal	Low	Normal	Factor VIII concentrates rich in VWF	
	2M Autosomal dominant	Mild–moderate	Normal	Lower than antigen	Normal	Normal	DDAVP, Factor VIII concentrates rich in VWF	
Type 3	Autosomal recessive	Severe	Near absent	Near absent	Near absent	Absent	Factor VIII concentrates rich in VWF	

gene mutation or combined heterozygotes, carrying a 2N mutation along with a type 1 mutation. Specific diagnosis either requires demonstrating the lack of binding of VWF to factor VIII or genetic analysis.

Though VWD is a defect in a soluble clotting factor, bleeding in patients with this disorder is more similar to that produced by a defect in platelets. The bleeding manifestations tend to be more of the “oozing and bruising” variety, with hematoma formation being rare. Bleeding in types 1 and 2 VWD is usually mild to moderate, although severe bleeding may occur with trauma and surgery. Some patients with type 1 VWD may be relatively asymptomatic. Table 39.2 lists the diagnostic features of the various types of VWD.

The diagnosis of VWD should be suspected in any patient with abnormal bruising, bleeding from mucosal surfaces, menorrhagia, or excessive bleeding after surgery, dental work, or trauma.²⁰ The PT and TCT should be normal. The PTT is variably prolonged, depending on the degree to which the factor VIII level is reduced. The BT is prolonged, except in patients with type 2N VWD. A new diagnostic test, the PFA-100®, is another test of primary hemostasis and has been reported to be more sensitive and specific for a diagnosis of VWD.²¹

Treatment of type 1 and type 2A VWD usually consists of administration of desmopressin (DDAVP), either parenterally or by intranasal administration. It may be used for 3–5 days, but in time, the drug loses its efficacy by depleting endothelial stores of VWF, a process termed “tachyphylaxis.” DDAVP is theoretically contraindicated in type 2B VWD, because thrombocytopenia may be worsened as release of type 2B VWF with enhanced affinity for platelet membrane glycoprotein 1b results in further *in vivo* platelet agglutination. In type 2b and type 3 VWD, factor VIII concentrates rich in VWF should be used for treatment.

Acquired Clotting Factor Deficiencies

In theory, an antibody may develop against any clotting factor, leading to loss of function and/or enhanced clearance. In practice, however, certain factors are more immunogenic than others, and acquired deficiencies of factor VIII, prothrombin, and von Willebrand factor occur much more frequently.

Acquired hemophilia is a sporadic disorder, which occurs primarily in adults with a frequency of approximately one case per million. Autoantibodies, typically of the IgG₄ subclass, are directed against various regions of the factor VIII molecule, impairing function. The antibodies bind factor VIII best at 37°C and require prolonged incubation for optimal binding and inactivation. Unlike in congenital hemophilia, hemarthroses are rare, and bleeding tends to be more mucocutaneous, although muscle hematomas and intracranial bleeds also occur. Because the median age at presentation is between 60 and 67 years, mortality for this disorder is high, ranging from 8 to 22%, likely because of comorbid illnesses. 50% of patients will have a predisposing

condition, usually autoimmune or malignant, but half of the cases are idiopathic. Diagnosis relies on finding an isolated prolonged PTT, with a normal PT, TCT, and BT. The mixing study should fail to completely correct the PTT, and incubating the mix of normal and patient plasma for 1–2 h at 37°C should re prolong the PTT. The Bethesda assay quantitates the strength of inhibitors to Factor VIII and is used to detect, quantitate, and follow these inhibitors. Patient plasma is mixed and incubated with serial dilutions of normal control plasma, and the residual activity of FVIII is measured. The assay is controlled for normal decay of FVIII by performing the assay in tandem using control plasma diluted in buffer or in FVIII-deficient plasma. One Bethesda unit is the amount of antibody that inactivates 0.5U of FVIII in normal plasma after incubation for 2 h at 37°C. Treatment is two-pronged. Immediate measures should be taken to halt the bleeding and will require use of high doses of FVIII infusions (if the Bethesda titer is >5), or bypassing agents, such as recombinant FVIIa or an activated prothrombin complex concentrate. Long term immunosuppression will be required for inhibitor eradication.

Factor II deficiency may be acquired as the result of antiprothrombin antibodies, usually in conjunction with a lupus inhibitor. These antibodies lead to accelerated clearance of factor II and will thus prolong both the PT and the PTT. The existence of a concomitant antiprothrombin antibody is the only instance in which a patient with a lupus inhibitor may exhibit a bleeding diathesis.

Inhibitors to Factor V are typically seen in patients who have undergone redo vascular or cardiac surgery and are provoked by use of bovine thrombin. This hemostatic agent is contaminated with a small amount of bovine FV, and antibodies to bovine FV will crossreact with human FV. This condition may be self-limited, but bleeding may be treated with platelets, since platelet FV may be less susceptible to inhibitors in plasma.²²

Acquired factor X deficiency may be seen in conjunction with amyloidosis, since the FX is adsorbed onto the amyloid protein. This may cause a severe hemorrhagic disorder, which has been reported to respond to splenectomy. This condition will also produce a mixing study, showing normal between the PT and the aPTT.²³

Acquired von Willebrand disease (AVWD) may be difficult to distinguish from acquired hemophilia, since both present with bleeding in later life, with no prior history of a bleeding diathesis. In both, the FVIII activity is low, but the BT will be prolonged in AVWD and normal in acquired hemophilia. Additionally, the VWF antigen and activity will be decreased in AVWD.²⁴ The most common associated conditions include lymphoproliferative and plasma cell dyscrasias, in which the malignant immunoglobulin produced binds to VWF/FVIII and clears it from the circulation.²⁵ This is a similar mechanism by which systemic lupus erythematosus causes AVWD. Hypothyroidism may lead to an acquired type 1 VWD, by causing decreased synthesis of VWF.²⁶ Myeloproliferative

disorders with greatly increased platelet counts (usually >1 million/dL) cause a type 2 VWD by increased proteolysis of the VWF on the platelet surface.²⁷ Wilm's tumor may cause AVWD by unknown mechanisms,^{28,29} and treatment or resection of the tumor usually causes resolution of the VWD.

Disorders of Fibrinolysis

There are two main inhibitors of the fibrinolytic system: the alpha 2 plasmin inhibitor (alpha 2 antiplasmin) and the plasminogen activator inhibitor 1 (PAI-1). The genes for both have been identified and sequenced, and mutations within each have been described.^{30,31} Deficiency of either inhibitor results in excessive fibrinolysis and hence, severe bleeding, including hemarthroses and hematoma formation, that often occurs following trauma or surgery. Both disorders are inherited in an autosomal pattern and, in most cases, bleeding occurs in homozygotes. The diagnosis is suspected when a patient gives a life long history of bleeding, often after trauma, and when the usual screening tests of coagulation are normal. In such cases, a euglobulin lysis test should be performed, and if the clot lyses within a few hours (normal lysis times are >24 h), specific assays for alpha 2 plasmin

inhibitor and plasminogen activator inhibitor 1 should be considered. Bleeding episodes respond to administration of antifibrinolytic agents, either epsilon aminocaproic or tranexamic acid.³²

Disorders of Platelet Number or Function

Disorders of Platelet Production

Inherited disorders causing thrombocytopenia are a heterogeneous group of conditions. Some are associated with a profound thrombocytopathy; some are associated with other somatic changes; and others have only thrombocytopenia (Table 39.3).

The MYH9-Associated Disorders

The May-Hegglin anomaly is the prototype of a family of disorders, now known to be due to a defect in the MYH9 gene.³³ May-Hegglin anomaly, Sebastian syndrome, Fechtner syndrome, and Epstein syndrome are autosomal dominant macrothrombocytopenias distinguished by different combinations of clinical and laboratory signs, such as sensorineural hearing loss, cataracts, nephritis, and Döhle-like bodies

TABLE 39.3. Defects in platelet production.

<i>MYH9-associated diseases</i>			
May-Hegglin anomaly	Autosomal dominant	Macrothrombocytopenia, Döhle-like inclusions in leukocytes composed of cytoplasm surrounding parallel microfilaments with clustered ribosomes	Defect in MYH9 gene encoding non-muscle myosin heavy chain
Sebastian syndrome	Autosomal dominant	Macrothrombocytopenia, Döhle-like inclusions in leukocytes composed of highly dispersed microfilaments with few ribosomes	Defect in MYH9 gene encoding non-muscle myosin heavy chain
Fechtner syndrome	Autosomal dominant	Macrothrombocytopenia, Döhle-like inclusions in leukocytes. Also, sensorineural deafness, nephritis, and ocular abnormalities	Defect in MYH9 gene encoding non-muscle myosin heavy chain
Epstein syndrome	Autosomal dominant	Macrothrombocytopenia, no leukocyte inclusions. Also, sensorineural deafness, nephritis, but no cataracts	Defect in MYH9 gene encoding non-muscle myosin heavy chain
<i>Defects in transcription factors</i>			
Paris-Trousseau syndrome	Autosomal dominant	Macrothrombocytopenia with giant alpha granules	Hemizygous loss of FLI1 gene due to deletion at 11q23
Jacobsen's syndrome		Macrothrombocytopenia with normal alpha granules. Also, congenital heart disease, trigonocephaly, dysmorphic facies, mental retardation, multiple organ dysfunction	11q23 deletion
X-linked congenital dyserythropoietic anemia and thrombocytopenia	X-linked	Macrothrombocytopenia with defective collagen-induced aggregation	Defects in GATA-1, leading to alteration in DNA-binding, or FOG interactions
Familial thrombocytopenia with predisposition to AML development		Defective aggregation, predisposition to AML	Mutation in RUNX1
<i>Other</i>			
Congenital amegakaryocytic thrombocytopenia		Severe thrombocytopenia, also defects in other cell lines	Defects in c-MPL gene encoding thrombopoietin receptor
Thrombocytopenia absent radius		Severe congenital thrombocytopenia with absent or shortened radii, platelets with defective aggregation	
Wiskott-Aldrich syndrome	X-linked	Immune deficiency, eczema, microthrombocytopenia	Defects in Wiskott Aldrich syndrome protein (WASP)

within new trophoblasts. Mutations in the MYH9 gene encoding for the nonmuscle myosin heavy chain IIA (NMMHC-IIA) have been identified in all these syndromes. The hallmark of these conditions is macrothrombocytopenia with characteristic Döhle-like inclusions within the leukocytes. The Fechtner syndrome has these findings, along with the triad of sensorineural deafness, ocular abnormalities, and nephritis seen in Alport syndrome.³⁴ The Epstein syndrome differs from the Fechtner syndrome by lack of cataracts and lack of the leukocyte inclusions.³⁵ Both the May-Hegglin anomaly and the Sebastian syndrome have macrothrombocytopenia with leukocyte inclusions. However, these two syndromes may be distinguished by ultrastructural analysis of the inclusions. In the May-Hegglin anomaly, the Döhle-like bodies are composed of cytoplasm surrounding parallel microfilaments with clustered ribosomes. The inclusion bodies in the Sebastian syndrome are comprised of highly dispersed microfilaments with few ribosomes.³⁶

Defects in Transcription Factors

Alterations in megakaryocyte development due to defective transcription factors underlie a large number of the familial thrombocytopenias. Derangements in the development of other cell lines as well as other somatic mutations may also occur. Mutations in HOXA11 have been described in two unrelated families with bone marrow failure and skeletal defects.³⁷

The Paris–Trousseau syndrome is an autosomal dominant condition, characterized by macrothrombocytopenia with giant alpha granules. It is caused by hemizygous loss of the FLI1 gene because of deletion at 11q23. Lack of FLI1 protein leads to lack of platelet production, because of arrested megakaryocyte development.³⁸ The 11q23 deletion is also seen in patients with Jacobsen syndrome, who also have congenital heart disease, trigonocephaly, dysmorphic facies, mental retardation, and multiple organ dysfunction, as well as macrothrombocytes with abnormal alpha granules.³⁹

Mutations in GATA-1 lead to the X-linked congenital dyserythropoietic anemia and thrombocytopenia syndrome.⁴⁰ The platelets are large and exhibit defective collagen-induced aggregation. The GATA-1 transcription factor has two zinc fingers. The C-terminal finger binds DNA in a site-specific fashion, while the N-terminal one stabilizes the DNA binding, as well as interacts with FOG-1 (Friend of GATA). Mutations within GATA-1 may alter DNA-binding, FOG-1 interactions, or both, and phenotypes may differ, depending on the site of mutation. X-linked thrombocytopenia without anemia is due to mutations within GATA-1 that disrupt FOG-1 interactions while leaving DNA-binding intact.⁴¹ By contrast, GATA-1 mutations which affect binding to DNA while not interrupting FOG interactions lead to a thalassemic phenotype.⁴² The acute megakaryoblastic leukemia, seen in conjunction with Down's syndrome, may be associated with mutations in GATA-1.

Mutations in RUNX1 lead to familial thrombocytopenic syndromes with a predisposition to development of acute myelogenous leukemia.⁴³ RUNX1 mutations cause an arrest in

megakaryocyte development with an expanded population of progenitor cells. The platelets that are produced show defects in aggregation. The development of acute leukemia likely requires a second mutation within RUNX1 or another gene.

Defects in Platelet Production

Congenital amegakaryocytic thrombocytopenia (CAMT) is due to defects in the c-MPL gene encoding the thrombopoietin receptor. Children born with this disorder have severe thrombocytopenia and go on to develop deficiencies in other cell lines.⁴⁴

Thrombocytopenia with absent radii (TAR) is a syndrome characterized by severe congenital thrombocytopenia along with absent or shortened radii.⁴⁵ The platelets produced show abnormal aggregation. Although thrombopoietin levels are elevated, no defect in c-MPL has been identified, and abnormal intracellular signaling pathways are postulated as the cause of this rare disorder.

Perhaps the most common hereditary thrombocytopenia with small platelets is the Wiskott–Aldrich syndrome (WAS), a disorder associated with the triad of immune deficiency, eczema, and thrombocytopenia.⁴⁶ Also see Chap. 2. This syndrome is X-linked and results from mutations in the gene for Wiskott–Aldrich syndrome protein (WASP). Platelets as well as T lymphocytes show defective function, and clinical manifestations vary widely. Definitive treatment requires allogeneic stem cell transplantation. As opposed to the macrothrombocytopenic defects, platelets from WAS patients are small and defective in function.

Disorders of Platelet Function

Defects in Platelet Adhesion

Bernard Soulier Syndrome (BSS) is a severe bleeding disorder characterized by macrothrombocytopenia, decreased platelet adhesion, abnormal prothrombin consumption, and reduced platelet survival. Deficient platelet binding to sub-endothelial von Willebrand factor is due to abnormalities (either qualitative or quantitative) in the GP-Ib-IX-V complex. Mutations in GPIbA binding sites for P-selectin, TSP-1, factor XI, factor XII, α M β 2, and high molecular weight kininogen may mediate variations in the phenotype seen. The product of four separate genes (i.e., *GPIBA*, *GPIBB*, *GP9* and *GP5*) assemble within the megakaryocyte to form the GP-Ib-IX-V on the platelet surface. Defects in any of the genes may lead to BSS.⁴⁷

Platelet-type von Willebrand disease is due to a gain of function mutation, such that plasma VWF binds spontaneously to platelets, and the platelets exhibit agglutination in response to low dose ristocetin. Mutations generally lie within the *GPIBA* gene. High molecular weight multimers of VWF and platelets are cleared from the circulation, and bleeding results. The phenotype is identical to that seen in type 2B VWD, in which the mutation lies within the VWF

rather than its receptor. It may be quite difficult to distinguish between type 2B VWD and platelet-type VWD. Gene sequencing of the VWF gene, the *GPIBA* gene, or both may be required.

Defects in Platelet Aggregation

Glanzmann thrombasthenia is a rare, autosomal recessive disorder characterized by absent platelet aggregation. It is due to absent or defective GP IIb/IIIa on the platelet surface. Patients have severe mucocutaneous bleeding, which becomes refractory to platelet transfusions as alloantibodies to transfused platelets form. Recombinant FVIIa has been used in this disorder and is postulated to work by enhancing thrombin generation on the platelet surface and allowing fibrin to cross-link platelets via an as yet undetermined receptor.⁴⁸ Although demonstration of absent platelet aggregation in response to all agonists will suggest the diagnosis, and definitive diagnosis relies on showing the absence of functional GPIIb/IIIa on the platelet surface, either by flow cytometry or by electron microscopy, using immuno-gold labeled fibrinogen imaging. Acquired Glanzmann thrombasthenia has been described in patients who develop auto-antibodies against GPIIb/IIIa. These patients may have immune thrombocytopenic purpura as an underlying condition, but the severity of their bleeding is out of proportion to their platelet number. These patients may respond to immunosuppression.⁴⁹

Some patients exhibit defects in aggregation in response to specific agonists. Platelets from these patients may show defects in either platelet receptors or in the intracellular signaling pathways leading to activation. Deficiency of GPVI leads to defective collagen-mediated aggregation.⁵⁰ Since ADP-mediated platelet secretion is required for generation of the second wave of platelet aggregation needed by weak agonists, mutations within the P2Y₁₂ ADP receptor leads to defective aggregation, not only to ADP, but also to weak platelet agonists.⁵⁰ The TxA₂ thromboxane receptor has been reported to lead to defective aggregation in response to arachidonic acid and the thromboxane receptor agonist U46619.⁵¹ Patients defective in enzymes within the cyclooxygenase pathway, including cyclooxygenase-1, prostaglandin H synthetase-1, thromboxane synthetase, and lipooxygenase, also exhibit defective aggregation in response to arachidonic acid, but their response to U46619 is retained. Patients deficient in the alpha subunit of the heterotrimeric GTPase protein Gq show defective aggregation in response to agonists, which utilize Gq for inside-out signaling.⁵² These disorders must be distinguished from the effects of drugs, such as aspirin, whose ingestion may produce similar effects on platelet function.

Disorders of Platelet Secretion: The Storage Pool Diseases

Platelets contain two families of intracellular granules: alpha and delta (or dense) granules. Alpha granules contain proteins, either synthesized within the megakaryocytes or endocytosed

from the plasma, including fibrinogen, factor V, thrombospondin, platelet-derived growth factor, multimerin, fibronectin, factor XIII A chains, high molecular weight kininogen, and VWF (among others). Their membrane contains molecules, such as P-selectin and CD63, which are translocated to the outer plasma membrane after secretion and membrane fusion. Dense granules contain ATP and ADP, as well as calcium and serotonin, and any deficiency of dense granules leads to a defective secondary wave of platelet aggregation.

Defects in Alpha Granules

The gray platelet syndrome (GPS) is an autosomal recessive condition leading to a mild bleeding diathesis. It may be recognized by the examination of a Wright–Giemsa stained peripheral blood smear showing platelets that appear gray without the usual red-staining granules. Electron microscopy is a better way to diagnose the syndrome and shows a depletion of alpha granules. The syndrome is thus classified with the other platelet secretion defects, but the disorder may also be classified with the macrothrombocytopenias. In this disorder, platelets may be slightly larger than usual, but they are not as large as those seen in the giant platelet disorders described earlier. Furthermore, the platelet count is only moderately depressed and bleeding symptoms are mild. Patients with this disorder may develop early onset myelofibrosis in addition to their defect in platelet function. The myelofibrosis is felt to be a consequence of the impaired storage of growth factors such as PDGF.⁵³

The Quebec platelet disorder is associated with a normal to slightly low platelet count with a mild bleeding disorder because of abnormal proteolysis of alpha granule proteins. It was first recognized as a deficiency of platelet factor V, with normal concentrations of plasma factor V. The platelets appear normal on the peripheral blood smear, and diagnosis depends upon showing decreased alpha granule proteins. It is inherited in an autosomal dominant fashion.⁵⁴

Defects in Dense Granules

The Hermansky–Pudlak syndrome is the association of delta storage pool deficiency with oculocutaneous albinism and increased ceroid in the reticuloendothelial system. There are several subtypes of the Hermansky–Pudlak syndrome resulting from at least seven different mutations. The syndrome is inherited in an autosomal recessive pattern. Granulomatous colitis and pulmonary fibrosis are also part of the syndrome. Mutations in at least eight genes (i.e., HPS-1 through HPS-8) lead to defects in HPS proteins responsible for organelle biosynthesis and protein trafficking.⁵⁵

The Chediak–Higashi syndrome is also associated with storage pool deficiency and is characterized by oculocutaneous albinism, neurologic abnormalities, immune deficiency with a tendency to infections, and giant inclusions in the cytoplasm of platelets and leukocytes. The disorder is rare, and bleeding manifestations are relatively mild. The syndrome is

due to mutations in the *LYST* (lysosomal trafficking regulator) gene. Affected patients are homozygous; while heterozygotes are clinically normal.⁵⁶

The Scott Syndrome

In this disorder, platelets, when activated, cannot translocate phosphatidylserine from the inner to the outer platelet membrane when the “flip-flop” of the membrane leaflet occurs, presumably because of defects in the “scramblase” enzyme activity.⁵⁷ Because of this defect, factor Xa and Va are unable to efficiently bind, and thrombin generation on the platelet surface is impaired. The Scott syndrome is characterized by a mild bruising and bleeding tendency. It may be detected using flow cytometry with antibodies against annexin V, which will show the defective microvesicle formation characteristic of this disorder.

Disorders of Platelet Destruction

Disorders of platelet destruction are too numerous to list here. This section will be limited to discussion, in which the molecular pathogenesis is better understood. Those readers looking for a more detailed description of these diseases are referred to several excellent references^{58,59} (Table 39.4).

Antibody-Mediated Platelet Destruction

Neonatal alloimmune thrombocytopenia (NAIT) is a bleeding disorder, caused by trans-placental transfer of maternal antibodies

directed against fetal platelet antigens inherited from the father. In Caucasians, the antigens most frequently implicated include HPA-1a (PL^{A1}) and HPA-5b (Br^a). In Asians, HPA-4a and HPA-3a account for the majority of the NAIT seen. NAIT occurs with a lower frequency in Caucasians than is expected by the incidence of HPA-1a negativity in the population, suggesting that other factors influence antibody development. Additionally, NAIT mediated by antibodies against HPA-1a is more clinically severe, perhaps because these antibodies may also block platelet aggregation, since HPA-1a is an antigen expressed on the platelet surface glycoprotein IIIa. Mothers who are negative for the antigen in question may develop anti-platelet antibodies that cross the placenta, leading to severe thrombocytopenia in the fetus. Even the first child may be affected, and intracranial hemorrhage is a feared and devastating complication. Subsequent pregnancies have a near 100% rate of NAIT, and various measures (i.e., prenatal IVIG with or without corticosteroids given to the mother, or in utero transfusions of matched platelets or IVIg) have been employed. After birth, affected infants may be treated with IVIg or matched platelets.

Posttransfusion purpura (PTP) is another disorder which causes thrombocytopenia because of a mismatch between platelet antigens. In this condition, patients previously sensitized against certain platelet antigens (the same ones that lead to NAIT) develop acute, severe thrombocytopenia 5–14 days after transfusion.⁶⁰ Although packed red cells are most commonly associated, transfusion of any blood component may precipitate this disorder. These blood components contain platelet microparticles, which express the offensive platelet

TABLE 39.4. Disorders of platelet destruction.

<i>Antibody-mediated platelet destruction</i>			
Neonatal Alloimmune thrombocytopenia (NAIT)	Transplacental transfer of maternal antibodies against fetal platelet antigens. HPA-1 and HPA-5 most commonly implicated in Caucasians, HPA-4a and HPA-3a seen more commonly in Asians	Severe fetal thrombocytopenia	Platelet transfusions to fetus from HPA-1 negative donors or maternal pheresed platelets. Mother can be treated with IVIg or steroids prenatally
Post-transfusion purpura	Recipient receives blood product from donor mismatched in platelet antigen. Antigens mismatched same as in NAIT	Severe thrombocytopenia occurs 7–10 days after transfusion (usually of red cells)	IVIg or plasmapheresis
Immune thrombocytopenic purpura (ITP)	Autoimmune condition characterized by antiplatelet antibodies and Fc-receptor mediated platelet consumption	Varies	Immunosuppression, thrombopoietin-stimulating agents
Drug-induced	Varies	Varies	Varies
<i>Thrombotic microangiopathies</i>			
Thrombotic thrombocytopenic purpura (TTP)	Autoimmune inhibitor to or deficiency of ADAMTS-13, the protease responsible for cleaving VWF. Ultra-large VWF multimers accumulate, leading to platelet activation and clearing	Thrombocytopenia, microangiopathic hemolytic anemia, renal manifestations, neurologic manifestations, and fever	Plasma exchange
Hemolytic uremic syndrome (HUS)	If diarrhea-positive, then shiga toxin binds to glycosphingolipid receptors on surface of renal cells, leading to cell death If diarrhea-negative, then autoantibodies against factor H, membrane cofactor protein, or complement factor I, lead to unregulated complement activation	Thrombocytopenia, microangiopathic hemolytic anemia, renal manifestations predominate; neurologic manifestations and fever are less prominent	supportive

antigen, leading to an anamnestic production of antibodies. However, paradoxically, these patients go on to develop antibodies directed against their own platelets, either by fusion of the exogenous microparticles with their own platelets or by a process in which exposure to foreign platelets leads to the formation of autoantibodies. Unlike NAIT, transfusion of matched platelets leads to only a transient improvement in this condition. IVIg has been reported to shorten the duration of thrombocytopenia, as has plasma exchange.

Thrombotic Microangiopathies

We have made more rapid progress in our understanding of the molecular pathogenesis underlying thrombotic thrombocytopenic purpura (TTP) than for almost any other thrombocytopenic disorder. In 1924, Moschowitz reported a case of a young girl with anemia, leucopenia, renal insufficiency, and fever.⁶¹ After her unfortunately rapid demise, autopsy showed hyaline thrombi within the microvasculature. The prognosis of this disorder remained grim until reports of whole blood exchange transfusion leading to remission in 1976 began a therapeutic revolution.⁶² Fresh frozen plasma was subsequently shown to be of therapeutic benefit,⁶³ and in 1991, the Canadian Apheresis Group published a randomized controlled trial showing plasma exchange to be superior to simple plasma infusion.⁶⁴ In 1982, Moake made a seminal observation that the plasma of patients with TTP contained “ultralarge” (UL) multimers of VWF, which were absent in normal plasma.⁶⁵ He hypothesized that TTP may be due to the absence of a protease or depolymerase responsible for cleaving the UL VWF multimers. The protease was identified in 1996 by Tsai⁶⁶ and Furlan⁶⁷; its gene was cloned⁶⁸, and the enzyme was named ADAMTS-13 when it was found to be a member of the “a disintegrin-like and metalloprotease with thrombospondin repeats” family of metalloproteases.^{69,70} ADAMTS-13 levels are found to be low in patients with both familial⁷⁰ and sporadic TTP, and an IgG auto-antibody inhibitor to ADAMTS-13 is found in a majority (but not all) of patients with sporadic TTP.^{71,72}

The hemolytic uremic syndrome (HUS) shares many clinical features with TTP, including microangiopathic hemolytic anemia, thrombocytopenia, and renal insufficiency. Renal findings are more prominent and neurologic findings are less. HUS is divided into diarrhea-associated HUS (D+HUS) and atypical (diarrhea-negative) HUS.⁷³ Diarrhea-positive HUS is triggered by infection with a Shiga-toxin-producing bacteria. *Escherichia coli* O157:H7 is implicated in 80% of the cases, but other bacteria, including other *E. coli* subtypes and *Shigella dysenteriae* serotype 1, may cause D+HUS. Shiga toxins bind to the glycosphingolipid receptor globotriaosylceramide (Gb3) on the surface of renal mesangial, glomerular, and tubular epithelial cells. Protein synthesis is impaired through the inhibition of 60S ribosomes and cell death occurs.⁷⁴ Plasma from patients with HUS demonstrates markers of abnormal thrombin generation. As compared with TTP, ADAMTS13 levels are typically normal in patients with HUS, and the fibrin microthrombi do not contain VWF strands.

Atypical HUS occurs in patients without a diarrheal prodrome. Secondary causes, such as organ transplantation or exposure to drugs, may be present. In as many as 30–50% of patients, mutations in one of three proteins involved in complement regulation occur.^{75–77} Factor H (CFH) and membrane cofactor protein (MCP or CD46) are regulators of complement factor I (CFI), which is a serine protease that cleaves and inactivates surface-bound C3b and C4b. Autoantibodies against these proteins have also been reported, suggesting that unregulated complement activation plays a role in the pathogenesis of HUS.⁷⁸

Heparin-Induced Thrombocytopenia

Heparin-induced thrombocytopenia (HIT) is a common iatrogenic thrombocytopenic disorder that may paradoxically lead to thrombosis. It occurs in 1–5% of patients treated with standard unfractionated heparin for at least 5 days and in <1% of those treated with low molecular weight heparin. Approximately 50% of patients develop venous and/or arterial thromboses. New thromboses develop in 25% of patients; amputations are required in 10%; and mortality rates vary between 10 and 20%.⁷⁹ The pathogenic autoantibodies that cause HIT are directed against neoepitopes on PF4 that are induced by heparin and other anionic glycosaminoglycans (GAGs). PF4 is an abundant protein stored in the alpha granules of platelets in complex with chondroitin sulfate (CS). Upon platelet activation, PF4/CS complexes are released and bind to the platelet surface. Heparin may displace CS, forming PF4/heparin complexes. Binding of IgG anti-PF4/heparin to the platelet leads to Fcγ receptor-mediated clearance of platelets, but also leads to platelet activation and generation of procoagulant microparticles via FcγRIIA. PF4/heparin complexes also form on the surface of monocytes and endothelial cells, and antibody binding leads to tissue-factor driven thrombin generation and hence to clot formation. The PF4 heparin complexes are most antigenic when PF4 and heparin are present at equimolar concentrations, where they form ultralarge molecular complexes.⁸⁰ Low molecular weight heparin forms these ultralarge complexes less efficiently and at concentrations that tend to be supratherapeutic, perhaps explaining the lower frequency of HIT in patients treated with LMWH, as opposed to standard unfractionated heparin.

Thrombophilia

Understanding how the coagulation system is regulated is necessary, when seeking to determine how it may become deranged. Thrombosis may occur when controls on the coagulation system slip and become less effective, or when the coagulation engine is running too fast to be completely controlled. This section will focus on the two major pathways for inhibiting coagulation: the protein C pathway and the antithrombin pathway.

The Protein C Pathway and Thrombosis

Protein C (PC) is a vitamin K-dependent protein, which is activated by thrombin. Thrombin is normally a procoagulant protein, but when it is bound to the endothelial cell surface protein thrombomodulin, it changes substrate specificity and cleaves PC to form activated protein C (APC). Activated protein C and its cofactor protein S (another vitamin K-dependent protein) work to inactivate factors Va and VIIIa, thus working to turn off thrombin generation. APC also alters gene expression profiles, downregulates inflammatory pathways, and inhibits p53-mediated apoptosis of ischemic brain endothelium.^{81,82} The endothelial protein C receptor (EPCR) serves to bind PC and APC and is localized on the surface of endothelial cells. EPCR binding to PC enhances the generation of APC by fivefold. EPCR is also found in a soluble form, and its levels are enhanced in such thrombotic conditions, such as disseminated intravascular coagulation and systemic lupus erythematosus. When APC is bound to EPCR, its substrate specificity shifts to prefer cleaving and activating the protease activated receptor-1 (PAR-1), thus switching from anticoagulant to procoagulant activity.⁸³

Protein C deficiency is known to cause thrombosis with an odds ratio of 6.5–8^{84–86}; a database of genetic mutations leading to this disorder has been published.⁸⁷ Most of these mutations are of the type I variety, that is they lead to an equal decrement in activity and antigen. These mutations affect protein folding and lead to unstable molecules that are either poorly secreted or degrade more rapidly. Type II defects lead to activity levels that are reduced disproportionately to the antigen levels and result in dysfunctional molecules with ineffective protein–protein interactions. Heterozygous protein C deficiency is found in 0.2–0.4% of normal individuals^{88,89} and in approximately 4–5% of outpatients with confirmed deep venous thrombosis.⁸⁶ Protein C-deficient individuals with personal and family histories of thrombosis may have inherited a second prothrombotic mutation, such as factor V Leiden, to account for the thrombotic tendency. Venous thromboembolic disease (VTE) occurs in 50% of heterozygous individuals in affected families by age 45, with half of the events being spontaneous.⁹⁰ Venous thrombosis at unusual sites (cerebral sinus and intra-abdominal) is a clinical hallmark. Arterial thrombosis is rare, although reported. Homozygous protein C deficiency with levels <1% leads to neonatal pupura fulminans and massive thrombosis in affected infants.⁹¹ Individuals with protein C deficiency are predisposed to develop warfarin skin necrosis when anticoagulated with vitamin K antagonists, such as coumadin. Since protein C has a much shorter half life (8 h) than the procoagulant vitamin K-dependent factors, such as prothrombin and factor X (24–48 h), a transient hypercoagulable state may occur in patients treated with coumadin without being bridged with an alternate anticoagulant such as heparin. This risk is magnified in patients with underlying deficiency of either protein C or vitamin K.⁹²

Protein S is a vitamin K-dependent protein, which is not a serine protease; rather, it acts as a cofactor for APC. In normal plasma, 60% of protein S is bound to C4b-binding protein (C4BP), and the remainder is free. Protein S also exhibits anticoagulant activities independent of APC by binding to and inactivating factors Va, VIIIa, and Xa.⁹³ Only the free protein S may function as the cofactor for APC. Protein C deficiency exists in three forms: type I has equal decrements of antigen and activity; type II has low activity but normal antigen levels; and type III shows low free protein S levels, with total protein S levels in the low to normal range. A database of implicated mutations leading to protein S deficiency has been published.⁹⁴ The odds ratio for VTE with protein S deficiency is variable, being reported as 1.6,⁸⁶ 2.4,⁹⁵ 8.5,⁸⁵ and 11.5.⁹⁶ Deep venous thrombosis and pulmonary embolism are the most common clinical manifestations of this deficiency, although superficial thrombophlebitis and thromboses at odd locations also occur. More than 50% of VTE events are unprovoked. Arterial thromboses occur at higher frequency, especially amongst smokers or those with other thrombotic risk factors.^{97,98} Laboratory testing needs to be done and interpreted with caution. Normal levels differ with age and gender, and may be falsely low in patients with activated resistance to protein C. Acquired protein S deficiency occurs in a variety of conditions, including acute thrombosis, inflammation, liver disease, nephrotic syndrome, vitamin K deficiency, disseminated intravascular coagulation, and in association with the lupus anticoagulant. Low levels of free protein S are found with the use of oral contraceptives and hormone replacement therapy, as well as during pregnancy. Antibodies to protein S may be seen in children with varicella or other viral illnesses.⁹⁹

Addition of APC normally causes a prolongation of the PTT by approximately twofold. In 1993, Dahlback reported a series of thrombophilic families, in which the plasma of the probands and their affected relatives all exhibited resistance to APC.¹⁰⁰ Mixing studies showed this defect to be due to a problem with factor V,¹⁰¹ and the genetic defect responsible for APC resistance was shown to be a mutation at the major cleavage site of APC on factor V from arginine to glutamine (R506Q).^{101–104} This mutation, now known as factor V Leiden (after the city in which it was first discovered), is the most prevalent inherited mutation leading to thrombophilia. It is found in approximately 5% of all Caucasians, and is felt to be the result of a founder mutation in a single Caucasian ancestor 21,000–34,000 years ago.¹⁰⁵ The mutant factor V is inactivated by APC tenfold more slowly, and thus leads to excess thrombin generation.¹⁰⁶ Factor V Leiden accounts for 20–25% of inherited thrombophilia. Heterozygosity for this mutation confers a relatively low risk for VTE in younger patients (OR 1.2 in those 40–50), but the risk increases steeply with age (OR 6 for those older than 70).¹⁰⁷ Homozygotes have an odd ratio for VTE of 50–100 and half of such individuals will have thromboses during their lives.¹⁰⁸ Coronary artery thrombosis may also be found with higher frequency in young men and women with other thrombotic

risk factors.^{109–112} The risk for thrombosis in individuals with factor V Leiden is greatly magnified when other risk factors for thrombosis are present. These risks may be either genetic or acquired, including PC deficiency, PS deficiency, the prothrombin G20210 mutation, elevated levels of factor VIII, antiphospholipid antibodies, hyperhomocysteinemia, or in the settings of prolonged immobility, surgery, malignancy, pregnancy, or oral contraceptive use.¹¹² APC resistance may be caused by conditions other than factor V Leiden, including pregnancy, lupus anticoagulants, inflammation, and the use of anticoagulants. Testing for APC resistance is best done using factor V-deficient plasma, which will eliminate the above conditions. Genetic testing for factor V Leiden is also available and is sensitive and specific for the disorder.

A mutation found in 1% of Caucasians is the second most frequent cause of inherited thrombophilia. A mutation (G20210A) in the 3'-untranslated region of the prothrombin gene results in elevated levels of prothrombin synthesis,¹¹³ perhaps increasing thrombotic risk by increasing thrombin generation or decreasing fibrinolysis by enhanced activation of TAFI, the thrombin activatable fibrinolysis inhibitor.^{114,115} The relative risk for VTE in heterozygotes is between 2 and 5.5, and 4–8% of patients presenting with their first VTE have this mutation.^{113,116–121} Homozygosity for the mutation appears to confer a higher risk of VTE.^{122,123} Venous clots in odd locations, as well as arterial clots, are found with increased frequency, especially in patients younger than 55 and in those with other thrombotic risk factors. PCR amplification of the pertinent region followed by DNA sequencing is required for the diagnosis. Measurement of factor II levels is neither sensitive nor specific for the disorder.

Antithrombin Deficiency

Antithrombin (AT) is a serine protease inhibitor that inactivates thrombin and clotting factors Xa, IXa, and XIa by irreversibly forming 1:1 complexes in reactions accelerated by glycosaminoglycans, such as heparin or heparan sulfate, on the surface of endothelial cells. Deficiency of antithrombin therefore results in less efficient neutralization of heparin and potentiation of thrombosis. Two types of antithrombin deficiency exist: type I deficiency decreases the antigen and activity levels in parallel; whereas, type II deficiency produces a dysfunctional molecule. Type IIa mutations affect the active center of the inhibitor, which is responsible for complexing with the proteases active site. Type IIb mutations target the heparin binding site, and type IIc mutations are a heterogeneous group. A database of AT mutations has been compiled.¹²⁴ Severe antithrombin deficiency with levels <5% is rare, resulting from one of several IIb mutations and leads to severe recurrent arterial and venous thromboses.^{125–128} The odds ratio for venous thrombosis in heterozygotes is approximately 10–20.^{85,129} Lower extremity deep vein thrombosis is common, and clots in unusual sites have been reported. Clots tend to occur at a younger age, with 70% presenting

before age 35 and 85%, before age 50.¹³⁰ Some patients with AT deficiency exhibit resistance to the anticoagulant effects of heparin. Other conditions associated with lowered levels of AT include treatment with heparin, acute thrombosis, disseminated intravascular coagulation, nephrotic syndrome, liver disease, treatment with the chemotherapeutic agent L-asparaginase, and preeclampsia.^{131–136}

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Sarcoidosis: Are There Sarcoidosis Genes?

Helmut H. Popper

Introduction

More than 100 years have passed since the first description of sarcoidosis by Hutchinson¹ and the identification of sarcoid granulomas by Besnier,² Boeck,³ and Schaumann,⁴ but the causative agent or agents of sarcoidosis still have not been identified. However, in these intervening years, considerable knowledge has accumulated about the pathogenesis and the molecular events that lead to the granulomatous reaction. Clinical evaluation has shed some light on this systemic disease; pathology and immunology have contributed to our understanding of the inflammatory process. More recently, genetics and molecular biology have opened new avenues of research for this still enigmatic disease. There is some hope that new techniques provided by molecular biology, employing samples from bronchoalveolar lavage and biopsies, might elucidate the causative agents behind this disease and define the genetic modifications that make some people prone to developing sarcoidosis.

Morphology and Its Implications

Sarcoidosis begins with local infiltration of alveolar septa and bronchial mucosa by alveolar macrophages and lymphocytes, predominantly of the T helper phenotype. This early infiltration is centered on blood and lymphatic vessels (Figure 40.1). It is most probable that the antigens, which induce the accumulation of these cells, enter or reenter the lung via the blood stream and the lymphatic system. This is in contrast to airway epithelium-centered granulomas as seen in infectious granulomatosis. At these early stages of sarcoidosis, the granulomas have ill-defined borders and abundant lymphocytes. As the granulomas mature, lymphocytes are less numerous and the granulomas organize with central epithelioid histiocytes and Langerhans giant cells surrounded by a smaller rim of lymphocytes (Figure 40.2). Within this outer rim of lymphocytes, T-helper lymphocytes (CD4⁺) predominate, whereas CD8⁺ T cells and B lymphocytes are found outside the granulomas.

This suggests a functional relationship. Within the granulomas, the secretion of cytokines and chemokines from CD4⁺ lymphocytes and epithelioid cells is necessary to maintain the immune reaction toward the antigen(s), develop the granulomas, and stimulate the differentiation of epithelioid and giant cells out of the macrophage/monocyte cell pool. The function of the CD8⁺ lymphocytes outside the granulomas is still not understood; maybe, they are downregulating inflammatory signals or are involved in limiting the immune reaction and inflammation.

Variants of Sarcoidosis

Löfgren's Disease/Acute Sarcoidosis

The Swedish physician Sven Halvar Löfgren described acute sarcoidosis as a variant of sarcoidosis presenting with erythema nodosum and hilar lymphadenopathy,⁵ and this disease now bears his name. Pulmonary involvement in Löfgren's disease is usually also present but radiographically not always detectable. As in the classic form of sarcoidosis, there is a lymphocytic infiltration and also early granuloma formation. However, the granulomas are usually smaller (500 µm in diameter) and less numerous. The course of acute sarcoidosis most often is benign with an acute onset, a short duration, and a spontaneous resolution. Muscular pain and joint swelling may be present and are probably caused by a granulomatous inflammation (unpublished personal observation), but most often no biopsies are performed.

Nodular Sarcoidosis

Romer⁶ and later Churg⁷ described nodular sarcoidosis as a variant of sarcoidosis. The main features are confluent sarcoid granulomas, absence of necrosis, and ischemic infarcts. The nodules are usually in the range of 1–7 cm, but usually 1–3 cm prevails. Besides the lung, several other organs can be involved. Most often, the macronodular pattern

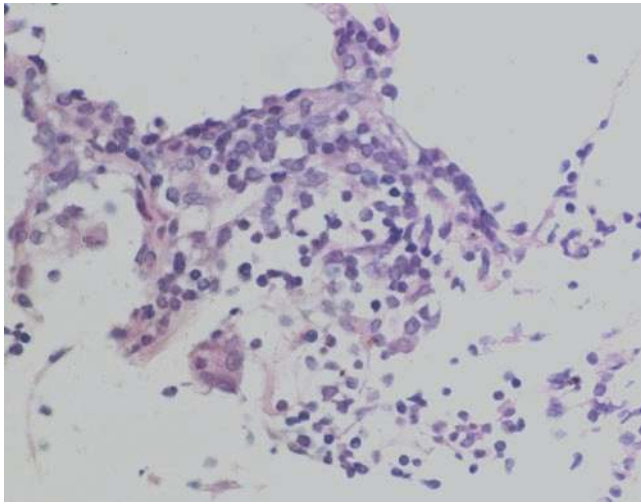


FIG. 40.1. Early epithelioid cell granuloma. Macrophages and a few developing epithelioid cells are infiltrating the lung together with lymphocytes (hematoxylin and eosin stain; $\times 200$).

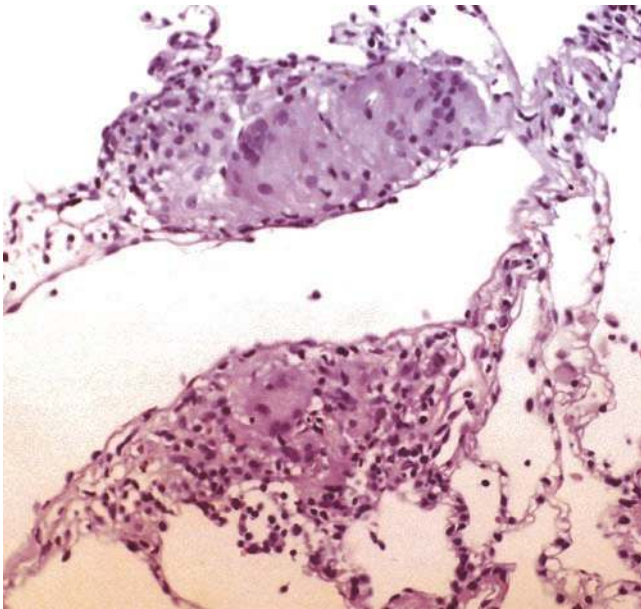


FIG. 40.2. Epithelioid cell granulomas in sarcoidosis. Note the intimate location of the granulomas to this dilated lymphatic, whereas there is a distance to the alveolar side. This is a typical finding in sarcoidosis and might be caused by antigens entering via bloodstream and lymphatics (hematoxylin and eosin stain; $\times 200$).

is accompanied by conventional microscopic granulomas as well. There is no known cause for nodular sarcoidosis. Treatment follows the same regimen as for sarcoidosis.

Necrotizing Sarcoid Granulomatosis

Necrotizing sarcoid granulomatosis (NSG) was originally described by Averill Liebow as a separate entity.⁸ For Liebow, NSG shared a mixture of patterns of sarcoidosis and Wegener's

granulomatosis, showing sarcoid granulomas, most often a macronodular pattern, and ischemic necrosis and vasculitis as in Wegener's granulomatosis. All of Liebow's original NSG cases presented in the lungs and hilar lymph nodes. In cases published subsequently by Andrew Churg and colleagues, extrapulmonary involvement was found in some cases, and the separation of NSG from sarcoidosis was questioned.⁹ In the largest series published so far, Popper et al demonstrated that NSG has features of sarcoidosis: sarcoid granulomas, nodular sarcoid aggregates, and epithelioid cell granulomatous vasculitis.¹⁰ The only difference was vascular occlusion by the vasculitis and subsequent ischemic infarcts.

All these variants of classic sarcoidosis might be caused by the same mechanisms, but disease modifiers might influence the reaction pattern. Some genetic differences from classic sarcoidosis are recognized in Löfgren's disease (see subsequent paragraphs); however, nodular sarcoidosis and NSG have not been investigated thus far.

What Is the Meaning of Indistinguishable Differentials?

Chronic allergic metal disease, such as chronic berylliosis and zirconiosis, and so-called sarcoid-like reactions in the lung and lymph nodes are indistinguishable histopathologically from sarcoidosis. Both present with nonnecrotizing epithelioid cell granulomas, and there are no organisms detectable within the granulomas. In sarcoid-like reaction, granulomas are distributed along lymphatics and blood vessels within the lung and in pulmonary and mediastinal lymph nodes in patients with primary pulmonary carcinomas and other malignancies in the lung. The mechanism of granuloma formation in this condition is not fully explored, but release of various cytokines and chemokines, like interleukin (IL)-1 β , IL-2, interferon (IFN)- γ , granulocyte-macrophage colony stimulating factor (GM-CSF), and tumor necrosis factor (TNF)- α from tumor cells might be responsible for this reaction.¹¹⁻¹⁴

Genetic susceptibility to chronic berylliosis has been associated with human leukocyte antigen (*HLA*)-*DP* alleles, possessing a glutamic acid at the 69th position of the β -chain. These *HLA*-*DP* molecules bind and present beryllium to pathogenic CD4⁺ T cells. These helper T cells secrete Th1-type cytokines upon beryllium recognition. The presence of circulating beryllium-specific CD4⁺ T cells directly correlates with the severity of lymphocytic alveolitis.¹⁵ In addition to *HLA*-*DP*-Glu69, *HLA*-*DR*-Glu71 is capable of inducing beryllium-specific proliferation and IFN- γ expression by lung CD4⁺ T cells.¹⁶ The metal oxide acts as a hapten, associates with *HLA*-*DP* and *HLA*-*DR* to form an allergen, and then induces a type IV immune reaction.¹⁷⁻¹⁹ In both berylliosis and sarcoidosis, CD4⁺ helper cells are the major immune cell population within the granulomas. CD8⁺ and B cells can be found outside the granulomas in much lesser quantity.

Other granulomatous diseases caused by infectious organisms can also mimic sarcoidosis. Good examples are granulomas caused by slow growing mycobacteria such as *Mycobacterium avium* and *M. intracellulare*.

These granulomatous reactions demonstrate that there can be different causes but one mechanism to induce nonnecrotizing sarcoid granulomas. This is primarily because many of these antigens, such as mycobacterial capsules, are of low solubility and are minimally degradable. Other antigens, such as BeO by its association with HLA molecules, can directly stimulate CD4⁺ T lymphocytes. Probably, many of these antigens are processed similarly by the dendritic cells and stimulate similar subpopulations of helper T cells by a selection of T-cell receptors and costimulatory molecules of the HLA system. The analysis of costimulatory molecules by comparing different types of epithelioid cell granulomas and their immune cells is also a focus of research. A few important findings in this field are emerging such as the butyrophilin-like 2 gene and Toll-like receptor 4 (TLR4; see later discussion).

The Cells in Sarcoidosis

The first cells to appear in a granuloma are alveolar macrophages followed by T lymphocytes. Not much is known about the phenotype and function of the macrophages. What morphologically appears as a macrophage might not always be a classic phagocytic cell. Antigen-presenting dendritic or Langerhans cells may resemble macrophages histologically. Even among CD68⁺ macrophages, there are phagocytic cells, as well as cells capable of antigen processing and stimulating an immune reaction. In experimental assays, immunostaining of multiple surface markers can easily define these cells, but in formalin-fixed tissue sections or in cytologic specimens, immunopositivity of many of these markers is lost. Given that alveolar macrophages are the primary cells, which induce a lymphocytic influx in sarcoidosis, these cells most probably have the capability to process antigens and present them to lymphocytes. A better characterization of the cellular compartments in this early granulomatous reaction is warranted. In addition, the participation of dendritic cells and Langerhans cells needs to be elucidated. Because macrophages and lymphocytes induce the differentiation of macrophages/monocytes into epithelioid cells and giant cells and release chemokines to maintain the granulomas, this granulomatous reaction is most likely not the primary antigen contact but recognition of antigens to which the patient has been previously sensitized.

T Lymphocytes

The major cell population in sarcoidosis is helper T cells. Most of these express the $\alpha\beta$ receptor, produce/secrete IFN- γ and IL-2, and therefore belong to the Th1 subset of helper cells.²⁰ The selection of a Th1-dominated reaction can be facilitated by the release of various chemokines/cytokines or by the

presence of antigens and costimulatory molecules. Interleukin-18 expressed by airway epithelial cells and macrophages drive a Th1 reaction with an increase of IFN- γ producing cells.²¹ Interleukin-27 induces the expression of the major Th1-specific transcription factor T-bet (T-box expressed in T cells) and its downstream target IL-12R β 2. Interleukin-27 suppresses expression of GATA-3, the Th2-specific transcription factor. Interleukin-27 induces phosphorylation of signal transducer and activator of transcription (STAT) 1, STAT3, STAT4, and STAT5. STAT1 is required for the suppression of GATA3.²² Another mechanism for selecting a Th1 or Th2 response is by activation of the aryl hydrocarbon receptor. Aryl hydrocarbon receptor activation by the addition of representative ligands suppresses naive helper T-cell differentiation into Th2 cells.²³ A further mechanism that suppresses Th2 differentiation and thus the balance of Th1/2 is by the Src kinase homology domain 2.²⁴ A Th2 cell differentiation can also be achieved by the ras/extracellular signal-regulated kinase/mitogen-activated protein kinase system, which facilitates GATA3 protein stability and GATA3-mediated chromatin remodeling at Th2 cytokine gene loci.²⁵

Some conflicting reports exist on the role of TNF- α : overexpression of TNF- α and IFN- γ together leads to persistence and progression in sarcoidosis,²⁶ whereas in animal models, TNF- α is required for Th1 accumulation, granuloma formation, and antigen clearance, which should lead to the downregulation of inflammation.²⁷

Epithelioid Cells

Epithelioid cells are the secretory cells within the granulomas. Epithelioid cells secrete many different chemokines and cytokines, such as migration inhibitory factor and various interleukins. Their major role is the maintenance of the granulomas and the immune reaction. In part, they support the helper T lymphocytes functionally.

Giant Cells (Foreign Body and Langerhans Type)

Giant cells form by fusion of macrophages as well as by nuclear division without concomitant cell division. Giant cells are specialized in phagocytosis and have large lysosomes capable of digesting poorly soluble and slowly degradable material. In tuberculosis and other infectious diseases, these cells are directed against the resistant capsules of mycobacteria and similar organisms. However, the reason for giant cell formation in sarcoidosis is not clear. A comparison with rheumatoid arthritis might shed some light on this phenomenon. Antigen-antibody complexes, sometimes combined with additional idiotypic and anti-idiotypic antibodies, form very robust complexes deposited in the synovial membrane in rheumatoid arthritis. In these usually seropositive cases, histiocytic granulomas with giant cells develop. One might speculate that antigen-antibody complexes in sarcoidosis are also insoluble and thus induce a giant cell reaction.

Mycobacteria and Other Trigger Mechanisms: Is Sarcoidosis an Infectious Disease?

Because of the close resemblance of granulomas in sarcoidosis to those in mycobacterial diseases, an infection caused by *Mycobacterium tuberculosis* or other mycobacteria has been proposed in sarcoidosis. Culture of mycobacteria from sarcoidosis granulomas of the skin has been reported,²⁸ but this finding was not confirmed in other investigations.^{28–30} Several investigators have reported mycobacterial DNA and RNA in sarcoidosis.^{31–37} However, other investigators have reported negative results for mycobacterial DNA or RNA.^{38–40} It has been speculated that cell wall-deficient mycobacteria, unable to grow in culture, might induce sarcoidosis.^{31,41,42} In some cases, DNA insertion sequences characteristic for *M. avium* were reported to be amplified from granulomas.³⁵ In three cases of recurrent sarcoidosis in lung transplant patients, mycobacterial DNA other than tuberculosis complex could be demonstrated in the explanted native lungs and in the transplanted lungs with recurrent sarcoidosis.³⁶ Other recent reports have shown that naked mycobacterial DNA is capable of inducing a strong immune response.^{43,44} It is known that mycobacteria can persist preferentially in macrophages. In accordance with these observations, heat shock protein 90 is upregulated in sarcoidosis, which is part of the initial immunologic response mechanism in mycobacterial infections. Heat shock protein together with TLR4 expression is one of the mechanisms by which the selection of HLA types and costimulatory molecules is facilitated in mycobacterial infections (unpublished observation).^{45–48}

In a working hypothesis, we assume that slow-growing mycobacteria or even breakdown products thereof might elicit an allergic reaction in the background of a host's hyperergic predisposition. These allergens could be distributed to different organ systems via the circulation, eliciting the well-known perivascular granulomatous reaction.^{35,49}

Other investigators demonstrated *Propionibacterium acnes* DNA in sarcoidosis. A scenario similar to that with mycobacteria was reported. Propionibacteria DNA or RNA could be amplified from tissue sections of sarcoidosis granulomas, and in experimental settings these bacteria can elicit a granulomatous reaction.^{50–52} Many other agents have been discussed as probable causes or trigger mechanisms of sarcoidosis, including herpesvirus and Chlamydia.^{53,54} None of these has attracted as much attention and controversy as mycobacteria and propionibacteria.

Risk Factors

The course of sarcoidosis remains unpredictable. Approximately two thirds of patients will recover from the disease. However, in one third the disease progresses. The most serious

complication is lung fibrosis, starting with fibrosis of the granulomas and extending into the surrounding lung parenchyma. Many studies have focused on factors predicting fibrosis, because these patients should be treated with corticosteroids.

The combination *DQB1**0602/*DRB1**150101 haplotype was found to be positively associated with severe pulmonary sarcoidosis indicated by radiographic stages II–IV. However, there was no association with fibrosis.^{55,56} In the study by Kruit et al, an association of the *TGF-β3*17369C allele was demonstrated more frequently in patients with fibrotic lung than in patients with acute/self-remitting and chronic sarcoidosis.⁵⁷ The *TGF-β2* 59941G allele also was more abundant in fibrosis combined with sarcoidosis. *TGFβ1* gene polymorphisms were not associated with fibrosis.⁵⁷

Prostaglandin-endoperoxide synthase 2 (*PTGS2*) is a key regulatory enzyme for the synthesis of the antifibrotic prostaglandin E₂ and is reduced in sarcoidosis lung. A promoter polymorphism –765G>C in *PTGS2* identified individuals who are susceptible to sarcoidosis and, more importantly, at risk of pulmonary fibrosis. An altered Sp1/Sp3 binding to the –765 region may reduce *PTGS2* expression.⁵⁸

In Löfgren's syndrome, which usually presents with sudden onset, short duration, and quick resolution of the disease, other genetic variations have been demonstrated that could be responsible for this disease modification. In Löfgren's syndrome, significantly increased frequency of the *CCR2* haplotype 2 was observed when compared with controls.⁵⁹ A prevalence of *DRB1**03, HSP(+2437)-C and (+2763)-G was observed in patients with Löfgren's syndrome and might confer a susceptibility toward this variant of sarcoidosis.⁶⁰

The Steps of an Immune Reaction and What Might Happen in Sarcoidosis

If we suppose a working hypothesis that is based on the induction of the disease by different immunogenic agents, such as mycobacteria, propionibacteria, chlamydia, and even viruses or other unknown antigens, there are several steps within the immune reaction that might bear a gene modification or mutation conferring susceptibility to sarcoidosis (Figure 40.3):

- Antigen recognition and take up by macrophages or dendritic cells
- Antigen handover and antigen processing usually within dendritic cells and/or macrophages
- Antigen presentation to effector cells together with preselected costimulatory molecules
- Finally, the immune reaction by a concerted action of T and B cells⁴⁵

An upregulated allergic or hyperimmune reaction could be buried within each of these steps and might be responsible for the heterogeneity of clinical courses in sarcoidosis. Allergic reaction may have already been induced at the antigen presentation site. For example, antigen presented in association with costimulatory molecules could induce prolonged stimulation

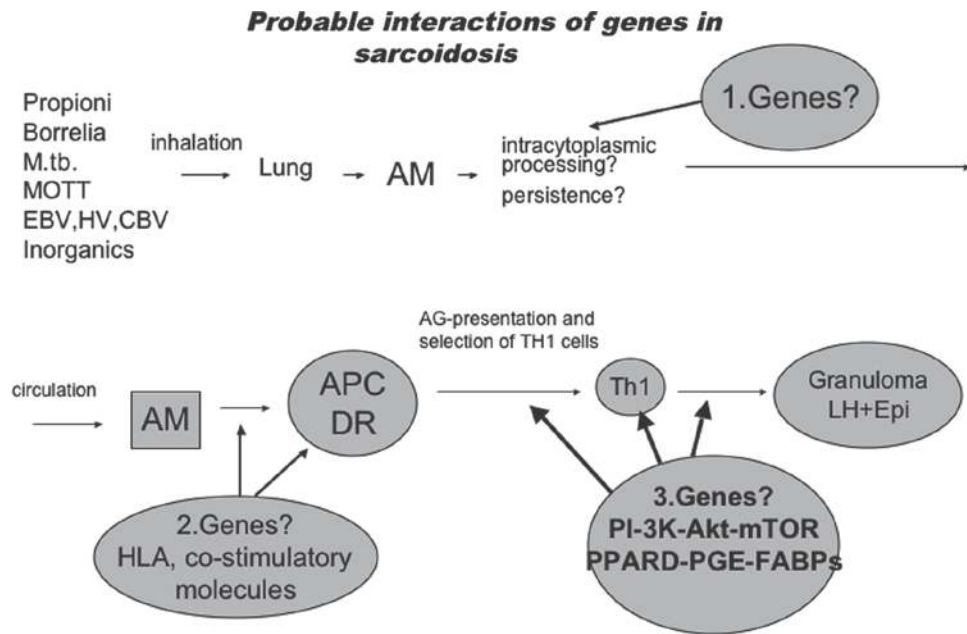


FIG. 40.3. Possible steps where in the pathogenesis of sarcoidosis genes might interact (probably the reason why this disease is so multifaceted). (1) Genes might be responsible for an inability to degrade invading organisms, thus leading to persistence of these organisms. The organisms might produce proteins and induce an autoimmune reaction. (2) The immune reactions might be altered

by a predisposition in the organism to select improper costimulatory molecules, thus inducing an adverse immune reaction. Here, also an alteration of antigen-presenting cells (APC) might come into play. (3) There might be a genetically abnormal and prolonged type IV immune reaction determined by genetic modification or mutations.

of lymphocytes as a result of phenotypic variations or point mutations in these molecules, resulting in increased susceptibility to sarcoidosis. Other possible explanations for a hyper-immune reaction include a hyperreactive effector cell system that has an exaggerated response to antigen stimulation or impairment of a counterbalance system such as apoptosis.

Antigen Uptake and Processing

It is reasonable to assume that the susceptible individual has had previous exposure to the antigen(s) that cause sarcoidosis. Otherwise, a short onset reaction as in Löfgren's disease would not be possible. It is well known that the tertiary antigen structure preselects the type of immune reaction. Selection of Toll-like receptors (TLRs), heat shock proteins, and so forth, determines the type of immune reaction toward an antigen as well as the cells for response: CD4 (Th1, Th2), CD8, or B cells.⁶¹ Antigens within the mycobacteria family, for example, dictate a Th1 reaction via an upregulation of various cytokines.^{62,63}

A failure in this selection process, or prolongation of the immune cell stimulation, could be one of the possible mechanisms of susceptibility to sarcoidosis. As noted previously, little is known about the regulatory mechanisms of activation and deactivation of the antigen-presenting cells.

Not much is known about the processing of antigens within macrophages or dendritic cells, although this might be another factor that influences disease susceptibility. If the antigens that

cause sarcoidosis are not degraded within a given time, they might persist intracellularly, resulting in protein transcription eliciting an allergic reaction.

Antigen Presentation, Costimulatory Molecules, and Gene Polymorphisms and Where They Come into Play

Antigen presentation is another potential step, where an allergic or hyperimmune reaction could occur. Processed antigens are presented to T cells in association with costimulatory molecules. This combination can exert opposite effects on the T cells. For example, CD28 activates phosphatidylinositol-3 kinase (PI3K) and Akt1 kinase, causing downstream impaired recruitment of procaspase-8 to the death-inducing signaling complex, inhibiting apoptosis.⁶⁴ Inducible costimulator (ICOS), another costimulatory molecule, augments PI3K levels, whereas only CD28 costimulation activates c-Jun N-terminal kinase. However, only CD28 induces high levels of IL-2 and Bcl-xL, which also prevent apoptosis.⁶⁵

Tripartite motif-containing genes (TRIMs) are costimulatory molecules and are able to stimulate proliferation of lymphocytes via an activation of the PI3K pathway. The TRIMs 5, 16, and 22 are upregulated in sarcoidosis and in Löfgren's disease.⁴⁹ This pathway might also contribute to the inhibition of apoptosis in sarcoidosis.

Toll-like receptors might play a role in the development of sarcoidosis. Toll-like receptor 4 is usually upregulated in response to certain substances present in the capsules of bacteria.^{51,66} An association of a TLR4 phenotypic mutation with chronic sarcoidosis has been found recently.⁶⁷ Toll-like receptors are an important mechanism for the maintenance of a balanced immune system. Toll-like receptors induce an upregulation of IL-12, IL-1 receptor associated kinase-M, and suppressor of cytokine signaling-1. Normally, an excess of these proinflammatory action proteins is limited by PI3K, which suppress IL-12.⁶⁸ An imbalance in this gate-keeping system might be another facet in the sarcoidosis mosaic.

Valentonyte et al have identified the butyrophilin-like 2 gene (*BTNL2*) as strongly associated with sarcoidosis.⁶⁹ The authors demonstrated a truncated protein whose function is impaired in sarcoidosis. In a subsequent study, a strong association with sarcoidosis susceptibility was shown for Caucasians but was weaker for African-Americans.⁷⁰ *BTNL2* is a member of the immunoglobulin superfamily of genes and is a homolog to B7-1. Recently, it was shown that *BTNL2* is a receptor expressed on activated B and T lymphocytes. It inhibits T-cell proliferation and T-cell receptor mediated activation of nuclear factor of activated T cells (NFAT), nuclear factor (NF)- κ B, and activator protein-1 (API) signaling pathways.⁷¹

Members of the HLA system, located on chromosome 6, are strongly associated with immune reactions as well as with autoimmune disorders. However, it can be concluded that HLA molecules associated with sarcoidosis have most often been identified as disease modifiers and, thus far, not as disease-causing agents. Polymorphisms have been identified in many of the HLA genes, and these are responsible for stimulation of the immune reaction and also for organotypic involvement.

Human Leukocyte Antigen Class I Genes

Human leukocyte antigen B8 (*HLA-B8*) has been found in sarcoidosis patients with acute onset and short duration of the disease.⁷² *HLA-B*07* and *HLA-B*08* have been associated with higher risk of developing sarcoidosis.⁷³ An association with persistent disease was conferred by a combination of the *HLA* alleles *A*03*, *B*07*, and *DRB1*15* (see also later discussion).

Human Leukocyte Antigen Class II Genes

In a genetic linkage study using 122 affected siblings from 55 families, Schtirmann et al have defined a locus close to major histocompatibility complex (MHC) II and III frequently altered in these affected persons.⁷⁴ In a follow-up study analyzing 63 families with affected siblings, this observation was expanded, and a peak was identified again at the D6S1666 marker.⁷⁵ In addition, other peaks were identified, including genes for chemokine receptor 2 (*CCR2*) and

5 (*CCR5*), T cell receptor B, TGF- β receptor 1, and IL-2 receptor. In a subsequent study focusing on *CCR2*, an association with sarcoidosis could not be confirmed.⁷⁶

In the ACCESS study, *HLA-DRB1* alleles were associated with sarcoidosis in Caucasians as well as African-Americans. The *DRB1*1101* allele confers risk in both ethnic populations, whereas the allele *DRB1*1501* was differently distributed among the tested patients and controls.⁷⁷ Another class II gene *DQB1*, namely, the allele **0602*, was significantly associated with progressive disease.⁵⁵ This was confirmed in a subsequent study within a European population.⁷⁸ *HLA-DR1* and *HLA-DR4* have been shown to be protective in Scandinavian, Japanese, Italian, English, Polish, and Czech populations.⁷⁹ A major problem with most of these studies is that they do not attempt to investigate the function of these HLA alleles, and thus the importance of mutations or phenotypic variations remains obscure.

Tumor necrosis factor genes are located on chromosome 6, in close proximity to most genes of the MHC complex. Tumor necrosis factor- α is a proinflammatory mediator found in most inflammatory conditions and also early in sarcoidosis.¹¹ A genetic variation within the TNF- α gene at positions 308 (G to A mutation, *TNFA*) has been shown to be associated with high TNF- α production and was found in Löfgren's disease,⁸⁰ but an 857 T mutation was observed in sarcoidosis and not in Löfgren's disease.⁸¹ This point mutation was associated with higher promoter activity and thus resulted in increased TNF- α release. Another variation in the first intron of the TNF- β gene (*TNFB*) was associated with prolonged disease but not with severity.⁸²

Effector Mechanisms, the Lymphocyte-Macrophage Network, and Gene Expression in Sarcoidosis

Once an immune reaction has been started, it is regulated through activation and deactivation. Regulatory T cells might be responsible for this fine tuning. However, as earlier, the susceptibility of patients to elicit a type IV granulomatous immune reaction might also be based on a hyperreactive T-cell system. Recently, it was shown that in bronchoalveolar lavage cells from sarcoidosis patients, predominantly composed of T-helper cells and macrophages, there was an upregulated signaling cascade for proliferation and a down-regulation of apoptosis. Conflicting reports have been published on the upregulation of apoptosis-associated ligands and receptors.⁸³⁻⁸⁵ Fas is highly expressed in sarcoidosis T lymphocytes, and high levels of soluble Fas ligand (FasL) are found,^{86,87} favoring apoptosis. However, once Fas and FasL are associated, they activate downstream death receptors, which subsequently cleave and activate caspase-8. Caspase-8 either cleaves Bid and thus activates the mitochondria mediated pathway or cleaves caspase-9 and activates the direct

apoptosis pathway finally leading to the activation of caspase-3. Petzmann et al found that the extracellular concentration of FasL or other proapoptotic molecules might have no effect, because the apoptosis signaling in sarcoidosis is blocked at the intracellular level of the mitochondria-mediated and the direct apoptotic pathways (Figure 40.4).⁴⁹ This was also confirmed in another study.⁸⁸ p21^{WAF1} is highly expressed in sarcoidosis⁸⁹ and inhibits apoptosis in alveolar macrophages and lymphocytes. p21 is predominantly regulated by IFN- γ and might be another mechanism by which macrophages and lymphocytes in sarcoidosis can escape apoptosis.

In addition to prolonged survival of CD4⁺ lymphocytes and macrophages in sarcoidosis, there is also an enhanced stimulation of proliferation by an activation of the PI3K/Akt2 kinase/mTOR/STAT3 pathway. The antagonist PTEN, which can interrupt the PI3K activation system, was down-regulated in sarcoidosis.⁴⁹ Moreover, in lavage cells of patients with Löfgren's, an additional stimulatory signal for proliferation was found. The mRNA for peroxisome proliferator-activated receptor (PPAR α) and its shuffle proteins fatty acid binding proteins 4 and 5 (FABP4/5) were all upregulated. The PPAR α needs FABPs for a translocation into the nucleus. If shuffled into the nuclei, PPAR α associates

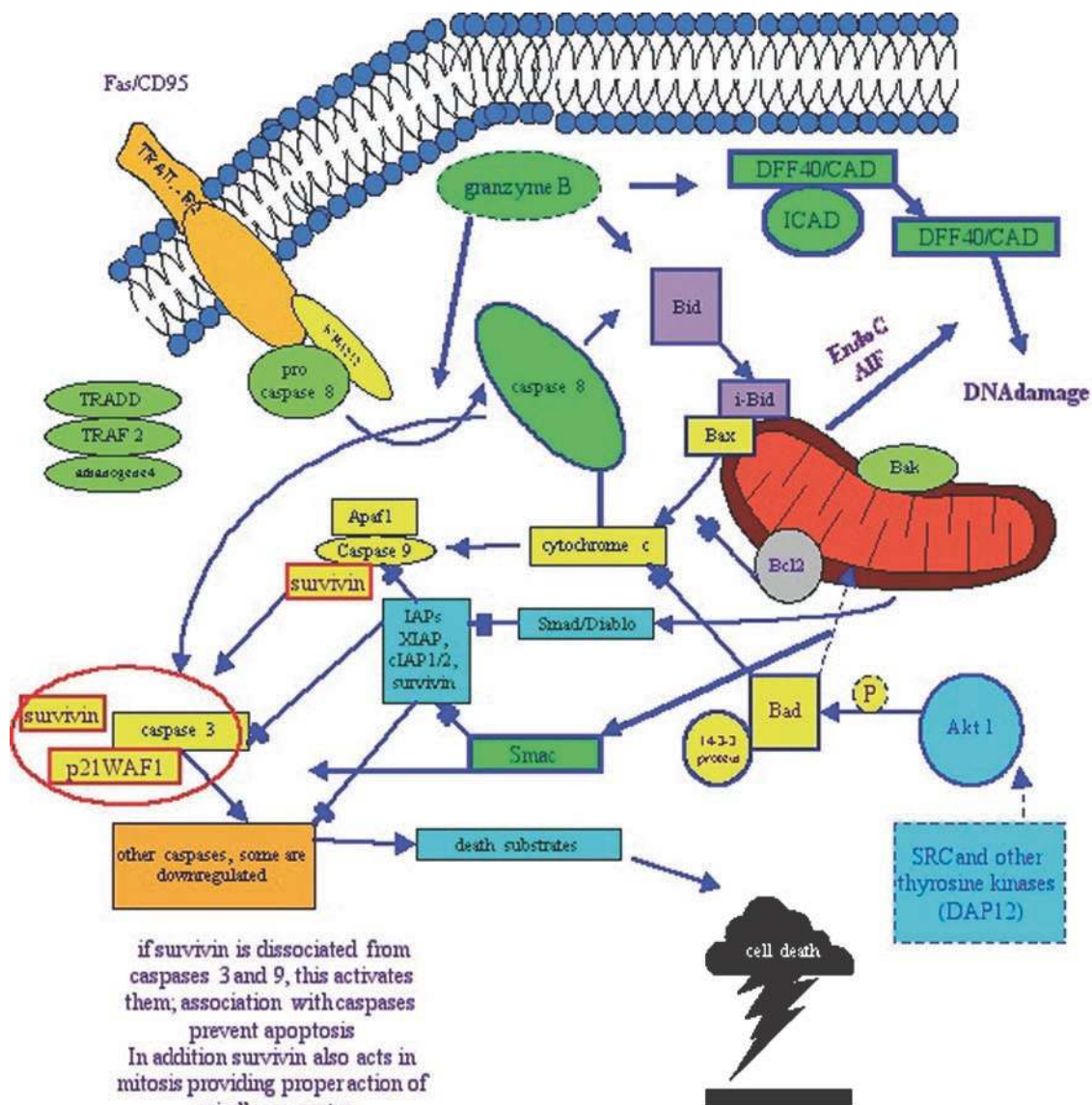
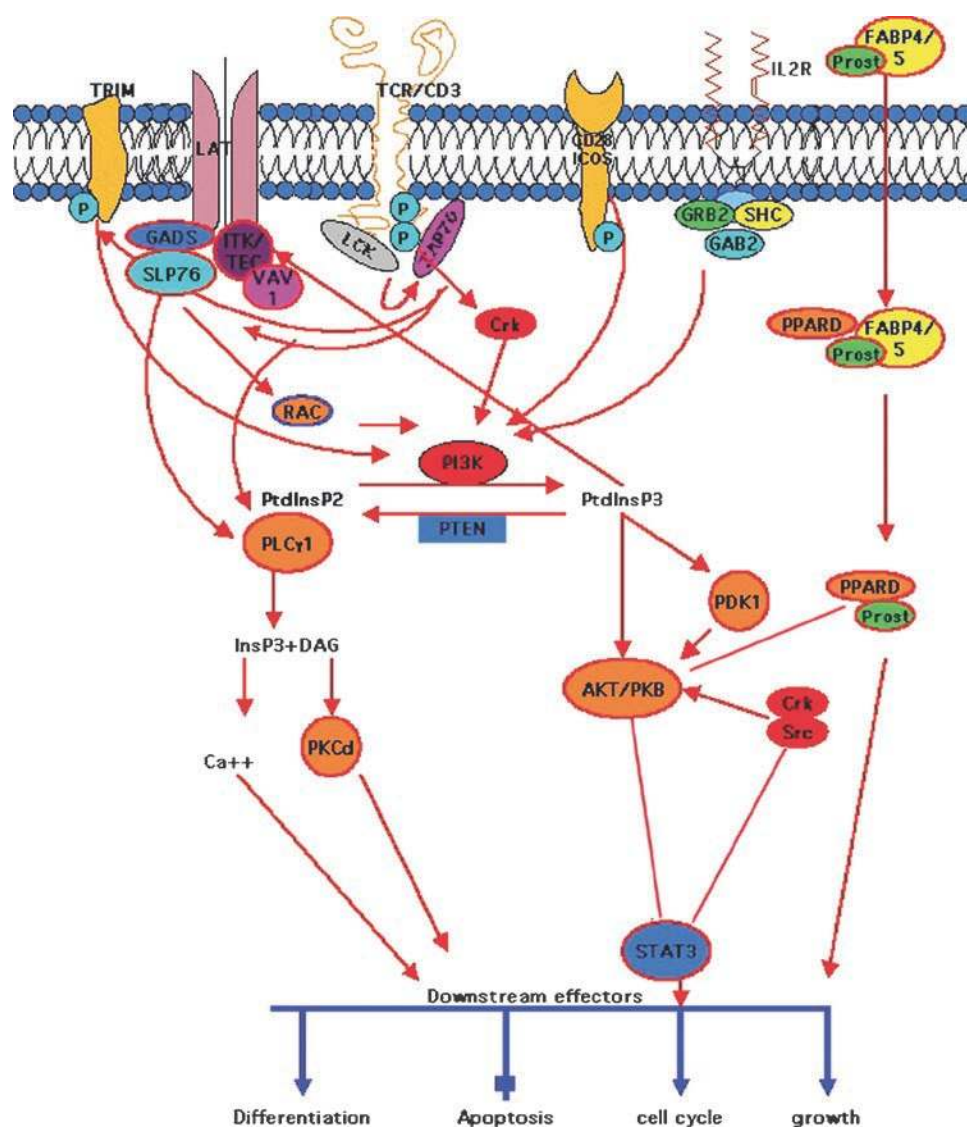


FIG. 40.4. Antiapoptotic mechanisms might prolong the lifetime of sarcoidosis lymphocytes. Independent of the concentration of outside proapoptotic ligands, there is a downregulation of key molecules within the mitochondrial apoptosis pathway (tumor necrosis factor receptor-associated death domain, tumor necrosis

factor receptor-associated factors, athanogene4, Bid), upregulation of antiapoptotic molecules (14-3-3, Bad, Akt), and upregulation of molecules that act antiapoptotic for the mitochondrial as well as intrinsic apoptosis pathways (surviving).

FIG. 40.5. Probable activation of proliferation pathways in sarcoidosis CD4⁺ lymphocytes and CD68⁺ macrophages. Tripartite motif molecules might be the main receptor type signaling into the phosphoinositol-3 kinase pathway and activating Akt and signal transducer and activator of transcription 3 downstream. Other receptors such as CD28 and inducible cosimulator and the T-cell receptor system might interplay here, because Crk is also upregulated. In Löfgrens disease (acute sarcoidosis), an additional proliferation pathway is turned on by prostaglandins and fatty acid binding proteins 4 and 5, which associate with peroxisome proliferator-activated receptor and again activate Akt.



with E-type prostaglandins or leukotrienes and stimulate cell proliferation.⁴⁹ This additional proliferation signal might be responsible for the rapid increase of macrophages and lymphocytes in this variant of sarcoidosis (Figure 40.5).

Disease Modifier Genes and Aspects of Organ Involvement in Sarcoidosis

A switch from Th1 to Th2 cells occurs in progressive sarcoidosis. There is an increase of IL-4, which can stimulate fibroblast proliferation. Interleukin-1 stimulates granuloma formation, fibroblast proliferation, and collagen deposition. Insulin-like growth factor I, TGF- β , and platelet-derived growth factor, produced and secreted by macrophages and epithelioid cells, might also be responsible for fibrosis.^{90,91}

Organs other than the lungs may be involved in sarcoidosis. It has long been recognized that organ involvement does not occur randomly. For example, sarcoid myocarditis

is common in Japan,⁹² whereas skin involvement is common in other ethnic groups.⁹³ This has prompted research for factors responsible for the distribution of specific organ involvement. Although at an early stage, several observations have been reported. Cardiac sarcoidosis probably is mediated by an *HLA-DQB1*0601* allele in addition to an unusual A2-allele of the *TNF* gene.^{94,95}

Cytotoxic T-lymphocyte antigen 4 (*CTLA-4*) with a phenotypic CC mutation at 318 and AG or GG mutation at position 49 was significantly associated with ocular involvement in association with involvement of three or more organs.⁹⁶ Cytotoxic T-lymphocyte antigen 4 is another costimulatory molecule that counterbalances T cell activation, serving as an antagonist to CD28.⁹⁷ Thus far, nothing is known about an impaired function as a result of phenotypic point mutations of the *CTLA-4* gene. No molecular genetic data are available regarding most other organ involvement in sarcoidosis, probably because these cases are rare and can only be investigated by multicenter studies to acquire sufficient numbers of patients for the study.

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Castleman's Disease

Richard Flavin, Cara M. Martin, Orla Sheils, and John James O'Leary

Introduction

Castleman's disease (CD) is a rare, benign lymphoproliferative disorder of lymph nodes that was first described by Dr. Benjamin Castleman in 1956. CD is also known as giant lymph node hyperplasia or angiofollicular hyperplasia.¹

Clinical Classification

Castleman's disease may be classified as:

- unicentric or multicentric, based on clinical and radiological findings
- hyaline-vascular, plasmacytic, or mixed variant based on histopathology.²

Unicentric Castleman's Disease

Patients with unicentric CD usually have a slow growing solitary mass typically located in the mediastinum or mesentery. There are no constitutional symptoms and no elevation of acute phase reactants (i.e., interleukin 6 (IL-6), erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP)). Symptoms, if present, are due to the mass effect of bulky lymphadenopathy. In general, there is no progression to lymphoma and there is no known association with other tumor types. In 90–95% of cases, surgical resection is curative and hence, the prognosis is excellent with a 5-year survival close to 100%.

Multicentric Castleman's Disease

Patients with multicentric CD typically present with widespread lymphadenopathy, with or without associated hepatosplenomegaly. "B" symptoms including severe fatigue, night sweats, fever, weight-loss and anorexia, are usually present. Typically, there is overproduction of IL-6 with elevation of acute phase reactants ESR, CRP, and fibrinogen with associated

anemia, thrombocytosis, hypergammaglobulinemia, and hypoalbuminemia. Approximately 20% of patients will have peripheral neuropathy. Multicentric CD is associated with a variety of conditions, including human immunodeficiency virus type-1 (HIV), autoimmune hemolytic anemia (AIHA), multiple myeloma, amyloidosis, and pemphigus vulgaris.^{3,4} It is important to recognize the association with HIV, since the prognosis is worst, and therapy is focused upon the same protocols used for treating the underlying HIV infection (described later in this chapter; also see Chap. 29). In addition, it may be associated with POEMS syndrome (i.e., Polyneuropathy, Organomegaly, Endocrinopathy, M-protein, and Skin).⁵ Multicentric CD tends to run an aggressive course, often requiring systemic therapy, and may in a few cases progress to non-Hodgkin lymphoma. Malignancies have been reported to arise in as many as 32% of patients with multicentric Castleman's disease. Clonal changes have been rarely described; however, these have been associated with discordant immunogenotypic and immunophenotypic findings, suggesting a type of lymphoproliferative disorder that lies between benign and malignant diseases.^{2,6} Subsequent infection seems to be the main cause of death in most cases.^{1,7,8}

HIV Status and Castleman's Disease

Today, multicentric CD is most commonly observed in individuals infected with HIV, who tend to present at a younger age.⁹ As such, the clinical course is less favorable than in HIV-negative patients, as mentioned previously.¹⁰

In HIV-positive patients, multicentric CD is associated with human herpesvirus type 8 (HHV8),¹¹ which is a Kaposi's sarcoma-associated herpesvirus. Most patients will also develop Kaposi's sarcoma (KS) (up to 70%).^{12–15} Even though life expectancy in multicentric CD seems to have significantly improved in the HAART (highly active anti-retroviral therapy) era, it remains a disease with a poor prognosis and an increased incidence of non-Hodgkin lymphoma in the HIV-context.¹⁶

Epidemiology

CD is very rare. It usually affects adults but may also occur in children. Pediatric patients appear to have a more favorable outcome than adults.¹⁷ About 70% of affected patients with unicentric CD are less than 30 years old. Males and females are affected in equal numbers. Patients with multicentric CD tend to be older individuals, in their fifth or sixth decade.

Histopathological Classification of Castleman's Disease

There are two main variants: the hyaline-vascular and plasmacytic subtypes. The hyaline-vascular variant is more common (90% of cases). This variant consists of poorly formed germinal centers (GCs) surrounded by an expanded mantle zone, consisting of small CD20 positive lymphocytes arranged in an onion skin manner (Figure 41.1). This is associated with a network of dysplastic/atrophic CD21 positive follicular dendritic cells. Follicles usually have a central targetoid vascular proliferation with thick and hyalinized blood vessel walls and hyalinization of the GCs themselves.

There is also increased interfollicular cellularity with hyperplastic post-capillary venules, plasma cells, eosinophils, immunoblasts, and plasmacytoid monocytes.

The rarer plasmacytic variant of CD is characterized by more numerous and larger hyperplastic follicles, which have more expanded mantle zones, when compared to hyaline-vascular CD. Sheets of plasma cells are present in the interfollicular areas, and Russell bodies are often found. The center of the follicle may have amorphous eosinophilic material, due to fibrin and immune complex deposition. The mixed cellularity form of CD has features of both hyaline-vascular and plasmacytic subtypes of CD but clinically behaves more like the plasmacytic subtype.

Relationship Between Histological Type and Clinical Classification

Classically, it was thought that unicentric CD is of the hyaline-vascular variant, and multicentric CD is of the plasmacytic subtype or mixed cellularity variant. As CD is so rare, these relationships were based on the analysis of small patient cohorts. A review of 37 patients with CD, treated at the University of Arkansas Medical Sciences (the largest single institution experience in the world with this disease), found that patients with unicentric CD typically have the hyaline-vascular variant of the disease. However, they found that both the hyaline-vascular variety and the plasmacytic/mixed cellularity variants are found with equal frequency in multicentric CD.

Pathogenesis of Castleman's Disease

Human Herpes Virus 8 (HHV8) is a γ -Herpes virus, which is homologous to Epstein-Barr virus and Herpes Virus Siamuri. It is tropic for B-lymphocytes and has been associated with KS and primary effusion lymphoma (also see Chap. 7).^{18,19} Recently, epidemiologic and polymerase chain reaction (PCR) studies have shown that CD is strongly associated with HHV8.^{20–22} The presence of HHV8 DNA in HIV-positive patients antedates development of multicentric CD (5–25% of cases) and may be detected in the lymph nodes and peripheral blood mononuclear cells. This finding may be of clinical use, as quantitative PCR for HHV8 DNA may be used for monitoring disease activity and therapy response in HIV-positive patients. Furthermore, the genome of this virus harbors an analog of the IL-6 gene. It has been proven that the introduction of HHV8 into mice via a retroviral vector causes polyclonal hypergammaglobulinemia with plasma-cell hyperplasia, and the resulting condition mimics multicentric CD.^{23,24} Moreover, treatment of multicentric CD with monoclonal antibodies against IL-6 confers therapeutic benefits.²⁵

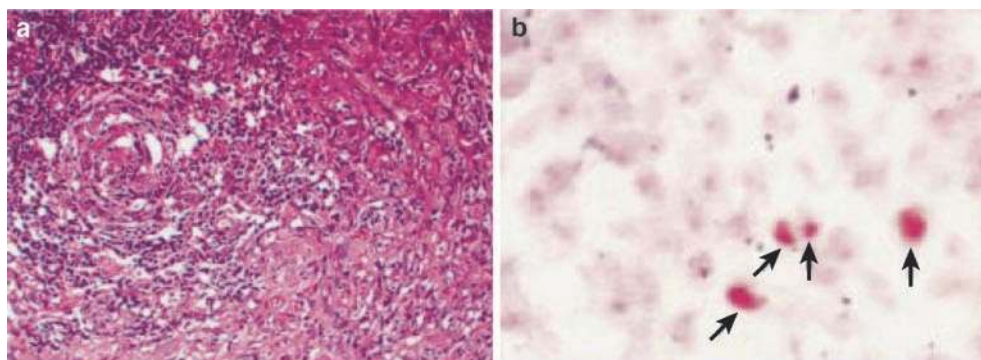


FIG. 41.1. (a) Hematoxylin and eosin section of hyaline-vascular type CD. (b) In-situ hybridization analysis of CD showing positivity for HHV-8 in lymphocytes. From O'Leary J, Kennedy M, Howells

D, et al. Cellular localization of HHV-8 in Castleman's disease: is there a link with lymph node vascularity? *Mol Pathol*. 2000;53: 69–76 with permission from BMJ Publishing Group Ltd.

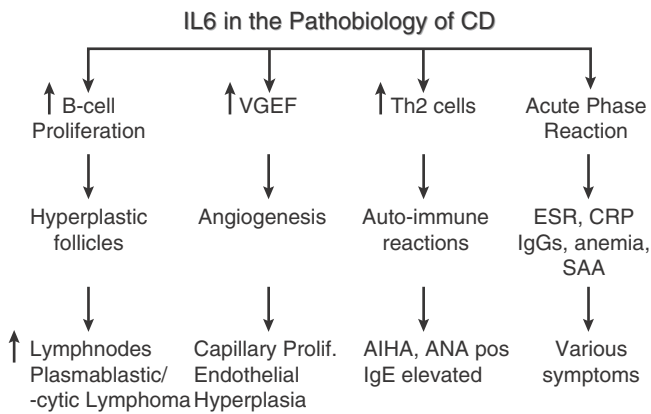


FIG. 41.2. The role of IL-6 in the pathobiology of CD. From O'Leary J, Kennedy M, Howells D, et al. Cellular localization of HHV-8 in Castleman's disease: is there a link with lymph node vascularity? *Mol Pathol.* 2000;53:69–76 with permission from BMJ Publishing Group Ltd.

The role of HHV8 in the pathogenesis of CD in HIV-negative patients is inconclusive.

IL-6 has been implicated in the pathophysiology of CD (see Figure 41.2).^{23,26–29} It has been postulated that HHV-8 produces IL-6. Increased levels of IL-6 result in B-cell proliferation, which is seen morphologically as hyperplastic follicles within enlarged lymph nodes. IL-6 also increases secretion of vascular endothelial growth factor (VEGF), causing angiogenesis and capillary proliferation with endothelial hyperplasia within lymph nodes.

IL-6 is responsible for polarization of T-lymphocytes to a type 2 cytokine profile, leading to autoimmune phenomena, including AIHA, ANA positivity, and elevation of IgE. IL-6 induces an acute phase reaction, comprising increases in ESR, CRP, IgG, serum fibrinogen, and serum amyloid A (SAA) protein. Increased SAA levels may result in AA amyloidosis, while hyperfibrinogenemia may play a role in venous thrombosis and various thrombotic sequelae. Finally, B-type symptomatology is virtually always associated with increased IL-6 levels.

Diagnosis

The diagnosis of CD is based upon a thorough clinical evaluation that includes a detailed patient history, laboratory studies (including IL-6, ESR, and CRP), histopathology of affected lymph node(s), and a variety of imaging techniques (i.e., CT, MRI, and PET-scanning).³⁰ A PET scan may complement CT-scanning by giving detailed information regarding the metabolic status of the affected lymph nodes. Usually the specific uptake values of FDG-avid lymphocytes are less in normal lymph nodes than those observed with active lymphoma. PET-scanning is also used in the assessment

of treatment response. No increase in metabolic activity is detectable by PET scan in patients responding to treatment with IL6-receptor antibody.

Therapy

Surgical excision is the preferred treatment in most cases of unicentric CD, and adjuvant therapy (i.e., steroids and/or rituximab before surgery) is useful to shrink bulky or inoperable disease. The highly vascular nature of the tumor makes surgical management challenging, and it warrants preoperative embolization whenever possible.³¹ In some cases, radiotherapy has proven effective, in unresectable cases.⁶

A number of therapies have been used for multicentric CD: intravenous immunoglobulin, rituximab, steroids, anti-herpes drugs (i.e., acyclovir; ganciclovir in HIV-positive and HHV8-positive CD), HAART (HIV-positive cases), combination chemotherapy (i.e., CHOP), and in intractable cases, autologous stem cell transplantation.^{16,32–34} Adjunct therapies include the anti-angiogenesis factor, thalidomide, and anti-IL6 therapy.

Anti-IL6 therapies include suramin, anti-IL-6, or anti-IL-6 receptor antibody.^{35,36} Suramin is a polysulfonated urea compound, originally used for the treatment of Trypanosomiasis. It acts through inhibition of viral reverse transcriptase and mediates a number of biological effects through inhibition of growth factor, modulation of cytokine secretion, and prevention of cytokine binding to their complementary receptors (i.e., IL-6, IL2, PDGF, and FGF). Anti-IL-6 antibody is particularly effective in controlling IL-6 related symptoms, but importantly it may induce disease regression with durable remissions. More recently, the use of the anti-CD20 monoclonal antibody, rituximab, in large single center cohorts has been associated with prolonged remissions, radiologic responses, as well as hematologic and serum chemistry normalization of the inflammatory picture, at the expense of B-cell depletion and flare of KS.⁹

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Molecular Pathology of Histiocytic Disorders

Mihaela Onciu

Introduction

Histiocytic disorders are diseases characterized by the proliferation and/or accumulation of histiocytic cells in bone marrow (BM), peripheral blood (PB), and a variety of extramedullary tissues, associated with variable local and systemic symptoms, which depend on the disease subtype. Histiocytes are hematopoietic cells that derive from BM progenitors and have important functions in the immune system, including antigen presentation and antigen processing. Normal histiocytes include multiple functional subsets with distinct morphology, immunophenotype, and tissue localization. Histiocytic disorders are currently classified according to their morphologic and immunophenotypic resemblance to these normal counterparts, as well as according to their clinical presentation and biologic behavior (Table 42.1). The immunohistochemical classification of the tumors of histiocytes and accessory dendritic cells proposed by the International Lymphoma Study Group^{1,2} comprises two major categories: disorders of varied biologic behavior and malignant disorders. This chapter will focus on the former category, which includes clonal and non-clonal entities, none of which may be definitively classified as malignant, although their clinical behavior may be aggressive and even fatal (Table 42.2). The malignant disorders include sarcomas with histiocytic or dendritic cell differentiation, as well as acute myeloid leukemias (AMLs) with monocytic or monoblastic differentiation. Due to the rarity and thus lack of a significant amount of molecular data, sarcomas with histiocytic or dendritic cell differentiation will not be addressed in this chapter. For a discussion of AMLs with monocytic or monoblastic differentiation, see Chaps. 34 and 35.

Langerhans Cell Histiocytosis

Clinicopathologic Features

Langerhans cell histiocytosis (LCH) is a disease characterized by accumulation of epidermal antigen-presenting dendritic cells [i.e., Langerhans cells (LCs)] in various organs

and tissues of the body. LCH may occur at any age, but most commonly in children, where it has an annual incidence of 1:200,000 per year, with a peak age of 1–3 years.³ It may occur as de novo disease or in association with a variety of malignant neoplasms, most often non-Hodgkin lymphoma and solid tumors, most commonly lung adenocarcinoma.^{4,5} Several forms of disease have been described that show similar histologic features, but differ in extent of organ involvement and associated systemic symptoms. Unifocal disease (formerly known as eosinophilic granuloma), manifested most often as a solitary bone lesion, occurs in approximately two thirds of the patients, typically older children or adults. This form may be treated with curettage of the lesion, intralesional steroid, and low-dose local radiation therapy, and has a 100% survival rate. Multifocal (and multisystem) disease with multiple organ failure (previously termed Abt-Letterer-Siwe disease) affects multiple organs (i.e., bones, skin, liver, spleen, and lymph nodes), occurs primarily in infants, and carries a 20% mortality rate despite combination chemotherapy. Lastly, an intermediate clinical presentation is that of multifocal single organ disease (previously named Hand-Schüller-Christian disease), that presents with several sites of involvement in one organ system, typically bone, affects older children and, while requiring chemotherapy, has an excellent survival (100%).⁶ A unique disease subset is that of pulmonary LCH, primarily affecting smokers in the third to fifth decade of life and typically women, that presents with innumerable bilateral lung nodules, less than 2 cm in size. The clinical course is characterized by spontaneous stabilization or regression, with progression to end-stage lung fibrosis in only 10–20% of the patients. Steroid therapy and smoking cessation are usually the only recommended treatment.⁷

Histologically, regardless of the clinical presentation, all of the LCH lesions have a pseudo-granulomatous appearance and contain numerous LCs, associated with variable numbers of eosinophils (sometime with eosinophilic abscesses), histiocytes (including osteoclast-like giant cells), neutrophils, and small lymphocytes. LCs may be recognized by their characteristic histologic features (i.e., moderately abundant lightly eosinophilic cytoplasm, indented,

TABLE 42.1. Immunophenotypic subtypes of histiocytic disorders.

Histiocyte subtype	CD45	CD1a	S100	CD21	CD35	FactorXIII	Langerin (CD207)/ Birbeck granules ^a
Monocyte/macrophage	+	–	+/-	–	–	–	–
Langerhans cell	+	+	+	–	–	–	+
Indeterminate cell	+	+/-	+	–	–	–	–
Follicular dendritic cell	–	–	-/+	+	+	–	–
Interdigitating reticulum cell	+	–	+	–	–	–	–
Dermal dendrocyte	+	–	–	–	–	+	–

^aIdentification of Birbeck granules requires electron microscopic examination.

TABLE 42.2. WHO classification of histiocytic disorders of varied biologic behavior.

Dendritic cell-related histiocytic disorders

- Langerhans cell histiocytosis
- Secondary dendritic cell processes
- Juvenile xanthogranuloma and related disorders
- Solitary histiocytoma of dendritic cell phenotype

Macrophage-related histiocytic disorders

- Hemophagocytic syndrome (primary/familial or secondary)
- Rosai-Dorfman disease (also see Chap. 43)
- Solitary histiocytoma with macrophage phenotype

folded or grooved nuclei with inconspicuous nucleoli), immunophenotype (see below), and the presence of Birbeck granules on electron microscopy. The latter, considered a hallmark of normal and disease-associated LCs, are 200–300 nm pentalaminar tennis racket-shaped cytoplasmic structures. While their function is yet unclear, they are associated with expression of a type II C lectin displaying mannose-binding specificity (i.e., langerin, CD207)^{8,9} that may be demonstrated using immunohistochemical staining. Expression of the langerin gene appears to be essential for Birbeck granule formation. Experimental evidence suggests that Birbeck granules may have a temporary storage function. They may retain antigens internalized by the LC via the langerin receptor, until the cells reach the T-cell dependent areas of the lymph nodes, at which point the Birbeck granules disappear and the antigen is exported back to the cells surface for presentation, in association with CD1a.⁸ CD1a, a MHC class I-like sialoglycoprotein that is also abundantly expressed in LC, appears to accumulate in the Birbeck granules following endocytosis and is again exported for antigen presentation in the lymph nodes.⁸

The biology of LCH is still being unraveled. Immune dysregulation, the contribution of yet unidentified host genetic factors, and a disturbed cytokine network associated with defective Langerhans cell maturation, have all been implicated in the pathogenesis of this disease. Furthermore, a subset of LCH appears to be clonal with chromosomal abnormalities and disrupted tumor suppressor genes, suggesting the possibility of a neoplastic process. All of these factors will be further discussed below.

Immunohistochemistry of LCS in Normal Tissues and LCH

Normal human LCs express a variety of myelomonocytic antigens and functional molecules to match their role in antigen processing and antigen presentation. They typically express CD1a, CD45, CD13, CD33, CD68, CD163, S-100 protein, integrins (i.e., CD11c and CD49), adhesion molecules (i.e., CD15, CD40, CD44, CD50, CD54, and cutaneous lymphocyte antigen – CLA), E-cadherin (CD324), CD95, CD95L, CD120b, MHC class I and II molecules, CD74, CD1c, CD80, CD83, and CD86. In addition, they express receptors for interleukins (i.e., IL-1, IL-6), TNF α , GM-CSF, IFN γ , Fc γ RI (CD64), Fc γ RII(CD32), and Fc ϵ RI (CD23).⁸ In practice, for diagnostic purposes, the antigens that define the proliferating cells in a lesion as LCs include CD1a, S-100 protein, and langerin, although the latter is not mandatory and CD1a is an acceptable surrogate.

LCs of LCH appear to best resemble the morphology and immunophenotype of immature LC cells, at least in the systemic and multifocal forms. This is best defined by the profile of cytokines and cytokine receptors expressed by these cells (see below). Like normal immature LCs, they also display higher levels of CD14 and CD68 than normal mature LCs, show cytoplasmic MHC class II expression, and are frequently negative for CD80, CD86, and DC-LAMP (mature LC markers).^{8,10} Normal immature LCs reside in skin and have antigen uptake and processing properties. As they move to draining lymph nodes, they lose these properties and become mature LCs with functions in antigen presentation. The LCs of LCH show a maturation block that seems to be most likely related to their cytokine-rich inflammatory milieu, rather than an intrinsic property.^{10–12} LCs in the unifocal and/or self-limited cutaneous LCH lesions have been found to have a more mature phenotype (i.e., CD14–, CD86+)¹² that may explain the benign clinical course of these forms of disease.

A rare entity that has significant clinical and immunophenotypic overlap with LCH is indeterminate cell histiocytosis. This disease, affecting children and adults, has a predominantly cutaneous presentation, with solitary or multiple nodules. A minority of patients may also have conjunctival

and bony lesions. Histologically, these lesions have features intermediate between LCs and macrophages with a variety of patterns, including xanthogranulomatous, spindled, scalloped, or foamy appearance.¹³ Immunophenotypically, the monocytic cells express CD68, S-100, and variable levels of CD1a. Importantly, all lesional cells lack Birbeck granules and, likely, langerin expression. Some of the cases with cutaneous involvement have been treated successfully with topical therapy.¹⁴ The postulated cell of origin is a subtype of cutaneous dendritic cell.

Host Genetic Factors in Langerhans Cell Histiocytosis

Epidemiologic Data

Increasing evidence supports a role for a genetic component, at least in a subset of LCH patients. Familial clustering has been reported in this disease. One study¹⁵ reports an affected relative (i.e., parent, sibling, or first cousin) in 1% of children with LCH. In addition, a striking concordance rate (86%) is reported in the same study for couples of monozygotic twins developing LCH, with a much lower rate (12%) reported for dizygotic twins. Furthermore, this concordance involved not only disease occurrence, but also clinical features, pattern of dissemination, and age of onset. Interestingly, LCH occurred simultaneously in five of the reported seven couples of monozygotic twins.

Association with congenital abnormalities also occurs with increased frequency in patients with LCH. Sheils and Dover¹⁶ found congenital abnormalities of variable severity in 23% of the LCH patients studied. These included malformations of the central nervous system, bones, and genitourinary system. The frequency of anomalies was even higher when only patients with multisystem presentation and some form of organ dysfunction were considered. There was also a trend toward an earlier age of LCH onset in patients with congenital abnormalities. The association of LCH with other tumors, preceding, concurrent with, or following LCH has been well described in the literature, although, due to the nature of the reported studies, it is not possible to infer the true incidence of associated malignancies in this disease.^{4,17} The malignancies associated with LCH include lymphoma (Hodgkin and non-Hodgkin), acute leukemia, and a variety of solid tumors [the most common of which include lung carcinoma (adeno- and squamous cell carcinoma) and brain tumors of various histologic subtypes]. Most of the cases of acute leukemia preceding LCH are acute lymphoblastic leukemias. Leukemias following LCH are mostly acute myeloid leukemias and fit the pathologic and clinical criteria for therapy-related disorders. An interesting occurrence is that of concurrent malignant tumors, especially non-Hodgkin lymphoma,^{5,17} often within the same tissues. While in many of these cases a reactive nature has been postulated for the LCH cells, occasional reports⁵ have also documented a clonal relationship

between the two processes, where microdissected LCH cells contained an immunoglobulin gene rearrangement similar to that found in the adjacent follicular center cell lymphoma cells. Such findings raise the possibility of a common progenitor cell for B-lymphocytes and LCs.

Increased Chromosomal Breakage in LCH Patients

Constitutional cytogenetic studies, using phytohemagglutinin-stimulated PB lymphocytes, have found increased numbers of chromosomal breaks and chromosomal pulverization in a high percentage of patients with LCH.¹⁸ Higher numbers of breaks were observed in patients with multisystem disease, when compared to those with single system disease. None of these patients showed features of known chromosomal instability disorders. It remains to be established to what extent these observed abnormalities were truly present in germline, or secondary to environmental or viral exposure. A rare pediatric patient with ataxia, ocular telangiectasia, chromosome instability, and LCH (but without ATM gene mutations) has also been reported.¹⁹

The Role of Cytokines and Cytokine Gene Polymorphisms in LCH

Accumulating evidence suggests that the pathogenesis of LCH involves, at least partially, the dysregulation of the normal chemokine network that directs the mobilization of LCs from skin to regional lymph nodes. It has been suggested that in LCH, LCs are at an arrested state of activation and/or differentiation, acting as functionally abnormal immature dendritic cells.^{10,12} Similar to immature and early maturing LCs, they express receptors for inflammatory cytokines, including CCR6, but not CCR7, and are a major source of CCL20-MIP-3 α , CXCL5/RANTES, and CXCL11/I-TAC, also inflammatory chemokines. This profile likely plays a role in the accumulation of LCs, the mobilization of other types of inflammatory cells at the sites of disease (i.e., CXCL5 for eosinophils, CXCL11 for CXCR-3-positive activated T-lymphocytes), and the systemic symptoms associated with LCH. It is then not surprising that single nucleotide polymorphisms in cytokine genes, correlating with altered levels of the corresponding cytokines, have been found to correlate with disease subtypes in LCH.²⁰ The IL-1R +970 T polymorphism was found with increased frequency in LCH patients with multi-system disease. Increased levels of IL-4 (IL-4 high producer phenotype), corresponding to the IL-4 -590C/T and T/T, have been associated with single system disease occurring in patients older than 2 years. The intermediate interferon (IFN) γ producer phenotype, corresponding to the IFN γ +874A/T polymorphism, was found to associate with single system disease in patients 2 years of age or younger. The association of these profiles of chemokine production with lower clinical aggressiveness may be due to their predominantly anti-inflammatory role, as noted in other settings (i.e., rheumatoid arthritis).

HLA Haplotypes in LCH Patients

The implication of genetic predisposition in the pathogenesis of LCH, possibly via immune mechanisms, is underscored by studies showing striking associations of this disease with certain human leukocyte antigen (HLA) haplotypes. It has been shown that in LCH patients of various ethnic backgrounds, there is an increased incidence of the HLA Cw7 type, as well as of the HLA B haplotypes B7 and B8, typically exhibiting linkage disequilibrium with Cw7. The Cw7 type has been associated with skin or single bone lesions, but not with the multisystemic type of LCH, also suggesting a possible role for the genetic background in the specific clinical presentation of this disease. In addition, Caucasian patients with single bone lesion LCH show a strong association with the HLA DR4 type when compared to normal American Caucasian controls (67% vs. 24%, respectively). Overall, Cw7 or DR4 are present in a high proportion of Caucasian LCH patients (86%) and in 100% of the Caucasian patients presenting with LCH as a single bone lesion.²¹ These HLA haplotypes may be associated with alterations in the effectiveness of antigen presentation and possibly with inhibition of NK cell function, thus inducing immune alterations that may lead to LCH.

CD45 Splicing Defects

Leukocyte common antigen (i.e., CD45) is a tyrosine phosphatase expressed on hematopoietic cells and essential for lymphocyte receptor signal transduction. Several polymorphisms have been described for the encoding gene (i.e., *PRPDC*, located on chromosome 1q31-21), some of which lead to altered splicing into CD45 isoforms (including CD45RA and CD45RO). One of the latter polymorphisms (i.e., C77G) has been associated with altered immunity and autoimmune disorders (i.e., multiple sclerosis, systemic sclerosis, and autoimmune hepatitis) and with rare cases of hemophagocytic lymphohistiocytosis. This polymorphism occurs with variable frequency in Caucasian populations (0.5–3.6%) and has not been found in any of 527 healthy African individuals studied. It also appears to occur with increased frequency (4.8%) in Italian patients with LCH.²² Individuals carrying the C77G polymorphism demonstrate altered proportions of CD45RA and CD45RO expression in their normal T-cells, with concurrent alterations in adhesion molecules (including CD11a, CD62L, CD27, and CD95). Additionally, increased expression of CD45RA (when compared to the normally expressed CD45RO) has been observed in the monocytes of these individuals. The few Italian LCH patients carrying this polymorphism that have been identified were all younger than 18 years at presentation but showed no other association with clinical presentation or outcome. The importance of this association with LCH in populations of different ethnic backgrounds remains to be established at this time.

Clonality Studies in LCH

Conventional Cytogenetics and Ploidy Studies

Cytogenetic analysis is unsuccessful in most cases of LCH.²³ Hence, there is only limited information available regarding karyotypic abnormalities in this disorder. A single study²⁴ reported successful karyotyping of lesional tissue and BM from five cases of LCH. In three of these cases, chromosomal breakage studies were also performed. The main findings were a translocation t(7;12)(q11.2;p13) (unknown partner genes) present in lesional tissue from one case and inv(13)(q21q33) present as constitutional abnormality in one patient. The remaining three cases had a normal diploid karyotype. Interestingly, complex non-clonal abnormalities were found in four of these five cases. These abnormalities correlated with an increased number of chromosomal breaks were also found in three of these four cases. Most of these breaks were small chromatin breaks, with 2q31 as the only recurring site of breakage.

Most studies have failed to demonstrate DNA aneuploidy in LCH. A single report²⁵ detected small (3% and 5%, respectively) aneuploid populations with DNA indices of approximately 1.5 in paraffin embedded and frozen tissues from 2 of 18 pediatric patients with disseminated LCH.

HUMARA Studies

Clonality studies using molecular analysis of X-chromosome inactivation patterns, and especially the highly polymorphic human androgen receptor (HUMARA) gene locus on this chromosome, have found evidence of clonality in LCs microdissected or purified by flow cytometry from small numbers of cases representative of each subtype of LCH (16 of 16 cases tested).^{23,26–28} In addition, sequential testing of one case of recurrent disease has shown a clone with similar pattern of HUMARA allele inactivation present at diagnosis and relapse.²⁶

Testing of microdissected lesional dendritic cells from pulmonary histiocytosis has rendered less consistent results, with some studies finding evidence of clonality in all of the cases tested,²³ while others found evidence of clonality in only approximately half of the patients examined.⁷ Interestingly, in the latter group, when more than one LC nodule was examined, some of the nodules from a given patient were found to be clonal and others polyclonal. These findings suggest the possibility of a clonal process emerging in the background of a reactive LC expansion, in this particular clinical subtype of LCH.

Comparative Genomic Hybridization (CGH) and Loss of Heterozygosity (LOH) Studies in LCH

CGH and LOH, applied to a small group of unifocal and bifocal lytic bone LCH lesions, identified a recurrent pattern of chromosomal imbalances, suggestive of clonality.²⁹ By CGH, losses predominated over gains and clustered at

chromosomes 1 (1p21-1p36), 5, 6, 7(7p), 9 (9p), 16, 17, and 22 (22q). Gains of genomic material were less common and were localized on chromosomes 2q, 4q, and 12. The same study performed LOH analysis using 85 polymorphic microsatellite markers corresponding to known loci mapped on chromosomes 1, 7, 9, and 22. Allelic imbalance was identified in four of the seven cases analyzed, involving at least one locus on the analyzed chromosomes, the most common of which was 1p35-36 segment (confirmed by 6 of 19 microsatellite markers used). These findings further support the view that LCH is a clonal disorder. Additionally, the genomic instability and frequent loss of genetic material suggest the putative role of tumor suppressor gene alterations in the biological behavior of LCH.

Gene Expression Profiling in LCH

Limited gene expression profiling studies have been performed in LCH. The most comprehensive study was reported by Rust et al,³⁰ who applied serial analysis of gene expression (SAGE) technology to LCs derived from normal cord blood stem cells, as well as to lesional tissue from 14 patients with LCH, encompassing all clinical subtypes of disease. The goal of this study was to identify LC-specific genes and investigate their expression in LCH. Seven LC-specific genes were identified and found to also be highly expressed in LCH, with some differences between clinical forms of disease and anatomic site of involvement. These genes included *FSCN1*, *GSN*, *CD207*, *MMP12*, *CCL22*, *CD1a*, and *CCL17*. These findings also correlated with protein expression, documented by immunohistochemistry. Expressions of *CD1a*, *CD207*, and gelsolin (*GSN*) were found to be highest in skin and bone LCH, fascin (*FSCN1*) expression was high in all LCH types and tissues. Chemokines (i.e., *CCL17* and *CCL22*) showed highest expression levels in LCH involving lymph nodes and skin and metalloproteinase 12 (*MMP12*) expression was most abundant in multisystem LCH. The expression levels of all seven genes were lower in adult eosinophilic granuloma than the other LCH types. These differences suggest that the different LCH subtypes might be associated with different mechanisms of pathogenesis, including differences in cell motility and extracellular matrix degradation. Furthermore, expression of the chemokines (i.e., *CCL17* and *CCL22*) offered a possible explanation for the presence of abundant activated T-helper 2 (Th2) lymphocytes observed in LCH lesions, as these factors bind strongly and specifically to the chemokine receptor CCR4, expressed by the latter lymphocytes.

Alterations of Cell Cycle Proteins in LCH

Limited information is available regarding alterations of cell cycle proteins in LCH. Immunohistochemical staining for Ki-67 has demonstrated variable levels of proliferative activity within the lesional LCs.³¹ Immunohistochemical

overexpression of p53, MDM2, Bcl-2, p21, p16, and retinoblastoma protein has been found in several studies, typically with heterogeneous distribution and intensity, suggestive of physiologic upregulation rather than clonal mutations in the corresponding genes.³¹⁻³⁴ Studies of p53, in particular, have demonstrated nuclear p53 expression in all LCH subtypes, present in variable proportions of the lesional LCs. The mechanism for p53 accumulation is unclear, as mutational analysis has failed to identify *TP53* gene mutations, at least in the exons 4-11 interrogated by polymerase chain reaction (PCR)/single-stranded conformation polymorphism (SSCP) techniques.³⁴ Possible suggested alternative mechanisms for nuclear accumulation of wild-type p53 include viral binding and MDM2 over-expression.^{32,34} Bcl-2 over-expression in LCH cells has been demonstrated by immunohistochemistry and in situ hybridization for mRNA.³³ As with other cell cycle proteins, no mutations were found in this gene by Southern blotting.³³

Erdheim-Chester Disease

Clinicopathologic Features

Erdheim-Chester disease (ECD) is a rare histiocytic disorder characterized by multifocal xanthogranulomatous tissue infiltrates and distinctive radiologic findings.³⁵ It affects predominantly adult patients over 40 years of age, with no gender predilection. The anatomic sites of involvement are diverse and include the orbit and retroorbital tissues (with exophthalmia), eyelids, pituitary (with diabetes insipidus and rarely, panhypopituitarism), cerebellum, bones, lungs (with significant, sometimes progressive respiratory failure), serosal surfaces, mediastinum, retroperitoneum (especially perirenal, leading to obstructive uropathy), and occasionally skin.^{35,36} The diagnosis is established based on the characteristic radiologic appearance (of bilateral symmetric sclerotic polyostotic lesions, usually present in the long bones, "polyostotic sclerosing histiocytosis")^{35,37} and the histologic findings. Histologically, the findings are non-specific, and are those of a xanthogranulomatous infiltrate that includes foamy, lipid-laden macrophages, Touton giant cells, lymphocytes, and an associated proliferation of fibroblasts with significant fibrosis and osteosclerosis, with only sparse eosinophils and plasma cells.³⁸⁻⁴⁰ Rare cases of ECD and LCH, occurring concurrently at the same anatomic locations, have been reported.^{41,42} Treatment includes a combination of corticosteroids, chemotherapeutic agents, radiation, and surgery. Due to the rarity of the disease, a standardized therapeutic approach has not been established. The prognosis is unfavorable, with a reported average survival of 32 months³⁵ and a 60-65% mortality rate,^{39,43} typically due to respiratory or heart failure, and less commonly to complications of the central nervous system involvement, including diabetes insipidus or tumor (mass) effect.^{36,39}

Immunohistochemistry

No distinctive markers have been described for the xanthogranulomatous infiltrates seen in this disease, which overlaps histologically with juvenile xanthogranuloma, as well as with other adult xanthogranulomatous diseases of the orbit and ocular adnexae, including adult onset xanthogranuloma, adult onset asthma and periocular xanthogranuloma, and necrobiotic xanthogranuloma. The differential diagnosis with all of these entities typically relies on correlation with the clinical and radiologic findings. The histiocytic cells that make up most of the infiltrate are typically positive for CD68 and are negative for CD1a and S100.^{38–40} When examined by immunohistochemical staining for Ki-67, the foamy histiocytes show a low proliferative index, which correlates with the low number of mitotic figures typically encountered in these infiltrates, and suggests that their accumulation may be due to alternative mechanisms.⁴⁴ Most of the infiltrating lymphocytes are CD3-positive T-cells, with a striking predominance of CD8-positive cytotoxic cells.³⁹

Chemokine Network in ECD

Limited immunohistochemical studies have further characterized the complex chemokine/chemokine receptor network that makes up the inflammatory milieu of the ECD lesions, regardless of their anatomic location.⁴⁴ As the lesional histiocytes do not appear to have significant proliferative activity, these autocrine loops may provide mechanisms for their migration and retention at the sites of disease, as well as for the recruitment of other inflammatory cells that make up the ECD tissue infiltrates. Most of the histiocytic cells, including the foamy macrophages, Touton giant cells, and epithelioid histiocytes, as well as the endothelial cells of the lesional blood vessels were found to express chemokine receptors (i.e., CCR1, CCD2, CCR3, and CCR5), as well as their ligands [i.e., CCL4/MIP-1 β , CCL2 (MCP-1), and CCL5/RANTES]. Also, an important subset of the histiocytic cells express CCR6 and its ligand, CCL19/MIP-3 β , and CCR7 and its ligand, CCL20/MIP-3 α . CCL19 is a well-described chemoattractant for B and T lymphocytes, dendritic cells, and macrophage progenitors. The lesions also strongly express IL-1 α , TNF α , and IL-6. Expression of RANKL, a chemokine involved in bone remodeling, has been found in a large percentage of the lesional histiocytes from a bony lesion, and in a smaller percentage of these cells present in a lung lesion. The expression of IL-6 (which is also elevated in the serum of ECD patients)⁴⁵ and of RANKL provides insights into the mechanisms of bone remodeling and osteosclerosis, associated with the osseous lesions characteristic for ECD. Lastly, some of the chemokine receptors/ligands expressed by the macrophages and the infiltrating lymphocytes indicate the presence of a Th1-polarized microenvironment. Many of the infiltrating lymphocytes express IFN γ (characteristic for Th1 lymphocytes), and many of the macrophages express CXCL10/IP-10 [specifically induced by IFN γ] as well as its receptor, CXCR3.

Clonality Studies

Conventional cytogenetic analysis has been reported in a single case of ECD, occurring in a 31 year old man with typical clinical presentation. In this case, clonal abnormalities were identified, with the following karyotype: 44,XY,-7,t(12;15;20)(q11;q24;p13.3),-19[4]/46,XY[19].⁴⁰ The significance of these abnormalities is unclear and suggests the presence of an underlying clonal proliferation in this morphologically heterogeneous disorder.

Clonality studies using the HUMARA assay have been performed in a limited number of ECD cases, and have yielded variable results. Chetritt et al analyzed microdissected lesional tissue from five female patients and found a monoclonal pattern in three of four informative cases, and a polyclonal pattern in the fourth case.⁴⁶ Interestingly, one of the clonal cases also presented with LCH involving a distinct anatomic site, which was also found to be clonal by HUMARA. (The authors did not provide any data regarding the clonal relationship between the LCH and ECD lesions in this latter patient.) Al-Quran et al analyzed material enriched in lesional cells from two biopsy samples obtained from a 35 year old female patient with recurrent disease and found no evidence of clonality.³⁸ Due to the mixed inflammatory nature of the ECD tissue infiltrates, it is possible that some of these results have been impacted by the methodology used for enriching the samples in lesional histiocytic cells.

In conclusion, ECD is a rare histiocytic disorder with an unfavorable prognosis, the etiology of which remains elusive. Biological studies of this disease are limited due to the rarity of this disorder. The information available thus far suggests that the multifocal systemic lesions of ECD are primarily chemokine-driven accumulations of histiocytic cells and lymphocytes that develop into self-maintained lesions through autocrine loops. They may also be responsible for some of the systemic manifestations of this disease, through the production of chemokines that target, for instance, bone remodeling processes. Clonal proliferations of cells of uncertain lineage may be associated with this rich inflammatory background, although it is not clear at this time whether they represent primary or secondary events. Last, as the optimal treatment of ECD remains unclear at this time, better insights into the chemokine network, that maintains these lesions, may provide rational targets for a better therapeutic approach.

Hemophagocytic Lymphohistiocytosis

Clinicopathology Features

Hemophagocytic lymphohistiocytosis (HLH) is a disease characterized by strong immunological activation of the monocyte/macrophage system, resulting from defects in cytotoxic lymphocyte homeostasis, with major systemic, metabolic, and hematological manifestations. The clinical presentation typically includes fever, hepatosplenomegaly,

and cytopenias. Very commonly, the patients also present with hypertriglyceridemia, coagulopathy with hypofibrinogenemia, liver dysfunction, elevated levels of ferritin and serum transaminases, and neurological symptoms. Less commonly, the patients may present with lymphadenopathy, skin rash, jaundice, and edema. Other features, which are also a part of the diagnostic criteria for HLH, include low or absent NK-cell activity and elevated levels of soluble interleukin-2 receptor (IL-2r, CD25) in serum or cerebrospinal fluid.⁴⁷ Histologically, organs such as liver, spleen, lymph nodes, and BM show a prominent infiltrate of histiocytes with hemophagocytosis and associated activated T-lymphocytes. HLH comprises two partially overlapping entities: primary or familial HLH (FHL) and secondary HLH (sHLH). Familial HLH, an autosomal recessive disease, occurs in 1:50,000 live-born children. It may be triggered by infectious episodes and, if left untreated, is rapidly and uniformly fatal, with a median survival of 2 months.⁴⁷ This form of disease may be associated with mutations in one of several genes related to immune regulation, which will be detailed below. sHLH is typically triggered by infections or malignant neoplasms. Infection-associated sHLH is typically triggered by viral infections [most commonly the Epstein–Barr virus (EBV)] occurring in both immunocompetent and immunocompromised patients. Malignancy-associated HLH has been described in association with a variety of hematopoietic and non-hematopoietic malignancies and may present at the time of initial diagnosis (sometimes obscuring the underlying cancer) or during therapy.⁴⁷ Lastly, HLH is also part of the clinical course in several genetic disorders that have in common defects in NK and cytotoxic T-cell function, including X-linked lymphoproliferative disorder (mutations in the SLAM-associated protein, *SAP* gene, chromosome Xq25; Also see Chaps. 2 and 38), Chediak-Higashi syndrome (mutations in lysosomal trafficking regulator gene, *LYST*, chromosome 1q42), and Griscelli syndrome type 2 (mutations in *RAB27a* gene, a key regulator of cytotoxic granule exocytosis). Therapy for HLH includes a combination of immunosuppressive drugs (such as cyclosporine and steroids) and chemotherapy agents (such as etoposide), leading to ~51% 3-year overall survival.⁴⁷ Hematopoietic stem cell transplantation has increased the survival of HLH patients to 64%.⁴⁷

Immunophenotypic and Immunologic Abnormalities

HLH of all subtypes is characterized by the uncontrolled proliferation and accumulation of activated macrophages and T-cells in tissues and in PB. A rare case report has documented the prolonged presence in PB of expanded populations of mature monocytes differentiating to macrophages, expressing dim CD14, bright CD16, as well as lower levels of CD11b, CD64, and CD35. These populations, which normally comprise less than 10% of the PB monocytes, correlated with disease activity.⁴⁸ Likewise, transient clonal or non-clonal proliferations of CD8+ T-lymphocytes are

commonly found during active disease, as a marker of the immune dysregulation characteristic of HLH.⁴⁹ These lymphocytes co-express CD3, CD8, and HLA-DR and show decreased levels of CD5 expression. In some of the EBV-related cases, the expanded T-lymphocytes may also be positive for this virus.⁵⁰ It is important to recognize these proliferations as a part of the HLH spectrum, and to differentiate them from true peripheral T-cell lymphomas, which may present with HLH as their first manifestation.⁴⁷

Host Genetic Factors in HLH

Linkage disequilibrium analysis initially identified two loci of importance in HLH:

1. A 9q21.3-22 locus (the FHL1 locus) has been found in four inbred Pakistani families and is estimated to occur in approximately 10% of FHL cases. The molecular basis for this disease subset remains to be determined.
2. A second locus linked to FHL was 10q21-22 (FHL2).⁵¹ The observation of markedly decreased cytotoxic cells activity in HLH, combined with the linkage to this locus, which harbors the perforin (*PRF1*) gene, has led to the discovery of mutations in this gene, now believed to be present in 20–40% of FHL patients.^{52–55} The *PRF1* mutations are typically homozygous in the index patients and heterozygous in their parents and other unaffected relatives. The mutations are nonsense, missense, or truncating, leading to variably decreased or absent levels of perforin in the PB NK cells and cytotoxic T-cells, that also correlate with a significant decrease in cytotoxic cell function.^{54,55} Rare mutations that lead primarily to decreased perforin function, through decreased ability to bind calcium, have also been described.⁵¹ In most of the cases tested, significantly reduced levels of perforin expression and of cytotoxic activity may also be found in the heterozygous unaffected relatives.⁵² Perforin, located within the cytoplasmic granules of the cytotoxic T-cell and NK cells, has been shown in mouse models to have an important role in downregulating cytotoxic T-cell responses in chronic infections. Perforin knock-out mice remain healthy when maintained in a pathogen-free environment but develop an HLH-like clinical and immunologic picture in the presence of viral infection.⁵⁶ It is postulated that perforin may play an essential role in the clearance of antigen presenting cells (APCs), such as macrophages. In the absence of a functional protein, persistent APCs may continue to provide activation and proliferation signals to the cytotoxic T-cells, thus maintaining HLH.

The study of a group of patients with perforin-positive FHL led to mapping of a new, FHL3 locus at 17q25.⁵⁷ Analysis of genes located at this site (and related to cytotoxic granule release) led to the discovery of mutations in the human homologue of the rat *Munc 13-4* gene (termed *hMunc 13-4* or *UNC13D*). All of the described mutations were found to associate with significantly decreased or

abolished hMunc13-4 function, resulting in defective cytolytic granule exocytosis from the cytotoxic and NK cells, and to a significant decrease in cytotoxic function. Decreased levels of hMunc 13-4 (CD107a) on the surface of PB NK cells were later found to be an acceptable surrogate marker for the FHL3 subtype of HLH.⁵⁸ These mutations are currently estimated to account for approximately 20% of FHL cases.⁴⁷

The study of a large consanguineous kindred of Kurdish descent, affected by FHL and lacking perforin mutations, uncovered linkage to a new locus, 6q24, designated FHL4. All members of this family were found to have a homozygous 5 base pair deletion in the *Syntaxin-11* gene located in this region.⁵⁹ NK cells with loss-of-function mutations of *syntaxin-11* fail to degranulate when encountering susceptible target cells. IL-2 stimulation partially restores degranulation and cytotoxicity by NK cells, which could provide an explanation for the less severe clinical course observed in the FHL4 patients, when compared to the FHL2 and 3 subtypes.⁶⁰ This subtype is currently estimated to represent 10% of all FHL cases.⁴⁷

CD45 Splicing Abnormalities

Similar to LCH, CD45 splicing defects, associated with homozygous C77G polymorphisms in the *PTPRC* gene, have been described in a small number of FHL patients (with or without perforin gene mutations).^{22,51,61} Their role in the pathogenesis of FHL remains unclear.

Host Genetic Factors in SHLH

A recent study addressing *TNFα* gene polymorphisms in sHLH Korean patients found a strong association between the presence of the -1031 allele and disease.⁶² This polymorphism was present in a significantly higher proportion in the sHLH patients than in normal matched controls, and it was also the most prevalent allele present in the sHLH patients, where it was found in 57% of the cases. This polymorphism, possibly located within a regulatory site for gene transcription, is known to be associated with enhanced gene transcription and with higher levels of *TNFα* production. This could provide a possible pathogenetic explanation for the propensity of the carriers to develop HLH. This same polymorphism has been related to other inflammatory and degenerative diseases in Asian populations (i.e., Crohn's disease and thyroid-associated ophthalmopathy in Japanese; Alzheimer's disease and peptic ulcer disease in Chinese).⁶² The role of this polymorphism in individuals of different ethnic backgrounds remains to be established.

Cytokines in HLH

Most of the clinical manifestations of HLH are likely mediated by inflammatory cytokines that are often increased in these patients and presumably originate from the activated

T-lymphocytes and macrophages. These include interleukin (IL)1 beta, IL-6, IL-8, IL-10, IL-18, tumor necrosis factor alpha (*TNFα*), and interferon gamma (*IFNγ*), GM-CSF, soluble IL-2 receptor (sCD25), and sCD95 ligand, respectively.^{58,63,64}

In conclusion, HLH is a disease with a dramatic, potentially fatal, clinical presentation that manifests by accumulation of activated macrophages and cytotoxic T-cells in the BM, spleen, liver, lymph nodes, and PB. This accumulation is caused by an abnormal activation of the immune system, resulting from defects in cytotoxic lymphocyte regulation, in the context of infection or malignancy. These defects may be related to inherited homozygous defects in genes that encode for key determinants of cytotoxic effector function, or to other yet unknown mechanisms that predispose to this type of abnormal inflammatory response. While tremendous progress has been made in the recent years in the understanding of the molecular mechanisms underlying this disorder, further studies will be necessary to uncover the most useful therapeutic targets.

Juvenile Xanthogranuloma

Clinicopathologic Features

Juvenile xanthogranuloma (JXG) is a histiocytic disorder characterized by solitary, or rarely, multiple nodular lesions with characteristic xanthogranulomatous histologic appearance. It affects predominantly patients in the first decade of life, with a large proportion of cases involving infants, and even neonates, with male predominance (1.3:1), most striking in the group of patients with systemic disease (12:1).⁶⁵ JXG most commonly presents as a solitary cutaneous lesion. Fewer cases may present as solitary subcutaneous, soft tissue (superficial or deep), or visceral or bone lesions. Rarely, patients, most often infants, may present with multifocal systemic disease. Few cases of association with neurofibromatosis type 1 have been reported.⁶⁵ The diagnosis is typically established based on a combination of the clinical presentation and the characteristic histologic and immunophenotypic features. Histologically, JXG is composed of a mixture, in variable proportions, of mononuclear monocytic cells, multinucleated giant cells (often with features of Touton cells), and spindled cells. In many cases, the monocytic cells may have a lipid-laden, foamy (or "xanthomatous") appearance. Some lesions may demonstrate degenerative nuclear atypia and increased numbers of mitotic figures.⁶⁵ The prognosis is favorable in most cases, with spontaneous regression of the lesions. Chemotherapy regimens similar to those used in LCH appear to be effective in patients with systemic disease.⁶⁶ In the few fatal cases reported, demise was due to either hepatic failure secondary to extensive infiltration of the liver, or to intractable hypercalcemia associated with a deep-seated lesion.⁶⁵

Immunohistochemistry

The monocytic cells of JXG (including mononuclear and multinucleated cells) are invariably positive for vimentin and CD68 and negative for CD1a and S100. In addition, in a large proportion of cases, all of the JXG cell types express Factor XIIIa.^{65,67,68} These findings have led to the conclusion that the normal counterpart of JXG may be a subtype of dendritic cell, the dermal dendrocyte. While this may explain the propensity of JXG for cutaneous sites, it provides a less satisfactory explanation for the deep-seated lesions. In a study of three cases of deeply located JXG lesions, de Graaf et al⁶⁷ found that all lesional cells expressed CD1a, but not S-100 protein, and did not contain Birbeck granules by electron microscopy. These findings led to authors to postulate that the normal counterpart for these deep lesions may be the indeterminate dermal dendrocyte that migrates to these locations. In a more comprehensive immunohistochemical assessment of JXG, Kraus et al⁶⁸ found all of the JXG cases to stain strongly for CD68 and fascin, and the majority of cases to be positive for HLA-DR, Factor XIIIa, leukocyte common antigen, and CD4. A minority of cases also expressed S-100 protein. All of the cases were negative for CD1a, CD3, CD21, CD34, and CD35. Based on their findings, these authors proposed the plasmacytoid monocyte as the normal counterpart of JXG.

Proliferation and Apoptosis in JXG

Very limited information is available regarding mechanisms of cell proliferation in this disorder. Immunohistochemical studies performed in a group of 13 JXG samples showed the histiocytic cells to stain for Ki-67, with variable proliferation rates that were higher in lesions from children younger than 2 years of age when compared to patients older than 10 years.⁶⁹ The same study found the lesional cell to express the antiapoptotic protein BCL-2, suggesting that this represents an additional mechanism of cell accumulation and lesional growth in JXG.

Other Molecular Findings

Only very limited molecular studies are available in JXG. Flow cytometric analysis of DNA content found no evidence of aneuploid populations in a few cases.^{67,70} Clonality assessment using the HUMARA locus documented clear-cut clonality in a single case of cutaneous JXG.⁷¹ Further studies will be necessary to assess the significance of these findings.

In conclusion, JXG represents a typically benign self-limited histiocytic proliferation with xanthogranulomatous features, which appears to be derived from dermal dendrocytes or plasmacytoid monocytes. Proliferation of the lesional cells, combined with increased survival, may be the main mechanisms of growth in the JXG lesions, which are possibly clonal, as documented in a single case. Further studies will be needed to clarify the biology of this disorder.

Kikuchi-Fujimoto Disease (See Also Chaps. 43 and 44)

Clinicopathologic Features

Kikuchi-Fujimoto disease (KFD), also termed histiocytic necrotizing lymphadenitis, is a typically benign self-limited lymphadenitis of yet unclear etiology. It was initially described in Southeast Asian women in their third and fourth decades of life. However, at the current time, this pattern of lymphadenitis has been found in patients of all ethnic backgrounds, genders, and ages.⁷² There is a striking female predominance (1:3–4). The clinical presentation typically involves limited lymphadenopathy, with or without fever, an inflammatory syndrome, and mild peripheral blood cytopenias, with circulating atypical lymphocytes. The affected lymph nodes may be peripheral (at any location, most commonly cervical) or intraabdominal. Extranodal sites, such as skin and bone, have also been reported. Rare cases of systemic involvement may occur in immunosuppressed patients and infants. The diagnosis is typically established based on the unique histologic appearance that consists of patchy histiocytic infiltrates with or without central necrosis, characteristically containing abundant karyorrhectic debris and lacking granulocytes. The histiocytic infiltrates contain typical pale, sometimes xanthomatous histiocytes with C-shaped nuclei, plasmacytoid monocytes and immunoblasts, and typically lack significant numbers of plasma cells. The underlying lymph node architecture is usually preserved, with paracortical expansion and proliferation of immunoblasts. Most patients do not require therapy. Steroid or non-steroidal anti-inflammatory therapy is used in patients with significant systemic manifestations. KFD may be fatal in the rare systemic cases associated with immunosuppression, as well as in a rare patient who also developed myocarditis. A subset of patients develop systemic lupus erythematosus (SLE). Malignant transformation has not been reported.

Immunohistochemistry

The immunoarchitecture of KFD is similar at all sites of involvement. The histiocytes are positive for CD11b, CD11c, CD14, CD68 (KP1), Mac387, Ki-MP1, and lysozyme. In addition, the histiocytes present in KFD lesions, as well as in the uninvolved areas of the lymph node, express myeloperoxidase, a feature unique to histiocytes present in this entity and in SLE.^{73,74} The plasmacytoid monocytes share monocytic markers with the histiocytic population, express CD10 (CALLA) and are negative for myeloperoxidase. Staining for S-100 protein highlights increased numbers of interdigitating reticulum cells in the uninvolved paracortical areas. The lymphoid population includes predominantly mature T-cells with a variable proportion of CD8+ positive and CD4+ cells, and only rare CD56+ cells. In most cases, the CD8+ lymphocytes predominate and express cytotoxic markers, such as

TIA-1 and granzyme B. Immunoblasts are CD30- and CD45-positive, negative for CD15, and are most often of T-cell lineage. Few B lymphocytes are usually present. Lymphocytes and histiocytes also exhibit a high rate of apoptosis, correlating with the morphologic appearance. Proliferation markers, such as Ki-67, are positive in the “activated”-appearing lymphoid population and negative within the histiocytic and plasmacytoid monocytic compartment.⁷⁵

HLA Subtypes in KFD

Patients of any ethnic background may be affected. However, many of the largest reported series originate from Southeast Asia, where the entity was first described. The reason for this geographic or ethnic association is not clear. Significant correlations with certain HLA class II alleles (i.e., DPA1*01 and DPB1*0202) have been identified using PCR-based DNA typing methods in Japanese KFD patients, when compared to Japanese individuals not affected by this disorder.⁷⁶ Interestingly, one of these alleles (i.e., DPB1*0202) is significantly more frequent in Asians than in Caucasians. A role for the genetic background is also suggested by a report of KFD occurring in HLA-identical non-twin Saudi Arabian sisters approximately 10 years apart.⁷⁷

Infectious Agents in KFD

Extensive testing for infectious agents, including viral serology and blood cultures, is usually negative. Associations with the EBV and other herpes viruses (i.e., HHV-6, HHV-7 and HHV-8) have been suggested by some studies. Other infectious agents occasionally associated with KFD, usually by positive serology, include parvovirus B19, HTLV-I, HIV, *Yersinia enterocolitica*, *Toxoplasma*, and *Brucella*.⁷²

Association with EBV has remained controversial, as many studies have failed to identify convincing evidence for the presence of EBV in lesional tissues.^{78–80} In rare patients, lymphadenopathy with typical features of KFD has been associated with positive EBV serology. Of these cases, a few have been evaluated PCR or in situ hybridization (ISH) methods for EBV and were found to be positive. In one case,⁸¹ relapse of KFD was accompanied by persistent high levels of EBV infection. Chiu et al,⁸² in their study of 10 EBV+ KFD cases, found a correlation between the number of EBV+ cells detected by ISH, using a probe for EBV small encoded RNA (EBER), and the histologic type of lesion. EBV was detected in nuclei of lymphocytes within the affected lymph node, while the histiocytes were negative in the early, pre-necrotizing phase of disease. In contrast, in the necrotizing phase, EBV was present in lymphocytes and karyorrhectic histiocytes. These findings suggest that the different phases noted morphologically may correspond to various stages of antigenic clearance.

Several studies have addressed the role of other human herpesviruses in KFD, using multiple modalities, including

immunohistochemistry, ISH, PCR, nested PCR, and Southern blotting (i.e., HHV-6, HHV-7, and HHV-8).^{78–80,83} While serology and lesional tissue may often be positive by one of these modalities for HHV-6 and/or HHV-7, it is notable that these viruses may be detected at comparable frequency in control samples from individuals not affected by KFD.⁸³ HHV-8 has been found by PCR in a rare case of KFD, but not in normal controls, suggesting the possibility of a real association with this disease.⁸³

In conclusion, KFD is a benign lymphadenitis with characteristic histologic appearance and unclear etiology. Various studies suggest that it may represent a pattern of histologic response to a variety of etiologies, including possible infectious agents, possibly occurring with increased frequency in individuals with certain genetic backgrounds.

Lysosomal Storage Disorders

Clinicopathologic Features

Lysosomal storage disorders are autosomal recessive or X-linked diseases, manifested most commonly by total or partial deficiencies of various lysosomal acid hydrolases, leading to the accumulation of their substrates (i.e., mucopolysaccharides, sphingolipids, glycoproteins) in cells whose functions they ultimately disrupt. The enzyme deficiencies may occur through a variety of mechanisms, including mutations in their encoding genes, leading to decreased specific activity or decreased stability, lack of formation of protector or activator proteins for the enzymes, or deficiency of mechanisms that target the enzyme to its lysosomal location.^{84,85} An alternative mechanism for these disorders is deficiency of molecules that ensure active export of substrates from the lysosomes. The genetic targets and resulting biochemical alterations of the most common lysosomal storage disorders are summarized in Table 42.3.^{85–95}

Storage disorders may have a range of clinical presentations, depending on the type of biochemical abnormality, rate of accumulation, the pattern of distribution of the accumulating compounds in normal tissues, and the metabolic needs of specific organs/tissue types for specific substrates. Clinical features include hepatosplenomegaly (in some cases progressing to cirrhosis), progressive neurologic impairment (that may consist of mental retardation, blindness, deafness, ataxia, and spasticity), skeletal abnormalities (leading to characteristic facial features in some disorders), myopathies, and renal dysfunction.^{84,85} The specific diagnosis and classification of these disorders, that often show an overlapping spectrum of clinical features, require laboratory testing. The latter involves testing for the enzymatic defects in PB leukocytes and erythrocytes, skin fibroblasts, and even more specifically-affected tissues (i.e., liver, muscle, or heart), depending on the disorders included in the differential diagnosis. Testing for the specific genetic defect is likely

TABLE 42.3. Main subtypes of lysosomal storage disorders and their biochemical and genetic defects.

Disorder	Accumulating product(s)	Deficient protein/process	Affected gene/chromosome
<i>Mucopolysaccharidoses</i>			
MPS I (Hurler, Scheie)	Dermatan and heparan sulfates	α -L-Iduronidase	<i>IDUA</i> /chr4p16.3
MPS II (Hurler)		Iduronate sulfatase	<i>IDS</i> /chrXq28
MPS IIIA (Sanfilippo A)	Heparan sulfate	Heparan N-sulfatase (heparan sulfate sulfamidase)	<i>SGSH</i> /chr17q25.3
MPS IIIB (Sanfilippo B)	Heparan sulfate	α -N-Acetylglucosaminidase	<i>NAGLU</i> /chr17q21
MPS IIIC (Sanfilippo C)	Heparan sulfate	Acetyl-CoA: α -glucosaminide acetyltransferase	<i>TMEM76</i> /chr8
MPS IIID (Sanfilippo D)	Heparan sulfate	N-Acetylglucosamine-6-sulfatase	<i>GNS</i> /chr12q14
MPS IVA (Morquio A)	Keratan and chondroitin-6-sulfates	N-Acetylglucosamine-6-sulfate sulfatase	<i>GALNS</i> /chr16q24.3
MPS IVB (Morquio B)	Keratan sulfate	β -Galactosidase (specific for keratan sulfate)	<i>GLB1</i> /chr3p21.33
MPS VI (Maroteaux-Lamy)	Dermatan and chondroitin sulfates	Arylsulfatase B	<i>ARSB</i> /chr5q11-q13
MPS VII (Sly)	Chondroitin, dermatan and heparan sulfates	β -Glucuronidase	<i>GUSB</i> /chr7q21.11
MPS IX (Hyaluronidase deficiency)	Hyaluronan	Hyaluronidase	<i>HYAL1</i> /chr30p21.3-p21.2
<i>Sphingolipidoses</i>			
GM ₁ gangliosidosis	GM1 ganglioside	β -Galactosidase	<i>GLB1</i> /chr3p21.33
<i>GM₂ gangliosidoses</i>			
Tay-Sachs disease	GM2 ganglioside	Hexosaminidase A	<i>HEXA</i> /chr15q23-q24
Sandhoff disease	GM2 ganglioside	Hexosaminidase B	<i>HEXB</i> /chr5q13
GM2 ganglioside activator protein deficiency	GM2 ganglioside	Hexosaminidase A	<i>GM2A</i> /chr5q31.3-q33.1
Fabry	Glycosphingolipids (globotriaosylceramide, galactosylceramide)	α -Galactosidase A	<i>GLA</i> /chrXq22.1
Schindler	Glycolipids	α -N-Acetylglucosaminidase (α -galactosidase B)	<i>NAGA</i> /chr22q11
<i>Glucocerebrosidoses</i>			
Gaucher	Glucocerebroside (glucosylceramide)	β -Glucocerebrosidase	<i>GBA</i> /chr1q21
Sphingolipid activator protein 2 (saposin C) deficiency	Sphingoglycolipids (glucosylceramide)	β -Glucocerebrosidase, galactosylceramidase, sphingomyelinase	<i>PSAP</i> /chr10q21-q22.1
<i>Ceramidoses</i>			
Farber	Ceramide	Ceramidase	<i>ASAH</i> /chr8p21.3-p22
Prosaposin (saposin precursor) deficiency	Sphingoglycolipids	Several lysosomal hydrolases (including ceramidase, galactosylceramidase, β -galactosidase, glucosylceramidase, β -glucosidase)	<i>PSAP</i> /chr10q21-q22.1
<i>Sphingomyelin-cholesterol lipidoses</i>			
Niemann-Pick types A,B	Sphingomyelin, sphingosylphosphocholine	Acid sphingomyelinase	<i>SMPD1</i> /chr11p15.4-p15.1
Niemann-Pick type C1	Unesterified cholesterol	NPC1/cholesterol transport and esterification	<i>NPC1</i> /chr18q11-q12
Niemann-Pick type C2	Unesterified cholesterol	NPC2/cholesterol transport and esterification	<i>NPC2</i> /chr14q24.3
Krabbe	Galactosylceramide	Galactosylceramidase	<i>GALC</i> /chr14q31
<i>Sulfatide lipidoses</i>			
Metachromatic leukodystrophy	3-O-sulfo-galactosylceramide (sulfatide)	Arylsulfatase A (cerebroside sulfatase)	<i>ARSA</i> /chr22q13.31-qter
Sphingolipid activator protein 1 (saposin B) deficiency	Sulfatides	Arylsulfatase A, α -galactosidase A	<i>PSAP</i> /chr10q22.1
Multiple sulfatase deficiency	Sulfatides	α -Formylglycine-generating enzyme (activator of all known sulfatases)	<i>SUMF1</i> /chr3p26

(continued)

TABLE 42.3. (continued)

Disorder	Accumulating product(s)	Deficient protein/process	Affected gene/chromosome
<i>Other storage disorders</i>			
Mucopolidoses			
Mucopolidosis II (I-cell disease)	Phospholipids, gangliosides, neutral lipids, mucopolysaccharides	N-Acetylglucosamine-1-phosphotransferase (α/β subunit)/impaired trafficking of multiple lysosomal hydrolases	<i>GNPTA</i> /chr4q21-q23
Mucopolidosis III	Phospholipids, gangliosides, neutral lipids, mucopolysaccharides	N-Acetylglucosamine-1-phosphotransferase (α/β subunit in type III A, γ subunit in type IIIC)/impaired trafficking of multiple lysosomal hydrolases	<i>GNPTA</i> /chr4q21-q23
Mucopolidosis IV	Phospholipids, gangliosides, neutral lipids, mucopolysaccharides	Mucopolin-1 (cation channel)/lysosomal membrane transport	<i>MCOLN1</i> /chr19p13.3
Glycoproteinoses			
Fucosidosis	Fucosylated glycoconjugates	α -L-fucosidase	<i>FUCA1</i> /chr1p34
α -Mannosidosis	Oligomannosides	Lysosomal α -mannosidase	<i>MAN2B1</i> /chr19p13.2-q12
β -Mannosidosis	Oligosaccharides	β -Mannosidase	<i>MANBA</i> /chr4q22-25
Sialidosis	Sialylated glycopeptides and oligosaccharides	α -Neuraminidase	<i>NEU1</i> /chr6p21.3
Galactosialidosis	Sialylated glycopeptides and oligosaccharides	Protective protein/cathepsin A (protects β -galactosidase and α -neuraminidase from degradation)	<i>PPCA</i> /chr20q13.1
Aspartylglucosaminuria	Glycoasparagines	Aspartylglucosaminidase	<i>AGA</i> /chr4q32-q33
Lysosomal membrane transport defects			
Free sialic storage disorders (infantile, Salla disease)	Sialic acid	Solute carrier family 17 (sodium phosphate) member 5 (sialin)/lysosomal membrane transport	<i>SLC17A5</i> /chr6q14-q15
Cystinosis	Cystin	Cystinosin/lysosomal membrane transport of lysylcystine	<i>CTNS</i> /chr17p13
Other lysosomal disorders			
Glycogen storage disease II (Pompe)	Glycogen	Acid α -glucosidase (acid maltase)	<i>GAA</i> /chr17q25.2-q25.3
Glycogen storage disease IV (Andersen disease, amylopectinosis)	Polyglucosan (amylopectin-like)	Glycogen branching enzyme	<i>GBE</i> /chr3p14
Acid lipase deficiency (Wolman disease, cholesteryl ester storage disease)	Cholesteryl ester, triglycerides	Lysosomal acid lipase	<i>LIPA</i> /chr10q24-q25

TABLE 42.4. Lysosomal storage disorders with manifestations in the hematopoietic system.

Disease	Pathologic findings
Gaucher	Infiltration of liver, spleen, bone marrow, lungs, CNS, by characteristic histiocytes (Gaucher cells)
Niemann-Pick	Infiltration of liver, spleen, bone marrow, tonsils, lymph nodes, by foamy histiocytes
α -Mannosidosis	Vacuolated lymphocytes
Free sialic acid disease	Inclusions in lymphocytes

CNS central nervous system.

to become the preferred diagnostic tool, as the affected genes are unequivocally identified and appropriate clinical testing becomes available. Depending on the severity of the defect, untreated patients often die of their disease during their childhood years. Various therapeutic modalities (including specific enzyme replacement, substrate deprivation, gene therapy, and hematopoietic stem cell transplantation) have greatly improved the outcome of patients with at least some of the lysosomal storage disorders.^{92,96,97}

Some of the storage disorders are associated with infiltration of hematopoietic tissues (i.e., BM, lymph nodes, tonsils, and spleen) by macrophages containing mucopolysaccharides or lipids. These cases may raise the differential diagnosis with histiocytic disorders. Other storage disorders may be associated with characteristic abnormalities in PB leukocytes. Occasionally, these abnormalities may constitute the first clue to the correct diagnosis and may be crucial to the initiation of the correct diagnostic studies.⁹⁸ The most common lysosomal storage disorders with hematopoietic manifestations are summarized in Table 42.4.

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Reactive Lymphadenopathies: Molecular Analysis

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Overview

The molecular analysis of benign and reactive processes in lymph nodes is important in several contexts. In terms of research into underlying pathophysiology, molecular analyzes of clonality may be crucial in establishing that a rare or enigmatic process, such as Rosai–Dorfman disease, is a non-clonal proliferative lesion. In addition, molecular testing for the presence of infectious agents may be critical in evaluating the causative effects of these infections in benign and reactive processes.

However, perhaps the most practical and important use of molecular studies in benign lesions is in evaluating them in contrast to neoplastic entities that may mimic them. In these cases, clonality testing by molecular techniques with a *negative result* may be supportive of a benign or reactive causation of a lymphoid process, while a *positive result* would often preclude a benign or reactive process (Table 43.1).

In spite of this, it is possible to obtain false positive results in tests of clonality, leading to spurious support for a neoplastic process.^{1,2} The circumstances and reasons for this will be discussed further in the chapter and may also be reviewed in Chap. 8.

B Cell Processes

In the Western world, B cell lymphomas comprise approximately 90% of the lymphomas encountered. As such, they are, practically speaking, the most common problem faced by the pathologist in routine practice. Likewise, most B cell processes are easily recognizable as benign, especially in normal or reactive lymph nodes.

Various hyperplasias of lymph nodes may cause occasional diagnostic difficulty; they may be mistaken for low grade lymphomas of various types. Many methodologies are necessary to clarify the myriad diagnostic circumstances that may arise, including cytology, histology, immunohistochemical stains, and flow cytometry. Particularly in very small biopsies, molecular evaluation for B- and T-cell gene

rearrangement studies may be of great benefit in supporting a diagnosis of lymphoma.

Molecular testing within B cell processes is most often accomplished by testing for clonality by polymerase chain reaction (PCR) methods of immunoglobulin heavy (IgH) chain gene rearrangement studies. Occasionally, similar PCR techniques for immunoglobulin light chain rearrangements may also be used to increase sensitivity. As outlined in previous chapters, this methodology attempts to determine if there is a significant population of B cells with the same immunoglobulin rearrangements, which would indicate a clonal population.

In the context of benign lymphadenopathies, it is important to review some of the basic immunologic concepts that underlie B cell development. In the unreacted lymph node, primary lymphoid follicles exist as quiescent nodules of naïve B cells awaiting signals to begin proliferation. These naïve B cells await exposure to an antigenic stimulus, which begins the complex chain of events to develop an adaptive immune response. When an antigen arrives at the site of the lymphoid follicle, it is processed by *antigen presenting cells* (APCs), and through a series of interactions of helper T cells, is presented to naïve B cells. The B cells then begin to proliferate. Simultaneously, the process of affinity maturation is occurring. This is a process of mutation of the binding site, in order to develop higher affinity for the antigen in question. Non-productive mutations do not survive the process (i.e., undergo apoptosis). At the end of the process, there is a population of B cells that have one or a few specific antigen affinities (i.e., specific for the presented antigen).

It should be emphasized that this is a process of *clonal selection*, and that at its most basic level, the adaptive immune response is responsible for creating clonal populations. However, taken in the broader context, it is unlikely that all sites of antigen processing and affinity maturation would select the same epitopes for a single antigen, so the process ultimately ends up with multiple discrete populations composed of many clonal populations, or polyclonality.

This digression into basic immunology has a purpose, in that it establishes the reasons for some false positive results

TABLE 43.1. Summary of reactive lymphadenopathies and molecular findings.

Diagnosis	Clinical	Morphologic	Molecular	Comments
Follicular hyperplasia	Variety of clinical situations	Reactive germinal centers	No clonal rearrangements ^a	In spleen, marginal zone hyperplasia may closely mimic splenic marginal zone lymphoma, which would have clonal B cell gene rearrangement studies in the majority of cases
Marginal zone/monocytoid B cell hyperplasia	Variety of reactive conditions. Most common include viral infections (CMV, EBV, HIV)	Increased pale lymphoid cells in paracortical or interfollicular locations	No clonal rearrangements	May precede, follow or be concurrent with nodular lymphocyte predominant Hodgkin lymphoma
Progressive transformation of germinal centers	Proposed as an "abnormal immune response"	Large nodules composed of mantle-type B cells with interspersed follicle center cells in central portion	No clonal rearrangements	
Immunoblastic hyperplasia (B cell)	Variety of clinical circumstance. Viral infections a common association	Increased enlarged, transformed B cells typical in interfollicular areas of lymph node	No clonal rearrangements	
T cell large granular lymphocytic proliferation	Strong association with autoimmune disease such as rheumatoid arthritis	Increased large granular lymphocytes in peripheral blood, spleen and bone marrow	Clonal rearrangement supports diagnosis of neoplastic process	
Nodular paracortical T cell hyperplasia	Variety of etiologies	Nodules of T cells in paracortical/interfollicular areas of lymph node with classic mottled appearance	No clonal rearrangements	
Immunoblastic/interfollicular hyperplasia (T cell)	Variety of clinical circumstance. Viral infections a common association	Increased enlarged, transformed T cells typical in interfollicular areas of lymph node	No clonal rearrangements	
Dermatopathic lymphadenitis	Variety of etiologies; typically associated with nodes draining sites of skin lesions	Nodules of T cells in paracortical/interfollicular areas of lymph node with classic mottled appearance. Pigmented macrophages and extracellular pigment seen	No clonal rearrangements	Identical morphologic appearance to nodular paracortical T cell hyperplasia with the addition of pigment
Kikuchi–Fujimoto disease	More common in females and Asian population. Typically presents as isolated enlarged lymph nodes in cervical region	Areas of necrosis without neutrophils. Increased immunoblasts, and proliferation of plasmacytoid dendritic cells	No clonal rearrangements	
Kimura disease	Isolated adenopathy. More common in males	Follicular hyperplasia, with increased eosinophils and polykaryotic dendritic cells	Clonal T cell rearrangements may be seen	
Angioimmunoblastic lymphadenopathy	Exceedingly rare or nonexistent. Essentially all cases represent angioimmunoblastic T cell lymphoma	Partial effacement of lymph node by T cells, with sparing of peripheral sinuses. Increase in plasma cells	T cell clonality associated with Angioimmunoblastic T cell lymphoma	May also see B cell clonality with concurrent EBV-related B cell lymphomas
Sinus histiocytosis with massive lymphadenopathy (Rosai–Dorfman disease)	Rare. Nodal or extranodal disease	Proliferation of histiocyte-derived cell population which expresses S-100 and have emperipolesis	No clonal rearrangements	
Rheumatoid arthritis (RA)	Often associated with lymphadenopathy	Features of florid follicular hyperplasia	No clonal rearrangements	Clonal disorders may develop associated with therapies for RA
Systemic lupus erythematosus	Adenopathy may be present	Follicular hyperplasia common. Necrosis common; may appear identical to Kikuchi–Fujimoto disease	Clonal T cell rearrangements may be seen	
Sjögren syndrome	Lymphoid proliferations in salivary glands	Range of morphologic findings ranging from lymphoid hyperplasia to frank lymphoma	Clonal B cell population suggests B cell neoplasm	
Methotrexate-related lymphadenopathy	Lymphoid proliferations may be associated with medication or underlying autoimmune disorders	Range of morphologic findings ranging from atypical lymphoid hyperplasia to frank lymphoma	Clonal B cell population suggests B cell neoplasm	
Anticonvulsant medication-related lymphadenopathy	Adenopathy may be present	Range of morphologic findings ranging from atypical lymphoid hyperplasia to frank lymphoma	Clonal B cell population suggests B cell neoplasm	

for clonality that may be seen in specific benign processes.³⁻⁵ Monoclonal or oligoclonal results may be seen in autoimmune processes.⁶⁻⁹ Likewise, false positive results for B cell clonality may be obtained, if samples are limited in size, thereby having only a small amount of amplifiable product for PCRs.

Follicular hyperplasia is one of the most commonly encountered processes in lymph nodes or lymphoid tissues. Increase in the size and number of follicles indicates an active immunologic process. These follicles show active secondary germinal centers. The main differential diagnosis to consider is follicular lymphoma (FL). Molecular testing for clonality by IgH rearrangements would not be positive in follicular hyperplasia, unless limited samples were tested.¹⁰

It has been shown that there is a “baseline” rate of IgH/bcl-2 positive cells in “normal” individuals.¹¹⁻¹⁵ The positivity in peripheral blood and “benign” lymphoid tissues is 24–50%, and the frequency is unrelated to age.¹⁶⁻¹⁸ It is postulated that only a small subset of these individuals go on to develop FLs, although the rate of transformation, and other possible genetic events required, is not clear. This highlights that the degree of sensitivity of testing by molecular methods needs to be considered when a positive result is obtained.

Further, it has been shown that if individual follicles are microdissected and PCR for IgH clonality is performed, oligo- and monoclonal results may be obtained.¹⁰ As mentioned previously, clonal selection in response to antigen is the function of germinal centers, and clonality in cases with limited samples is thus not an unusual finding.

Marginal zone/monocytoid B cell hyperplasia (MZH) is an uncommon manifestation of a lymphoid immune response that may occasionally present as a diagnostic difficulty. MZH is classically associated with some specific lymph node infections, most notably *Toxoplasma* lymphadenitis, cytomegalovirus lymphadenitis, and human immunodeficiency/acquired immunodeficiency syndrome (HIV/AIDS) lymphadenitis. Frequently, marginal zone hyperplasia may be confused with marginal zone lymphoma, or other low grade B cell lymphomas.^{19,20} In these circumstances, evaluation of clonality by molecular studies may be of benefit. Two circumstances of particular benefit are marginal zone expansions in the spleen and marginal zone proliferations in pediatric patients.

Splenic marginal zone lymphoma (SMZL) is a relatively common diagnosis when evaluating patients with splenic enlargement. However, there are frequent cases of MZH that may closely mimic SMZL.²¹⁻²³ The marginal zones become expanded; a marginal zone layer wider than 12 cells has been arbitrarily defined as “hyperplasia.” In these circumstances, the presence of a clonal population by molecular studies supports a diagnosis of SMZL. In pediatric patients, atypical marginal zone hyperplasia with lambda light chain restriction may be a difficult diagnosis, especially in light of the existence of a pediatric marginal zone lymphoma.²⁴ Molecular studies of the former show no clonality, while the latter, although showing lambda light chain restriction by flow

cytometry or immunohistochemistry, lack clonality by PCR or Southern blot.

Progressive transformation of germinal centers (PTGC) is thought to represent an unusual presentation of the lymphoid immune response, which is characterized by enlarged and fused follicles with large, expanded mantle zones, which is then followed by regression and involution of follicles.^{25,26} This is a benign process, and with the exception of possible pitfalls noted in the introduction of this chapter, PTGC should not be clonal. The differential diagnosis includes two lymphomas, the floral variant of FL and nodular lymphocyte predominant Hodgkin lymphoma (NLPHL).

In NLPHL, the neoplastic cells (lymphocytic and histiocytic cells; L&H cells) have clonal immunoglobulin gene rearrangements. However, in whole section evaluation of IgH clonality by PCR studies, NLPHL does not typically appear clonal because of the relative paucity of the neoplastic cells. Microdissection of the L&H cells is necessary to evaluate clonality of these cells. As such, when the differential of PTGC versus NLPHL is considered, immunohistochemistry is a more practical approach.

Distinction of the floral variant of FL from PTGC by molecular methods is more straightforward. Floral variant of FL will have IgH/bcl-2 translocations in most cases, which may be evaluated by PCR, FISH, or routine cytogenetic studies. In a small subset, other translocations involving IgH (i.e., Bcl-6) may be present, which may also be evaluated. Regardless, the presence of clonality by any method is not supportive of a diagnosis of PTGC.

Immunoblastic hyperplasia, composed of proliferations of immunoblasts, or large transformed lymphocytes, are most often seen in association with viral infections, although frequently the etiology is unknown. Most often, these types of proliferations will be confused with large cell lymphomas, such as diffuse large B cell lymphoma (DLBCL), or occasionally involvement by T cell lymphoma. As with other types of hyperplasias, negative studies for T- and B-cell gene rearrangements may be used to support a benign result, although, as in all cases, these results should be correlated with other ancillary studies, such as flow cytometry and immunohistochemistry.

T Cell Processes

Analogous to the process that occurs with B cells, T cells also undergo a process of mutation and selection associated with the T cell receptor (TCR; see Chap. 8 for additional details). Testing for T cell clonality is accomplished by similar processes using PCR testing for TCR gene rearrangements. Like B cells, the presence of clonal bands is strongly supportive of a clonal, and hence neoplastic, process. However, like B cell processes, false positive results may occur.²⁷ This is especially common in cases where there are limited numbers of cells (or very small samples are available) for PCR studies, and pseudoclonal bands may be discovered.

Large granular lymphocyte (LGL) proliferations represent a spectrum of disorders, due to increases in cytotoxic T lymphocytes. Most commonly associated with autoimmune diseases, especially rheumatoid arthritis, these proliferations may either be non-clonal proliferations of these cells, or a true clonal process, which is most appropriately termed as LGL leukemia/lymphoma. Peripheral blood and bone marrow are most frequently involved, but nodal involvement (and tissue involvement) are not uncommon. Proving T cell clonality in LGL proliferations is a significant finding and may guide therapy.²⁸ Evaluation of T cell clonality may be accomplished by TCR gene rearrangements, with a sensitivity of 70–80%, as described previously. In addition, there are other methodologies, based on flow cytometry, that yield comparable results.

T cell clonality may be analyzed by flow cytometry using V-beta testing (also see Chap. 8). Using a group of probes to different V-beta families, normal T cell populations would have a polyclonal pattern. Clonal T cell populations would show restricted expression of V-beta. While valuable, these results may also show false positive results, as with other techniques. In addition, T and NK cells may be evaluated by flow cytometric analysis of killer cell immunoglobulin-like receptor (KIR) testing (also see Chap. 8). KIRs are stably expressed throughout the life of T and NK cells. In cases of clonal LGL proliferations and other T cell disorders, restricted expression or complete lack of expression of KIRs on a T cell population supports clonality.²⁸

T cell lymphoma involvement, especially mycosis fungoides (MF), a relatively indolent cutaneous T cell lymphoma, may be difficult to distinguish from benign processes in lymph nodes. In its earliest stages, lymph node involvement by MF appears indistinguishable from the morphologic findings seen in nodular paracortical T cell hyperplasia (NPTCH) and/or dermatopathic lymphadenitis. In these circumstances, molecular testing for TCR gene rearrangement is a necessary adjunct to morphologic examination. A positive result would support a finding of involvement by MF.²⁹ In later stages, with clear evidence of abnormal T cell populations, molecular testing may not be necessary. Although not necessary in every case, it may be of some benefit to compare TCR gene rearrangement results from the primary skin lesion(s) to those of other sites of involvement; clonal bands of the same size would confirm clonal identity between the two sites.

NPTCH is a relatively common morphologic pattern seen in lymph nodes. It is a hyperplasia of T cell elements along with macrophages and sometimes dendritic cells (see below – dermatopathic lymphadenitis). It is a non-specific response pattern that may be seen in a variety of conditions. Classically, this pattern of reaction has been associated with viral infections, post-vaccinal lymphadenitis, and other causes. Although rarely a cause for diagnostic concern, there are specific circumstances in which involvement by a lymphoproliferative process may mimic these benign entities.

The key differential diagnosis to be considered is that of nodal involvement by T cell lymphomas, or less likely

histiocytic/dendritic cell processes. Lymph node involvement by the most common cutaneous T cell lymphoma, MF may have a morphologic appearance that is quite similar to NPTCH/dermatopathic lymphadenitis. In fact, both may occur in lymph nodes of MF patients.³⁰ The International Society for Cutaneous Lymphomas and the European Organization of Research and treatment of cancer specifies that clinically abnormal lymph nodes should be evaluated for the presence of clonal T cell populations (by PCR evaluation of TCR rearrangements) and, if possible, the clone/band size should be compared to those of skin biopsies with MF from the patient.³⁰

Immunoblastic/interfollicular hyperplasia is also a relatively common pattern of lymph node reaction, characterized by a proliferation of enlarged, transformed lymphocytes (i.e., immunoblasts) in the interfollicular regions of the lymph node. These processes may be composed of either T or B cells, depending on the nature of the inciting cause. Although most often of unknown etiology, viral infections may also be seen with this pattern. The main differential diagnosis is that of a large cell lymphoma, of either B or T cell type. In these cases, the finding of a clonal population by IgH or TCR studies would support a diagnosis of lymphoma.

Specific Entities

Dermatopathic lymphadenitis is a specific entity with morphologic findings that exactly overlap those of NPTCH with the addition of pigment-laden macrophages. This process is presumably due to regional dermatologic conditions and proliferation of T cells in response to dermatologic antigenic stimulus. As with NPTCH, dermatopathic lymphadenitis may exactly mimic early phases of nodal involvement by T cell lymphomas (NPTCH, see above). TCR studies are warranted in suspicious cases, and a clonal population would support a diagnosis of lymphoma.

Kikuchi–Fujimoto disease (KFD) is a rare and enigmatic cause of lymphadenitis. Most commonly seen in young females, it is more common in Asians, although it is seen in all racial groups. It is typically present in cervical lymph nodes. The morphology is characteristic with zones of necrosis with central areas of apoptotic debris, lack of neutrophils, and a periphery with large transformed lymphocytes and unusual monocytoïd dendritic cells (so-called plasmacytoid monocytes). The appearance is such that large cell lymphoma, especially DLBCL, is frequently a diagnostic concern. Molecular studies for clonality are negative in KFD.³¹

The etiology of KFD remains a puzzle, although it is likely of viral etiology.³¹ Numerous studies have attempted, through molecular means, to characterize a single viral etiology for KFD.^{32–34} Although several candidate viruses have been presented and ultimately rejected, it is likely that a single or possibly multiple viral agents will be found to cause KFD. This disease has been discussed in more detail in Chap. 42 and is also discussed in Chap. 44.

Kimura disease is a rare cause of lymphadenopathy. Clinically, most often seen as isolated cervical adenopathy in Asian males, it tends to be of a limited course, although it may be associated with systemic symptoms, such as fever. The lymph nodes have very typical and distinctive findings that support the diagnosis: follicular hyperplasia, a marked increase of eosinophils, and the presence of multinucleated dendritic cells, termed polykaryocytes. It is unlikely that Kimura disease would be confused with lymphomas, although the presence of eosinophilia and atypical lymphocytes is not uncommon in T cell lymphomas. There are reports of T cell clonality seen in this disorder, including the suggestion that this may be associated with the underlying pathology of the disorder.^{35–38} Further studies are necessary to clarify this relationship. Also, a single study has shown the presence of EBV by PCR studies,³⁹ although it is unlikely that this is the inciting agent of Kimura disease. This disease is also discussed in Chap. 44.

Angioimmunoblastic lymphadenopathy with dysproteinemia (AILD) is important mostly as a historical artifact. AILD was originally postulated to be a clinicopathologic syndrome, consisting of fever, weight loss, lymphadenopathy, and a number of other symptoms. It was ultimately felt that all cases of AILD actually represented a specific T cell lymphoma, angioimmunoblastic T cell lymphoma. This lymphoma will almost always have clonal rearrangements of the TCR and in some cases may also have clonal B cell populations as well (see Chap. 25).

Sinus histiocytosis with massive lymphadenopathy (SHML) (or *Rosai–Dorfman disease*) is a rare disorder of childhood and early adulthood.⁴⁰ It presents as nodal and extranodal proliferations of distinctive histiocytic/phagocytic cells. Although the exact pathogenesis of SHML is not known, it has been shown that the process is polyclonal and not a neoplastic proliferation. As such, molecular analysis, such as human androgen receptor (HUMARA) studies, of the process yields a non-clonal result.⁴¹ Although rarely necessary for the diagnosis of SHML, circumstances may arise where the differential diagnosis includes other, neoplastic proliferations of histiocytic, dendritic/accessory, or lymphoid cells. In these circumstances, the presence of clonality, by recurrent cytogenetic abnormality or molecular study, would support a diagnosis other than SHML. Rarely, SHML is seen in children with autoimmune lymphoproliferative syndrome, a diagnosis that is confirmed by mutations in the genes encoding Fas (see Chap. 38).⁴²

Rheumatoid arthritis (RA) and other autoimmune disorders are frequently associated with lymphoid proliferations. Patients with RA will often present with florid follicular hyperplasia, presumably as a result of activation of the immune system by the disease itself. As a result, prominent lymphadenopathy may lead to biopsy of an enlarged node. The degree of follicular hyperplasia, and the possibility of some atypical appearance, may lead to concerns of FL. As such, ancillary studies to evaluate these nodes are frequent. As in typical follicular hyperplasia, bcl-2 by immunohistochemistry would be

expected to be negative, there would be no evidence of light chain restriction by flow cytometry, and studies for IgH/bcl-2 translocation and IgH rearrangements would be negative.

Systemic lupus erythematosus (SLE) lymph node findings may mimic neoplastic disorders. Follicular hyperplasia is frequent, and so a differential diagnosis may include FL. Occasionally, foci of necrosis and large cells may be seen mimicking large cell lymphomas. Increases in plasma cells may suggest a diagnosis of marginal zone lymphoma. Perhaps related to the underlying pathology of the disease, clonally restricted populations of T cells may be seen in patients with SLE.^{43,44} This may be a potential diagnostic pitfall when examining diagnostic samples from patients with SLE.

Sjögren syndrome (SS) is another autoimmune disorder that is associated with both benign lymphoid proliferations and the development of lymphoma, most often extranodal marginal zone lymphoma. In SS, there is a stepwise progression of lymphoid hyperplasia, due to the antigenic stimulus of the autoimmune process, followed by atypical lymphoid proliferations (termed myoepithelial sialadenitis with haloes), and the ultimate development of extranodal marginal zone lymphoma.^{6,45} Molecular studies for clonality, using PCR analysis for an IgH gene rearrangement, may be critical in making the distinction of when the process crosses the border to a clonal, and hence neoplastic, disorder.

Medication-Related Lymphoid Proliferations

Methotrexate use has been associated with an increased incidence of lymphoma and lymphoid proliferations. Methotrexate therapy is most commonly used for treatment of autoimmune disorders, such as RA, psoriasis, and dermatomyositis.⁴⁶ Methotrexate therapy may be associated with the development of EBV-associated lymphoid proliferations,^{46,47} and cessation of therapy may lead to resolution of the lymphoid proliferation. Studies of B cell clonality may be of use in defining the process and following the course of disease. ISH studies for Epstein–Barr virus infection may be of value in diagnosis.

Anticonvulsant medications may also be associated with the development of lymphoid proliferations, ranging from paracortical hyperplasia to outright lymphoma.⁴⁸

See Table 43.1 for a summary of the reactive lymphadenopathies and their molecular findings.

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Molecular Pathology of Infectious Lymphadenitides

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Introduction

In its role in the presentation of foreign antigens to the immune system, the lymph node is frequently involved in both local and systemic infections. This may manifest as lymphadenopathy with reactive changes or localized infectious lymphadenitis, with or without necrosis and granulomatous inflammation, depending on the infectious agent. The vast majority of these infections are routinely diagnosed using nonmolecular methods, including culture, serology, and antigen testing. However, in some cases, other methods may be unavailable, or the presentation and appearance may be atypical, and direct confirmation of the infecting agent by in situ hybridization (ISH) or amplified nucleic acid detection is desired in the lymph node tissue itself.

Molecular methods are a powerful tool in the diagnosis of infectious disease; properly designed assays may be highly sensitive and specific, surpassing other methods to become a new “gold standard” test for a disease. They also allow the detection of fastidious and nonviable organisms in a specimen, including after initiation of antimicrobial therapy. However, the strengths of molecular methods are also their primary weaknesses. A high degree of specificity may mean that multiple individual assays must be performed to arrive at a diagnosis (the exception being broad-range amplification and sequencing of bacterial and fungal sequences). Likewise, exquisite sensitivity surpassing that of “classic” methods makes clinical validation of assays more difficult, yet also more critically important. These methods are also usually more susceptible to contamination than other diagnostic methods, due in part to their high sensitivity as well as inherent aspects of target amplification methods, such as polymerase chain reaction (PCR); this is especially true of broad-range assays. Careful work practices, attention to reagent purity, and other methods to prevent contamination are essential. Nucleic acid extraction methods may drastically affect the performance of molecular techniques in tissue specimens, and extraction controls and internal controls (often human genes, such as GAPDH or β -globin),

demonstrating the presence of extractable and amplifiable nucleic acid in the specimen, are essential. Due to fragmentation of nucleic acids in tissue processing, assays targeting shorter sequences are more likely to be successful in tissue specimens, particularly formalin-fixed, paraffin-embedded (FFPE) tissue.

It is vital to be aware of the performance characteristics of any given assay, particularly in tissue specimens, in order to properly interpret the results. Unfortunately, for many assays described in the literature, this may be difficult to assess, due to differences in patient populations and in the “gold standard” used in the assignment of diagnosis. Generally, the sensitivity of molecular methods in tissue specimens (lymph node as well as other tissues) is often significantly lower than sensitivity in other specimens; this is particularly true for FFPE tissues, due to the effects of tissue processing and nucleic acid fragmentation. While a positive result may be useful (in a well controlled assay without contamination), negative results are usually less helpful, as many assays are not sufficiently sensitive to completely rule out disease (particularly if the organism number is low), especially in tissue specimens.

This chapter will discuss the most common causes of infectious lymphadenopathy and reported use of molecular methods for their diagnosis in lymph node tissues, with a focus on the most common and useful tests in an effort to guide diagnosis. In cases where there are few data regarding lymph nodes, descriptions of studies using tissues from other sources will also be described. This discussion is not intended to offer a complete description of the histologic appearance of various infectious lymphadenitides; for this the reader is referred to Reference 1. For a complete discussion of the diagnosis of specific infectious agents, including the use of molecular methods on specimens other than lymph node tissue, the reader is referred to References 2 and 3. Microbial taxonomy is currently in near constant flux, as organisms are frequently reclassified based on molecular relatedness. While this has a direct impact on the use of molecular techniques to identify (and therefore name) pathogens in clinical

specimens, detailed discussion of microbial taxonomy will be limited to instances where it is necessary for clarification of specific molecular methods for organisms; the reader is referred to the references listed above for a more complete discussion.

Bacterial Lymphadenitis

Many etiological agents of bacterial lymphadenitis, such as *Staphylococcus aureus*, *Yersinia* spp., and *Francisella tularensis*, are cultured relatively easily from pus or tissue. However, other agents such as *Bartonella* spp., *Tropheryma whippelii*, LGV serovars of *Chlamydia trachomatis*, and *Treponema pallidum* are fastidious, proving difficult or impossible to culture in the routine clinical laboratory. It is these fastidious agents that often cause a diagnostic dilemma in the examination of lymph nodes. Molecular testing for these organisms by nucleic amplification or ISH may aid in the diagnosis of these infections. Selected assays are summarized in Table 44.1.

Bartonella spp.

Bartonella spp. infections cause a number of clinical manifestations in humans. The predominant manifestations pertinent to lymph node examination are cat scratch disease (CSD), caused by *Bartonella henselae* (and rarely other organisms, such as *Afipia felis*⁴) and bacillary angiomatosis (BA), caused by both *B. henselae* and *Bartonella quintana*. These, in addition to other less common *Bartonella* species, are also responsible for a number of other clinical manifestations in humans, including endocarditis, neurologic disease, ocular disease, and others.⁵ Cat scratch disease presents primarily as local lymph node swelling, typically showing necrotizing lymphadenitis microscopically; while the clinical presentation and histologic appearance is characteristic, it is not specific, and the differential diagnosis may also include lymphogranuloma venereum (LGV, see below), yersiniosis, brucellosis, tularemia, mycobacterial infection, fungal infection, and others.^{6,7} The organism is fastidious and grows very slowly in culture. In addition, serologic testing may be difficult to interpret in practice; extensive cross-reactivity exists between the *Bartonella* species, as well as other organisms such as *Chlamydia* and *Coxiella* for some assays, and background seroprevalence may make interpretation difficult.⁵ In addition, individuals who are immunocompromised may be seronegative. Warthin-Starry staining has historically allowed visualization of the organisms within tissue sections, but is nonspecific and poorly sensitive. Immunohistochemical methods have been described for *Bartonella*, but generally show less sensitivity than nucleic acid methods.⁶ Molecular detection has become an important tool in the diagnosis of these infections.⁵ In the evaluation of the performance characteristics of various molecular assays for *B. henselae*, it is

important to keep in mind that, due to the difficulties in confirming CSD due to *B. henselae* by other methods, there is not a true “gold standard,” and the assignment of clinical sensitivity and specificity should be interpreted with caution; in a recent large study, only 31.2% lymph nodes submitted for suspected CSD (the standard from which sensitivity was calculated in most studies listed below) showed *Bartonella* sp.; other diagnoses included malignancy, mycobacterial infections, and other bacterial infections.⁷

Bartonella was first identified as the cause of bacillary angiomatosis by identification and sequencing of 16S rRNA gene sequences in lesions (Table 44.1).⁸ Numerous studies have since established *B. henselae* as the primary cause of CSD^{5,9,10} (with a rare role for *A. felis*⁴), and both *B. henselae* and *B. quintana* as causative agents in bacillary angiomatosis.⁵ Initial studies focused on the 16S rRNA gene as a target; these primers are not completely specific for *Bartonella*, however, and generally require probe, sequence, or restriction fragment length polymorphism (RFLP) confirmation.^{4,11–14} In addition, due to a high degree of similarity of the region in *Bartonella*, it is not as effective for the differentiation of *Bartonella* species (i.e., *B. henselae* vs. *B. quintana* or *Bartonella elizabethae*). The 16S–23S intergenic region (ITS) has also been used for detection of *Bartonella* spp.,⁶ but has shown nonspecific amplification when used alone¹⁵; the use of these highly conserved regions as targets for the detection of *Bartonella* (and indeed, any bacteria) should be accompanied by confirmation of the specificity of the amplicon,¹⁶ either by probe, RFLP analysis, other specific confirmation, or sequencing.

Another target described in early studies was *htrA*, a 60 kDa heat shock protein conserved in *B. henselae*, as well as *B. quintana*. Anderson and colleagues¹⁷ designed a conventional PCR assay using degenerate primers and dot blot hybridization with specific probes to *B. henselae* and *B. quintana*, showing 84% overall sensitivity in fresh lymph node biopsies and aspirates from 25 patients with clinically diagnosed CSD and 100% specificity in non-CSD specimens; *B. quintana* was not identified in any of the clinical specimens from patients with CSD. This same assay, in original form or modified, has been used in a number of additional studies. Mouritsen designed a hemi-nested assay based on the Anderson assay, using the RH1 probe as the inner primer, detecting *B. henselae* in 6 of 12 FFPE lymph node tissue samples for which CSD was in the differential diagnosis; nonspecific amplification occurred with a single *Bartonella vinsonii* isolate.¹⁸ Hansmann and colleagues used this assay in fresh lymph node tissue, with 76% sensitivity and 100% specificity, applying a combined diagnostic algorithm as a gold standard.¹⁹

Another commonly used *Bartonella* target is the citrate synthase gene *gltA*. Norman and colleagues²⁰ cloned and sequenced this gene in *B. henselae*, developed a PCR assay to detect *B. henselae*, and tested it on culture isolates. The assay also amplifies both *B. quintana* and *Rickettsia prowazekii*, requiring

TABLE 44.1. Molecular methods for diagnosis of bacterial infections in lymph nodes: selected assays.

Reference	Target	Method	Primers/probes (5' → 3')	Specimens tested (#), references
<i>Bartonella</i> spp.				
8	16S rDNA	Conventional PCR	p24E: 5'-CCT CCT TCA GTT AGG CTG G-3' p12B: 5'-GAG ATG GCT TTT GGA GAT TA-3'	Refs. 4, 11, 13, 22
17	<i>htrA</i>	Conventional PCR, dot blot hybridization	<i>Primers</i> CAT1: 5'-GAT TCA ATT GGT TTG AAG/A GAG GCT-3' CAT2: 5'-TCA CAT CAC CAG GA/GC GTA TTC-3' <i>Probes</i> <i>B. henselae</i> RH1: 5'-GGT GCG TTA ATT ACC GAT CC-3' <i>B. quintana</i> RQ1: 5'-GGC GCT TTG ATT ACT GAT CC-3'	25 fresh lymph node biopsies and aspirates. Ref. 18: 12 FFPE lymph node specimens Ref. 11
20	<i>gltA</i>	Conventional PCR, RFLP	BhCS.781p: 5'-GGG GAC CAG CTC ATG GTG G-3' BhCS.1137n: 5'-AAT GCA AAA AGA ACA GTA AAC A-3'	Ref. 11
21	<i>gltA</i>	Semi-nested PCR, RFLP	TN-2: 5'-TGG TGG AGC TAA TGA AGC ATG-3' TN-1: 5'-GCA ACA AAC CTG CCA TGA GG-3' IP: 5'-GTT CTG TTG AAA GAA TTC CTG A-3'	8 FFPE (and 1 fresh) lymph nodes
<i>Tropheryma whipplei</i>				
40	16S rDNA	Conventional PCR, Southern blotting	W3FE: 5'-GGA ATT CCA GAG ATA CGC CCC CCG CAA-3' W2RB: 5'-CGG GAT CCC ATT CGC TCC ACC TTG CGA-3' Probe: 5'-ATA CCG ACC TTG CGG GGG GCG TAT CTC TAC GCC TTT CCG GTA TAT A-3'	30 FFPE tissues with histologically diagnosed Whipple's disease.
42	16S rDNA	Conventional PCR, Southern blotting	W3AF: 5'-TAC CGG AAA GGC GTA GAG ATA CGC C-3' W4AR: 5'-CAG TCT CCT GTG AGT CCC CGC CAT T-3' Probe: 5'-ACC CTC GTC CTG TGT TGC-3'	
43	16S rDNA	Real-time PCR, FRET probes	<i>Primers</i> Tw1: 5'-AGA GAT ACG CCC CCC GCA A-3' Tw2: 5'-ATT CGC TCC ACC TTG CGA-3' <i>Probes</i> Tw5fl: 5'-CGG GAC TTA ACC CAA CAT CTC ACG-fluorescein-3' Tw4Lc705: 5'-red705-ACG AGC TGA CGA CAA CCG TGC AC-3'	
44	16S rDNA	In situ hybridization	Tw16S-652: 5'-TTC CGC TCT CCC CTA TCG CAC TCT-3' Control probe Tw16S-Cnt: 5'-AAG GCG AGA GGG GAT AGC GTG AGA-3'	8 FFPE tissues (including 2 lymph nodes) and 19 control tissues.
51	<i>hsp65</i>	Real-time PCR, FRET probes, melt curve analysis	<i>Primers</i> TW704: 5'-AAA GAG GTT GAG ACT G-3' TW899: 5'-ATC GGT TAC AAA ATA AGC-3' <i>Probes</i> TW795 (anchor): 5'-AGA AGG TTG GCA AGG AAG GC-3' TW 817 (donor): 5'-TGT CAC TGT CGA GGA GTC AAA TAC T-3'	321 specimens, including 82 tissue specimens
<i>Chlamydia trachomatis</i> , serovars L1–L3				
64	<i>omp1</i>	Conventional nested PCR, (RFLP or sequencing), AGE	<i>Outer primers</i> NLO: 5'-ATG AAA AAA CTC TTG AAA TCG-3' NRO: 5'-CTC AAC TGT AAC TGC GTA TTT-3' <i>Inner primers</i> PCTM3: 5'-TCC TTG CAA GCT CTG CCT GTG GGG AAT CCT-3' VD42: 5'-TGC AAG GAA ACG ATT TGC AT-3'	Note: Inner primers given are for sequencing. Ref. 67

(continued)

TABLE 44.1. (continued)

Reference	Target	Method	Primers/probes (5' → 3')	Specimens tested (#), references
72	<i>pmpH</i>	Real-time PCR, TaqMan probe	<i>Primers</i> LGV-F: 5'-CTG TGC CAA CCT CAT CAT CAA-3' LGV-R: 5'-AGA CCC TTT CCG AGC ATC ACT-3' <i>Probe:</i> LGV-P: 5'-6FAM-CCT GCT CCA ACA GT-MGB-3'	Ref. 74: 7 FFPE tissue samples (rectal biopsies, lymph node biopsies). Schaeffer: (see below)
<i>Treponema pallidum</i>				
81	<i>tpn47</i>	Conventional PCR (multiplex with <i>H. ducreyi</i> and HSV), probe capture	Primer KO3A: 5'-GAA GTT TGT CCC AGT TGC GGT T-3' Primer KO4: 5'-CAG AGC CAT CAG CCC TTT TCA-3' Probe KO17: 5'-CGG GCT CTC CAT GCT GCT TAC CTT A-3'	Ref. 88: blood, ear scrapings. Ref. 78: 12 fresh skin biopsies
82	<i>tpn47</i>	Conventional nested PCR, AGE, confirmation by RFLP	<i>Outer</i> Trep-339: 5'-CAG CAG GGG AAG AAA AAA GTG GG-3' Trep-716: 5'-AAG GTC GTG CGG GCT CTC CAT-3' <i>Inner</i> Trep-375: 5'-GAC CCA AGC GTT ACT AAG ATG G-3' Trep-564: 5'-ACC GCA ACT GGG ACA AAC TTC AT-3'	13 FFPE tissue specimens from patients with 2° or 3° syphilis. Ref. 84: 3 lymph node aspirates
86	<i>polA</i>	Conventional PCR, AGE and capillary electrophoresis	<i>Assay 1</i> F1: 5'-TGC GCG TGT GCG AAT GGT GTG GTC-3' R1: 5'-CAC AGT GCT CAA AAA CGC CTG CAC G-3' <i>Assay 2</i> F2: 5'-CGT CTG GTC GAT GTG CAA ATG AGT G-3' R2: 5'-TGC ACA TGT ACA CTG AGT TGA CTC GG-3'	Genital ulcer specimens. Note: two assays, both with equivalent performance.
89	<i>polA</i> , <i>tpn47</i> (two assays)	Real-time PCR, TaqMan probe	<i>tpn47 assay</i> Primer TP-7: 5'-CAA CAC GGT CCG CTA CGA CTA-3' Primer TP-8: 5'-TGC CAT AAC TCG CCA TCA GA-3' Probe TP-13: 5'-ROX-ACG GTG ATG ACG CGA GCT ACA CCA-BHQ3-3' <i>polA assay</i> Primer TP-1: 5'-CAG GAT CCG GCA TAT GTC C-3' Primer TP-2: 5'-AAG TGT GAG CGT CTC ATC ATT CC-3' Probe TP-3: 5'-FAM-CTG TCA TGC ACC AGC TTC GAC GTC TT-BHQ1-3'	FFPE gastric tissue (1)
92	<i>tmpA</i>	Conventional PCR, AGE, confirmation by sequencing, RFLP	TP1: 5'-GGC GGC TCG CTC ATA AAG GAA TAC-3' TP2: 5'-TAC GCC GGA GTA CAC AAG CGT ATG-3'	FFPE gastric biopsies (2)

PCR polymerase chain reaction, AGE Agarose gel electrophoresis, RFLP restriction enzyme length polymorphism, BAL bronchoalveolar lavage, FFPE formalin fixed, paraffin embedded, BHQ black hole quencher.

confirmation by RFLP. This assay has been used in a number of other studies,¹¹ including an adaptation to small amounts of sample, including fine needle aspirates and unstained tissue on glass slides.¹⁴ A new set of primers to *gltA* was designed by Margolis and colleagues²¹ for use in a seminested assay with RFLP confirmation, yielding a smaller amplicon than the

Norman assay. The assay did not show any nonspecific amplification (as was seen with the original Norman assay) and showed 100% sensitivity and specificity in 8 FFPE lymph node specimens from patients with clinical CSD.

These assays have been compared to each other on the same material, allowing some point of reference for their

performance relative to each other. Avidor and colleagues¹¹ compared 16S rRNA, *htrA*, and *gltA* assays in 32 fresh clinical samples (including 29 lymph node aspirates and 2 biopsies). The 16S assay was confirmed by dot blot hybridization with a *B. henselae*-specific probe, and the other two assays were confirmed by RFLP. 16S and *gltA* amplification were 100% and 94% sensitive, respectively, while *htrA* was only 64% sensitive; this difference in sensitivity between *htrA* and *gltA* assays was confirmed by DNA dilution experiments. All three assays were 100% specific. Sander and colleagues²² compared the performance of 16S rRNA⁸ and *htrA*¹⁷ assays in 60 FFPE lymph node specimens from patients with suspected CSD, finding 60% positive with the 16S assay (with a slightly smaller amplicon size), and only 43.3% positive with *htrA*. This group subsequently designed a modified PCR-enzyme immunoassay based on the same 16S primers, showing 100% sensitivity in FFPE tissues and demonstrating the increase in sensitivity that may be accomplished, using the same primers and more sensitive detection methods.

Importantly, a number of these assays have been used to test FFPE tissue, the most challenging of samples. Formalin fixation usually leads to DNA fragmentation, decreasing the sensitivity of nucleic acid amplification tests (NAAT), particularly those producing large amplicons. While an assay may be designed with excellent analytical sensitivity (by dilution experiments) and analytical specificity when using bacterial isolates, it must be validated in the specimen types for which it will be used, to account for effects such as nucleic acid degradation (FFPE tissue), inhibitory substances (i.e., pus), or differences in distribution of the organism in an infected patient (i.e., when sensitivity in tissue is poor and sensitivity in blood is high, or vice versa).

Some of the previously-described assays have been further modified, including adaptation to real-time PCR methods^{23–25}; real-time PCR is faster, less labor intensive, and has less risk of contamination due to the use of a closed system, although it is not necessarily always more sensitive.²⁶ In addition, the 16S–23S intergenic spacer target has been used for the in situ detection of *B. henselae* cells,²⁷ and a 16S probe was used to diagnose endocarditis by detecting *B. quintana* in heart valve tissue. While in situ assays do not appear to be as sensitive as amplification, they are another potential tool for diagnosis.²⁸

While the most commonly used and cited *Bartonella* targets are 16S, *htrA*, and *gltA*, a number of conventional and real-time assays have been designed using other gene targets, including the 16S–23S intergenic spacer region (ITS), *rpoB*,²⁹ *ribC*,^{25,30,31} heat shock protein *groEL*,^{32,33} and *pap31*.^{7,32} The latter two targets have shown sensitivity in a large number of fresh lymph node tissue and aspirate specimens, while also allowing for differentiation of *Bartonella* species (*groEL*) and subtyping of *B. henselae* strains (*pmp31*).³²

Summary

While clinical history, histology, and serology are useful in the diagnosis of CSD, their limitations make molecular techniques an important additional tool for the confirmation

of the diagnosis.^{7,19} Several targets for NAAT testing, most frequently 16S rRNA, *htrA*, and *gltA* genes, have been successfully used in both fresh and FFPE lymph node tissue, in addition to lymph node aspirates; it is important to note that assay sensitivities are uniformly lower in FFPE material, as expected. The number of different targets that have been used successfully is particularly fortunate. This provides the option of confirming results using a second target, an important step when results are questioned due to the amplification of a highly conserved target or suspicion of contamination. Testing for other *Bartonella* species, such as *B. quintana*, may be useful in bacillary angiomatosis lesions that are difficult to differentiate from other lesions, such as Kaposi's sarcoma.¹² *Bartonella* NAAT testing on tissue (fresh/frozen, including FFPE for some) is available at several reference laboratories in the United States, as well as at many clinical laboratories in large academic centers globally, making this testing available for those without local access.

T. whipplei (Whipple's Disease)

Whipple's disease is a rare systemic bacterial disease caused by *T. whipplei*, with manifestations including lymphadenopathy, fever, weight loss, diarrhea, central nervous system involvement, and arthritis.^{34,35} Lymphadenopathy with systemic symptoms may predominate,^{35,36} bringing into the differential diagnosis other infectious lymphadenitides, sarcoidosis,³⁷ or, in some cases, malignant lymphoma.³⁸ The diagnosis of Whipple's disease is usually made on upper endoscopy with small intestinal biopsy(ies), which show(s) PAS-positive, diastase-resistant material within macrophages in the lamina propria.³⁵ However, biopsies may be PAS-negative in true cases proven by electron microscopy (and PCR)³⁹; in addition, the finding of PAS-positive material in macrophages is not always specific for Whipple's disease.^{35,39} The organism does not grow in conventional culture, and was discovered through amplification and then sequencing of the 16S rDNA gene using broad-spectrum primers.⁴⁰ Once the organism had been identified, molecular testing became an important tool for the diagnosis of this disease; molecular testing is especially important in the confirmation of disease in extraintestinal sites or in unusual presentations. As with CSD (see above), the lack of a true "gold standard" makes the interpretation of sensitivity and specificity of molecular assays for *T. whipplei* difficult.

In 1992, Relman and colleagues⁴⁰ reported the identification of the causative organism of Whipple's disease in a duodenal biopsy using broad range primers to the 16S rRNA gene followed by sequencing. Specific primers and a hybridization probe were then designed which identified identical sequences in five additional patients with the disease. Based on the findings, they proposed the organism be named *T. whipplei*. These same primers (Table 44.1) were used to confirm a case of Whipple's disease in a patient presenting with low grade fever, weight loss, malaise, and massive lymphadenopathy, highly suspicious for malignant

lymphoma; an abdominal lymph node (FFPE) was positive by PCR, and the patient improved on long-term antibiotic therapy.³⁸ These primers have been subsequently used in several series of fresh and FFPE lymph node biopsies from patients with Whipple's disease.^{37,41} Amplicons generated using these primers are confirmed by probe hybridization or sequencing due to the conserved nature of the primers used and potential cross-reactivity with other organisms.

Ramzan and colleagues⁴² modified the Relman primers for improved sensitivity and specificity (Table 44.1). Non-specific amplification was identified with *Rhodococcus* and *Nocardia* isolates, and so a *T. whipplei*-specific probe was used (as in the original assay). This assay was used to study FFPE archival tissues of patients with Whipple's disease, including several lymph nodes. The assay had a sensitivity of 96.6% and specificity of 100% when testing tissues with histologic evidence of Whipple's disease (PAS positive). They were also able to identify *T. whipplei* DNA in several cases of suspected Whipple's disease without histologic findings (PAS negative). A real-time PCR assay with fluorescence resonance energy transfer (FRET) probes targeting the 16S rDNA was designed and tested in 263 samples, including intestinal and lymph node biopsies, showing a calculated 95.7% sensitivity and 97.8% specificity.⁴³ Fluorescent ISH (targeting 16S rRNA sequences) has also been performed in both intestinal and lymph node FFPE tissues.⁴⁴ Additional gene targets for amplification in the ribosomal gene complex include the 16S–23S rDNA intergenic spacer region⁴⁵ and the 23S rRNA gene.⁴⁶

Once *T. whipplei* was finally cultured,⁴⁷ other gene targets could be sequenced, allowing the development of other, non-ribosomal targets for detection, most of which have been compared to 16S amplification assays. The development of these alternate targets was particularly important with the reporting of *T. whipplei* DNA detection in the saliva and other tissues of healthy patients.^{35,48} Drancourt and colleagues⁴⁹ sequenced the conserved beta-subunit of the RNA polymerase (*rpoB*), and designed a *T. whipplei*-specific conventional PCR assay that detected the organism in three tissue samples that were previously positive by 16S amplification. In an attempt to improve specificity, Fenollar⁵⁰ adapted this *rpoB* assay, as well as an intergenic spacer assay,⁴⁵ to a real-time PCR format, using SYBR green for detection of amplification. They were successfully used in tandem to detect and confirm *T. whipplei* in frozen tissue samples.⁵⁰

In 2000, Morgenegg and colleagues reported the cloning and sequencing of the *T. whipplei* heat shock protein *hsp65* gene from a patient specimen (heart valve) and the subsequent development of a seminested PCR assay targeting this gene.⁵¹ This assay showed good performance compared to the 16S assay in 17 clinical specimens (including 7 fresh and 1 FFPE tissue). The *hsp65* sequence was then subsequently used by Sloan and colleagues⁵² in the design of a real-time PCR assay on the LightCycler platform. This assay was tested on specimens from 321 patients, including 82 fresh and FFPE tissues,

showing excellent sensitivity and specificity, both clinically and in comparison to a conventional 16S assay.

In an attempt to further enhance sensitivity, Fellonar and colleagues⁵³ targeted a repeat element found in the *T. whipplei* genome sequence for the design of another real-time PCR assay (with SYBR green detection). In 98 clinical samples previously tested by both *rpoB* and ITS assays,⁵⁰ they found improved clinical sensitivity and 100% specificity after testing 40 other bacterial strains; all amplicons were sequenced for confirmation.

Summary

Due to the dearth of other specific laboratory tests available, molecular testing is the only method besides electron microscopy for direct confirmation of *T. whipplei* in patients with Whipple's disease. Testing on tissue specimens is available in a number of reference laboratories and academic centers (some accepting FFPE tissues). Unfortunately, *T. whipplei* DNA has been detected in specimens from healthy individuals^{34,48}; it is unclear whether this represents colonization with *T. whipplei*, or contamination (particularly when using nested PCR assays).³⁵ It is recommended that in unusual cases and atypical presentations (which would include testing of lymph nodes), positive results should be confirmed with a second, nonribosomal *T. whipplei*-specific target.^{34,51} Fortunately, assays targeting other genes with good performance have been described. However, even using multiple assays, diagnosis may still be difficult; clinical correlation and repeated sampling may be indicated in such cases.⁵⁴

C. trachomatis, Serovars L1–L3 (Lymphogranuloma Venereum, LGV)

Lymphogranuloma venereum (LGV) is a sexually-transmitted disease (STD), caused by the lymphoinvasive serovars of *C. trachomatis* (i.e., serovars L1, L2, and L3). It usually begins with a small, painless ulcer or ulcers at the site of inoculation, followed by regional lymphadenitis. If left untreated, it may lead to extensive morbidity due to rectal scarring and fistula formation.^{55,56} LGV is endemic in tropical and subtropical parts of Asia, Africa, central and South America, and the Caribbean, but has been uncommon in other parts of the world until recently. Since 2004, there has been an evolving outbreak of LGV in the United States and Europe among men who have sex with other men (MSM), making the prompt recognition and diagnosis of LGV in these countries vital.^{57,58}

The diagnosis of LGV is usually clinical, but findings are relatively nonspecific and patients may rarely present with isolated lymphadenitis. LGV may not be suspected until histologic examination of biopsy material; the differential diagnosis of its appearance includes CSD,^{59,60} syphilis, tularemia, as well as mycobacterial and fungal infections.⁶¹ Culture is not routinely available, is labor

intensive, and shows poor sensitivity. Serologic testing is available, but extensive cross-reactivity between *C. trachomatis* serovars (and also other *Chlamydia* spp.) makes interpretation difficult; in addition, serologic responses take time to develop, and may be attenuated in immunocompromised patients. While direct antigen testing (DFA) for *C. trachomatis* is available, it is less sensitive than molecular techniques and cannot differentiate LGV serovars.^{56,62}

The new gold standard for the diagnosis of *C. trachomatis* infections is NAAT, and a number of kit-based assays approved for patient testing are commercially available, all with excellent performance characteristics in the diagnosis of *C. trachomatis* urogenital infections; primary amplification targets include ribosomal RNA or the cryptic plasmid.⁶² These assays detect the LGV serovars, but they will not differentiate them from the other less invasive serovars. In addition, these assays are not validated for lymph node specimens (aspirates or tissue) in most clinical laboratories. Few studies have reported molecular detection of LGV serovars in lymph node material, as most diagnoses of LGV using molecular methods are based on testing of rectal or urogenital swab specimens. While 16S rDNA gene amplification and sequencing has been performed to identify *C. trachomatis*,⁶³ there is not sufficient sequence variability to distinguish LGV serovars. Therefore, when 16S rDNA detection is used, it may only identify the organism as *C. trachomatis*⁶¹; additional genotyping for LGV serovars is required for specific identification.⁶⁰

The standard approach to molecular identification of *C. trachomatis* serovars is to amplify the major outer membrane protein gene (*omp1*) and then identify serovars by restriction fragment length polymorphism (RFLP) or sequencing. The *omp1* gene of *C. trachomatis* has both highly conserved regions (useful for detection) and variable regions (useful for genotyping). Lan and colleagues⁶⁴ designed a nested PCR assay amplifying the *omp1* gene; serovars could be identified by RFLP or sequencing (Table 44.1). *Omp1* gene detection has subsequently been used by a number of other investigators for the detection of LGV serovars of *C. trachomatis*, including the development of a real-time PCR assay for use with rectal swabs.⁶⁵ A specific real-time PCR assay targeting *omp1* has also been developed to identify serovars L1, L2, and L3 individually in oral, rectal, and urogenital swabs,⁶⁶ and an L2 specific *omp1* real-time PCR assay was developed to detect the epidemic serovar in New York.⁶⁷ Assays using this target have also been used on fresh and FFPE lymph node tissue with success.^{68–71} Kellock⁶⁸ used *omp1* amplification according to the Lan assay on FFPE lymph node tissue to confirm the unexpected diagnosis of LGV in a low-risk woman from a nonendemic region. Bauwen also used *omp1* amplification and sequencing on lymph node material (fresh lymph node aspirates) to make the diagnosis of LGV, confirming an epidemic of LGV in the Bahamas.⁶⁹

Another promising target for LGV detection is the polymorphic membrane protein H gene (*pmpH*), which has a unique sequence gap in LGV serovars that is not present

in other serovars; this major difference may be exploited to identify these strains.⁷² Morre and colleagues developed a real time PCR assay with a sensitivity of 0.01 inclusion-forming units per reaction and 100% specificity when testing other serovars as well as other nonchlamydial bacteria.⁷² These same primers could be adapted to conventional PCR, with identification of LGV serovars based on amplicon size after gel electrophoresis.⁷³ This assay has been used with success not only on rectal swabs,⁶⁶ but also on a small number of FFPE tissues, including lymph nodes.⁷⁴ Another study used the cysteine-rich outer membrane protein (*CrP*) gene as a target for differentiation of LGV serovars in genital ulcers.⁵⁵

Summary

Molecular testing for confirmation of the diagnosis of LGV is usually performed on rectal or urogenital swabs. However, testing of fresh and FFPE lymph nodes has met with success in several published reports. It has been recommended that the diagnosis of LGV be confirmed by NAAT, to include testing on lymph node aspirates and biopsy material when other specimens are not available.⁵⁷ This may be particularly useful when LGV is not initially suspected or the presentation is atypical. This testing may become increasingly important as global epidemiology of LGV evolves to include more frequent infections in previously nonendemic regions. Definitive diagnosis by molecular methods in unclear histologic cases of lymphadenitis allows for the initiation of appropriate specific antibiotic therapy,⁵⁹ as well as public health investigation.

T. pallidum (Syphilis)

Syphilis is a systemic STD caused by the spirochete *T. pallidum*. It is known as “the great imitator,” and the lymph node manifestations are no exception. Lymph node involvement may predominate,⁷⁵ mimicking lymphoma or other malignancy.^{76,77} Direct detection of the spirochetes in smears by darkfield examination may be performed in primary or secondary lesions, but is insensitive and difficult to interpret; silver staining of tissues is also insensitive.^{78,79} Immunohistochemical methods (IHC) show promise for the detection of *T. pallidum* in tissues, but false positivity has been described with other spirochetes including *Borrelia*.^{78,79} Serologic testing with nontreponemal (i.e., rapid plasma reagin (RPR) and Venereal Disease Research Laboratory (VDRL)) and treponemal (*T. pallidum* particle agglutination (TP-PA) and fluorescent treponemal antibody absorbed (FTA-ABS)) antibody tests is the mainstay of diagnosis, but may show decreased sensitivity in both early syphilis and in latent disease. Molecular testing for *T. pallidum* is an increasingly important tool in the confirmation of *T. pallidum* infection in unusual or atypical infections; the vast majority of testing has been performed on genital ulcer specimens and blood, but some studies have investigated small numbers of fresh and FFPE tissue, including lymph nodes.

One of the most common targets used in the molecular detection of *T. pallidum* is the 47-kDa protein gene (*tpn47*). This target was used in a conventional PCR assay with Southern blot confirmation to test blood, CSF, amniotic fluid, and FFPE rabbit testes; nonspecific priming was identified, but was negative by probe hybridization.⁸⁰ Orle and colleagues developed an assay targeting *tpn47*, along with *Haemophilus ducreyi*- and herpes simplex virus-specific targets, to develop a multiplex PCR reaction for the diagnosis of genital ulcers.⁸¹ These same primers and probe were used to compare the performance of darkfield examination, IHC, and conventional PCR with Southern blotting in 12 skin biopsy specimens; polyclonal IHC had a sensitivity of 90%, while PCR (performed on frozen tissue) was 75% sensitive.⁷⁸ Another group of investigators redesigned primers to the *tpn47* target in a nested PCR reaction with a smaller amplicon for use in FFPE skin tissues.⁸² Kouznetsov used this modified assay to identify *T. pallidum* in three lymph node aspirates from patients with suspected secondary or latent syphilis and lymphadenopathy,⁸³ as well as fresh skin biopsies in patients with secondary syphilis.⁸⁴ Another assay targeting *tpn47*⁸⁵ was used in the postmortem confirmation of syphilitic aortitis in FFPE tissues from a patient who had been suspected of having a mediastinal malignancy.⁷⁶

Liu and colleagues used an alternative target, the DNA polymerase I gene (*polA*), in a new assay for diagnosis in genital ulcer specimens, showing excellent sensitivity and specificity compared to the Orle multiplex PCR assay.⁸⁶ However, Behrhof and colleagues attempted to use this assay in FFPE tissues (skin), and found markedly decreased sensitivity. They designed a new semi-nested PCR assay targeting a different region of *polA*, producing a smaller amplicon more suited to use in FFPE tissues, and they were able to increase the sensitivity in these specimens.⁷⁹ Additional assays targeting *polA* have been described, including a real time PCR assay.⁸⁷

A comparison of Orle's *tpnA* assay and Liu's *polA* assay was performed on 87 clinical samples (blood, sera, and ear scrapings) from patients with latent and treated syphilis, showing significantly increased relative sensitivity of the *tpnA* assay in these specimen types.⁸⁸ On the other hand, Chen and colleagues⁸⁹ described the development of two different real-time PCR assays targeting *tpn47* and *polA*, respectively, with equivalent analytical sensitivity and specificity (Table 44.1). These assays were used in the evaluation of FFPE tissue in a patient who had undergone partial gastrectomy for suspected malignancy; both real-time PCR assays confirmed the diagnosis of gastric syphilis.⁸⁹

Additional assays have also been described targeting the 16S rRNA gene,⁸⁵ *bmp* gene,⁹⁰ *rpoB* gene,⁹¹ and *tmpA* gene, including a study confirming gastric syphilis in FFPE from two patients with suspected lymphoma.⁹²

Summary

Molecular testing for *T. pallidum* is increasingly used in the diagnosis of genital ulcers, most commonly with assays targeting the *tpn47* gene. While studies of lymph node material

are scarce, successful molecular testing has been reported using other fresh and FFPE tissues. The small numbers of specimens tested, in addition to differences in timing of collection (secondary versus latent syphilis) and other factors make interpretation of sensitivity difficult, but specificity has been excellent with the commonly used assays. Thus, as with molecular testing for many other pathogens in tissues, a positive result may be considered confirmatory, although a negative result will not rule out disease. This tool, in conjunction with immunohistochemistry, may be useful for the confirmation of syphilitic lymphadenitis.

Broad Range Detection of Bacteria

Bacterial pathogens may also be detected in tissues using broad range amplification of highly conserved targets; indeed, two of the agents discussed above were discovered in this way.^{8,40} This has been particularly useful in tissues such as excised heart valves, where the infecting organism cannot be identified by other means,⁹³ but has also been shown to be useful for the diagnosis of lymphadenitis.⁷ The most common approach has been to amplify the 16S rDNA gene,^{63,94,95} but other conserved targets include heat shock proteins and *rpoB*. In addition, array-based technology has been applied to the diagnosis of granulomatous lymphadenitis in FFPE lymph node tissue.⁹⁶ Because of the ubiquitous presence of bacteria and their nucleic acid in the environment (including some reagents), great care must be taken to prevent contamination, and stringent negative controls must be used.^{15,95} The conserved nature of these targets requires confirmation of products, usually by sequencing¹⁶; if mixed bacteria are present, amplicons may need to be separated prior to sequencing (usually by cloning) for a reliable result.⁹⁴

One vital aspect of broad range detection and sequencing of bacteria is the quality of the sequence database used to assign identity of the organism. Even with a reliable database, the criteria used for assignment of a genus and/or species must be defined; not all bacterial genera may be reliably differentiated at the species level using 16S sequences, due to high levels of similarity; for these, amplification of a different conserved target may be indicated.⁹⁵ While broad-range amplification is a powerful tool that can cast a broad "net" (not unlike routine culture), the conserved nature of the targets, the need for valid sequence databases, and the potential for contamination have led to the suggestion of a two step process.⁷ Once an organism is identified using broad range PCR and sequencing of a highly conserved target, such as the ribosomal RNA gene complex, the result should be interpreted in the clinical context, and perhaps confirmed using a second, organism-specific assay that amplifies a second target.

Mycobacterial Lymphadenitis

Mycobacterial lymphadenitis causes a necrotizing granulomatous lymphadenopathy, which may be histologically similar to CSD, fungal lymphadenopathy, or other infections. The frequency of *Mycobacterium tuberculosis* (MTB)

complex-associated lymphadenitis versus mycobacteria other than tuberculosis (MOTT) lymphadenitis depends on population, geographic location, and endemicity of tuberculosis. As antimicrobial and surgical treatment differs depending on the mycobacterial species causing disease, confirmation of diagnosis is important. Diagnosis may be made by acid fast staining of tissues, but this is relatively insensitive, and does not differentiate between mycobacterial species. Culture is also relatively insensitive, and may be very slow (several weeks). Many investigators have turned to molecular methods in an attempt to improve both the sensitivity and speed of the diagnosis of mycobacterial lymphadenopathy. While success has been more limited than had been hoped, these methods are useful in conjunction with conventional diagnostic methods and clinical criteria to aid in the diagnosis.

M. tuberculosis Complex

Tuberculosis is most frequently caused by *M. tuberculosis*. However, other members of the *M. tuberculosis* complex (MTBC) can also cause tubercular disease, and include *Mycobacterium bovis* (including the attenuated vaccine BCG strain), *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium canettii*, *Mycobacterium caprae*, and *Mycobacterium pinnipedii*. Most molecular methods do not differentiate between the closely related members of this complex.⁹⁷ There is a proliferation of assays described and in use on lymph node material, with multiple targets, multiple primer sets, and different extraction methods, including several commercial methods that have been variously modified for use on lymph node aspirates and tissues. In addition, the patient populations and gold standard against which these assays are compared varies widely, making interpretation of the performance of these assays in lymph nodes and their comparison to each other very difficult. Several of these assays are described below.

One of the most common targets used in the detection of MTB is IS6110, a multicopy insertion sequence present in MTB (and also present as a single copy in *M. bovis*). One of the most commonly used primer sets to the IS6110 target was developed by Eisenach and colleagues,⁹⁸ producing a 123bp amplicon. In the initial studies, a single strain of *Mycobacterium simiae* was also detected by the Eisenach primers; although this has not been found in subsequent studies, it is an important consideration in geographic locations where *M. simiae* is a common clinical isolate. Diaz and colleagues⁹⁹ used these primers and confirmation by Southern blotting to detect MTB in 64 FFPE tissue specimens from patients in Mexico, including lymph nodes, as well as liver, lung, and other tissues. They found complete agreement with culture in all 6 lymph nodes tested (100% sensitivity compared to culture). Yang and colleagues investigated molecular detection of MTB in 8 FFPE lymph nodes with histologic evidence of TB from patients who responded to antituberculous therapy.¹⁰⁰ They compared three conventional assays to three targets (i.e., IS6110, 16S ribosomal RNA, and protein antigen B, finding

50% sensitivity compared to the clinical gold standard protein anti standard in all three assays. Additional investigators have used IS6110 targeted assays on frozen lymph node material,^{101–103} including a study by Heinmoller demonstrating the importance of sampling issues when selecting FFPE vs. fresh/frozen lymph node tissue as a specimen for testing.¹⁰⁴

Several investigators have also used IS6110 assays in the detection of MTB in lymph node aspirates, particularly in high prevalence areas. Kidane and colleagues studied lymph node aspirates from 40 Ethiopian patients with tuberculous lymphadenitis by clinical and cytological criteria,¹⁰⁵ using three assays targeted to antigen 85 complex, IS6110, and *pncA* (for identification of *M. bovis*). Sensitivity of both IS6110 and antigen 85 assays were 87.5% for MTB complex (35/40); six aspirates were identified as *M. bovis*. Gong and colleagues used a one-step nested PCR to the IS6110 target to test 118 fresh lymph node aspirates in Korean patients with lymphadenitis.¹⁰⁶ Compared to the gold standard of culture or clinical evidence of disease (including response to anti-tuberculosis therapy), PCR detected 77.8% of 63 patients with TB lymphadenitis, while acid fast staining identified only 39.7%. In addition, *rpoB* gene mutation, indicating rifampin resistance, was detected by PCR-single strand conformation polymorphism in 7 of 22 specimens tested.

Another common target used for molecular diagnosis of tubercular lymphadenitis is MPB64, particularly in India, Pakistan, and other areas with a significant percentage of IS6110-negative MTB.^{105,107} In 1999, Manichopisit described the use of MPB64 PCR on fresh lymph node aspirates in a high incidence area (i.e., Bangkok, Thailand), where MTB infection was responsible for approximately 24% of cervical masses, showing 84% sensitivity and 75% specificity compared to a combined gold standard of culture, acid fast staining, histopathology, and response to therapy.¹⁰⁸ One group of investigators used a nested MPB64 assay to detect MTB in fresh lymph node aspirates from Indian patients with clinically confirmed tuberculosis, demonstrating 100% specificity and increased sensitivity over culture.¹⁰⁷ Conversely, another Indian group also used a MPB64 assay to test lymph node aspirates, showing only 89.5% sensitivity and 86.1% specificity compared to culture. Singh and colleagues compared PCR to IS6110 and MPB64 targets in fresh lymph node material from patients in India with clinically suspected disease.¹⁰³ PCR positivity was 48.2% for MPB45 vs. 69.1% for IS6110, but 77.8% for both, supporting a role for testing more than one target, particularly in regions with a high percentage of MTB with low- or zero-copy IS6110.

As with molecular assays for other bacteria, a number of investigators have used ribosomal DNA gene complex targets for the detection of MTB (as well as other mycobacteria; see below), including commercially available assays. In 1998, Yang (see above) used 16S, as well as IS6110 and protein antigen B, for detection of MTB in FFPE lymph nodes, with 50% sensitivity compared to histologic and clinical diagnosis.¹⁰⁰ Bruijnesteijn and colleagues developed a

real-time PCR assay, based on an assay described by Roth¹⁰⁹ and targeting the 16S–23S spacer region (ITS), to detect mycobacteria in lymph node aspirates and tissue from children with lymphadenitis, with probes specific for MTB and *Mycobacterium avium* (see also below).

Two common commercial nucleic acid amplification tests targeting 16S have been modified by investigators to detect MTB in lymph node tissue. Baek and colleagues used the COBAS AMPLICOR *M. tuberculosis* Test (Roche Diagnostic Systems, Inc.) to detect MTB in fresh lymph node aspirates from Korean patients with clinically diagnosed tuberculous lymphadenitis.¹¹⁰ Sensitivity of PCR was 76.4% and sensitivity of cytologic findings was 52.9%, with combined sensitivity of 82.4%; specificity was 100%. Another group compared this assay with an IS6110 assay on fresh/frozen lymph node tissue in German patients,¹¹¹ showing decreased analytical sensitivity as well as decreased sensitivity (vs. 87.5%) and specificity (87.5% vs. 100%) compared to the IS6110 assay using culture as the gold standard. Investigators in Peru¹¹² used the COBAS assay on lymph node aspirates and tissues, showing only 58.2% sensitivity in 55 patients with MTB by histology and/or culture; sensitivity was higher in patients more likely to have higher bacillary loads, including acid fast stain positive specimens (83%) and HIV patients (94%). Fernstrom and colleagues were able to increase the sensitivity of the COBAS assay on tissues from 63% to 92% by first incubating the specimen in culture medium for several days.¹¹³ O'Sullivan and colleagues used a commercially-available transcription-mediated amplification assay targeting the ribosomal gene complex (Amplified *M. tuberculosis* Direct Test, AMTD, Gen-Probe, Inc.) on multiple specimens including lymph node material, showing 73% sensitivity compared to a combined clinical and culture diagnostic standard.¹¹⁴ In lymph node aspirates from patients in Djibouti, another group found disagreement between culture and the AMTD test, but found sensitivity of 93% and specificity of 100% using a calculated gold standard based on clinical data.¹¹⁵

A number of other sequences have been targeted by investigators to detect MTB in lymph node specimens, including *devR*,^{102,116,117} protein antigen B,^{100,118} 65 kDa heat shock protein (*hsp65*),¹¹⁹ and the antigen 85 complex (*Ag85*).¹⁰⁵ In addition, specimens such as archival material from lymph node aspirate slides¹²⁰ and peripheral blood mononuclear cells in patients with tuberculous lymphadenitis¹²¹ have been used.

Overall, when evaluated based on a clinically confirmed diagnosis of tuberculosis, nucleic acid amplification is specific, but not very sensitive in lymph node aspirates and biopsies, and should not replace culture, which is still considered the gold standard. Paucibacillary disease, presence of inhibitors, inadequate lysis of the organism, specimen volume, and sampling issues have all been suggested as reasons for disappointing performance of molecular testing in the diagnosis of MTB infection, and it does appear that the processing and nucleic acid extraction method matters greatly.

However, conventional culture is slow and has relatively low sensitivity, and nucleic acid amplification is another tool that may aid in the confirmation of the diagnosis of tuberculous lymphadenitis; a combination of diagnostic assays (culture, histology/cytology, and nucleic acid testing) may give the greatest sensitivity.¹¹⁹ Because of the many different assays in use, it is important to know the performance of the test being used, and to understand its limitations. While sensitivity has been limited, specificity for most assays has been excellent. Therefore, in clinical practice, while a positive test is usually specific, a negative test does not rule out tuberculous disease.

Mycobacteria Other Than Tuberculosis (MOTT)

Mycobacteria other than tuberculosis (MOTT) are a common cause of mycobacterial lymphadenitis, with prevalence varying with geographic location. While *M. avium* is one of the more common isolates, a number of other mycobacteria have been identified in lymph node tissue, both in culture as well as by molecular techniques. Several general mycobacterial detection assays have been described (see section below). In addition, specific assays have also been developed. In 1990, Williams designed a PCR assay for detection of *Mycobacterium leprae*, targeting a specific 18 kDa protein, for use in FFPE skin biopsies¹²²; the assay was 100 times more sensitive than direct microscopic observation. The primers and hybridization probe were used to detect *M. leprae* in a FFPE lymph node (and skin biopsy tissue) in an Indian patient with lepromatous leprosy.¹²³ As *M. leprae* cannot be grown in routine culture, this could potentially be a valuable tool for the confirmation of disease in some cases.

Additional specific assays for MOTT have been described in lymph node material. *Mycobacterium paratuberculosis* was identified with an assay targeting the IS900 insertion sequence in FFPE lymph node tissue from a patient who ultimately was diagnosed as having Crohn's disease.¹²⁴ Haas and colleagues designed a PCR assay targeting IS1245 to detect *M. avium* in three patients with mycobacterial lymphadenitis.¹²⁵ Amplification of *hsp65*, followed by RFLP and sequencing, was used to detect *Mycobacterium chelonae* in FFPE lymph node tissue from a patient with lymphadenopathy not responding to antibiotic therapy (see also section below).¹²⁶ Bruijnesteijn and colleagues used their previously described real time PCR assay targeting the ITS region, combined with a probe specific for *Mycobacterium haemophilum*, to detect this species in 16 of 89 lymph nodes for which mycobacterial infection was suspected, but a species could not previously be identified; results were confirmed by sequencing.¹²⁷ A number of investigators have also developed assays, or assay algorithms, for the identification and differentiation of the *M. bovis* BCG vaccine strain in FFPE LN, using IS6110 in combination with other targets, such as *pncA* and *oxyR*¹²⁸ or *hsp65*.¹²⁹ Other investigators have targeted the RD1 region to differentiate vaccine strain from wild type *M. bovis* in culture isolates.^{130,131}

General Mycobacterial Detection

A number of approaches have been developed for the general detection of mycobacteria in lymph nodes. Some investigators have targeted the 16S¹³² or ITS¹⁰⁹ regions of the ribosomal RNA gene complex. However, some mycobacterial species cannot be distinguished using these targets, including *Mycobacterium marinum* vs. *Mycobacterium ulcerans*^{111,133} as well as *M. chelonae* vs. *Mycobacterium abscessus* and *Mycobacterium gastri* vs. *Mycobacterium kansasii*,¹³² requiring additional testing for species identification. As described above, Bruijnesteijn and colleagues developed a real-time PCR assay targeting the ITS region to detect mycobacteria in lymph node aspirates and tissue from children with lymphadenitis, with confirmation of species by sequence analysis.¹³³ Other investigators have used the single copy gene *hsp65*, followed by RFLP, as a general mycobacterial assay,¹³⁴ including extensive algorithms for identification of species.¹³⁵

Fungal Lymphadenitis

Introduction

Lymphadenopathy is a frequent clinical manifestation of some disseminated and systemic fungal diseases. In addition, patients may present with isolated fungal lymphadenitis. In the vast majority of cases, the diagnosis of fungal lymphadenitis is made using nonmolecular methods (i.e., serology, antigen tests, or culture). However, if a fungal etiology is not initially considered, a lymph node biopsy or excision may be performed for suspected malignancy without submission of tissue for fungal culture. In these cases, the diagnosis may usually be made based on additional patient testing, and characteristic morphology of organisms on histology. Gomori silver staining (GMS) is considered the most sensitive stain for fungal elements in tissue, comparing favorably to some molecular methods (see below). However, occasionally, specific confirmation is sought because of unusual clinical presentation, atypical or inconclusive morphology, or conflicting laboratory results. Several molecular methods for the diagnosis of fungal infections are described in Table 44.2.

At the time of writing this chapter, molecular methods for the identification of fungal culture isolates are undergoing rapid development. However, molecular methods are not widely used in clinical practice outside of reference and academic centers, especially in tissue specimens such as lymph nodes. While there are data on various targets for molecular testing, well-standardized assays have not been developed for the common agents of lymphadenitis, possibly in part because other methods of diagnosis are frequently used.

There are two general approaches to the use of molecular methods for the detection of fungal organisms in clinical

specimens: (1) molecular targeting (via amplification and/or probe) of genes or other sequences specific to the pathogen of interest, or (2), use of molecular targets common to all fungi (such as the ribosomal RNA gene complex), often combined with either probes or sequencing for specific identification of the pathogen. Each has its advantages and disadvantages. A description of the common approaches to fungal detection will follow the discussion of specific agents.

Histoplasma capsulatum

Histoplasmosis, caused by the dimorphic fungus *H. capsulatum*, is one of the endemic mycoses, with a geographic distribution focused in the Ohio and Mississippi River valleys of North America, as well as some areas of South America, with rare reports of sporadic disease in nonendemic regions.¹³⁶ In addition, *H. capsulatum* var. *dubosii* is found in Africa. *H. capsulatum* usually causes either asymptomatic or self-limited disease in immunocompetent individuals, but it may cause potentially life-threatening disseminated disease in those with compromised immune systems. Lymph node involvement includes isolated lymphadenitis, mediastinal lymph node involvement in acute pulmonary histoplasmosis that may evolve into granulomatous mediastinitis, and lymphadenopathy as a component of disseminated histoplasmosis.¹³⁷ *Histoplasma* infection is diagnosed by culture, serology, and antigen testing of urine and other body fluids. It may also be diagnosed by histologic examination, typically causing a necrotic granulomatous reaction in lymph nodes. In clinical specimens, including tissue, the organism appears as a small (2–5 µm) budding yeast, which may be found intracellularly; the most sensitive stain for detecting the organism in histologic sections is Gomori Methenamine Silver (GMS) staining.^{138,139} These structures may be confused with small yeasts in the *Candida* genus (especially *Candida glabrata*), with the endospores of *Coccidioides*, or with other fungal organisms, such as *Sporothrix* or *Penicillium marneffei*.

Unfortunately, the mould form of *Histoplasma* cultivated in the clinical microbiology laboratory grows quite slowly (especially in the development of its diagnostic fruiting structures); in addition, it is a potential cause of laboratory-acquired infections, and must be handled with special care. A commercial nonamplified probe test targeting ribosomal RNA (AccuProbe, Gen-Probe, Inc.) is available for the rapid confirmation of *H. capsulatum* in culture, prior to the development of morphologically characteristic structures; this test is in widespread use in endemic areas. Other than probes used for culture confirmation, molecular methods have not been routinely used or recommended for diagnosis and monitoring of *Histoplasma* infection, and their current and future role remains unclear.¹⁴⁰ Fortunately, diagnosis is usually not an issue, even when culture has not been performed. However, additional confirmation may be desired when culture is not available and morphology is atypical or in conflict with laboratory or clinical findings.¹⁴¹

TABLE 44.2. Molecular methods for diagnosis of fungal infections: selected assays.

Reference	Target	Method	Primers/probes (5' → 3')	Specimens tested (#), other references
Panfungal and multiplex assays				
172	rDNA gene complex (ITS1-5.8S-ITS2)	PCR	ITS1 (18S region): 5'-TCC GTA GGT GAA CCT GCG G-3' ITS2 (5.8S region): 5'-GCT GCG TTC TTC ATC GAT GC-3' ITS3 (5.8S region): 5'-GCA TCG ATG AAG AAC GCA GC-3' ITS4 (28S region): 5'-TCC TCC GCT TAT TGA TAT GC-3'	Refs. 136, 146, 173, 174
171	18S rRNA gene	Real-time PCR, melt curve analysis, sequencing (LightCycler, SYBR green)	Forward: 5'-ATT GGA GGG CAA GTC TGG TG-3' Reverse: 5'-CCG ATC CCT AGT CGG CAT AG-3' Note: These primers will not detect some zygomycetes	Fresh/frozen tissue samples (9) (liver, lung, brain, bone marrow, soft tissue)
138	18S, 28S rRNA genes	In situ hybridization	<i>Histoplasma</i> 18S: 5'-GCA GAC GCG GGT CGG GCT-3' <i>Histoplasma</i> 28S: 5'-CCC GGC CGC GGG GGA TTG-3' <i>Coccidioides</i> 18S: 5'-GAA CGG GTT ATT CAA AGT TGC CCG T-3' <i>Coccidioides</i> 28S: 5'-TAA CCA ACC GCC AGA ACT GAT G-3' <i>Cryptococcus</i> 18S: 5'-CGA CCC AGT CAG AGA TTG ACG TGG G-3' <i>Cryptococcus</i> 28S: 5'-ACA CCA GCA GAA CTG GCT GAA CCC AAT AGA-3' Panfungal 18S: 5'-CCG ATC CCT AGT CGG CAT AG-3'	98 FFPE tissue samples (mostly lung, 3 lymph nodes)
<i>Histoplasma capsulatum</i>				
139	Hc100	Nested PCR, AGE, confirmation by sequencing	<i>Outer</i> Hc I: 5'-GCG TTC CGA GCC TTC CAC CTC AAC-3' Hc II: 5'-ATG TCC CAT CGG GCG CCG TGT AGT-3' <i>Inner</i> Hc III: 5'-GAG ATC TAG TCG CGG CCA GGT TCA-3' Hc IV: 5'-AGG AGA GAA CTG TAT CGG TGG CTT G-3' <i>Outer</i> Hc2: 5'-GCG GGG TTG GCT CTG CTC T-3' Hc3: 5'-TTG GAA ACC CCG GGC TTG-3' <i>Inner</i> Hc1: 5'-TCA TAG TAG GCT GTT CAC CCC CG-3' Hc2 (as above)	100 FFPE tissue samples (including 13 lymph nodes). Ref. 143: Fresh/frozen tissue (24, including 2 lymph nodes), blood, serum, CSF, BAL fluid
144	H antigen	Semi-nested PCR, AGE	<i>Outer</i> Hc2: 5'-GCG GGG TTG GCT CTG CTC T-3' Hc3: 5'-TTG GAA ACC CCG GGC TTG-3' <i>Inner</i> Hc1: 5'-TCA TAG TAG GCT GTT CAC CCC CG-3' Hc2 (as above)	Tissue (2), blood (24), tissue scrapings (4)
145	M antigen	PCR, AGE, confirmation by sequencing	Msp1F: 5'-ACA AGA GAC GGT AGC TTC ACG-3' Msp1R: 5'-GCG TTG GGG ATC AAG CGA TGA GCC-3' Msp2F: 5'-CGG GCC GCG TTT AAC AGC GCC-3' Msp2R: 5'-ACC AGC GGC CAT AAG GAC GTC-3'	Culture isolates
147	ITS	Real-time PCR, quantitation, melt curve analysis (LightCycler platform)	<i>Primers</i> Hcap-F: 5'-TTG TCT ACC GGA CCT G-3' Hcap-R: 5'-TTC TTC ATC GAT GTC GGA AC-3' <i>Probes</i> Hcap HP1: 5'-ACG ATT GGC GTC TGA GC-3'-Fluorescein Hcap HP2: 640-5'-GAG AGC GAT AAT AAT CCA GTC AAA AC-3'	Culture extracts, three clinical specimens (incl. FFPE bone marrow). Ref. 141: FFPE tissue, after microdissection (case report)

158	<i>Coccidioides immitis</i> , <i>C. posadasii</i> ITS2	Real-time PCR (LightCycler platform), confirmation by sequencing	<i>Primers</i> Forward: 5'-CGA GGT CAA ACC GGA TA-3' Reverse: 5'-CCT TCA AGC ACG GCT T-3' <i>Probes</i> Anchor probe: 5'-GAG CGA TGA AGT GAT TTC CC-3'-Fluorescein Donor probe: 640-5'-TAC ACT CAG ACA CCA GGA ACT CG-3'	266 Respiratory specimens (100% sensitivity), 64 fresh tissue samples (92.9% sensitivity), 148 FFPE tissue samples (73.4% sensitivity)
			<i>Outer primers</i> Cocci I: 5'-GTA CTA TTA GGG AGG ATA ATC GTT-3' Cocci II: 5'-GGT GTC AAC TGG TGG GAT GTC AAT-3' <i>Inner primers</i> Cocci III: 5'-ATC CCA CCT TGC GCT GTA TGT TCG A-3' Cocci IV: 5'-GGA GAC GGC TGG ATT TTT TAA CAT G-3' <i>Probes</i> Cocci-HP-1: 5'-CCA AAT TCT TGC ATC TCG CCC A-3'-Fluorescein Cocci-HP-2: 640-5'-ATG GGA TAA GAT GAG AAG ATG GAA AG-3'	120 clinical isolates ³ FFPE tissue samples (lung, skin) Ref. 156: Sputum (1) (nested PCR, AGE)
163	<i>Cryptococcus neoformans</i> ERG11	Conventional PCR, AGE	<i>Primers</i> P450₁: 5'-ATG ACT GAT CAA GAA ATY GCT AA-3' P450₂: 5'-TAA CCT GGA GAA ACY AAA AC-3' <i>Probe</i> pCry: 5'-GGT TGA TCA TCG ACC ATG TC-3'	140 samples including CSF, blood, urine, vaginal swabs, pharyngeal exudate
			<i>Primers</i> Cryp I: 5'-TCC TCA CGG AGT GCA CTG TCT TG-3' Cryp II: 5'-CAG TTG TTG GTC TTC CGT CAA TCT A-3' <i>Probes</i> Cryp-HP-1: 5'-TCC TGG TTC CCC TGC ACA C-3'-Fluorescein Cryp-HP-2: 640-5'-CAG TAA AGA GCA TAC AGG ACC ACC-3'	

PCR polymerase chain reaction, AGE Agarose gel electrophoresis, BAL bronchoalveolar lavage, FFPE formalin fixed, paraffin embedded.

Several investigators have designed assays specific for *H. capsulatum*. Bialek and colleagues began by designing a nested PCR assay targeting the 18S rRNA gene, which was extensively tested in experimentally infected mice.¹⁴² They discovered that there was no significant difference between PCR and GMS staining; in addition, they expressed concern about the close similarity of the 18S sequences of two other endemic fungi, *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis*. Bialek then developed a nested PCR assay to the Hc100 protein (Table 44.2) and compared it to the 18S assay on 100 FFPE tissues, including 7 positive lymph node tissues.¹³⁹ Results were confirmed by sequencing, and human GAPDH was used as an extraction control. The sensitivity of the Hc100 assay was equivalent to that of the 18S assay, detecting 69% of positive specimens with a valid extraction control result. While the 18S assay produced many false positives, the Hc100 assay was 100% specific. Another investigator used this same assay successfully on two fresh/frozen lymph node tissue specimens; both tissues were PCR-positive and culture-positive.¹⁴³ Other specific molecular targets have included the H antigen gene, encoding beta-galactosidase,¹⁴⁴ and the M-antigen gene, which also detects *H. capsulatum* var. *dubosii*.¹⁴⁵

Most of the published assays used to detect *Histoplasma* have targeted the ribosomal DNA gene complex (see also Panfungal section below). Benefits to using this target are its conserved nature, combined with the fact that there are approximately 100 copies of this gene complex,¹⁴⁶ potentially increasing sensitivity. Due to the conserved nature of the entire complex, while some investigators have used *Histoplasma*-specific probes, others have suggested sequencing of amplification products. The fungal ribosomal RNA genes (18S, 5.8S, 28S) are highly conserved, which may cause problems with specificity (such as the 18S assay described above). Fungal internal transcribed spacer (ITS) sequences (i.e., ITS1 and ITS2), however, are more variable, allowing greater specificity (see also Panfungal section below).

Several assays have been developed using *Histoplasma*-specific primers/probes based on ITS sequences. Martagon-Villamil and colleagues designed a real-time PCR assay on the LightCycler platform targeting the ITS region with primers specific for *H. capsulatum* (Table 44.2), using quantitation and melt curve analysis for confirmation of amplicons produced.¹⁴⁷ The assay was originally designed for the identification of culture isolates, but has been tested on a small number of clinical specimens,¹⁴⁷ including successful detection in FFPE gastrointestinal tissue.¹⁴¹ Other investigators used a conventional nested PCR assay with *Histoplasma*-specific ITS primers to identify *Histoplasma* in FFPE skin tissue in an unexpected case of histoplasmosis outside of an endemic area (Italy).¹³⁶ Collins and colleagues have also used *Histoplasma*-specific ITS primers in FFPE tissues (including two lymph nodes) from pediatric patients with granulomas on histologic examination.¹⁴⁸ Another approach is to use primers to target conserved portions of the ribosomal DNA gene com-

plex (i.e., 18S, 5.8S, and 28S) to amplify ITS1 and/or ITS2 sequences of any fungal organism (see Panfungal section). Then, specific probes or sequencing may be used to identify the specific organism. This approach was used in an unusual case of a patient in a nonendemic area (Taiwan) with a history of previous histoplasmosis who presented with culture-proven *H. capsulatum* pulmonary disease nine years later.¹⁴⁹ The ITS1 and ITS2 regions were amplified from the current isolate, as well as FFPE laryngeal tissue from the previous infection, and the sequences were identical, proving recurrent disease. However, use of highly conserved targets followed by identification of the amplicon sequence may be a problem with mixed or contaminated specimens. An example of this is the description by Rickerts and colleagues of a case in which pulmonary samples from a patient in a nonendemic area were tested with Hc100 and *Histoplasma*-specific 18S assays, as well as other fungal assays.¹⁵⁰ One specimen was positive by both Hc100 and 18S, but when they sequenced the amplification product, they were unable to interpret the results. Further testing showed that an *Aspergillus*-specific 18S assay was also positive, indicating a mixed population in the specimen. This is an important issue when using any broadly-reactive primer set to detect and identify organisms.

Published studies have not shown superiority of molecular methods for diagnosis of *Histoplasma* infection over conventional methods such as culture, serology, and antigen testing,¹⁴⁰ and there are few studies with thorough evaluation of assay performance in clinical specimens. Until standardized molecular assays with performance equal or superior to existing diagnostic tests are developed that can add to the current diagnosis and management of patients, molecular testing for *Histoplasma* will remain the exception rather than the rule.

Coccidioides

Coccidioidomycosis is a fungal infection caused by the dimorphic fungus *Coccidioides immitis* in California and *Coccidioides posadasii* in other locations; it is endemic in the southwestern United States, northern Mexico, and parts of South America. *Coccidioides* infection is either asymptomatic or causes pulmonary disease in immunocompetent patients. Disseminated infection is seen in immunocompromised patients, and otherwise immunocompetent individuals of Black and Asian ethnic backgrounds are also at high risk for development of disseminated disease for unknown reasons. Handling this organism places laboratory workers at high risk for laboratory-acquired infection, and isolates should be handled under BSL3 conditions; in addition, it has been designated a select agent in the United States, restricting access to active cultures.¹⁵¹

Coccidioidomycosis is typically diagnosed by culture, serology, and/or histologic examination of tissues. Molecular identification in clinical specimens, including lymph nodes, has only rarely been described.¹⁵¹ Identification of *Coccidioides* in culture may be expedited using a commercial nonamplified probe (AccuProbe, Gen-Probe, Inc.). While a

mainstay of diagnosis, serology may be slow to become positive, and may be falsely negative in immunocompromised patients. Lymph node involvement in coccidioidomycosis is relatively common. As with histoplasmosis, hilar and mediastinal lymph nodes are frequently involved as part of an acute primary pulmonary infection. The other most frequently involved lymph nodes are supraclavicular and cervical lymph nodes, which may show necrosis and granulomatous inflammation.¹⁵² Rarely, patients may present with isolated lymphadenopathy.¹⁵³ In tissues, the organism appears as large spherules containing endospores. Histologic identification is not straightforward, even in areas of endemicity; in areas outside endemic zones, unfamiliarity with its appearance in tissue may make it even more problematic. Appearance of both spherules and endospores from ruptured spherules may be quite misleading in tissue,¹⁵⁴ where structures may resemble *B. dermatitidis*, *Histoplasma*, *Candida*, or other organisms.

A small number of studies have described molecular detection of *Coccidioides* in clinical specimens. Bialek and colleagues developed a *Coccidioides*-specific real-time PCR assay and conventional nested PCR assay, both detecting the Ag2/PRA target in *C. immitis* and *C. posadasii* strains, as well as three tissue specimens (lung and skin).¹⁵⁵ The nested PCR Ag2/PRA assay was subsequently used to detect *Coccidioides* in sputum specimens.¹⁵⁶ Other investigators have used *Coccidioides*-specific primers to the ITS region to detect the organism in human serum and CSF.¹⁵⁷ One of the most thoroughly evaluated (and most promising) assays is a real-time PCR assay specific for *Coccidioides*, targeting the ITS2 region¹⁵⁸ (Table 44.2). Analytical validation has shown a limit of detection of less than 50 copies per reaction, detecting both *C. immitis* and *C. posadasii* with no cross reactivity in a panel of 114 other organisms. The assay has been evaluated further, using multiple clinical specimens, including respiratory specimens ($n=266$, 100% sensitivity, 98.4% specificity), fresh tissues ($n=64$, 92.8% sensitivity, 98.1% specificity), and FFPE tissues ($n=148$, 73.4% sensitivity, 100% specificity); all positive results were confirmed by sequencing.

Very few assays have described the detection of *Coccidioides* nucleic acid in clinical specimens, even fewer in lymph nodes. Until more well-characterized assays are available, molecular techniques are unlikely to play a significant role in the diagnosis of *Coccidioides* infection.

Cryptococcus neoformans

C. neoformans causes both pulmonary disease in immunocompetent patients and disseminated infection in immunocompromised patients. The vast majority of cryptococcal infections are readily diagnosed by nonmolecular methods. The organism is easy to identify in culture and grows relatively quickly on routine fungal and bacteriologic media. In addition, serum antigen testing is useful in immuno-

compromised patients with disseminated disease. Rarely, cryptococcal lymphadenitis may be the primary presentation in immunocompromised patients,¹⁵⁹ and cryptococcal infection may present as isolated granulomatous lymphadenitis in immunocompetent children¹⁶⁰; both presentations may mimic malignancy or MTB disease.

A number of specific assays for detection of *Cryptococcus* have been developed, but few have been tested on tissues (lymph node or other tissues). Bialek designed a nested conventional PCR and a real-time PCR assay for *C. neoformans*, both targeting 18S gene, and tested the assay on fresh-frozen brains of experimentally infected mice, confirming results with sequencing. Sensitivity was excellent, but there were some cross-reactivity issues (as might be expected when using a conserved target), requiring confirmation of products by sequencing or other methods (Table 44.2).¹⁶¹ Another group tested clinical specimens, including fresh tissue, with a nested PCR assay targeting the URA5 gene, finding poor sensitivity in tissue specimens.¹⁶² Other targets used to test other clinical samples and culture isolates have included a cytochrome P-450 lanosterol demethylase¹⁶³ and the ITS regions.¹⁶⁴ Zou described the successful use of nested PCR to detect *C. neoformans* in abdominal lymph node tissue from two children presenting with abdominal lymphadenitis, clinically suspected to be tubercular disease or malignant lymphoma.¹⁶⁵ However, Takahashi and colleagues evaluated an ITS PCR assay on bronchoalveolar lavage fluid, finding its performance no better than cytology or culture,¹⁶⁶ demonstrating that the role of molecular testing in cryptococcal disease, as with histoplasmosis and coccidioidomycosis, is still unclear.

Panfungal Approaches to Molecular Mycology

The most common approach to panfungal molecular detection methods has been to target the ribosomal DNA gene complex. In fungi, the gene complex is arranged as follows¹⁶⁷:

5'-18S-ITS1-5.8S-ITS2-28S-3'

The 18S and 28S genes have both conserved and variable regions, but are overall relatively conserved (due to the nature of their function); because variability is low, large sequences are required to differentiate based on these genes. The 5.8S gene is highly conserved, making it unsuitable for differentiation. On the other hand, the internal transcribed spacer (ITS) region sequences have evolved at a much faster rate than the 18S, 5.8S, and 28S genes, and are highly variable. The nature of the structure of the gene complex is therefore ideally suited to the design of primers to conserved regions in the 18S, 5.8S, and 28S genes, allowing amplification of variable sequences in between, which may then be identified via RFLP, probe, or most specifically, sequencing.¹⁶⁷ When sequencing is used to identify organisms, however, the size and quality of the database used to assign identity is

critical¹⁴⁶; if there are limitations in the database, the accuracy of identification may suffer.¹⁶⁸ Some fungal organisms, such as *Fusarium*, may not be well differentiated using these sequences.¹⁶⁹ In addition, multiple organisms present in the specimen may make interpretation of sequencing impossible without separation of amplification products by cloning or other methods. Strict control practices are necessary in any broad range assay.¹⁴⁶

Several investigators have targeted the more conserved 18S and 28S genes within the ribosomal DNA gene complex to identify fungal isolates,¹⁶⁸ as well as fungi in clinical specimens.^{170,171} Hayden and colleagues designed multiple probes to 18S and 28S for use in an in situ hybridization assay, including probes for *Histoplasma*, *Coccidioides*, *Blasatomyces*, *Cryptococcus*, and *Sporothrix*, as well as a panfungal probe (Table 44.2).¹³⁸ They performed ISH on 98 FFPE tissues from archival materials (including two lymph nodes containing *Coccidioides*). While GMS staining was more sensitive than ISH, morphologic diagnosis was not always correct, leading to poor specificity. In situ hybridization was 100% specific, with sensitivity greater than 90% for all fungi except *Histoplasma*, for which sensitivity only reached 50% (apparently related to interference due to tissue necrosis in specimens).

While the conserved nature of the 18S and 28S targets has led to concerns about specificity, ITS regions are more variable, and are well suited to the differentiation of fungal organisms. White and colleagues developed primers to the fungal ITS regions that have been very commonly used in subsequent studies, followed by either probe or sequence identification of the amplification products (Table 44.2).¹⁷² Lau and colleagues targeted ITS1, using primers ITS 1 and ITS2,¹⁷² for amplification, followed by sequencing on 62 tissue specimens (37 fresh, 38 FFPE), with sensitivity of >97% in fresh tissue and 68% in FFPE.¹⁴⁶ They successfully identified the correct fungal organism in 93.6% of culture-proven cases; in addition, they were able to identify the organism in 64.3% of cases identified by histologic morphology only. Hendolin and colleagues used the White universal fungal primers, ITS1 and ITS4, in multiple frozen tissue specimens, followed by specific probes or sequencing.¹⁷³ Lindsley also used the ITS1 and ITS4 primers to amplify culture isolates, followed by EIA hybridization with probes for ITS2 region of dimorphic fungal organisms, with the hope of ultimately using the assay in clinical specimens.¹⁷⁴ Unfortunately, there was cross reactivity among the probes, including the *Histoplasma* probe.

While probe confirmation of broad range amplification products may allow multiple methods of detection (singly or in an array), including ISH, there are specificity issues when relying on short probes in a conserved region, such as the ribosomal DNA gene complex (as seen in the study of Lindsley and colleagues). While sequencing is more specific, there are a number of caveats. As previously mentioned, the use of a broadly specific amplification followed by sequencing may be

problematic in the case of a dual infection, presence of additional colonizing fungi in the specimen, or environmental contamination.¹⁵⁰ In addition, the database quality is of paramount importance, as is the data integrity and correct taxonomy and nomenclature of sequences deposited in the database.^{146,169} As databases continue to grow and develop, there will be improved accuracy of identification of organisms; in addition, we may find that some regions are more informative for some genera than others. As future work continues on the sequencing and evaluation (and reclassification) of culture isolates, it will provide data allowing investigators to identify the best candidate targets for testing on clinical samples, such as lymph node material.

Summary

Molecular diagnostic methods for detection and identification of fungi have been described and are available at reference and academic center labs. However, while the identification of fungal isolates by sequence analysis is rapidly becoming a new gold standard, the role of molecular assays on clinical specimens in patient care remains uncertain,¹⁴⁰ and their role in the diagnosis of lymphadenitis is doubly so. Additional studies testing clinical specimens with well standardized, well-characterized molecular assays to detect fungal pathogens are needed.

Parasitic Lymphadenitis

Toxoplasma gondii

Acute infection with *T. gondii* in the immunocompetent host presents primarily as lymphadenopathy, with or without constitutional symptoms. While the clinical differential diagnosis may include other lymphadenitides and malignancy, the histologic appearance is usually characteristic.^{175,176} Diagnosis is generally made using serologic testing in conjunction with characteristic histologic findings; while visualization of the organism is uncommon in lymph nodes (in either cyst or tachyzoite stage), they may occasionally be seen on cytologic examination of fine needle aspirates.^{175,177,178} The infection is self-limited, although the organism remains latent in cyst form. In immunocompromised patients, the picture is quite different. Acute infection or reactivation may lead to severe, potentially fatal disease, including encephalitis and myocarditis; these patients may not mount an appropriate serologic response. In addition, acute infection during pregnancy may lead to congenital toxoplasmosis in the infant. Molecular testing for *T. gondii* is commonly performed for the diagnosis of toxoplasmosis in immunocompromised patients (on specimens including tissues, CSF, respiratory specimens, and blood), and on amniotic fluid for the diagnosis of congenital toxoplasmosis.^{179,180}

The most common and well-studied molecular target for the detection of *T. gondii* is B1, a sequence repeated 35-fold

in the organism's genome.¹⁸¹ This original assay has been used in many studies, as have other assays designed to target this same sequence, with generally excellent performance.¹⁸⁰ Several conventional and real-time assays have been described.^{182–184} Another assay target used in some laboratories is the 529 bp repeat element (RE), a repeat element present 200–300 times within the genome.^{185–187} Studies comparing the two assays have shown slightly improved sensitivity over B1 assays, but the clinical significance of this increased sensitivity is unclear.^{188,189} Other studies have used stage-specific targets, such as tachyzoite- (Sag-1) and bradyzoite- (Sag-4, Mag-1) specific proteins,¹⁹⁰ but these assays are less commonly used.

Although reports of molecular detection of *T. gondii* have proliferated, there are very few reports that include testing of lymph nodes in toxoplasma lymphadenitis, and those that do exist show contradictory findings. In 1992, Weiss and colleagues¹⁹¹ reported testing FFPE lymph node tissue from patients with acute toxoplasmic lymphadenopathy using the B1 assay described by Burg and colleagues. While there was excellent sensitivity in FFPE heart and brain tissue controls (confirmed by dilution testing), they only detected *T. gondii* DNA in 1 of the 9 lymph nodes; this node was from the patient with the shortest duration of symptoms (4 weeks). Conversely, in 2001, Lin and colleagues reported excellent sensitivity of the same B1 PCR assay in comparison to classic histologic findings in 12 frozen lymph nodes.¹⁹² On the other hand, several investigators have demonstrated parasitemia in patients with toxoplasmic lymphadenitis,^{193,194} detecting *T. gondii* DNA in the blood of up to 76.4% patients,¹⁹⁵ and suggesting that this may be a future tool in the diagnosis of toxoplasma lymphadenitis.^{193–195}

The role of molecular methods in the diagnosis of toxoplasma lymphadenitis remains unclear.¹⁷⁹ Diagnosis is generally made using well-defined histologic criteria in conjunction with serologic testing. However, while organisms are not typically visualized in lymph nodes, they are occasionally seen, indicating their presence in the tissue. With the initial poor results reported in lymph nodes,¹⁹¹ efforts in molecular testing for *T. gondii* were quickly focused (appropriately) on clinical situations in which serologic interpretation is difficult, such as in immunocompromised patients and in pregnancy (with testing of amniotic fluid specimens), and with few exceptions,¹⁹² molecular testing of lymph nodes has not been revisited. However, there are now a number of well-described sensitive and specific assays for *T. gondii* described that could be adapted to test a lymph node, or the blood^{193–195} of patients with acute lymphadenitis; while a positive result may confirm a diagnosis, a negative result would not exclude toxoplasma infection.

Leishmania spp.

Leishmaniasis is a vector-borne disease caused by multiple species of the protozoal parasite *Leishmania*, and is endemic

in Asia, the Middle East, South America, and Africa. It typically manifests as either cutaneous leishmaniasis or visceral leishmaniasis; either may be accompanied by lymphadenopathy, which may precede the development of skin lesions and may be massive.¹⁹⁶ Leishmaniasis may also rarely present with isolated localized cervical, axillary, or inguinal lymphadenitis mimicking toxoplasmosis, cat scratch disease, sarcoidosis, or lymphoma,^{197,198} placing leishmaniasis in the differential diagnosis of granulomatous lymphadenitis in a person with a history of residence or travel in an endemic area. Organisms may be seen on histology or cytology as Leishman-Donovan (LD) bodies; in addition, other characteristic findings may be seen when LD bodies are not present.¹⁹⁸ However, histologic and cytologic examination of lymph node material has poor sensitivity.¹⁹⁶ Culture is difficult and may not be readily available,¹⁹⁹ and serology may be difficult to interpret due to cross-reactivity, and background seroprevalence (i.e., previous infection). Molecular testing using multiple gene targets has been widely reported on multiple specimens, including blood, bone marrow, skin tissue, and lymph node aspirates, in the diagnosis and monitoring of leishmaniasis²⁰⁰; a brief selection with a focus on use in lymph node aspirates is described here.

Targets for the molecular detection of *Leishmania* have included the intergenic transcribed spacer region of the rRNA complex (ITS), 16S rRNA, kinetoplast DNA (kDNA), *GP63* gene, and others. Mathis designed a 16S rRNA assay capable of detecting all medically important *Leishmania* species, and found 100% sensitivity and specificity in 16 lymph node aspirates from dogs with leishmaniasis.¹⁹⁹ During an outbreak of cutaneous leishmaniasis with massive lymphadenopathy in Brazil, Harms and colleagues targeted the ITS in a conventional PCR assay, detecting *Leishmania* in 48 of 76 lymph node aspirates negative for organisms on cytology; RFLP analysis allowed the specific identification of *Leishmania braziliensis* in the outbreak cases.¹⁹⁶ Kinetoplast DNA (kDNA) PCR has been used to identify *Leishmania donovani* in the blood, bone marrow, and lymph node aspirates of Sudanese patients with microscopically confirmed visceral leishmaniasis.²⁰¹ Tupperwar and colleagues developed a real-time PCR assay, targeted to the highly conserved *GP63* gene, for the detection of a wide variety of *Leishmania* species with one assay; melt curve analysis is used to distinguish among several species.²⁰² PCR of lymph node aspirates has been used not only to diagnose visceral leishmaniasis and its recurrent forms, such as post-kala-azar dermal leishmaniasis,^{203,204} but also as a test of cure.²⁰⁵

In contrast to *Toxoplasma* lymphadenitis, molecular testing, when available, has great value in the diagnosis and monitoring of leishmaniasis. A plethora of assays have been reported, including testing of lymph node material for diagnosis, although standardization is sorely needed.²⁰⁰ Increased international travel makes leishmaniasis an important consideration in the differential diagnosis of lymphadenitis.

Viral Lymphadenitis

Introduction

While viruses may cause a large proportion of cases of lymphadenitis, they typically present as part of a recognizable, self-limited viral syndrome. Diagnosis is most frequently made using serology, viral culture, or direct antigen testing of lesions, blood, or body fluids; lymph node aspiration and biopsy are uncommon. While molecular methods are now the new gold standard for the diagnosis of many viral diseases, published reports using molecular methods to diagnose viral lymphadenitis in lymph node tissue are uncommon. However, for rare cases when viral diseases cause localized lymphadenopathy, they may be misdiagnosed as a number of disorders, including malignant lymphoma.^{206,207} These cases may be particularly severe in immunocompromised patients, and prompt initiation of appropriate therapy (including antivirals, if indicated) depends on an accurate diagnosis of the causative agent. In these atypical cases, molecular testing, along with immunohistochemistry and/or in situ hybridization, may confirm the diagnosis.

Please note that this discussion covers the nonneoplastic manifestations of viral diseases. For a discussion of viral oncogenesis, including the association of various herpesviruses and lymphoproliferative diseases, see Chap. 7.

Herpesviruses

The herpesviruses are a large family of viruses infecting many vertebrates. There are eight human herpesviruses: herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), and human herpesviruses 6, 7, and 8 (HHV-6, HHV-7, and HHV-8). The herpesviruses are ubiquitous causes of human infection, many causing primary infection in early childhood. After acute infection they remain latent in host tissues, and may reactivate later in life, particularly under conditions of immunosuppression. Diagnosis of primary herpesvirus infections is frequently made by serology, culture, and antigen staining of infected cells and tissues. Molecular studies are routinely used in the diagnosis of herpesvirus infections, particularly for the diagnosis of reactivation or central nervous system infection; this testing is most frequently performed on blood or plasma, bone marrow, and cerebrospinal fluid, although other specimens may be tested. A detailed description of the clinical presentation and diagnosis of each virus is beyond the scope of this chapter. Several investigators have searched for herpesvirus nucleic acid material in cases of Kikuchi-Fujimoto disease and Kimura disease; these studies are discussed in a separate section below. Only a few other studies have described the detection of selected herpesviruses in lymph node material of patients with viral lymphadenitis.

HSV and VZV infections may rarely present with localized lymphadenitis in the absence of concurrent skin lesions, which may mimic malignant lymphoma, prompting biopsy and histologic evaluation.^{206,207} Of the few reported cases of localized HSV lymphadenitis, the majority are in immunocompromised patients.²⁰⁸ Characteristic viral intranuclear inclusions are usually seen in the tissue; however, the histologic appearance of HSV and VZV lymphadenitis may be identical.²⁰⁹ Immunohistochemical stains are commonly used to detect these viruses in FFPE tissues; however, unusual or atypical presentations may prompt molecular testing for further confirmation. Identification of either virus as the etiologic agent in severe cases is essential, because specific antiviral therapy (acyclovir) is available. Identification of HSV by both IHC and ISH has been reported in localized HSV lymphadenitis in two immunocompromised patients²⁰⁹ and two immunocompetent patients.²¹⁰ In addition, a striking case of massive, progressive, and destructive cervical lymphadenopathy due to HSV was described in a patient with both combined variable immunodeficiency (CVID) and NK cell deficiency.²⁰⁸ Workup for all other potential causes of lymphadenitis were negative, and continued progression of his disease led to an excisional biopsy, which showed characteristic herpetic viral inclusions. HSV-2 was confirmed by IHC, electron microscopy, culture, and PCR targeting the g8 major glycoprotein. The patient was started on acyclovir therapy with improvement and ultimate resolution of the neck mass.

HHV-6 is a ubiquitous virus that infects most individuals in early childhood. Two types have been identified, type A and type B; type B seems to be associated with more severe disease. Primary HHV-6 infection has also been described as a cause of acute lymphadenitis in adults. Sumiyoshi described a 20-year old woman with an infectious mononucleosis-like syndrome, but without evidence of EBV infection. HHV-6 was identified in lymph node tissue by Southern blot, PCR, and ISH.²¹¹ In 2004, Maric and colleagues reported a series of three immunocompetent patients, all adults, with febrile illness and lymphadenitis due to HHV-6, confirmed by PCR on FFPE, IHC, and electron microscopy.²¹² Many inclusions were seen in CD4-positive T cells on histologic examination. Studies for HIV, CMV, EBV (including ISH), and HHV-7 (by PCR) were all negative. Another report describes HHV-6B lymphadenitis and aortitis in a 9-month old child²¹³; replicating HHV-6 virus was identified using RT-PCR for the *gp105* gene for the major structural antigen,²¹⁴ differentiating it from latent HHV-6 in tissues.²¹⁵

Several investigators have also studied sites of latency for various herpesviruses by molecular methods. Chen and colleagues²¹⁵ screened 40 major anatomic sites for herpesviruses in autopsy tissue from eight patients without evidence of active disease at the time of death. EBV, HHV-6, and HHV-7 were identified in many tissues from all patients, while HSV, VZV, CMV, and HHV-8 were less common and more restricted by anatomic site. Niedobitek undertook a detailed study of

the distribution of EBV by ISH with EBER-1 and EBER-2 probes in lymph nodes and tonsillar tissues from patients with infectious mononucleosis (acute EBV infection), normal lymph nodes, and various other reactive conditions. Of note, EBV sequences were identified in 4 of 12 normal lymph nodes.²¹⁶ Another study used semiquantitative nested PCR for early and late genes of CMV to identify lymph nodes as a significant site of latency for CMV in heart and lung transplant donors and recipients.²¹⁷ Kempf and colleagues demonstrated increased frequency of HHV-7 in the lymph nodes of HIV-positive patients, compared to HIV-negative patients, using a nested PCR assay specific for HHV-7 sequences.²¹⁸

Other Viruses

A number of other viruses are known to cause lymphadenopathy that could prompt histologic or cytologic investigation, including the live attenuated vaccine strain of measles virus²¹⁹ and other vaccines.²⁰⁷ However, outside of the herpesviruses, reports of molecular detection of other viruses in lymph node tissue are even more uncommon. In 2006, investigators in Sweden described a young girl with severe facial cellulitis and necrotizing lymphadenitis due to cowpox virus, acquired from a domestic cat.²²⁰ After complete investigation for other infectious agents, viral culture revealed cowpox virus, which was confirmed by electron microscopy and PCR for the viral thymidine kinase gene. A lymph node that persisted 2 years after presentation was removed and was also PCR positive for cowpox virus sequences.

Lymphadenitides of Suspected Infectious Origin

Two inflammatory lymphadenopathies of unknown etiology, Kikuchi–Fujimoto disease and Kimura disease, have been described with an increased incidence in Asian patients. Signs and symptoms resemble infectious etiologies, but despite many investigations, no specific infectious agent has been conclusively tied to either entity. These two entities are also discussed in Chap. 43.

Kikuchi–Fujimoto Disease

Kikuchi–Fujimoto disease (also known as Kikuchi disease or histiocytic necrotizing lymphadenitis) is a self-limited lymphadenitis syndrome associated with fever, malaise, other systemic symptoms, and occasional lymphocytosis; it is not responsive to antibiotics.^{221,222} These features have led many to suspect that Kikuchi–Fujimoto disease (KFD) has a viral etiology. However, despite a number of studies, the evidence is still unclear. The agents of most interest have been EBV and HHV-6, but other viruses have been investigated as well.

Evidence of active EBV infection by serology, ISH, and PCR testing has been described in case reports of pediatric patients diagnosed with KFD,^{223,224} and EBV genomic material was identified in lymph nodes of every case in a series of

10 Taiwanese patients with KFD.²²⁵ However, other studies of EBV in KFD have been inconclusive,²²⁶ or have identified EBV by ISH and/or PCR in a significant number of cases, but at the same rate as in control specimens.^{227,228} Additional studies were unable to identify EBV sequences in lymph node material from any of the KFD cases studied.^{221,229,230} The story is very similar for HHV-6. While some studies show promising association of HHV-6 virus by ISH and PCR methods,²³⁰ others are inconclusive,²³¹ fail to demonstrate the presence of HHV-6,²²⁶ or show HHV-6 present at the same rate as controls.^{232,233} Interestingly, in one study, quantitative real-time PCR identified a single case of apparent active HHV-6-related lymphadenitis with high HHV-6 viral load,²³² similar to cases of HHV-6 lymphadenitis described above.^{211,212} A review of all reported cases of KFD and results related to HHV-6 was undertaken by Dominguez and colleagues.²³⁴ Results supporting an association with HHV-6 by ISH or PCR methods were present in 112 of 158 cases of KFD (70%). However, the authors cite poor standardization, use of cross-reacting reagents, and other methodologic problems, making the interpretation of this finding difficult, although intriguing. Studies of HHV-8 in KFD have also yielded conflicting results, although they do not appear to support any role in the disease.^{232,235,236} Two groups of investigators looked for the presence of all eight herpesviruses (HSV-1, HSV-2, VZV, EBV, CMV, HHV-6, HHV-7, and HHV-8), and found no molecular evidence of herpesvirus infection in KFD lymph nodes.^{222,237}

Additional viruses studied in association with KFD include HHV-7,^{232,233} HTLV-1,^{225,238} parvovirus B19,^{225,239} and hepatitis B virus,²²¹ without any consistent evidence of association with the disease. Finally, Chung and colleagues investigated 20 cases of KFD for evidence of *B. henselae*, using primers to both *gltA* and *pap31* targets, identifying 4 (20%) that were positive for *B. henselae*, representing cases of CSD.

The clinical presentation and histologic findings in KFD closely resemble an infectious process. Undoubtedly, some patients diagnosed with KFD actually have necrotizing lymphadenitis due to an identifiable infectious cause,^{224,232,240} and a search for infectious etiology (including the use of molecular methods, where appropriate) would be indicated for individual patients with this clinical presentation. The search for a single common viral (or other) infectious agent to account for the remaining “unexplained” cases has yielded inconsistent results. Future studies may clarify whether KFD is an infectious disease for which we have not identified the pathogen, a common presentation caused by a number of infectious agents, or, as has been postulated, an autoimmune or hyperactive reactive process of some sort. For additional discussion of Kikuchi–Fujimoto lymphadenitis, see Chaps. 42 and 43.

Kimura Disease

Kimura disease is a rare chronic inflammatory disease most commonly found in middle-aged Asian men, presenting with deep soft tissue masses of the head and neck, lymphadenopathy,

eosinophilia, and elevated IgE. The etiology of this disorder is unknown, although it is currently believed to be immunologically mediated.^{241,242} The disease was often confused with angiolymphoid hyperplasia with eosinophilia in the past, but the two are now considered as separate clinicopathologic entities.²⁴³ A few studies have looked for the presence of infectious agents in Kimura disease. Jang and colleagues searched for, but did not find, evidence of HHV-8 in affected tissues from patients with Kimura disease.²⁴⁴ EBV was identified in a FFPE lymph node biopsy of a man diagnosed with Kimura disease, but as EBV may also be found in lymph nodes of healthy individuals,²¹⁵ the interpretation of this finding is unclear.²⁴⁵ Parasitic etiologies have also been sought in vain.²⁴⁶ Since patients with Kimura disease typically present with local head or neck mass and lymphadenopathy without prominent systemic symptoms, an infectious etiology or malignancy may be considered in the differential diagnosis of these patients. For further discussion of Kimura disease, see Chap. 43.

Summary

Molecular diagnostics is a valuable tool in the diagnosis of infectious diseases. As investigation and clinical studies continue, the application of this tool to various diseases and specimen types continues to develop and evolve. With a few exceptions (described in this chapter), however, application of molecular techniques in the investigation of infectious lymphadenitis is limited to unusual or atypical presentations, and the performance of the assays is not ideal, leading to difficulty in interpretation of negative results on lymph node tissue (particularly FFPE tissue). Conversely, the presence of certain latent herpesviruses in normal lymph nodes may make interpretation of positive results for those pathogens equally difficult. Performance characteristics and limitations of an assay (particularly for the specific material tested, e.g., FFPE tissue) must be carefully considered. While clinical correlation is important for any diagnostic test, correlation of molecular testing results on lymph node tissue or aspirate materials with clinical presentation and other laboratory findings is critical for proper interpretation.

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Gene Therapy for Nonneoplastic Hematologic and Histiocytic Disorders

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Introduction

Over the past decades, gene therapy as a tool to cure disease has blossomed into an exciting clinical possibility. Gene transfer technologies have progressed immensely since the field's inception (Figure 45.1). The first replication-defective retroviral vector was described in 1983,¹ and by 1990 clinical studies using retroviral vectors began. Although initial efforts were robust, by 1996, none of the clinical gene "therapy" trials had shown clinical efficacy. After a change in policy and focus instituted by a panel convened by the National Institutes of Health, efforts to develop gene transfer technologies shifted toward targeting diseases that were early and obvious targets for therapeutic intervention. The simultaneous experimental advances in our understanding of hematopoiesis and the significant advances in gene transfer technology have led to success in humans, at least for disorders requiring modest transfer rates of genes not requiring complex regulation to hematopoietic stem cell (HSC) targets.

Gene therapy is defined as the transfer of a gene or genetic material into a cell with the intent of therapeutically altering the cell's phenotype. The concept of genetic correction of affected cell populations, ideally somatic stem cells of the target tissue, is a focus of many gene therapy trials. Table 45.1 references the proportion of disorders targeted for gene therapy clinical trials. Autologous hematopoietic targets, such as HSCs or lymphocytes, are frequently utilized for ex vivo gene therapy approaches, because these cells may easily be harvested, are renewable sources, may be manipulated in culture, and re-infused intravenously into the patient.^{2,3} Figure 45.2 depicts the ex vivo procedure utilized in many gene therapy applications. This process allows for a controlled exposure of only the desired target cells to the vehicle for transferring the new genetic material into a target cell, termed a vector. The process, termed transduction, occurs when there is a successful interaction between vector and target cell, leading to an alteration in that cell's genotype.

Most vectors derive from viruses, taking advantage of their known properties to deliver genes to cells. The types

of viral vectors utilized in gene therapy applications are shown in Table 45.2 and Figure 45.3, along with their unique properties and potential applications. Viral vectors (targeting HSCs) must integrate into the chromosome, or replicate with cell division, in order for the newly introduced genetic material to pass to their progeny in the circulation, and retroviral vectors have thus emerged as the most commonly employed vector systems for HSC applications.

Vectors for HSC Applications

Gammaretrovirus

Viruses are modified into clinically useful vector systems, in general, by recombinant incorporation of a gene, or genes replacing the genome of the virus, but retaining the necessary viral sequences required for packaging vector nucleic acids into viral particles without the production of replication-competent viral particles that could continue to propagate beyond transduction. The first practical retrovirus vector system was based on the gammaretrovirus, Moloney murine leukemia virus (MLV). These simple mouse retroviruses contain two single strands of linear coding and regulatory sequences flanked on each end by long terminal repeats (LTRs) sequences. There are three genes necessary for viral replication and packaging: *gag*, *pol*, and *env*. The *env* gene product mediates entry into cells after binding. When inside the cytoplasm, the viral RNA is reverse transcribed via the *pol* gene product into proviral cDNA which then translocates into the nucleus, where the LTR sequences allow integration of the viral cDNA into the host chromosomes. Recombinant retroviral vectors are made by removing the *gag*, *pol*, and *env* gene sequences from the viral backbone and replacing them with genetic material of interest, retaining only the LTRs and the packaging signal. The viral vector is then produced by introducing the necessary *gag*, *pol* and *env* sequences into a cell line separately, creating a producer cell line. In this way, producer cell lines release vector particles containing full-length vector RNA, consisting of the viral LTRs flanking

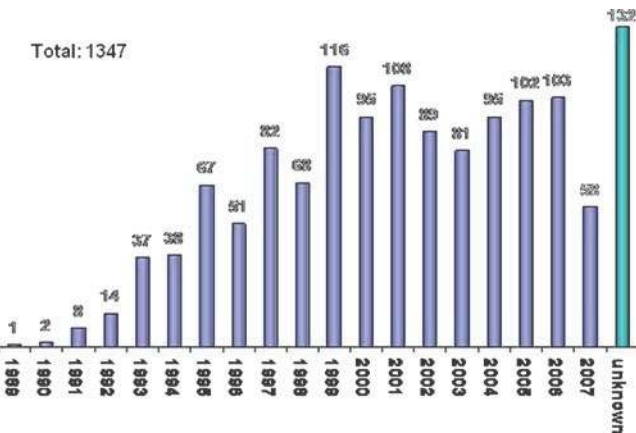


FIG. 45.1. Number of gene therapy clinical trials worldwide, 1989–2007.

TABLE 45.1. Indications for gene therapy clinical trials.

Disease	Percent	Number of trials
Cancer	66.5	896
Cardiovascular	9.0	121
Monogenic	8.2	110
Infectious	6.6	89
Neurological	1.3	17
Ocular	0.9	12
Other	1.9	26
Gene marking	3.8	50
Healthy volunteers	1.9	26

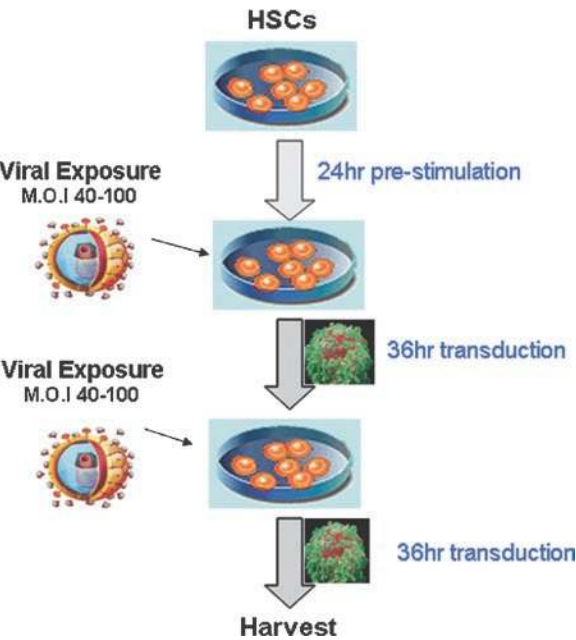


FIG. 45.2. Transduction schema.

the transgene, into cell culture media at a titer of up to 10⁷ particles/ml. The resulting viral vector can integrate and express the gene or genes of interest, but cannot produce new

retrovirus once within a target cell, because it lacks the *gag*, *pol*, and *env* genes within its genome. Gene transfer strategies, utilizing gammaretroviral vectors, have now proven clinically efficacious in the immunodeficiencies,⁴ but the development of leukemia in several individuals⁵ as a result of MLV-vector mediated insertional activation of neighboring oncogenes (insertional mutagenesis – see below) has mandated the development of additional safety measures, as well as the exploration of alternative vector systems.

Lentivirus

There has been real progress toward the development of alternative retroviral systems that may overcome some of the limitations of the gammaretroviral vectors. Lentiviral vectors, based on HIV-1, have essentially the same three key replication and packaging genes (i.e., *gag*, *pol*, and *env*) in addition to other accessory gene products, such as viral infectivity factor (VIF) genes, that encode proteins which contain a nuclear localization signal (NLS), such as matrix (MA), integrase (IN), VpR, and the *cis*-acting element, termed the central polypurine tract (cPPT), that enhances HIV’s infectivity of nondividing cells. A number of advantages of HIV-based lentiviral vectors include not only the ability to transduce nondividing cells, but the production of relatively high-titer vectors with numerous safeguards against recombination events that could produce wild-type HIV,^{6–8} improved transduction of a wider range of cells via pseudotyping with vesicular stomatitis virus-G or other envelope proteins, and production and delivery of nonrearranged genes with complex regulatory elements.⁹ The long held goal of achieving erythroid-specific globin gene expression has now been achieved by delivering the human β -globin gene along with large portions of the globin locus control region to bone marrow cells with an HIV-based vector, allowing (for the first time) regulated human β -globin expression sufficient to revert the phenotype in a murine model of β -thalassemia.⁹ Confirmation in a number of murine models of both sickle cell disease and thalassemia followed.^{10–12} Additionally, HIV and lentiviral vectors, based upon HIV, do not have the same propensity to integrate near transcription start sites as described for MLV-based vectors, and thus may ironically prove safer (insertional mutagenesis – see below).

Alternate Retroviruses

Another member of the *retroviridae*, under development as a gene transfer vector, is the human foamy virus (HFV). As a vector system, HFV has three potential advantages: it has never been associated with pathology in animals or humans, it infects a wide variety of primate cell types, and it has the capacity to package longer transgene(s).¹³ It does not appear, however, to transduce nondividing cells, although it may be more stable than conventional retroviruses within a target cell, tolerating a more prolonged period before cell

TABLE 45.2. Gene therapy viral vectors and utility.

Vector system	Integration	Cell cycle dependence	Insert size limit	Clinical experience	Advantages	Disadvantages	Major applications
Murine retrovirus	Yes	Yes	8–10 kb	Extensive	Stable producer lines No viral genes in vector Low immunogenicity Well-understood biology Efficient entry and integration in many cell types Proven clinical safety Faithful delivery of complex genes Well understood Efficient entry and integration Pseudotyping allows broad tissue range	Low titer, fragile vector Requirement for cycling Erratic expression Insertional mutagenesis	Ex vivo – stem cells, lymphocytes, myoblasts In vivo – producer cell or vector injection into tumors
HIV-based lentivirus	Yes	No		None	High titer, stable vector High level transgene expression Efficient entry into many cell types	Production labor intensive Erratic expression Insertional mutagenesis Recombination with wild-type HIV	Ex vivo – stem cells, lymphocytes, tumor cells, nondividing cells
Adenovirus	No	No	8–10 kb	Moderate		No stable producer lines Potential for recombination and replication-competent virus from vector Multiple viral genes expressed High immunogenicity (may be advantage as a vaccine vector!) Preexisting immunity Inflammatory responses	In vivo – pulmonary epithelium, tumor cells, muscle, liver
AAV	Yes – inefficient	Yes – controversial	4.5 kb	Minimal	Stable vector – extra and intracellularly High titer High level transgene expression No expressed viral genes in vector Ease of production High level of safety No extraneous expressed vector genes	No stable producer cell lines High percentage of defective particles Requirement for helper adenovirus during production Very limited insert size Pre-existing immunity Inefficient cell entry, uptake into nucleus Poor stability within cell Low level expression	Undefined
Naked DNA	No	No	No limit	Moderate			In vivo – tissues accessible to injection, for transient expression or vaccination
Facilitated DNA (liposomes, polylysine conjugates, inactivated adenovirus, etc.)	No	No	No limit	Moderate	No immunogenicity of vector Same as naked DNA plus: Can be targeted to specific cell types More efficient uptake and intracellular stability	No mechanism for persistence	Same as naked DNA, plus in vivo tumor cells, vascular endothelium

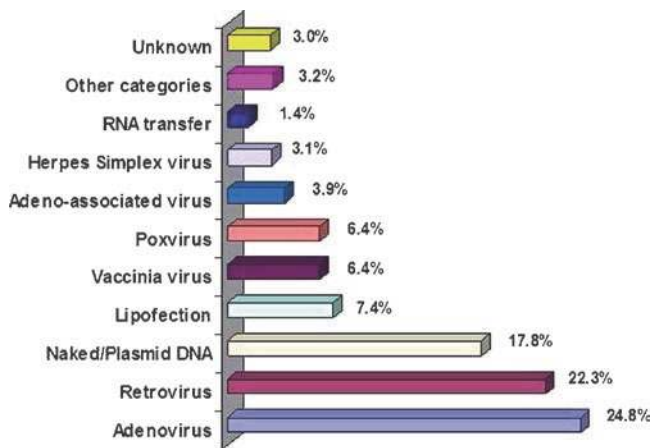


FIG. 45.3. Vectors used in gene therapy clinical trials.

division and integration,¹⁴ and a method for the production of helper free vectors stocks has recently been described.¹⁵ These foamy virus vectors appear efficient for transducing HSCs, with high gene transfer rates observed in human cord blood in vitro, and in murine bone marrow in vivo after a single, overnight vector exposure.¹⁶ A large-scale analysis of over 1,800 HFV integration sites among transduced human CD34⁺ cells suggested a safer pattern, when compared to that observed from gamma retrovirus-transduced cells.¹⁷ A therapeutic HFV vector has recently been tested in a canine model of leukocyte adhesion deficiency, with correction observed in four of five treated dogs.¹⁸

Adenoviral Vectors

Adenoviruses are nonenveloped double-stranded large DNA viruses. The linear adenovirus genome contains 36 kb with an inverted terminal repeat (ITR) of 100–165 base pairs at each terminus. A set of early genes encode for regulatory proteins, that serve to initiate cell proliferation, DNA replication, and down-modulation of host immune defenses, while the late genes encode for structural proteins. Most of the more than 50 adenovirus serotypes target epithelial cells of the respiratory and gastrointestinal tracts, and as such, are known to largely cause respiratory and gastrointestinal infections in immunocompetent humans. Recombinant adenovirus vectors have been engineered from mostly serotype 5 adenovirus, by removal of the E1 and E3 genes (regulating replication and immune recognition) and replacement of up to 7–8 kb with the gene or genes of interest.^{19–23} Because of their natural tropism for epithelial cells, these vectors were initially investigated for the treatment of pulmonary diseases,^{24,25} and later to disorders for which liver expression of the transgene could be therapeutic. The major drawback of these vectors is that they elicit a strong immune response, limiting expression, and adding potential toxicity. Unfortunately, severe toxicity with an early adenoviral vector lead to the tragic death of a subject, who received a high dose of

vector encoding the ornithine transcarbamylase (OTC) gene mediated in a large part by the “cytokine storm” and resultant systemic immune response, that led to widespread capillary leak multiorgan failure (Risks of Gene Therapy – see below).²⁶ Nonessential adenoviral sequences have gradually been eliminated from these vectors^{27,28} and these functions, provided by accessory plasmids through transfection of vector producing cells, much like that performed to make retroviral producers. These highly deleted vectors are often called “helper-dependent” or HdAd vectors. The fact that most early adenoviral gene transfer systems resulted in only transient transgene expression (in contrast to retroviral-mediated gene transfer) limited their use; however, these newer HdAd vectors may direct expression for years.²⁹

Adeno-Associated Virus Vectors

Adeno-associated viruses (AAV) are small nonenveloped single stranded DNA viruses in the *parvovirus* family, *deparvovirus* subfamily, that require a helper virus (typically a double-stranded DNA virus, such as adenovirus or herpes simplex virus) for the production of new viral particles.^{30,31} The linear AAV genome is approximately 4.7 kb long and consists of two homologous ITRs of 145 bp flanking two groups of genes: the *rep* or nonstructural genes and the *cap* or structural genes. There are at least 14 serotypes of AAV that differ mainly on the basis of their external capsid proteins. AAV-2 is the best characterized and enters host cells primarily through interaction with the heparin sulfate proteoglycan receptor on the cell and traffics to the nucleus.^{32–34} In the absence of helper functions, AAV integrates within a relatively small area on chromosome 19; this site specificity appears to require *rep* protein.^{35–39} Recombinant AAV vectors contain the AAV ITRs flanking a gene of interest replacing the *rep* and *cap* genes. This plasmid is introduced into a cell line permissive for adenovirus, along with a helper plasmid containing the AAV *rep* and *cap* genes but no ITRs. Upon exposure to adenovirus, or transfection with adenovirus genes such as E4, the cell line packages the recombinant vector sequences and vector particles are released.^{40–44} Many cell types may be efficiently transduced, including non-dividing cells, such as neurons, but integration and increased efficiency of transduction still appears to depend on cell division or other DNA-disrupting events, although this conclusion remains controversial.⁴⁵ Recently, there has been a great deal of interest in using other serotypes, especially AAV-3, AAV-6, AAV8, and AAV-9, based upon differences in tropism demonstrated by these serotypes.^{46–48} However, integration may prove to be limiting, as tumor development, presumably the result of insertional mutagenesis, has been described in mice.⁴⁹

Nonviral Vectors

Purified DNA may be introduced into target cells by a variety of physical and chemical means, such as microinjection into individual cells, enclosure in phospholipid bilayers (liposomes),

bombardment of the cell membrane after complexing with gold microparticles, electroporation, and calcium phosphate precipitation. However, these techniques have little clinical utility, due to high toxicities, but novel techniques are continually sought as nonviral mediated delivery has a number of advantages, including simplicity in manufacturing, inability to generate dangerous replication-competent infectious particles, and a lack of dependence on cell cycle for delivery.

Early Studies

Transplantation of HSCs following transduction with retroviral vectors as a gene delivery method was introduced decades ago, with high levels of circulating progeny carrying the transferred gene easily attainable in mouse models.^{50,51} These early studies identified conditions that would permit division and maintenance of the HSC population, while increasing the efficiency of transduction through the use of newly discovered hematopoietic growth factors and other manipulations. Long-term persistence of vector sequences in 10–100% of cells from all hematopoietic lineages^{52,53} in both primary and secondary recipients represented indirect evidence for transduction of a true primitive progenitor; and clonal tracking of progeny among different lineages supported this notion.^{49,54–56} These studies set the stage for the human clinical trials and the high expectations that preceded their initiation.

Despite efficient gene transfer to human progenitor cell populations assayed *in vitro* using nearly identical conditions to those used in mouse models, circulating levels of vector containing cells in patients undergoing autologous transplantation for malignancies with bone marrow or peripheral blood derived progenitor cells genetically modified using marker genes were too low to predict clinical benefit, indeed, even too low to measure by conventional means.⁵⁷ These important early marking studies demonstrated that murine models and human HSC progenitor cell *in vitro* assays poorly predict human engraftment by retrovirally transduced HSCs.

The value of large animal models for the preclinical testing of HSC gene transfer technology became apparent, and work performed in these models helped to move the field back to the clinic. Given their phylogenetic proximity to humans, as well as the cross-reactivity of reagents employed for human application, nonhuman primates have been employed most extensively.⁵⁸ High level marking was first demonstrated in 1989, following infusion of marrow cells transduced with a vector cell line that produced a very high concentration of viral particles.³⁷ Recombination between vector and helper sequences in the cell line, however, generated a virus capable of continued replication in the animals *in vivo*, and high-grade T cell lymphomas arose because of insertional mutagenesis in some of the recipient animals, and mandated testing for such replication competence in all clinical grade products.^{50,59,60} The advent of newly characterized hematopoietic growth factors allowed improved gene transfer

rates by systematically comparing these and other modifications to retroviral transduction methods, and clinically relevant levels of genetically modified cells at 5–10% or greater were eventually reached in this model.^{50,59–62} The common ancestry demonstrated among myeloid and erythroid marrow progenitors, as well as peripheral blood T and B cell populations, along with the prolonged contribution of some of these clones to short lived myeloid progeny satisfied the more strict criteria for transduction of the true HSC and provided further optimism for eventual clinical application.^{51,52} The large animal canine transplantation model has proven equally useful, with the added benefit that some breeds also allow testing in a disease-specific context.^{18,49,54,55,63–69}

Clinical Trials

Undaunted by the disappointing results seen in several early gene marking trials,⁷⁰ clinical trials in disease states continued in parallel, based in part on evidence for selective enrichment for genetically corrected cells in the context of diseases affecting hematopoiesis. Blaese et al. used a retroviral vector to transfer the adenosine deaminase (ADA) gene into T cells of two children with ADA-severe combined immunodeficiency (SCID), and the genetically modified T cells were infused without conditioning over 2 years.⁷¹ Integrated vector was detected at 0.3 vector copies per cell in one and 0.1–1% in the other patient. Ten years later, up to 20% of the lymphocytes in the first patient, and 0.1% in the second patient, continued to express the retroviral gene.⁷² Both patients improved clinically, evidenced by improved lymphocyte counts and function, although the advent of ADA enzyme replacement was no doubt contributory to these improvements. Bordignon et al. also used two similar retroviral vectors to distinguish ADA gene transfer to bone marrow cells and peripheral blood lymphocytes.⁷³ Infusions of the genetically modified cells were administered over 2 years in one child and 10 months in another. Early posttransplant, the vector used to transduce peripheral blood lymphocytes was detected. By 2 years, the vector used to transduce bone marrow cells was contributing 2–5% of the peripheral lymphocytes, granulocytes, monocytes, and erythroid cells, gradually replacing that from transduced lymphocytes. Similar results were described in two other patients.⁷⁴

Since early treatment in immunodeficiency diseases offers patients the best chance to avoid long-term irreversible complications, Kohn et al. applied this therapeutic approach to transfer ADA and a marker gene into CD34⁺ selected cord blood cells and infused these cells back to three neonates 4 days after delivery.⁷⁵ These cord blood cells not only engrafted without conditioning, but retroviral sequences were present for more than 18 months at 0.03% in granulocytes and mononuclear cells. Additionally, an oligoclonal integration pattern was demonstrated and persisted for more than 10 years.⁷⁶ While the gene marking levels were lower than expected,

these trials provided additional evidence that gene therapy may phenotypically revert these target cell populations.

Another such early pioneering trial focused on Fanconi Anemia (FA). FA is a rare recessive disorder, in which bone marrow cells are abnormally sensitive to DNA cross-linking agents, and over time progresses to aplastic anemia and the late development of cancer. Liu et al. successfully applied a recombinant AAV vector carrying FANCA, one of the defective genes in FA, to lymphoblastoid cell lines derived from FA patients⁷⁷ and peripheral blood CD34⁺ selected mononuclear cells.⁷⁸ Such correction could theoretically allow for the selection of genetically modified cells in vivo and prompted another of the earliest clinical gene therapy trials. However, these encouraging preclinical results did not translate into long-term FANCA correction in four FA patients who received retrovirally transduced bone marrow cells,⁷⁹ or in two other patients later transplanted elsewhere.⁴ A major barrier to the lack of long-term persistence of genetically modified cells in this disorder is the low number of bone marrow progenitors obtainable from FA patients, hence the lower number of transducible stem cells.

Recent Success

The most common form of SCID is the X-linked deficiency of the common gamma (γ c) chain (SCID-X1), without which T and NK cells cannot develop and mature. Successful gene transfer incorporating the γ c chain by MLV-vector mediated gene transfer to autologous bone marrow CD34⁺ selected cells using optimized techniques, was achieved in four of five treated children.^{80,81} There were increases in CD3⁺ T cell numbers to that of age matched controls, development of a diverse T cell repertoire, and the γ c gene was documented in all CD3⁺ T cells, and ~1 and 0.1% of B and myeloid cells, respectively. An increased number of mononuclear cells developed and matured in the thymus and full phenotypic correction was observed. These results were later extended to five additional children.⁸² This same group of investigators applied the same approach to a 16- and 20-year-old patients.⁸³ Despite the presence of the γ c transgene (though only 1% or lower), the lack of thymic function or thymic damage from chronic infection in these older subjects was suspected as the main barrier to higher levels of gene transfer and clinical improvement as observed in younger subjects.

The success of γ c gene transfer in young children was duplicated by Gaspar et al.; four children with SCID-X1 received autologous marrow CD34⁺ selected cells after exposure to γ c-retroviral vector, pseudotyped with gibbon-ape-leukemia virus envelop.⁸⁴ Again, there was normalization of lymphocyte numbers, development of a diverse T cell repertoire, and clinical improvements. The expression of the γ c chain was high in lymphocytes, and low in B and myeloid cells, as in the previous trial. These results were groundbreaking, as the phenotype of a genetic defect in a cytokine receptor was corrected by gene therapy, with very clear clinical benefit, demonstrated by reduction in infections, withdrawal

of prophylactic antibiotics and immunoglobulins, and fully functional lymphocytes.

Chronic granulomatous disease (CGD) is another inherited immunodeficiency, in which one of the four proteins in the NADPH oxidase complex, with gp91^{phox} being most common, is missing or defective. The clinical result is that of severely reduced antimicrobial killing of phagocytes, increased susceptibility to *Aspergillus* and certain catalase-positive bacteria, and frequent infections and abscesses in the soft tissues and organs. Ott et al. employed a γ -retroviral vector encoding gp91^{phox} to transduce G-CSF mobilized CD34⁺ cells, and reinfused the cells into two adults with CGD.⁸⁵ Vector positive myeloid cells were detectable at 10–20% levels in the circulation over the first 6 months, then gradually increased to 40–60% beyond 1 year. The percent of gene modified cells were approximately 10% in B and 5% in T cells. There was a reconstitution of NADPH oxidase activity, restoration of microbicidal activity, and clinical resolution of bacterial and fungal infections in these two patients. A growth advantage, deriving from integration sites near genes involved on cell proliferation and survival, was touted as one mechanism to explain the high level contribution of genetically modified cells in this context (insertional mutagenesis – see below).

Hemophilias

The hemophilias, resulting from genetic mutations leading to severely deficient clotting factors, are a group of bleeding disorders, for which clinical gene therapy trials have made significant progress, utilizing a broad array of viral vector and target cell populations. Retroviruses, adenoviruses, and AAV have been used to transfer factor 8 or 9 genes for hemophilia A and B, respectively, to cell lines, in mouse models, dog models, or nonhuman primates, with skin fibroblasts, myocytes, hepatocytes, and bone marrow cells as the targets. These vector systems have all progressed to testing in patients. Escalating doses of retroviral vector encoding Factor VIII have been infused intravenously in 13 hemophilia A patients.⁸⁶ Viral sequences were demonstrated in peripheral blood lymphocytes, and transient Factor VIII levels of approximately 1% were noted in nine patients on at least two occasions in the 4 month monitoring period. In another approach, a “gutted” adenoviral vector⁸⁷ encoding Factor VIII was tested in one patient, but the changes were insufficient to prevent the significant thrombocytopenia and transaminitis observed within 1 week after administration. Because of safety concerns, no additional patients were enrolled.

Nonviral gene delivery was tested in six patients with hemophilia A.⁸⁸ Skin fibroblasts were transfected with Factor VIII plasmids ex vivo, and these fibroblasts were then implanted in the abdominal omentum. Three of the six patients (with bleeding records available) had decreased frequency of bleeding, and had approximately 1% of Factor VIII levels up to 4 months.

AAV vector mediated gene transfer has shown great promise in animal models, and human clinical trials have in part

mirrored these success. Three patients with severe hemophilia B received intramuscular injection of AAV vector encoding Factor IX. Approximately 3–4% of normal Factor IX levels were measured with an associated reduction in joint bleeding, yet a return to baseline levels at 6 months was observed.⁸⁹ Targeting liver cells, Manno et al. infused AAV encoding Factor IX via the hepatic artery in seven patients in a dose escalation study. Peak Factor IX levels of 3 and 11% were detected in two patients who received the highest vector dose.²⁶ Unfortunately, Factor IX levels were no longer detectable beyond 6 months in blood; and T cell mediated clearance of AAV vector capsid protein was implicated. The culmination of these results was encouraging, but dictated the need to address the limitations posed by an intact human immune system, an active current area of research.

Risks of Gene Therapy

The first reported serious complication from gene therapy trial occurred in an 18-year-old individual with partial OTC deficiency, who participated in a dose escalating adenoviral vector (containing OTC) given via the hepatic artery. Unfortunately 4 days after the viral vector infusion, the patient expired from a systemic inflammatory response syndrome that led to multiorgan failure.⁹⁰ These results pointed out the limitations of adenoviral vectors in this context and sparked important debate about the way in which gene therapy and other clinical trials should be carried out.

The great enthusiasm shared by all surrounding the first successful gene therapy trial in humans was overshadowed by the subsequent observation of the development of acute lymphoid leukemias in several subjects, approximately 3 years after treatment.⁵ To date, four of the ten treated children treated for X-SCID have developed leukemia, one of whom died from leukemia and antileukemia treatment. These clonal lymphoproliferative events have been directly attributed to insertional mutagenesis by the transferred therapeutic gene.^{53,91} In another successful gene therapy trial in two individuals treated for CGD, both developed clonal expansion from activating integration events near several proliferative genes (i.e., *MDS-EVII*, *PRDM16*, and *SETBP1*), and one of these patients died from overwhelming infection. Functional studies revealed loss of expression of the therapeutic gene prior to death. These studies revealed a higher risk of insertional mutagenesis as a complication of gene therapy than had been envisioned, based upon random retroviral integration, and much effort has been devoted to its understanding over the last several years.

Retrovirus Integration and Insertional Mutagenesis

The ability of retroviruses to integrate efficiently into target cells was the primary reason for their development as gene transfer vectors, targeting HSCs and their progeny.

Although the Moloney murine leukemia retrovirus (MLV) was known to induce leukemia via insertional activation of adjacent proto-oncogenes, or inactivation of tumor repressor genes in mice (Figure 45.4a, b), safety features of viral vectors were designed to, in part, mitigate this risk, which was felt to be quite low when human clinical trials were initiated. In vitro studies of integration of retroviruses into synthetic DNA templates confirmed a random pattern,⁹² and based on evidence that only one or two integrations would occur per target cell, the risk of vector-associated tumorigenesis was estimated to be very low.

The availability of the human and murine complete genome sequences along with the observed insertional events in human clinical trials has prompted a reexamination of the integration patterns of retroviruses. In contrast to prior studies, strikingly nonrandom integration patterns have emerged in large-scale integration site surveys. In a study carried out in T cell lines, wild-type HIV and HIV-based lentiviral vectors were shown to prefer transcription units.⁹³ Distinct patterns of integration were found for the two major vector types: MLV vectors, favoring transcription start sites and HIV vectors, favoring sites within active genes.⁹⁴ These patterns were confirmed in the nonhuman primate in vivo model.⁹⁵

Integration site analyses performed on samples from recent recipients of genetically modified HSCs supported these observations. Analysis of integrations in the subjects of the

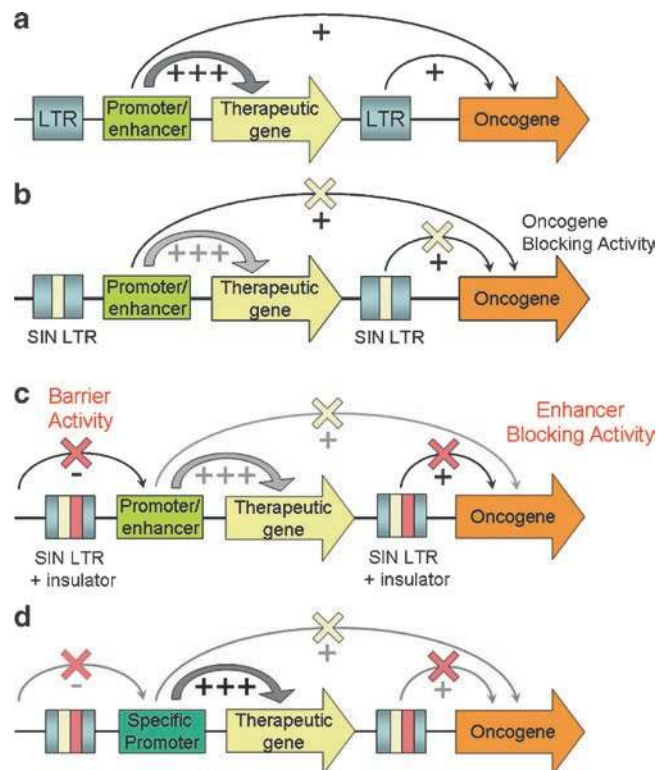


FIG. 45.4. (a, b) Vector design and insertional mutagenesis. (c, d). Future vector delivery system with both enhancer-blocking and barrier activities.

first successful gene therapy trial for X-SCID⁹⁶ again demonstrated a preference toward transcription start sites with a number of potentially troublesome sites (*CCND2*, *ZNF217*, *LMO2*, *NOTCH2*, *RUNX 1* and 3), many of which are highly expressed in CD34⁺ progenitor cells. These results suggested that this pattern of integration may have played a role in engraftment, survival, and proliferation of these genetically modified cells. However, there was no major difference in the pattern of integrations when comparing cells from healthy patients and from those with insertional events. This lack of difference suggests that there was no specific pattern of integration that would predict malignant transformation.

Future Directions

Transgene expression should ideally be long-term, nontoxic, tissue specific, differentiation and stage dependent, and position independent. Therapeutic vectors have suffered from low level expression by biochemical effects of position of integration to epigenetic interference. The *cis*-acting elements that protect neighboring genes from activation by enhancers are known as enhancer-blocking elements, whereas those that protect against heterochromatin-mediated silencing are known as barrier elements. The specific DNA sequence elements that establish and maintain inter-domain boundaries are generally termed insulators that may possess both enhancer-blocking and barrier activities. There are also enhancer-blocking insulators which protect against transcriptional repression. To develop more efficient viral vectors that promote high level expression and at reduced risk of insertional mutagenesis, an emphasis on the transcriptional control of gene regulation by enhancers and insulators has emerged as a major mechanism for control of transgene expression. In order to restrict expression of transgenes to a specific target cell population derived from a particular cell lineage, lineage or tissue-specific transcriptional promoter and enhancer elements are used to drive proviral expression. For instance, hemoglobin gene regulatory sequences may be used to drive transgene expression specifically in erythroid cells.

Enhancers serve to raise the level of basal transcription from the particular gene, while enhancer-blocking insulators interfere with enhancer–promoter interactions when placed between the gene and enhancer, hence development and usage of physiologic promoters may reduce the risk of integrating vectors. In 1999, Li et al. demonstrated that the murine beta-globin locus control region (muLCR) and promoter may be altered for efficient transgene expression in a retrovirus vector. By deleting the beta-globin gene promoter to –127 bp and utilizing a combination of the hypersensitive region, HS2 and HS3 core elements of the LCR, they established an optimized expression cassette which exhibited enhanced transgene expression in the murine erythroleukemia cell line, MEL585.⁹⁶ After further refinement, May et al. successfully established a lentiviral vector system, that

exhibited lineage-specificity and elevated therapeutic levels of beta-globin expression (in vivo), using proximal and distal transcriptional control elements to regulate hemoglobin production from erythroid progeny of transduced HSCs.^{97,98} Moldt et al. were able to produce a fourfold increase in episomal formation, by packaging lentiviral genomic RNA in integrase-defective lentiviral vectors.⁹⁹ Future vector delivery systems will likely contain core enhancer and insulator elements, like the HS4 element of beta-globin, which possess both enhancer-blocking and barrier activities, that inhibit inappropriate activation of surrounding or integrated genes and, at the same time, protect the transgene from epigenetic mediated silencing (Figure 45.4c, d).

Nonintegrating Vectors

Circular forms of viral genomic DNA are generated during infection and transfection of cells with retroviruses, like HIV-1. These extrachromosomal, or episomal, elements persist in the nucleus of transduced cells and offer an alternative tool for gene therapy, since they do not cause physical disruption in the host genome and, thus cannot induce insertional mutagenesis. To overcome the loss of nonreplicating retroviral episomal DNA as a result of cell division, Pich et al. used the latent origin of DNA replication of Epstein–Barr virus, oriP, to drive nuclear retention and replication of plasmid DNA.¹⁰⁰ Further advances in nonintegrating LVs (NILVs), which display efficient reverse transcription and transgene expression in dividing cells and prolonged expression in nondividing myotubes, have been made by introducing point mutations into the catalytic site, chromosome binding site, and viral DNA binding and attachment (att) sites of viral integrase (IN).^{101,102} A new nonintegrating, nonviral gene expression system being developed is the artificial chromosome expression system (ACE system).¹⁰³

Another possible safe and reliable genetic modification for gene therapy is to use Scaffold/matrix attachment region-based vectors, pEPI, as nonviral expression systems, that replicate autonomously in mammalian cells. Manzini et al. showed approximately 80% reporter gene expression in all tissue from 9 of 12 genetically modified pig fetuses when the reporter is delivered in pEPI vector into embryos by sperm-mediated gene transfer.⁹⁹ These vectors provide preliminary steps in developing and advancing animal transgenesis and provide the basis for the future development of germ-line gene therapy.

Targeted Integration

The development of controlled integrating retroviral vectors, which have integration preferences based on attributes of the targeted site, such as primary DNA sequence and the physical structure of the DNA, or through tethering to certain DNA sequences by host-encoded cellular factors, is an important development, that may help prevent insertional mutagenesis, epigenetic mediated transrepression,

as well as differentiation restricted expression. Naturally pathogenic integrating elements that are of interest as targeted gene-delivery vectors for human gene therapy are retrotransposons, DNA transposons, and parvoviruses. Moldt et al. showed that nonviral yeast Flp recombinase may direct insertion of transgenes in targeted episomes containing the recognition sites for the yeast Flp recombinase.¹⁰⁴ A Flp-mediated recombinant transgenic episome was shown to be stably transduced and have limited integration; 44% of all integration events occurred in gene regions, when insertion was directed by bacteriophage-derived integrase, PhiC31.¹⁰⁵ The phiC31 integrase is a protein from *Streptomyces* phage, which catalyzes the integration of a plasmid containing attB into pseudo attP sites. phiC31 based vectors are being developed as a nonviral site-specific gene therapy vector. As site-specific integrating vectors, phiC31 based vectors in cultured cells have been shown to modify type I tyrosinemia in a mouse model and two forms of epidermolysis bullosa in keratinocytes from patients.¹⁰⁶

Sleeping Beauty (SB) transposons have the potential for use as chromosome-integrating vectors for nonviral gene therapy. Recent evidence demonstrating efficacy as a therapeutic gene transfer system comes from preclinical data in the treatment of hemophilia, tyrosinemia type I, junctional epidermolysis bullosa, and type 1 diabetes mouse models of human disease.¹⁰⁷ Type II pneumocytes in mouse lung alveolar region showed long-term expression of SB mediated insertion and expression of transgenes.¹⁰⁸ The utility of SB vector system was tested by modifying GHOST-R3/X4/R5 cells to stably express both CCR5 and CXCR4 siRNA transgene constructs. Cotransfection of siRNA CCR5/CXCR4 with a construct expressing hyperactive transposase (HSB5) to downregulate HIV-1 coreceptor proteins conferred stable resistance against HIV-1 infection in vitro.

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