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RESEARCH ARTICLE

AN APPROACH IN THE ASSESSMENT OF HEMATOLYMPHOID NEOPLASMS

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ABSTRACT

Accurate assessment of tissues for hematolymphoid neoplasms requires an integrated multiparameter approach. Although morphologic examination by light microscopy remains the mainstay of initial assessment for hematolymphoid neoplasms, immunophenotypic analysis by immunohistochemistry and/or flow cytometry is essential to determine the pattern of differentiation and to detect minimal disease when morphology is inconclusive. In some cases, immunophenotypic analysis provides additional information for targeted immunotherapy and prognostication. Genotypic studies, including cytogenetics, fluorescence in situ hybridization, DNA microarray, polymerase chain reaction, and/or next-generation sequencing, are also imperative for subclassification of the genetically defined disease entities in the current World Health Organization classification of hematolymphoid neoplasms 2016. Moreover, genotypic studies can establish clonality, stratify patients to determine appropriate treatment, and monitor patients for treatment response. In the last several decades, technologic advances have dramatically increased our understanding of the biology of normal hematopoiesis and the pathogenesis of hematolymphoid neoplasms. In parallel, a variety of powerful analytic tools, such as immunohistochemistry, flow cytometry, fluorescence in situ hybridization (FISH), DNA micro-array, polymerase chain reaction (PCR), and next-generation sequencing (NGS), have become available in the clinical laboratories. These tools have been integrated into the routine evaluation of tissues for hematolymphoid neoplasms and have, over time, revolutionized the clinical practice of hematopathology. In this article, an approach to the assessment of hematolymphoid neoplasms is discussed.

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INTRODUCTION

Morphologic examination of tissues by light microscopy is still the most cost effective approach to the initial pathologic evaluation of hematolymphoid neoplasms (Figure 1, A). It includes assessment of cytomorphologic and histomorphologic features of cells present in the tissue. Cytomorphologic assessment encompasses evaluation of the maturation of cells (eg, the shape of the nuclei, the quality of chromatin and its distribution, and presence or absence of distinct nucleoli) and of the morphometric features of cells (eg, the nuclear to cytoplasmic ratio and size of cells). Histomorphologic assessment involves examination of the tissue architecture (intact versus partial or complete effacement), the pattern of growth (nodular versus diffuse), the presence or absence of necrosis and fibrosis, and the frequency of mitotic figures, among other features.

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To achieve accurate and comprehensive morphologic evaluation, hematopathologists must be familiar with the normal range of variations in the cytomorphology and histomorphology of the hematolymphoid cells and tissues. It is also important to have viable, well-fixed, adequately processed and stained, and representative tissue available for evaluation. The commonly used basic stains in hematopathology practice are Wright-Giemsa or the equivalent for cytologic preparations of blood, bone marrow aspiration, body fluid, fine-needle aspiration, and tissue imprints, and hematoxylin-eosin for histologic preparations of tissues and cytology cell blocks. The criteria for tissue adequacy may vary depending on the anatomic site, the nature of disease, and the clinical indications. For example, a bone marrow biopsy core that is adequate for evaluation of myeloid neoplasms may not be sufficient for lymphoma staging. Although needle core biopsy material can prove useful for initial screening morphologic assessment, ultimate diagnosis and classification of malignant lymphomas usually require incisional/excisional biopsy. Morphologic examination by light microscopy is the first step in the diagnostic assessment of tissues for hematolymphoid

neoplasms.¹ The cytomorphologic and histo-morphologic findings are the basis for the initial differential diagnosis between benign and neoplastic proliferations of hematolymphoid cells. For example, effacement of lymph node architecture by a nodular and diffuse proliferation of large lymphoid cells suggests involvement by follicular lymphoma and diffuse large B-cell lymphoma (DLBCL), respectively. In addition, morphologic findings are critical in triaging and planning additional immunophenotypic (immunohistochemistry and/or flow cytometry) and/or genotypic (cytogenetics, FISH, PCR, DNA microarray, and/or NGS) studies that help to exclude a neoplastic hematolymphoid cell proliferation or to further classify a hematolymphoid neoplasm. It should be emphasized, however, that not all tools have to be used in every case. The hematopathologists should have the necessary knowledge about these diagnostic modalities, their strengths, weaknesses, and potential pitfalls, in order to practice cost-effective, high-quality hematopathology, particularly in the current health care environment.

Paraffin immunohistochemistry: Immunohistochemistry performed on formalin-fixed, paraffin-embedded tissue sections has long been an essential diagnostic modality in the evaluation of hematolymphoid neoplasms, where it often complements flow cytometry for immunophenotypic profiling. Many antibodies are available to determine the presumptive cell of origin, a critical component in the current World Health Organization (WHO) classification of hematolymphoid neoplasms.¹⁻⁴ A major advantage of immunohistochemistry over flow cytometry is the ability to evaluate the cytomorphologic appearance and the architectural pattern of the positively and negatively staining cells. Knowledge of the expected staining patterns for normal and the various neoplastic hematolymphoid cells is essential for correct interpretation of the staining results. For example, the antiapoptotic protein BCL2 is expressed by benign T cells and mantle zone B cells but is not expressed by benign germinal center B cells (GCBs; centrocytes and centroblasts), a subset of which must undergo apoptosis for optimal selection of antibody-producing B cells. This knowledge is vital when evaluating tissue with nodular/follicular architecture. Positive staining of the follicles using a BCL2 immunohistochemical stain is consistent with follicular lymphoma, but only if the positive cells display GCB differentiation (ie, express CD10 and BCL6).

Of note, benign primary lymphoid follicles, which by definition lack germinal centers, are expected to be BCL2⁺, similar to naive B cells in mantle zones. T cells residing in the germinal center will also be positive for BCL2. Antigen not associated with the origin of the neoplastic cells can occasionally be detected by immunohistochemistry (Figure 1, B through F). Thus, careful morphologic evaluation in conjunction with assessment using a battery of antibodies and other diagnostic tools is essential to avoid misdiagnosis. New antibodies with diagnostic and clinical relevance, many of which have been identified from gene expression profiling studies, are constantly being developed for paraffin immunohistochemistry.^{5,6} For example, subtyping of DLBCL based on GCB differentiation or non-GCB differentiation is now standard practice for prognostic relevance and as an aid for therapeutic decision making. Several algorithms for selecting immunohistochemical stains have been developed as surrogates for gene expression profiling, making the

classification of GCB versus non-GCB practical for routine use.⁷⁻¹¹ We employ CD10, BCL6, and IRF4/MUM1 according to the algorithm set forth by Hans et al⁷ for this purpose. More recently, there has been clinical interest in expanding the immunohistochemical stain panel for DLBCL cases to include evaluation of MYC and BCL2 because overexpression of these 2 proteins, referred to as a “double expresser” phenotype, has been postulated to denote an aggressive form of DLBCL akin to “double-hit” lymphomas, even in the absence of concurrent MYC and BCL2 gene rearrangements.¹²⁻¹⁴ As new monoclonal antibody therapies continue to be developed for the treatment of lymphoma, immunohistochemistry will likely be used to demonstrate the presence of the corresponding protein targets, as has been done for years with CD20 and the anti-CD20 monoclonal antibody rituximab.¹⁵ Some cases of DLBCL, particularly relapsed disease, are evaluated for CD30 immunoreactivity because CD30⁺ cases may benefit from treatment with the anti-CD30 antibody–drug conjugate brentuximab vedotin.^{16,17}

Hematopathologists must be aware of such therapies because they can dictate the panel of immunohistochemical stains used and interpretation of those stains. For example, the use of rituximab to treat CD20⁺ B-cell lymphomas may yield post treatment B-cell proliferations that downregulate CD20 expression. Knowledge of previous rituximab therapy is important so that other antibodies, such as CD79a and PAX5, can be used to identify B cells that are negative for CD20. Another example of progress in immunohistochemistry is the identification of SOX11 as a marker of mantle cell lymphoma (MCL), including the occasional cases that are negative for cyclin D1.¹⁸⁻²⁰ Cyclin D1 has long served as a reliable marker for MCL, but the stain is not specific for this diagnosis, and some cases will be negative. Furthermore, approximately 5% of DLBCL cases express cyclin D1, and thus this stain should not be used indiscriminately. Fortunately, most cases of cyclin D1-positive DLBCL are negative for CD5 and t(11;14) IGH/CCND1, permitting their distinction from MCL, although rare DLBCL cases may have the t(11;14).^{21,22} Cyclin D1 expression is also observed in a subset of plasma cell dyscrasias and hairy cell leukemias, and sometimes in the proliferation centers of chronic lymphocytic leukemia/small lymphocytic lymphoma. SOX11 may be more sensitive than cyclin D1 for detecting MCL, but likewise it is not specific for this diagnosis. Some cases of Burkitt lymphoma, lymphoblastic lymphoma, and T-prolymphocytic leukemia will be SOX11⁺, although usually these are easily distinguished from MCL based on review of the hematoxylin-eosin stain in conjunction with flow cytometric immunophenotyping and/or a panel of immunohistochemical stains.¹⁹

In situ hybridization performed on formalin-fixed, paraffin-embedded tissue sections, a variant form of paraffin immunohistochemistry, currently has a limited role in the assessment of hematolymphoid neoplasms. One application is the evaluation for clonal immunoglobulin light chain gene expression in plasma cells (Figure 1, G and H), particularly when nonspecific high-background staining renders the interpretation of immunohistochemistry difficult.^{2,23} Another application is the detection of Epstein-Barr virus–encoded RNA to identify Epstein-Barr virus infection, because it is more sensitive than Epstein-Barr virus LMP1 immunohistochemistry.

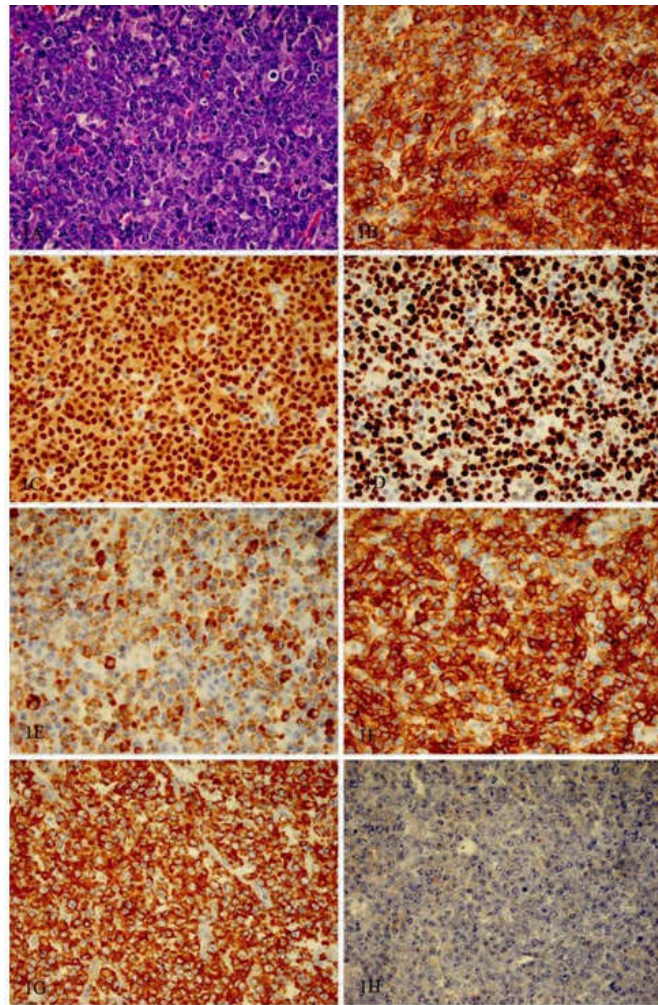


Figure 1. Hematoxylin-eosin stain shows a characteristic plasmacytic morphology in a plasmablastic lymphoma

(A). Paraffin immunohistochemistry shows that the lymphoma cells are positive for CD138 (B) and MUM1 (C), with a high proliferation rate (D), and aberrant expression of CD3 (E) and CD5 (F). These cells show cytoplasmic j (G) but not k (I) light chain restriction by paraffin in situ hybridization (original magnification 340).

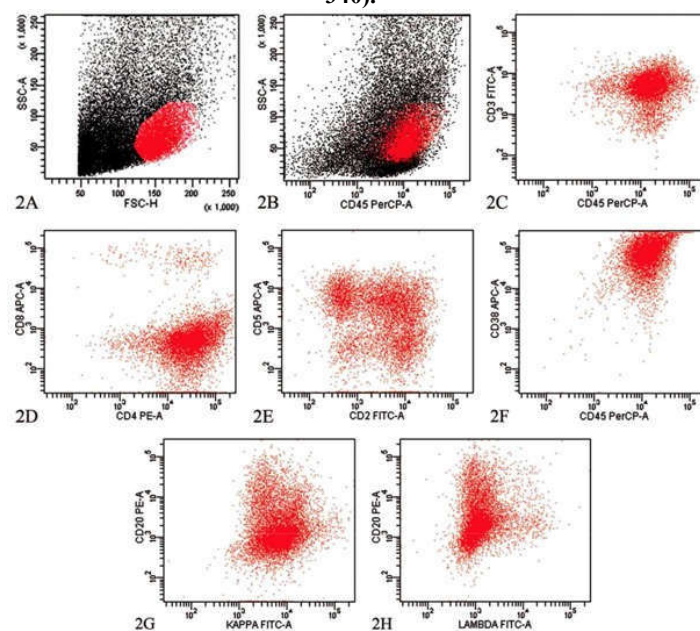


Figure 2. Flow cytometric immunophenotyping performed on the same plasmablastic lymphoma case as shown in Figure 1 demonstrates that the lymphoma cells are large and positive for CD45 (A and B; highlighted red in the dot plots), with expression of dim surface CD3 (C), CD4 (D), heterogeneous CD2 and CD5 (E), strong surface CD38 (F), and dim CD20 and surface j (G) but not k (H) light chain protein



Figure 3: Conventional cytogenetics shows 46,XX, t(3;3)(q21;q26) in an acute myeloid leukemia with marked dyspoiesis (arrows indicating the break points)

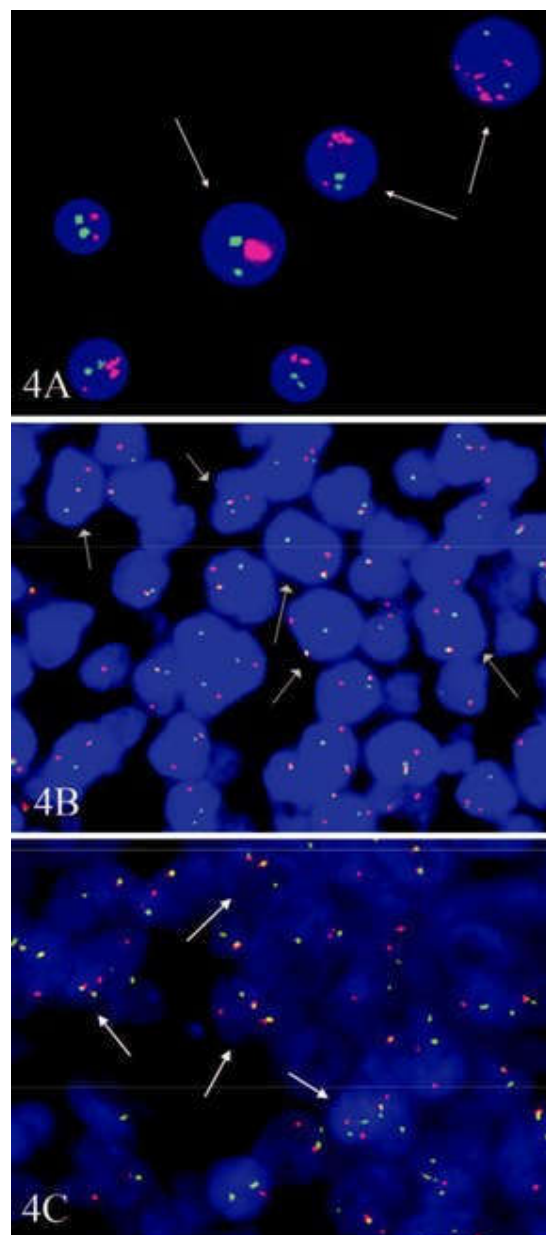


Figure 4. Fluorescence in situ hybridization shows iAMP21 in a B-lymphoblastic leukemia/lymphoma (A; RUNX1-ETV6 dual-color, dual-fusion probe; arrows indicating positive cells with multiple red signals and 2 green signals). Fluorescence in situ hybridization shows MYC rearrangement (B; dual-color breakapart probe; arrows indicating positive cells with 1 red, 1 green, and 1 fusion signal) and translocation t(14;18)(q32;q21)(IGH-BCL2) (C; dual-color, dual-fusion probes; arrows indicating positive cells with 1 red, 1 green, and 2 fusion signals) in a double-hit lymphoma

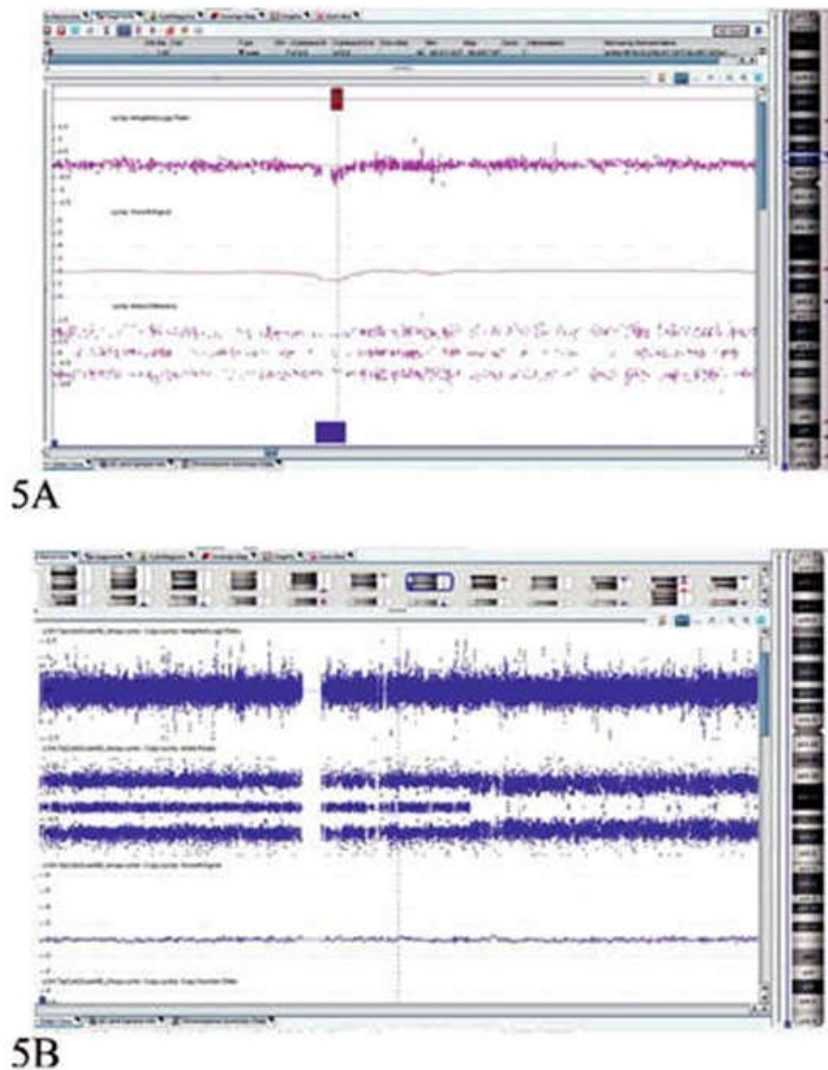


Figure 5. DNA single-nucleotide polymorphism microarray shows IKZF1 gene deletion on chromosome 7q12 in a B-lymphoblastic lymphoma/leukemia (A). Each dot represents a single-nucleotide polymorphism or a unique oligonucleotide probe. The top dotted line is the weighted log₂ ratio with a portion of the signals below the baseline of 0 (the red box above indicating the deleted region). The center line is the smooth signal (average copy number), showing a dip in the copy number to 1 for the deleted region. The next area is the allele peaks, showing alleles AA, AB, and BB, respectively, for most of chromosome 7 but only alleles or BB at the deleted region. The purple box at the bottom shows that the deleted region contains the IKZF1 gene. DNA single-nucleotide polymorphism microarray shows copy-neutral loss of heterozygosity on chromosome 7 (7q21.3q36.3) in a patient who has myelodysplasia with a normal karyotype and a negative fluorescence in situ hybridization panel for myelodysplastic syndrome (B). The top dotted line of the weighted log₂ ratio and the bottom line of the smooth signal show 2 copies of the entire chromosome 7. The central area is the allele peaks, showing alleles AA, AB, and BB, respectively, for most of chromosome 7. The distal portion of chromosome 7q has only alleles AA and BB without allele AB, consistent with copy-neutral loss of heterozygosity due to duplication of the homologous chromosome

Flow Cytometry: Flow cytometry was initially introduced to the clinical laboratories for lymphocytic subset analysis in patients with congenital or acquired immunodeficiencies. With the development of numerous commercially available antibodies, fluorochromes, multilaser flow cytometers, and sophisticated analytic software, it has now become a widely used diagnostic tool in the phenotypic analysis of hematolymphoid neoplasms.²⁴⁻²⁸ Flow cytometry has several important strengths over paraffin immunohistochemistry. Major strengths include the ability to analyze the expression of multiple antigens as well as the physical properties (eg, size and cytoplasmic complexity) of individual cells, and to identify normal and abnormal cell populations present in the same specimen simultaneously.

Additionally, the intensity and/or aberrancy of antigen expression, which are of diagnostic, prognostic, and therapeutic importance, are best evaluated by flow cytometry. Flow cytometers currently employed in many clinical laboratories can assess up to 10 different surface or intracellular antigens with a panel of antibodies in a single tube, making it possible to evaluate limited fine-needle aspiration or body cavity fluid specimens for abnormal hematolymphoid cells. These abnormal cells may be difficult if not impossible to identify by light microscopic examination, even with the help of paraffin immunohistochemistry. In addition, some antigens, such as CD103 in hairy cell leukemia and enteropathy-associated T-cell lymphoma, can only be detected by flow cytometry because of the lack of effective

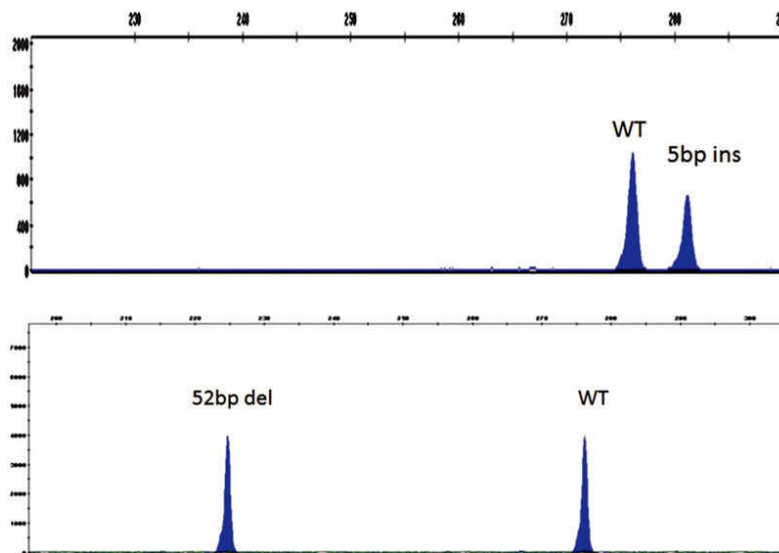


Figure 6. Real-time polymerase chain reaction assay shows CALR exon 9 mutations (mutant alleles with 5-bp insertion in the upper panel and 52-bp deletion in the lower panel compared with the wild-type alleles [WT]) from 2 different patients with myeloproliferative neoplasm

Fluorescence In Situ Hybridization Panels for Assessment of Common Hematolymphoid Neoplasms

Disease Category	Probes	Targets
B-lymphoblastic leukemia	12p13 ETV6, 21q22 RUNX1	t(12;21)(p13;q22)
	9q34 ABL1, 22q11.2 BCR	t(9;22)(q34;q11.2)
	11q23 KMT2A (MLL)	t(11;?)(q23;?)
	Centromeres 4, 10	Hyperdiploidy
Acute myeloid leukemia	5p15.31 TAS2R1, 5q31.2 EGR1	del 5q, 5
	7q31 D7S486, D7Z1	del 7q, 7
	11q23 KMT2A (MLL)	t(11;?)(q23;?)
	8q22 RUNX1T1, 21q22 RUNX1	t(8;21)(q22;q22)
Myelodysplastic syndrome	16q22 CBFB	inv(16)(p13q22)/t(16;16)
	5p15.31 TAS2R1, 5q31.2 EGR1	del 5q, 5
	7q31 D7S486, D7Z1	del 7q, 7
	D8Z2, 8q MYC	þ8
Chronic lymphocytic leukemia	11q23 KMT2A (MLL)	t(11;?)(q23;?)
	11q13 CCND1, 14q32 IGH	t(11;14)(q13;q32)
	13q14 D13S25, 13q34 LAMP	del 13q, 13
	6q23 MYB, D6Z1	del 6q
Plasma cell neoplasm	11q22.3 ATM, D11Z1	del 11q22.3, ATM
	D12Z3, 12p13 ETV6	þ12
	17p13.1 TP53, CEP17	del TP53
	1p32.3 CDKN2C, 1q21 CKS1B	loss 1p, gain/amp 1q
	Centromeres 3, 7, 9	Hyperdiploidy
	11q13 CCND1, 14q32 IGH	t(11;14)(q13;q32) or IGH rearrangement
13q14 D13S25, 13q34 LAMP	del 13q, 13	
	17p13.1 TP53, CEP17	del TP53
	4p16.3 FGFR3, 14q32 IGH	t(4;14)(p16.3;q32)
	14q32 IGH, 16q23 MAF	t(14;16)(q32;q23)

antibodies that work on fixed tissue in immunohistochemistry. The development of hematolymphoid cells is a complex and highly regulated process that is characterized by a unique antigen expression profile according to the stage of their maturation. For example, normal myeloblasts express CD13, CD34, and CD117, but not CD15. The expression of CD13 is down-regulated in promyelocytes and returns to normal in myelocytes, metamyelocytes, and granulocytes. In the bone marrow, normal precursor B cells strongly express CD10 and CD34, and they only dimly express CD45 and the other B-cell-associated markers CD19, CD20, and CD22, whereas mature B cells lose expression of CD10 and CD34, and gain expression of CD45 and other B-cell-associated markers.

In the peripheral lymphoid tissues, the mature B cells exposed to antigens express CD10 again after migrating to germinal centers. Normal double-negative, early precursor T cells are present at a very low number in the bone marrow, which precludes reliable detection by flow cytometry as routinely performed in many clinical laboratories. These cells express CD34, terminal deoxynucleotidyl transferase (TdT), cytoplasmic CD3, dim CD5, and dim CD7; they do not express CD1a, CD4, or CD8. After migrating to the thymus, they become double positive for CD4 and CD8, and they express CD1a, CD2, CD5, and bright CD7. The expression of CD10, CD34, and TdT is heterogenous. The double-positive precursor T cells eventually differentiate into either CD4^þ or CD8^þ T cells, with loss of CD10, CD34, and TdT expression.

Neoplastic hematolymphoid cells generally recapitulate the immunophenotypic profiles of their normal counterparts with abnormalities in the expression of some antigens that can be detected by multiparameter flow cytometry. These abnormalities include abnormal intensity of the normally expressed antigens (increased or decreased intensity, homogenous versus heterogenous antigen expression, or complete loss of antigen expression, such as absence of CD7 expression in some peripheral T-cell lymphomas), asynchronous antigen expression (expression of mature antigens in the progenitor cells, such as CD15 expression in leukemic myeloblasts), and lineage infidelity or cross-lineage antigen expression (expression of antigens that are not normally expressed in that lineage, such as expression of CD19, CD7, or CD56 in leukemic myeloblasts).

Thus, knowledge about the immunophenotypic profiles of normal hematolymphoid cells is essential to differentiate benign from neoplastic cells.^{24,27,29–31} Flow cytometry has also replaced cytochemical stains in lineage assignment and subcategorization of immature or precursor hematolymphoid neoplasms. With a comprehensive panel of antibodies, flow cytometry can reliably distinguish acute myeloid leukemia (AML) from acute lymphoblastic leukemia (ALL) of either T or B lineages based on the distinct immunophenotypic profiles of the leukemic blasts. Specific antigens can be demonstrated by flow cytometry that may be used to further subclassify AML, such as CD64 in acute monocytic/monoblastic leukemia and CD61 in acute megakaryoblastic leukemia.³² The characteristic immunophenotypic profile in some acute leukemias can be used to predict the presence of recurrent genetic abnormalities, and to guide further genotypic studies for subclassification. For example, aberrant expression of B-cell markers, such as CD19, is commonly seen in AML with t(8;21). The absence of CD34 and HLA-DR expression suggests the diagnosis of either acute promyelocytic leukemia with t(15;17) or AML with normal cytogenetics and NPM1 mutation. B-ALL with expression of CD15 but not CD10 will likely harbor the chromosomal translocation t(4;11). Moreover, flow cytometry facilitates the diagnosis of some leukemias that are difficult to evaluate by cytochemical stains and/or immunohistochemistry. Examples include acute undifferentiated leukemia, mixed-phenotype acute leukemias, blastic plasmacytoid dendritic cell neoplasm, and, in particular, early T-precursor ALL, a disease entity that is defined by flow cytometric immunophenotyping.^{33,34}

Flow cytometry additionally plays an important role in the diagnosis and classification of mature hematolymphoid cell neoplasms. For example, skewed or absent immunoglobulin light chain protein expression in B cells by flow cytometry has long been used to infer monoclonality and aid in the diagnosis of B-cell non-Hodgkin lymphomas. Similarly, analysis of the T-cell receptor b repertoire by flow cytometry is performed in a few clinical laboratories to determine T-cell monoclonality and help diagnose T-cell non-Hodgkin lymphomas. Because a variety of reactive conditions, particularly herpes virus infections, can induce a dominant T-cell clone, demonstration of a prominent T-cell receptor epitope is less specific for neoplasm than aberrant antigen expression, such as loss of CD7 or dim surface CD3 expression. Leukemia-associated immunophenotypes in myeloid stem cells and abnormal maturation patterns in mature myeloid cells as demonstrated by flow cytometry have recently been used to help diagnose

myelodysplastic syndrome (MDS) and myeloproliferative neoplasms.^{35–37} Hairy cell leukemia cells express CD25, CD103, and bright CD11c and CD22, a pattern not observed in other B-cell non-Hodgkin lymphomas. Mantle cell lymphoma, an aggressive lymphoma requiring more intense chemotherapy, can be distinguished from other, more indolent mature B-cell non-Hodgkin lymphomas with similar morphology.³¹ Burkitt lymphoma and aggressive B-cell lymphoma with MYC and BCL2 rearrangements (also called “double-hit” lymphoma) display some characteristic antigen expression profiles by flow cytometry that can be used in their differential diagnosis from DLBCL.^{38,39} Furthermore, flow cytometry can provide prognostic and therapeutic information for patients with hematolymphoid neoplasms.

For example, CD38 and ZAP70 are poor prognostic markers in patients with chronic lymphocytic leukemia/small lymphocytic lymphoma. The power of flow cytometry to detect minute quantities of specific cell populations in a mixed-tissue specimen has led to the discovery of early hematolymphoid lesions, such as mono-clonal B lymphocytosis, and in situ follicular and mantle cell neoplasms.⁴⁰ Patients with these lesions are asymptomatic but may have an increased risk of developing lymphomas, similar to patients with monoclonal gammopathy of unknown significance who have an increased, albeit overall small, risk of developing plasma cell myeloma. Further studies with long term follow-up, however, are necessary to determine the actual risk of developing overt lymphomas. The potential for flow cytometry to detect minute cell populations has also been used to monitor for minimal residual disease, providing one of the most important predictive markers for early disease relapse and poor treatment outcome in AML, B-ALL, and plasma cell myeloma.²⁸ The ability of flow cytometry to determine the expression of surface antigens with high accuracy and specificity has facilitated the development of targeted therapy with monoclonal antibodies and chimeric antigen receptor T cells. Examples include rituximab in CD20^b B-cell lymphomas, the anti-CD33 antibody–drug conjugate gem-tuzumab ozogamicin, and the anti-CD123 antibody CSL362 in refractory AMLs; the anti-CD38 antibody daratumumab in plasma cell myeloma; and CD19–chimeric antigen receptor T cells in B-ALLs.^{41–44}

Flow cytometry, however, has its own limitations and challenges. The technique requires fresh tissues with viable cells, such that frozen, fixed, or necrotic tissues preclude analysis. Before flow cytometry can be performed, the tissue needs to be disaggregated to form a single-cell suspension. As a result, architectural features are completely lost. The neoplastic cells, such as those of Hodgkin lymphoma, T-cell/histiocyte-rich large B-cell lymphoma, and a few DLBCLs, may not survive this processing step in some cases, rendering flow cytometric immunophenotyping results noninformative and potentially misleading. Dilution of neoplastic cells by normal hematolymphoid cells is another factor that may lower the sensitivity of flow cytometry (eg, in acute leukemias with a “dry-tap” bone marrow aspirate specimen, marginal zone lymphomas with residual benign lymphoid follicles, and some cases of angioimmunoblastic T-cell lymphoma). Some antibodies used in paraffin immunohistochemistry, moreover, are not available for flow cytometric immunophenotyping (eg, Ki-67 and cyclin D1). In vivo treatment with monoclonal antibody or chimeric antigen receptor T cells can mask the antigens of interest on neoplastic cells, and thus hide residual

or recurrent diseases from detection by flow cytometry. Importantly, demonstration of a clonal B-cell population by flow cytometry does not always indicate lymphoma.⁴⁵ Flow cytometric detection of a reactive clonal B-cell population is most commonly encountered in cases of florid follicular hyperplasia, which are usually easily distinguished from follicular lymphoma or other lymphoma based on evaluation of the hematoxylin-eosin section and a limited panel of immunohistochemical stains. In addition, lineage infidelity or cross-lineage antigen expression may lead to erroneous lineage assignment by flow cytometric immunophenotyping. For example, the neoplastic cells in a plasmablastic lymphoma display aberrant expression of multiple T-cell lineage antigens by flow cytometry (Figure 2). Without morphologic correlation and if a limited panel of antibodies was employed, this case can potentially be misdiagnosed as a peripheral T-cell lymphoma. Therefore, flow cytometric immunophenotyping results should be interpreted in the context of microscopic and other findings, and new strategies of flow cytometric immunophenotyping should be developed to meet the challenges in the era of ever increasing targeted immunotherapies.

Cytogenetics: Morphologic examination by light microscopy in conjunction with immunophenotypic analysis by flow cytometry and/or immunohistochemistry is sufficient to diagnose and classify most hematolymphoid neoplasms. However, genotypic studies are essential for further subtyping or subclassification of the genetically defined distinct disease entities of hematolymphoid neoplasms in the current WHO classification.¹ Genotypic studies may also facilitate the diagnosis of clinically suspected hematolymphoid neoplasms for which morphologic and/or immunophenotypic data are inconclusive. In addition, genotypic studies often have a significant impact on prognosis, monitoring of disease progression, and stratification of patients with various treatments, including those with small molecule inhibitors.

Conventional cytogenetics with G-banding technology provides a broad overview of the entire genome and allows us to identify genome-wide nonrandom recurring abnormalities and nonrecurring changes that include balanced rearrangements (translocations and inversions) as well as copy number changes. It also allows us to investigate the mechanisms of genotypic abnormalities and their role in the pathogenesis of hematolymphoid neoplasms. The first recurrent genotypic abnormality identified by chromosome analysis, more than half a century ago, was the Philadelphia chromosome. The discovery of Philadelphia chromosome associated with chronic myelogenous leukemia (CML) provided the first scientific evidence to support the concept that cancer is a genetic disease. Philadelphia chromosome is the result of reciprocal translocation between chromosomes 9 and 22, t(9;22)(q34;q11.2), with generation of the BCR-ABL1 fusion gene and constitutive activation of the ABL1 kinase, which is the underlying molecular mechanism for the development of CML and the basis for targeted therapy with small-molecule tyrosine kinase inhibitors, such as imatinib. Many balanced rearrangements and numeric changes of chromosomes have been identified by chromosome analysis since the discovery of Philadelphia chromosome. Some of the abnormalities have become the defining diagnostic features of specific hematolymphoid neoplasms, such as t(15;17)(q22;q21) (PML-RARA) in acute promyelocytic leukemia.¹ In other cases, specific abnormalities can help confirm

the diagnosis and provide prognostic information, such as del5q/5 or del7q/7 in myelodysplasia. They have also been used to aid in the assessment of risk and selection of chemotherapy in patients with hematolymphoid neoplasms. The ability of conventional cytogenetics to identify new genotypic abnormalities that were not present at diagnosis can provide important information in the follow-up for disease progression or relapse. Conventional cytogenetics is currently considered the standard of care in the routine workup of bone marrow specimens for MDS, acute leukemias, and plasma cell myeloma. It is less frequently used to evaluate extramedullary tissues because of the requirement for fresh tissue. Conventional cytogenetics is labor intensive and requires dividing cells to obtain metaphases for analysis. As a result, the karyotype in some cases may be noninformative when the neoplastic cells are scant or nondividing. The other limitation of conventional cytogenetics is the inability to detect genotypic changes shorter than 10 Mb in length. Nevertheless, conventional cytogenetics remains the method of choice to evaluate rare genotypic abnormalities. For example, the t(3;3)(q21;q26) is a recurring abnormality seen in AML and denotes a poor response to conventional chemotherapy (Figure 3). Because this rearrangement is rare, most laboratories do not routinely test for this rearrangement by FISH or molecular studies. Chromosome analysis is the only readily available test that will detect the genetic abnormality that defines this subtype of AML.

Fluorescence in situ hybridization: Fluorescence in situ hybridization is an excellent technique for revealing specific genotypic abnormalities, such as deletions/duplications, gene amplifications, and rearrangements. Although it is often part of the routine workup along with conventional cytogenetics, FISH has several distinct advantages. FISH is less labor intensive, and the results can be available within hours. This advantage is particularly notable when rapid confirmation of a suspected diagnosis of acute promyelocytic leukemia is required, because this diagnosis warrants a unique therapeutic approach that differs from other AMLs. FISH can detect genotypic abnormalities with a resolution of 0.1 to 2 Mb and an overall sensitivity of about 10- to 100-fold higher than conventional chromosome analysis. Its sensitivity can be further increased by enriching the sample for neoplastic cells. For example, enrichment of plasma cells using CD138-labeled magnetic beads and a magnet-activated cell sorter has greatly increased the yield of meaningful results in patients with less than 5% plasma cells in the bone marrow. FISH can also quantify the percentage of neoplastic cells per clone, and can be extremely useful when determining the level of amplification, such as intrachromosomal amplification of chromosome 21 (Figure 4, A). This abnormality occurs in about 2% of B-ALLs in older children with lower white blood cell counts, and confers an adverse outcome when treated with standard risk regimen, but an improved outcome when treated as high-risk B-ALL.⁴⁶⁻⁴⁸ Most importantly, FISH does not require fresh tissue or dividing cells to obtain metaphases for analysis. It can be performed on smears, fresh cells, or fixed cells in suspension, air-dried touch imprints, and formalin-fixed, paraffin-embedded tissue sections, an advantage that has greatly increased the application of FISH in hematopathology. However, FISH routinely performed in most clinical laboratories will not be able to detect unknown genotypic abnormalities. More sophisticated FISH studies, such as spectral karyotyping or multiplex FISH, can overcome this

limitation. But these techniques are expensive, and therefore are not routinely used in the clinical laboratories.⁴⁹ The identification and characterization of genotypic abnormalities by conventional cytogenetics and other methods have facilitated the development of numerous validated FISH probes. Most of these FISH probes are commercially available and are being used clinically to aid in the diagnosis and subclassification of hematolymphoid neoplasms. It is important, however, to know how the probe is designed (eg, break-apart versus fusion) in order to correctly interpret the hybridization pattern in the cells of interest. FISH is particularly efficacious in the identification of specific known genetic abnormalities, such as the cryptic del(4)(q12), that cannot be detected by conventional chromosome analysis. This specific abnormality results in formation of the FIP1L1-PDGFR α fusion gene and confers sensitivity to the tyrosine kinase inhibitor, imatinib, in the myeloid and lymphoid neoplasm with eosinophilia disease category. Another example is the t(12;21)(p13;q22)(ETV6- RUNX1), which cannot be identified by routine karyotyping but confers a good prognosis in pediatric B-ALL. Many clinical laboratories have simplified workflow by developing FISH panels. Although the probes in the panels may vary in each laboratory, they are usually based on the most common abnormalities with diagnostic or prognostic utility. The Table lists the FISH panels and probes that we use at Emory for the evaluation of various hematolymphoid neoplasms.

Most FISH studies for hematolymphoid neoplasms are performed on bone marrow smears or cell suspensions. FISH can also be performed on formalin-fixed, paraffin-embedded tissue sections from extramedullary sites, such as lymph nodes. This flexibility is highly useful because many institutions do not routinely send fresh tissues from these sites for conventional cytogenetics. In these cases, the FISH results can provide important information in the differential diagnosis, classification, and treatment of various non-Hodgkin lymphomas. For example, the differential diagnosis of B-cell lymphoma with a nodular pattern may include follicular lymphoma, marginal zone lymphoma, and MCL. Demonstration of t(11;14)(q13;q32)(CCND1-IGH) or t(14;18)(q32;q21)(IGH-BCL2) by FISH will support the diagnosis of MCL or follicular lymphoma, respectively. The presence of concurrent MYC rearrangement and t(14;18)(q32;q21)(IGH-BCL2) helps distinguish “double-hit” lymphoma from DLBCL and Burkitt lymphoma (Figure 4, B and C), which can have a significant impact on patient management. As more chromosomal rearrangements have come to be identified, new FISH probes have been developed to characterize the hematolymphoid neoplasms that harbor them. Chromosomal rearrangement of DUSP22 is present in about 30% of ALK anaplastic large cell lymphomas, and these cases have a much better prognosis than other ALK anaplastic large cell lymphomas, particularly those with TP63 rearrangement.⁵⁰ Immunoglobulin (IG)- IRF4 translocations are predominantly observed in GCB-type DLBCL and follicular lymphoma grade 3B that lack t(14;18)(IGH-BCL2) rearrangement. IG-IRF4 positivity is associated with young age and a favorable outcome. In addition, 11q aberrations may define a new variant of Burkitt lymphoma that shares the same gene expression profiles but lacks the characteristic rearrangements involving MYC.^{51,52} CRLF2 translocations have more recently been found in 7% to 14% of de novo B-ALL and 53% of Down syndrome-associated B-ALL.^{53,54}

These translocations are associated with JAK1/2 mutations, IKZF1 deletions, Hispanic ethnicity, and a very poor prognosis. It is apparent from these recent studies that some of these genetic abnormalities may be used to define distinct clinicopathologic disease entities that will likely be included in an updated WHO classification of hematolymphoid neoplasms.

Dna microarray: For many of the hematolymphoid neoplasms, conventional cytogenetics and FISH are still the standard tests to evaluate genotypic abnormalities. However, use of genome-wide DNA single-nucleotide polymorphism microarrays has been critical in identifying abnormalities that are not detectable by conventional G-banding and FISH, such as copy number aberrations and copy-neutral loss of heterozygosity or uniparental disomy.⁵⁵⁻⁵⁸ In contrast to conventional cytogenetics, the microarray affords examination of the entire genome at the kilobase or gene level. However, single-nucleotide polymorphism microarrays cannot detect balanced chromosomal rearrangements, nor can they detect alterations that are present in less than 20% of the cells from which the DNA is extracted. Microarray analysis has been particularly useful in childhood B-ALL, where the genetic information it yields can be used to stratify patients into prognostic groups. High-risk patients can benefit from more rigorous therapies, including stem cell transplants, whereas lower-risk patients can receive less aggressive treatment regimens. A good example of a recurrent finding from microarray testing that has changed patient risk assessment is a deletion in the IKZF1 locus in B-ALL^{48,53,59} (Figure 5, A).

IKZF1 is located on chromosome 7q12 and encodes the zinc finger transcription factor IKAROS. Deletion of IKZF1 confers a very poor outcome, independent of other prognostic markers, such that patients are generally treated with more aggressive therapy than those with a normal karyotype and no common B-ALL-associated abnormalities by FISH analysis. Interestingly, the gene expression signature in these cases is similar to that of the more aggressive Philadelphia chromosome-positive B-ALL. Another advantage of single-nucleotide polymorphism microarray is the ability to detect copy-neutral loss of heterozygosity. This abnormality has been observed in many regions of the chromosomes and affects the function of regulatory genes, such as EZH2 (7q), TP53 (17p), CBL (11q), and TET2 (4q). In MDS or myelodysplastic/myeloproliferative neoplasms, loss of 7q is commonly seen as the sole abnormality and is associated with a poor prognosis. Multiple reports have shown that copy-neutral loss of heterozygosity of 7q has a prognostic implication similar to del7q, which places this patient in a high-risk MDS category (Figure 5, B). Therefore, evaluation of copy-neutral loss of heterozygosity can provide additional useful information in the evaluation of myeloid neoplasms.⁶⁰⁻⁶²

Polymerase chain reaction: Polymerase chain reaction (PCR) is currently the most widely used molecular technique in clinical laboratories to evaluate genotypic abnormalities in hematolymphoid neoplasms.^{63,64} Various PCR-based assays have been designed to detect rearrangements, translocations, inversions, and mutations, with high sensitivity and specificity. They can be performed on a small amount of fresh tissue and on formalin-fixed, paraffin-embedded material as well. Either genomic DNA or RNA can serve as the starting material.

In the latter situation, however, RNA has to be transcribed to complementary DNA with reverse transcriptase before PCR amplification. Because of the high sensitivity, care should be taken at every step of the PCR assay to avoid contamination, from which false-positive results can be obtained. During normal lymphopoiesis, T and B cells undergo rearrangements of the T-cell receptor (TCR) and immuno-globulin (IG) genes, respectively, to generate diverse lymphoid populations with broad antigen detection capacities. Detection of a dominantly rearranged IG or TCR gene by PCR has been used as a surrogate marker for mono-clonality to aid in the diagnosis of non-Hodgkin lymphomas. Multiplex PCR with the BIOMED-2 primers has greatly increased the yield of finding clonal IG or TCR gene rearrangements in non-Hodgkin lymphomas,^{65,66} but a negative result does not exclude the diagnosis of malignant lymphoma. It is also true that detection of a clonal IG or TCR gene rearrangement does not necessarily equate to a diagnosis of malignant lymphoma. IG or TCR gene rearrangements have been observed in a variety of reactive conditions, such as aplastic anemia, autoimmune diseases, nonhematolymphoid malignancies, and viral infections, particularly Epstein-Barr virus. Moreover, detection of clonal IG or TCR gene rearrangements in a sample does not always correlate with detection of B or T lineage differentiation, respectively, as determined by phenotypic assessments of neoplastic cells in the same sample. For example, rearrangement of IGH genes has been seen in some histiocytic/ dendritic cell neoplasms due to presumed lineage plasticity, and rearrangement of TCR genes has been reported in B-ALL and some poorly differentiated AML. Thus, T- and B-cell clonality testing by PCR should only be performed if indicated, and the results should always be interpreted in the context of morphologic and immunophenotypic findings.

The PCR assay has also been used to detect chromosomal translocations and inversions. The method is less effective than conventional cytogenetics and FISH at initial diagnosis because of its lower yield.⁶⁷ In some cases, however, PCR may be the only method able to identify cryptic translocations that cannot be detected by either FISH or chromosome analysis. For example, rare patients with typical clinical, morphologic, and immunophenotypic features of acute promyelocytic leukemia lack the classic t(15;17)(q22;q21) detectable by FISH or conventional cytogenetics. These patients respond well to molecularly targeted therapy with ATRA and arsenic trioxide, and have a favorable prognosis similar to those with the classic translocation. In these rare cases, the PML-RARA fusion gene is formed as a result of inversion events or more complex rearrangements that can only be identified by PCR.⁶⁸

The ability of PCR to detect extremely low quantities of fusion genes or their transcripts has been extensively exploited to assess minimal residual disease and to monitor for treatment response or disease relapse, and some of the assays have become routine tests in most molecular diagnostic laboratories. For example, a quantitative real-time reverse transcription-PCR assay for BCR-ABL1 fusion gene transcript is now standard practice to monitor the response of CML patients after the initiation of treatment with tyrosine kinase inhibitors. Three important levels of the BCR-ABL1 fusion transcript have been defined for the monitoring of molecular response to treatment in CML, using a method that is sensitive to at least a 4.5-log reduction from baseline.^{69,70} An early molecular response is defined as a BCR-ABL1 fusion transcript level less than 10% International Standard (IS) at 3 and 6 months after

initiation of treatment, and it correlates well with conventional cytogenetics and FISH responses. A major molecular response is defined as a BCR-ABL1 fusion transcript level less than 0.1% IS, whereas a complete molecular response is defined as an undetectable BCR-ABL1 fusion transcript. Patients who achieve a major molecular response are more likely to reach a complete molecular response and are less likely to develop resistance to inhibitors or progress to acute leukemias. Reappearance of the fusion transcript after achieving molecular remission or increase in its level during therapy is often the earliest indicator of disease relapse or acceleration.⁷¹ Polymerase chain reaction also offers a more efficient and cost-effective way than Sanger sequencing to detect specific point mutation or small insertions/deletions in genes that are of diagnostic and prognostic significance in hemato-lymphoid neoplasms. In the 2008 WHO classification, mutations in the JAK2 gene (most commonly p.V617F, less frequently exon 12 mutations) are a major diagnostic criterion for polycythemia vera, and the KIT p.D816V mutation is listed as one of the minor diagnostic criteria for systemic mastocytosis.¹ KIT mutation status also provides prognostic data for treatment decisions.^{72,73} Demonstration of MPL (p.W515L or p.W515K) or CALR (insertions or deletions with a β 1-bp frame-shift, most commonly a 5-bp insertion or a 52-bp deletion) mutations helps support the diagnosis of JAK2 mutation-negative myeloproliferative neoplasms.⁷⁴⁻⁷⁶ CSF3R mutations are found in most chronic neutrophilic leukemia cases and have been associated with sensitivity to tyrosine kinase inhibitors and JAK inhibitors.^{77,78}

Frequent mutations were identified in the SETBP1 gene in BCR-ABL1-negative atypical CML and some secondary myeloid neoplasms.⁷⁹ The BCR-ABL1 p.T315I mutation is associated with resistance of CML to small-molecule tyrosine kinase inhibitors. The clinical significance of mutations in FLT3, NPM1, and CEBPA is well established and included in the 2008 WHO classification of AML. Furthermore, independent groups have reported that mutations in KMT2A/MLL (specifically partial tandem duplication), RUNX1, ASXL1, TET2, and PHF6 are associated with less favorable clinical outcome, and TP53 mutations predict the worst clinical outcomes in AML.⁸⁰ Recurrent mutations have also been identified and well characterized in some non-Hodgkin lymphomas, such as BRAF p.V600E in hairy cell leukemia,⁸¹ myeloid differentiation factor 88 (MYD88) p.L265P in lymphoplasmacytic lymphoma/ Waldenstrom macroglobulinemia,⁸² and RHOA p.G17V in angioimmunoblastic T-cell lymphoma.⁸³⁻⁸⁵ These mutations are relatively specific, and their detection may thus aid in the diagnosis and differential diagnosis of non-Hodgkin lymphomas. A variety of PCR assays have been developed in many molecular diagnostic laboratories to detect point mutations and small insertions/deletions. The regions containing hot spots of mutation can be amplified by PCR, and the amplicons can then be examined for sequence variation from the wild type by various methods, such as direct sequencing, high-resolution melting assay, and capillary fragment-length analysis. Figure 6 depicts the 2 most common frameshift mutations in exon 9 of CALR gene. Primers flanking the mutations were used for PCR, and the amplification products were separated by capillary electrophoresis followed by fragment-length analysis. The wild-type amplicons can be easily distinguished from the PCR products amplified from the mutant alleles with an insertion of 5 bp or a deletion of 52 bp based on the size

differences. This method can also detect the less common in-frame deletion mutations in exon 9 of the CALR gene. As a note of caution, these mutations should not be interpreted in isolation as pathologic, because they are benign germ line variants present in approximately 1% of healthy people.

Next-generation sequencing: The emergence of NGS technologies has facilitated the interrogation of genotypic abnormalities at a sensitivity and scope never achieved before. Whole-genome or whole-exome sequencing analysis has not only confirmed the previously characterized mutations in AML, but it has also identified many new genotypic abnormalities. Additionally, based on the mutational profiles, AML can be stratified into 5 risk groups with different predicted overall survival.⁸⁶ Mutation profiles are different between de novo AML and secondary AML, with different prognostic effects. Approximately 90% of MDS patients harbor at least 1 myeloid-related mutation. These patients can be stratified into different risk groups based on their genetic abnormalities. In fact, there may be a link between mutations of genes involved in epigenetic regulation and a patient's response to epigenetic therapies.^{89,90} These same myeloid-related mutations, such as TET2, DNMT3A, ASXL1, and JAK2, have interestingly been seen in elderly individuals without overt hematologic abnormalities and in whom the number of mutations increases with age. In aplastic anemia, the presence of these mutations confers a worse prognosis than those with only PIG-A mutation.⁹⁵ Studies have also started to reveal more complicated mutations and their clinical significance in some unique categories of lymphoid neoplasms. For example, deregulation of targetable tyrosine kinases was found in BCR-ABL1-like B-ALL⁹⁶; deletions, amplifications, and/or mutations in NF1, NRAS, KRAS, MAPK1, FLT3, and PTPN11 were detected in hypodiploid B-ALL⁹⁷; NOTCH1/FBXW7 mutations were associated with T-ALL⁹⁸; genetic lesions in DNMT3A, FLT3, or NOTCH1 may play a role in adult early T-cell precursor ALL⁹⁹; and recurrent mutations in chronic lymphocytic leukemia/small lymphocytic lymphoma, MCL, marginal zone lymphoma, DLBCL, and multiple myeloma may have diagnostic and/or prognostic utility in selected patients.^{80,100}

With marked reductions in the cost of sequencing, NGS technology is starting to replace traditional molecular tests for hematolymphoid neoplasms in the clinical laboratories. Unfortunately, the clinical significance of many newly identified abnormalities has not been well established, and our search for novel targeted therapies still faces many challenges. Although there is a desire from oncologists and patients to identify as many abnormalities as possible, there are practical considerations in the clinical laboratories when deciding the scope of mutation profiling. Currently, most clinical laboratories take a targeted gene panel approach instead of performing whole-exome or whole-genome sequencing to identify clonal markers to aid in diagnosis, evaluation of prognosis, choice of molecular targets, and treatment monitoring.¹⁰¹⁻¹⁰⁶ For example, at Emory we are validating and will soon implement a targeted gene panel for myeloid neoplasms. This laboratory-developed NGS test protocol is based on a well-validated research use-only kit that targets 54 genes, including all exons of 15 genes (BCOR, BCORL1, CDKN2A, CEBPA, CUX1, DNMT3A, ETV6, EZH2, IKZF1, KDM6A, PHF6, RAD21, RUNX1, STAG2, and ZRSR2) and hot spot exons/regions of 39 genes (ABL1, ASXL1, ATRX, BRAF, CALR, CBL, CBLB, CBLC, CSF3R, FBXW7, FLT3,

GATA1, GATA2, GNAS, HRAS, IDH1, IDH2, JAK2, JAK3, KIT, KMT2A/MLL, KRAS, MPL, MYD88, NOTCH1, NPM1, NRAS, PDGFRA, PTEN, PTPN11, SETBP1, SF3B1, SMC1A, SMC3, SRSF2, TET2, TP53, U2AF1, and WT1). These genes/exons cover essentially all of the recurrent mutations associated with AML, MDS, and myeloproliferative neoplasms reported in the extant literature. Several well-defined mutations associated with lymphoid neoplasms, such as CDKN2A, NOTCH1, and IKZF1, are also included in this panel, and detection of these mutations may be used to guide clinical trials for refractory lymphoid malignancies. At present, there are no established standards or guidelines for incorporating newly discovered genetic abnormalities into the routine clinical diagnosis or management of hematolymphoid neoplasms. Although we anticipate the recognition of more clinically relevant novel genetic abnormalities, it will be crucial to reach consensus on how to incorporate such complex and continually evolving information into patient management. The targeted sequencing approach displays limitations as well. For example, copy number information can be challenging to obtain from targeted sequencing, and special bioinformatics tools are required to identify long insertions or deletions. Nevertheless, we believe that use of NGS methods will continue to increase in the clinical laboratories, and continual improvement of NGS technology and bioinformatics pipelines will produce comprehensive molecular diagnostic tools that cover most, if not all, of the clinically relevant genetic abnormalities in a cost-effective manner.

Conclusion

In summary, an integrated approach using multimodality technologies is the current state-of-the-art method for hematopathology practice. Hematopathologists should be familiar with the strengths, weaknesses, and limitations of each modality to be efficient and cost-effective in the assessment of hematolymphoid neoplasms.

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