Review

Molecular Diagnostic Approach to Non-Hodgkin's Lymphoma

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The evaluation of hematopoietic neoplasms now requires a variety of methods to use the modern classification systems. Morphological features remain the cornerstone of the evaluation of leukemias and malignant lymphomas, but ancillary studies are needed in many, if not most, cases. Immunophenotyping is helpful in both the diagnosis and classification of these tumors and is essential for the proper use of recently described classifications of malignant lymphomas.^{1,2} The vast majority of leukemias and lymphomas can be diagnosed without the use of molecular genetic or cytogenetic studies. However, some cytogenetic abnormalities define a disease. For example, detection of the Philadelphia chromosome is an essential part of the diagnosis of chronic myelogenous leukemia. In the acute leukemias, cytogenetic and molecular genetic findings have marked prognostic significance, but they are not usually necessary to determine whether a proliferation is neoplastic or reactive. Most of the significant acute leukemia abnormalities are detectable by routine karyotype analysis. In contrast, the molecular genetic abnormalities of malignant lymphoma are often not easily detectable by routine karyotype analysis, and molecular diagnostic tests are necessary for evaluation. In addition, the detection of specific chromosomal translocations has helped to define clinically relevant lymphoma entities.^{1,2} This is particularly true in the low-grade lymphomas. The molecular genetic associations have resulted in improved recognition of the morphological and immunophenotypic features of these lymphomas. Despite these improved criteria for diagnosis, however, some cases still require molecular testing for proper classification. In lymphoid proliferations, molecular diagnostic tests have two primary uses: to demonstrate a clonal abnormality when the differential diagnosis is between a reactive or neoplastic proliferation, and to identify a disease-associated finding, such as an associated virus or specific chromosomal translocation, that is useful in subclassification of the lymphoma.

Materials and Methods

A variety of methods can be used for molecular diagnostic testing, and no one methodology is ideal for all tests. A detailed review of the different methods used for testing is beyond the scope of this review, but a brief summary of some of the methods will be given. In some instances, karyotype analysis is of limited use, because obtaining adequate growth of low-grade lymphoma cells may be difficult and a normal karyotype, from non-neoplastic cells, may result. In addition, immunoglobulin heavy and light chain and T cell receptor chain gene rearrangements of malignant lymphomas are not detectable by karyotype analysis.

Southern blot analysis has been the traditional gold standard for most molecular diagnostic testing. This procedure requires fresh tissue in fairly large amounts and is a labor-intensive, time-consuming method. A large percentage of the cells in the sample (5–10%) must harbor the suspected abnormality for this method to detect it. Despite these limitations, Southern blot analysis remains a useful methodology for some testing.

Procedures using the polymerase chain reaction (PCR) have replaced many of the traditional Southern blot tests. This methodology requires only a small amount of DNA or RNA, is relatively rapid, and can detect abnormalities at a very low level. Direct PCR amplifies genomic DNA, and this method can be used for many of the common lymphoma translocations. When a translocation site is variable, requiring a larger area of DNA to be amplified, reverse transcriptase (RT) PCR can be used. RT-PCR amplifies complementary DNA (cDNA), usually made from an RNA fusion product that does not contain all of the regions of the original genomic DNA. Direct PCR tests can usually be performed on paraffin-embedded tissues, as well as fresh and frozen tissues. Due to RNA degradation, most RT-PCR tests do not work on paraffin-embedded tissue unless the RT-PCR product is very small.

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Table 1. Most Common Molecular Abnormalities Studied in Non-Hodgkin's Lymphoma

Gene studied	Chromosomal site	Most common disease associations
Immunoglobulin heavy chain (<i>IgH</i>) rearrangements	14q32	B cell neoplasms*
Immunoglobulin kappa light chain (<i>Igκ</i>) rearrangements	2p11	B cell neoplasms
<i>J_H/BCL-1</i>	t(11;14)(q13;q32)	Mantle cell lymphoma
<i>J_H/BCL-2</i>	t(14;18)(q32;q21)	Follicular lymphoma, some diffuse large B cell lymphomas
<i>PAX5/IgH</i>	t(9;14)(p13;q32)	Lymphoplasmacytic lymphoma
<i>API2/MLT</i>	t(11;18)(q21;q21)	Extranodal marginal zone lymphoma
<i>BCL-6</i> translocations	t(3;n)(q27;n)	Some diffuse large B cell lymphomas
<i>C-MYC</i> translocations	t(8;n)(q24;n)	Burkitt's lymphoma
T cell receptor β chain (<i>TCRβ</i>) rearrangements	7q34	T cell neoplasms*
T cell receptor γ chain (<i>TCRγ</i>) rearrangements	7q15	T cell neoplasms*
<i>NPM/ALK</i>	t(2;5)(p23;q35)	Anaplastic large cell lymphoma

*Lineage infidelity may occur in some neoplasms, particularly lymphoblastic leukemias and lymphomas, which may result in detection of aberrant gene rearrangements (see text).

In situ hybridization studies allow for probing of tissue on a glass slide or cell suspension so that the intact positive cells can be directly visualized. This methodology is particularly useful in determining a viral association with a specific cell type. Fluorescence *in situ* hybridization (FISH) also allows for direct visualization of a specific chromosomal abnormality. FISH studies are less sensitive than PCR-based methods, but can detect abnormalities, such as monosomies and trisomies, that cannot be studied by PCR analysis.

In situ PCR is a method in which the polymerase chain reaction actually takes place in the cell on a slide, and the product can be visualized in the same way as in traditional *in situ* hybridization. The methodology is technically difficult, is often inconsistent, and is not used in most diagnostic laboratories.

Microarray technology allows for a large number of genetic abnormalities to be screened on a single chip that is then scanned and analyzed by a computer. Although recent studies have shown the power of this methodology in recognizing prognostically significant trends in large cell lymphoma, it currently remains a research tool.^{3,4}

The best method for testing depends on the question that is being asked and the abnormality that is being tested for. The advantages and limitations of the commonly used techniques will be discussed below in the context of the abnormality being evaluated. The most common abnormalities are listed in Table 1.

B Cell Neoplasms

Gene Rearrangements

Rearrangement of the immunoglobulin heavy chain region on chromosome region 14q32 occurs in all normal developing B lymphocytes.⁵⁻⁷ This chromosomal region contains over 100 variable (V), 30 diversity (D), and 6 joining (J) regions. When the B cell undergoes immunoglobulin heavy chain gene rearrangement (Figure 1), one V, one D, and one J region move into close proximity to each other. Because each normal B cell undergoes a unique rearrangement, there are differences among each

cell resulting in a polyclonal B cell population. Following rearrangement of the immunoglobulin heavy chain gene, the immunoglobulin kappa light chain region of chromosome 2p11 rearranges in a similar fashion with the exception that it does not contain diversity (D) regions. If this rearrangement is not productive in either allele (approximately one third of cases), the kappa light chain constant region locus is deleted and the immunoglobulin lambda light chain region on chromosome 22q11 undergoes rearrangement. Because mature B cell lymphomas are clonal neoplasms, immunoglobulin heavy chain and kappa light chain rearrangements are detectable in essentially all cases. Many precursor B cell malignancies (lymphoblastic lymphomas and leukemias), however, will demonstrate only immunoglobulin heavy chain rearrangements because the neoplastic transformation occurs before rearrangement of the immunoglobulin kappa light chain region. Because lambda light chain rearrangements do not always occur and occur later in B cell development when present, this region is not a good initial target for clonality testing.

Immunoglobulin gene rearrangements are usually detected by Southern blot analysis or by use of the polymerase chain reaction. The Southern blot procedure requires a large amount (at least 10 μ g) of high quality DNA

Figure 1. Immunoglobulin heavy chain gene rearrangement. Most PCR tests for this rearrangement use consensus primers directed against the framework three (FRIII) region and the heavy chain joining (*J_H* or FRIV) region of the rearranged product.

and requires fresh or frozen tissue. The DNA is cut with restriction enzymes, size electrophoresed, transferred to a membrane, and then probed for a specific portion of the immunoglobulin heavy chain or kappa light chain joining regions. If the B cells in the specimen are polyclonal, the restriction enzymes will cut different sized segments that are too few in number to be detected by the probe. The remaining non-rearranged cells (non-B cells) will not have undergone gene rearrangements for the area probed and will show bands of expected sizes (germline) on the probed membrane or radiograph. If a large number of polyclonal B cells is present in the sample, a weak smear without distinct rearranged bands may occur. Specimens with a monoclonal B cell population will have a prominent cell population that cuts to a specific size with the restriction enzymes, usually different from the non-rearranged germline cells, and will demonstrate additional bands on the membrane or radiograph. Criteria are published for the interpretation of Southern blots; generally, they require exclusion of bands due to partial digestion of DNA and require that rearrangements be seen with two of the three enzymes, or that two rearrangements be observed with a single enzyme for an interpretation of a clonal gene rearrangement.^{8,9} Very detailed and useful guidelines for specimen collection, transport, performance, and interpretation of immunoglobulin and T cell receptor gene rearrangement assays are published by National Committee for Critical Laboratory Standards (document MM2-A).⁹

The use of PCR for the detection of immunoglobulin heavy chain gene rearrangements allows for the use of smaller amounts of DNA and even DNA from paraffin-embedded tissue. This method uses consensus primer pairs that anneal to the V and J regions of the rearranged chromosome 14.¹⁰ Certain nucleotide sequences are similar among the different V and J regions, and the consensus primers are made to anneal to these sequences even if they are not a perfect match. Because different, polyclonal rearrangements result in slightly different-sized PCR products, a smear or ladder is seen on the gel in polyclonal specimens, and one or two discrete bands on a gel (or peaks on a capillary electrophoresis instrument printout) are seen with a monoclonal proliferation (Figure 2). The primers with the highest detection rate for the immunoglobulin heavy chain gene rearrangements are directed against a region termed the framework (FR) III region of the various V_H genes. FRIII-directed primers detect approximately 60% of clonal B cell malignancies.¹¹ The addition of other framework regions, particularly FRII primers, will increase the detection rate of this test. Framework I is composed of multiple families of regions, which require multiple PCR reactions to detect reliably. A combination of FRII and FRIII primers will detect 70 to 90% of B cell neoplasms depending on the type of disease. In one study using only FRIII primers, 35% of follicular lymphomas were positive, compared to 82% of non-follicular B cell lymphomas (including 72% of diffuse large B cell lymphomas, 86% of small lymphocytic lymphomas and 100% of mantle cell and Burkitt's/Burkitt-like lymphomas).¹¹ Somatic mutations of the immunoglobulin heavy chain gene of some mature B disorders,

Figure 2. Different methods for analyzing the immunoglobulin heavy chain PCR product are illustrated. **A:** A polyacrylamide gel illustrates both polyclonal and monoclonal results using FRIII/VLJH primers. Specimens 1–3 are run in duplicate and show a polyclonal pattern resulting in a smear pattern. Specimen 4 shows two reproducible, discrete bands. This biclonal pattern is considered evidence of a clonal population. Negative samples with, including a water control, a sample with no B lymphocytes (both with no amplifiable products), and a polyclonal B cell specimen (resulting in a smear pattern) are illustrated as lanes marked H₂O, –, and 10^{–2}. A monoclonal B cell line control and a 1:100 dilution of that control are labeled + and 10^{–2}. Both show a distinct band (arrow) of approximately 130 kb. MW lanes indicate molecular weight controls. **B:** The figure illustrates detection with a capillary electrophoresis instrument. Both demonstrate results of a monoclonal B cell population showing a large distinct peak, mixed with a polyclonal B cell population (multiple smaller peaks). In the upper portion, FRIII/VLJH primers amplify a 243-kb clonal product; at bottom, FRIII/VLJH primers amplify an 82-kb clonal product.

especially follicular lymphomas and plasma cell malignancies, alter the sequence of the region amplified by the primers so that primer hybridization is suboptimal or does not occur, resulting in false negative PCR results.¹⁰ Therefore, a negative PCR result does not exclude the presence of a monoclonal B cell proliferation. In addition, consensus primers are not a perfect match to the sequence being amplified and result in less efficient amplification. Therefore, they are less sensitive in the detection of minimal residual disease than PCR primers specific to a region of a translocation or primers made specifically against a patient's gene rearrangement. This limits the use of the immunoglobulin heavy chain PCR test in the evaluation of minimal residual disease. Most tests that employ consensus primers can detect only one clonal cell in 100 polyclonal cells.

PCR tests directed against rearrangement of the kappa light chain gene or the kappa-deleting segment are also useful in the detection of B cell clonality in mature B cell proliferations and are reported to detect clonality in up to 50% of B cell lymphomas.^{12,13} Although this method does not detect as many B cell neoplasms as the immunoglobulin heavy chain PCR test, Ig κ PCR is useful as a second line test. It is particularly helpful in detecting a clonal population in plasma cell disorders that give false negative results for the IgH PCR test due to somatic hypermutation of the immunoglobulin heavy chain gene. Ig κ PCR testing also uses consensus primers that limit

Figure 3. *BCL-2/J_H* rearrangements usually involve the major breakpoint region (MBR) of the *BCL-2* gene, but may also involve the minor cluster region (MCR) of the gene. *BCL-1/J_H* rearrangements of t(11;14)(q13;q32) (not shown) rearrange in a similar fashion with the *BCL-1* gene of chromosome 11q13 fused 5' to the *J_H* region of the immunoglobulin heavy chain.

the ability to detect minimal residual disease at a level below one clonal cell in 100 polyclonal cells.

T cell receptor gene rearrangements (see below) may also be detectable in B cell malignancies.¹⁴ This occurs most commonly in the precursor B cell lymphoblastic malignancies, and in these cases the gene rearrangement studies are not helpful in assigning lineage. Immunophenotyping studies, however, are usually adequate to resolve the lineage of most of these neoplasms. In mature B cell tumors, the addition of immunoglobulin kappa light chain Southern blot analysis or PCR analysis can aid in confirming the B-lineage of the tumor, as this locus is uncommonly rearranged in T cell malignancies.

Specific cytogenetic translocations are also associated with some types of malignant lymphoma. Unlike the translocations of acute leukemia, many of the more common lymphoma translocations do not involve large introns and can be reliably amplified at the DNA level. Therefore, PCR tests for these can be performed on paraffin-embedded tissues. Molecular changes, other than gene rearrangements, seen with specific disease types will be discussed below.

Translocations

J_H/BCL-2

Due to somatic hypermutation of the immunoglobulin heavy chain gene in follicular center cells, only 35 to 50% of follicular lymphomas will have a detectable immunoglobulin heavy chain rearrangement by PCR analysis.^{11,15,16} Because these mutations do not affect the overall gene rearrangement, virtually all follicular lymphomas will show a rearrangement by Southern blot analysis. Despite the relatively high false negative rate for immunoglobulin heavy chain gene rearrangement by PCR analysis, most (70–80%) follicular lymphomas will demonstrate t(14;18)(q32;q21) involving the immunoglobulin heavy chain gene on chromosome 14 and the *BCL-2* gene on chromosome 18 (Figure 3),¹⁷ and 70 to 90% of these translocations are detectable by PCR analysis.^{18,19} Over expression of *bcl-2* protein, which results from this translocation, is associated with a loss of apoptosis. This translocation is detectable by either Southern blot or by

PCR (*J_H/BCL-2*) analysis.¹⁸ Most translocations involve the major breakpoint region (MBR) of *BCL-2*, but 5 to 10% involve a minor cluster region (MCR) that requires the use of different PCR primers and Southern blot probes to detect.^{19–21} Although most *J_H/BCL-2* translocations can be detected from paraffin-embedded tissues, some breakpoints result in PCR products that are very large and may not be detectable after fixation.²² A recent study has suggested an improved prognosis in patients with follicular lymphoma with the MCR translocation,²³ but this test is not used as a prognostic marker in most laboratories at this time.

A variable cluster region (VCR) of the *BCL-2* gene is also present approximately 225 kb 5' to the MBR region. The VCR is occasionally involved in translocations involving the kappa light chain or lambda light chain genes on chromosomes 2 and 22, respectively, in cases of small lymphocytic lymphoma/chronic lymphocytic leukemia.²⁴

The t(14;18) has also been reported to be detected by *J_H/BCL-2* PCR analysis in normal peripheral blood and in reactive lymph nodes.^{25–27} These reports suggest that this translocation can occur in small numbers of cells without the development of malignant lymphoma. Non-nested PCR tests for *J_H/BCL-2* that do not amplify over 45 cycles do not usually get these "false positive" results.²⁸

The t(14;18)(q32;q21), identical to the translocations of follicular lymphomas, is identified in 17 to 38% of diffuse large B cell lymphoma, and the detection methods are identical to those described above.^{11,29–31} Some studies have suggested that the presence of t(14;18) in large cell lymphoma is an indicator of a poor prognosis.^{30,31} In both follicular lymphomas and diffuse large B cell lymphomas, detection of this translocation does not correlate completely with *BCL-2* protein expression.

Detection of t(14;18) by molecular methods is not necessary for the diagnosis of most cases of follicular lymphoma. However, such testing may be valuable in the detection of minimal residual disease, such as in bone marrow material aspirated after chemotherapy or bone marrow transplantation for follicular lymphoma (see below).

J_H/BCL-1

The t(11;14)(q13;q32), which involves the immunoglobulin heavy chain gene of chromosome 14 and the *BCL-1/PRAD1* gene of chromosome 11, is detected in approximately 60% of mantle cell lymphoma cases.^{32,33} The *BCL-1* gene encodes a cell cycle protein (termed cyclin D1, PRAD1, or BCL-1) and over expression is associated with the aggressive behavior of this tumor, and has been useful in further defining this disease. The major translocation cluster (MTC) region is involved in 40 to 50% of cases, but the remaining translocations involve a multitude of different sites that are not easily detectable by PCR analysis.³⁴ Methods for detection of *BCL-1* mRNA are described that detected over 95% of cases of mantle cell lymphoma, and the mRNA expression presumably occurs with translocations that involve the MTC as well as other breakpoints.^{35,36} This method requires a quantitative reverse transcriptase PCR procedure that is

Figure 4. **A:** Nuclear detection of BCL-1 (a.k.a. cyclin D1) protein overexpression by immunohistochemistry in mantle cell lymphoma is an excellent surrogate marker for the t(11;14) and reduces the need for the PCR detection method. **B:** ALK-1 immunohistochemistry is specific for abnormalities of the *ALK* gene in lymphoid neoplasms. **C:** *In situ* hybridization for EBER-1 RNA of the EBV demonstrates numerous EBV positive tumor cells in a case of nasal natural killer/T cell lymphoma. **D:** Some EBV-infected tumor cells, including the neoplastic cells of EBV-positive Hodgkin's disease, express the EBV latent membrane protein. Detection of this protein by immunohistochemistry is comparable to the *in situ* hybridization method in those cases.

not readily available in most laboratories, but may be a useful test in the future. Mantle cell lymphomas also demonstrate nuclear overexpression of BCL-1/cyclin D1 protein, related to the translocation involving *BCL-1/PRAD1*. Although detection of BCL-1 protein by immunohistochemistry is technically difficult, it is a more sensitive test than direct PCR for mantle cell lymphoma (Figure 4A).³⁷ However, weak expression of BCL-1 protein has been described in other lymphoid tumors, including hairy cell leukemia,³⁸ and a subgroup of cases of splenic lymphoma with circulating villous lymphocytes (SLVL) and multiple myeloma are t(11;14) positive by PCR or cytogenetics.^{39,40} FISH detection of t(11;14) is offered by some laboratories and is a more sensitive method for the detection of this abnormality than the direct PCR test that is offered in most laboratories.⁴¹ In one study,⁴¹ all 51 cases of mantle cell lymphoma tested by FISH were *J_H/BCL-1*-positive, and this methodology may be more commonly offered in the future.

PAX-5/IgH

The t(9;14)(p13;q32) is detected in approximately half of lymphoplasmacytic lymphomas.⁴² This translocation involves the *PAX-5* gene on chromosome 9 and the immunoglobulin heavy chain gene on chromosome 14. The

site of the translocation on chromosome 14 differs from the region involved in the *J_H/BCL-1* and *J_H/BCL-2* translocations, occurring 3' to the constant region of the immunoglobulin heavy chain locus in the switch μ region. *PAX-5* normally encodes a B-cell-specific transcription factor, known as B-cell-specific activator protein, that is involved in the control of B cell proliferation and differen-

Figure 5. FISH analysis for the t(8;14) of Burkitt's lymphoma may confirm this translocation (arrows) on metaphase spreads (left) or within intact nuclei (right), including nuclei from paraffin-embedded tissue (kindly provided by M. L. Slovak, Ph.D., City of Hope National Medical Center).

tiation.⁴³ Involvement of this gene may result in the plasmacytoid differentiation of these tumors. *PAX-5/IgH* translocations have also been reported in rare cases of marginal zone lymphoma and diffuse large B cell lymphoma.^{42,44} Southern blot analysis, RT-PCR, or FISH may be used to detect *PAX-5* rearrangements; however, this lymphoma type is less common than some of the other types with recurring translocations, and none of these methods are offered in most diagnostic laboratories at this time. Such testing may become more common if detection of the translocation is found to have prognostic significance.

API2/MLT

The t(11;18)(q21;q21) is detected in approximately one-third of marginal zone lymphomas by classic karyotype analysis.^{45,46} Recently, this translocation has been shown to involve the apoptosis inhibitor gene (*API2*) on chromosome 11 and the *MLT* gene (also known as *MALT1*) on chromosome 18.⁴⁷ *API2/MLT* translocations appear to be specific for only the non-splenic, extranodal marginal zone lymphomas, occurring in approximately 40% of gastric and lung marginal zone lymphomas, but are not detected in splenic marginal zone lymphomas and the primary nodal marginal zone lymphomas that were previously termed monocytoid B cell lymphomas.⁴⁸⁻⁵¹ In addition, the extranodal marginal zone lymphomas with increased large cells or evidence of large cell transformation do not demonstrate this translocation, even in the accompanying low-grade component. These findings suggest that the categories of marginal zone lymphoma in the REAL and proposed WHO classifications of malignant lymphomas represent biologically heterogeneous diseases.

Multiple breakpoint sites are described for *API2/MLT*, and RT-PCR or FISH analyses are usually needed to detect this the abnormality. Because most of these tumors are now diagnosed based on small tissue biopsies that usually do not have saved frozen tissue, FISH analysis on paraffin-embedded tissue may be the optimum means of detecting this translocation.

BCL-6 Translocations

Up to one-third of diffuse large B cell lymphomas, including some with t(14;18), have abnormalities involving the *BCL-6/LAZ3* gene on chromosome region 3q27.⁵²⁻⁵⁶ Translocations involving *BCL-6* involve the immunoglobulin heavy chain region of 14q32, the kappa light chain region of 2p11, or the lambda light chain region of 22q11. Translocations involving chromosomes 1, 9, 11, and 12 have also been reported with *BCL-6* in diffuse large B cell lymphoma. Rearrangements of *BCL-6* have also been reported to occur infrequently in other types of B cell lymphoma, particularly follicular lymphomas and marginal zone lymphomas. The clinical significance of the detection of *BCL-6* rearrangements in large cell lymphoma is controversial,^{30,57} but larger studies have not found a significant survival difference related to this ab-

normality. PCR-based detection methods are limited by the large number of translocations that occur with this gene, the high frequency of somatic mutations of the gene and because the translocations usually take place within an intron adjacent to the coding exons of the gene.^{56,58} Because of this, long range PCR, RT-PCR, or FISH methods are needed. Most methods require fresh or frozen tissue, but FISH analysis may be performed on paraffin-embedded tissue. Southern blot detection of *BCL-6* abnormalities is the most commonly performed test, but testing for *BCL-6* abnormalities is not offered in most diagnostic laboratories because of the current lack of definite prognostic significance of detection.

C-MYC Translocations

Burkitt's lymphoma is usually associated with translocations involving the *C-MYC* gene of chromosome region 8q24, particularly the t(8;14)(q24;q32) that is identified in approximately 80% of cases.^{59,60} The remaining cases demonstrate t(8;22)(q24;q11) or t(2;8)(p11;q24). The site of translocation differs between endemic and sporadic Burkitt's lymphoma.⁶¹⁻⁶⁴ In endemic disease, the t(8;14) occurs up to 300 kb 5' from the coding region of the *C-MYC* gene, whereas sporadic Burkitt's characteristically involves a translocation within the actual *C-MYC* gene. These translocations may also occur in the Burkitt-like lymphomas and in a small number of diffuse large B cell lymphomas. Variations in these translocations, including translocations involving the constant regions rather than joining regions of 14q32, make them poor targets for detection by routine PCR. Southern blot analysis for *C-MYC* is the most commonly used method of detecting this abnormality. FISH studies may also be performed and can be used on paraffin-embedded tissues (Figure 5).

Other Abnormalities

A variety of other cytogenetic abnormalities may be identified in malignant lymphomas using molecular techniques. Deletions of chromosome band 13q14 and 11q are probably the most common cytogenetic abnormalities in small lymphocytic lymphoma/chronic lymphocytic leukemia.⁶⁵⁻⁶⁷ These deletions are not routinely tested using diagnostic molecular methods. Trisomy 12, originally thought to be common in chronic lymphocytic leukemia, is more commonly associated with cases with atypical features or cases undergoing transformation to a higher-grade process. FISH studies are a reliable means of detecting this abnormality.

In addition to the relatively common *API2/MLT* translocation and the less common *PAX-5/IgH* translocation in marginal zone lymphoma, trisomy 3 and t(1;14)(q21-22;q32) have been reported. Several genes implicated in lymphomagenesis are present in the involved regions of chromosome 1, but *BCL-10* and *MUC1* appear to be the ones most commonly involved in marginal zone lymphomas.⁶⁸⁻⁷¹ The *BCL-9* gene at chromosome region 1q21 is also involved in a variety of malignant lymphoma types,

Figure 6. The T cell receptor γ chain locus on chromosome region 7p15 contains a limited number of variable and joining region genes that make it ideal for PCR amplification of the rearrangements.

other than marginal zone lymphoma.⁷² Although trisomy 3 may be detected by FISH analysis,^{73,74} the t(1;14) abnormalities are not offered as a diagnostic tests in most laboratories.

Some diffuse large B cell lymphomas have abnormalities of the p16 tumor suppressor gene *CDKN2* of chromosome region 9p21,⁷⁵ and 3 to 4% have translocations involving the chromosome region 15q11–13, the site of the *BCL-8* gene.⁷⁶

Precursor B cell lymphoblastic lymphoma has the same biological features of precursor B cell acute lymphoblastic leukemia and will not be covered in detail. Cases will demonstrate an immunoglobulin heavy chain rearrangement and 50% or more will also demonstrate some form of T cell receptor gene rearrangement. A variety of cytogenetic translocations occur with these disorders, including t(9;22)(q34;q11)-*BCR/ABL*, t(12;21)(p13;q22)-*TEL/AML1*, t(1;19)(q23;p13)-*E2A/PBX* and abnormalities of 11q23-*MLL*.⁷⁷ RT-PCR or FISH analysis best detects all of these, and routine karyotyping may miss *TEL/AML1* and *MLL* abnormalities.

T Cell Neoplasms

Gene Rearrangements

The T cell receptor (TCR) genes undergo VDJ or VJ rearrangements similar to the immunoglobulin heavy and kappa light chain genes in the sequential order of TCR δ (chromosome 14q11), TCR γ (7q15), TCR β (7q34), and TCR α (14q11).^{6,78,79} Approximately 95% of circulating T cells are of the α/β type, but a small population of γ/δ T cells do not undergo TCR β and TCR α rearrangements. These γ/δ T cells are preferentially located in the splenic red pulp.⁸⁰ Southern blot analysis of the TCR β chains will detect >90% of T cell malignancies, but will not usually detect gene rearrangements in malignancies of γ/δ T cells or natural killer cells. The DNA may be hybridized with probes directed against the TCR β constant region (C β) or with a cocktail of probes directed against TCR β joining regions 1 and 2 (J β 1 and J β 2).

PCR-based assays for T cell clonality are usually directed against either TCR β or TCR γ . Because of the complexity of the TCR β locus, PCR for these rearrangements require a large number of primers.⁸¹ The TCR γ region is less complex, with only 4 V region families

containing 11 genes and 5 J region genes (Figure 6). Because the TCR γ locus is consistently rearranged before the TCR β locus, PCR analysis with primers directed against the V γ 1–8, V γ 9, V γ 10, and V γ 11, coupled with a multiplex of J region primers will detect over 90% of clonal T cell neoplasms.^{82,83} Because it is a PCR-based test directed against genomic DNA, TCR γ PCR can be performed on paraffin-embedded tissue. In addition, TCR γ rearrangements can be detected in lymphomas of γ/δ T cells that may not demonstrate evidence of clonality on Southern blotting for TCR β . In contrast to the PCR for IgH gene rearrangements, if all of the TCR γ variable and joining regions sequences are covered by the PCR reactions, this test will result in very few false negative reactions when compared to Southern blot analysis.

Translocations

The t(2;5)(p23;q35) is the only recurring translocation that is routinely tested in T cell lymphomas. It is the most common cytogenetic abnormality in noncutaneous forms of anaplastic large cell lymphoma. Anaplastic large cell lymphoma, as it is defined in the REAL and proposed WHO classifications, is a T cell or null cell lymphoma.^{1,2} The t(2;5)(p23;q35) results in a fusion transcript of the nucleolar phosphoprotein (*NPM*) gene of chromosome 5 and the anaplastic lymphoma kinase (*ALK*) gene of chromosome 2.^{84,85} Although these lymphomas were originally termed “Ki-1 lymphomas” because of their expression of CD30, such antigen expression is not specific for this disease or for this cytogenetic translocation. The t(2;5) fusion product can be detected by RT-PCR, by amplifying a fairly small cDNA fragment.⁸⁶ Because the fusion product is small, it may also be detected in paraffin sections in some cases. The abnormality may also be detected by FISH analysis, and this is a more sensitive test than RT-PCR on paraffin sections.⁸⁷ This translocation results in expression of the ALK protein, which is not normally expressed in lymphoid cells. ALK expression can be detected by immunohistochemistry,⁸⁸ and in the right morphological setting, ALK expression correlates well with FISH or other detection of t(2;5) (Figure 4B).⁸⁷ ALK expression has been shown to correlate with improved survival in this disease, compared to ALK-negative anaplastic large cell lymphoma.^{87,89} ALK expression may be nuclear, cytoplasmic, or both, and translocations involving the *ALK* gene, other than t(2;5), that are described in anaplastic large cell lymphoma are also associated with ALK immunoreactivity.^{90,91} The improved survival of ALK-positive lymphomas is independent of the translocation partner.⁹¹ Because all *ALK* translocations, including many *NPM/ALK* translocations, are not detectable by RT-PCR analysis and the protein expression has such clinical relevance, ALK immunohistochemistry is the preferred test for this disease. The RT-PCR test may still have utility in monitoring for minimal residual disease. The t(2;5) and ALK expression are usually not detectable in primary cutaneous anaplastic large cell lymphoma.⁹²

Other Abnormalities

T cell prolymphocytic leukemia is associated with cytogenetic abnormalities of chromosome regions 14q, 8q, and 11q. The most common abnormality is inv(14)(q11q32). Chromosome 8 abnormalities include iso(8q) or trisomy 8.⁹³ Chromosome 11 abnormalities include 11q23 abnormalities that do not appear to involve the *MLL* gene. Several reports have identified the combined cytogenetic abnormality of isochromosome 7q and trisomy 8 in hepatosplenic $\gamma\delta$ T cell lymphoma.⁹⁴ None of these abnormalities are routinely tested for diagnostic purposes, but FISH analysis is the best method for detecting many of the changes.

Over 90% of T lymphoblastic lymphoma/leukemia cases demonstrate evidence of T cell receptor gene rearrangements. Approximately 20% of cases will also have immunoglobulin heavy chain rearrangements. A variety of cytogenetic translocations occur with T-cell acute lymphoblastic leukemia and usually involve one of the TCR genes.⁹⁵ Translocations or interstitial deletions involving the *SCL/TAL-1* gene on chromosome region 1p32 and abnormalities of the *HOX11* gene on 10q24 are common.^{96,97} Deletions of the p16/*CDKN2* gene of 9p21 are also common.⁹⁸ Molecular testing for these types of abnormalities will probably become more common in the future.

Viruses in B and T Cell Neoplasms

Several viruses are commonly associated with lymphoid neoplasms. The Epstein-Barr virus (EBV) is detectable as a latent infection in most healthy adults; however, clonal integration of the virus within tumor cells occurs in a variety of tumors. Molecular detection of Epstein-Barr virus RNA is seen in 90% of endemic cases of Burkitt's lymphoma compared to a frequency of 20 to 30% in sporadic cases. Nasal type natural killer/T cell lymphoma has a high association with clonal EBV in the tumor cells, and *in situ* hybridization detection of the virus in many cells may be diagnostically useful in the usually small biopsy specimens that may be obtained to evaluate for this disease. The angiocentric lesions of lymphomatoid granulomatosis are also EBV-positive, but these tumors are actually B cell neoplasms and will frequently demonstrate evidence of immunoglobulin heavy chain gene rearrangements.⁹⁹ Approximately 40% of cases of Hodgkin's disease will demonstrate evidence of EBV in the neoplastic cells by *in situ* hybridization.¹⁰⁰ The EBV-positive cases are usually of the mixed cellularity type and involve the head and neck region. EBV infection may be associated with other T cell malignancies, including some angioimmunoblastic T cell lymphomas, lymphoepithelial carcinomas, and some other tumor types.¹⁰¹

EBV infection is best detected by Southern blot analysis or *in situ* hybridization.^{102,103} Southern blot analysis is useful to demonstrate a clonal proliferation of EBV, but requires a large amount of tissue and is not routinely performed in most laboratories. *In situ* hybridization for EBER-1 RNA of the Epstein-Barr virus will demonstrate

evidence of EBV in virtually all of the tumor cell nuclei (Figure 4C). Because latent EBV infection is common in most adults, PCR amplification of EBV may not be specific for the tumor cells and this test is usually not reliable for determining an association between the virus and a particular tumor. Many EBV-infected cells will express the latent membrane protein (LMP), which is detectable by immunohistochemistry. There is high correlation between LMP immunohistochemistry and EBV EBER-1 *in situ* hybridization in Hodgkin's disease, and the immunohistochemical test is cost-effective and a reliable alternative to *in situ* hybridization in that setting (Figure 4D). However, not all EBV-positive tumors, particularly most natural killer/T cell lymphomas and EBV-positive Burkitt's lymphoma, are LMP-positive, and the *in situ* hybridization test is the preferred method when those tumors are suspected.

There is a strong association between HTLV-1 infection and adult T cell leukemia/lymphoma (ATLL).¹⁰⁴ Clonal integration of the virus occurs in almost all ATLL patients, but *in situ* hybridization studies for this virus are difficult to perform and are not routinely offered. The virus may be detectable by serological studies or PCR analysis.¹⁰⁵

Some investigators have reported an association between multiple myeloma and bone marrow dendritic cell infection by Kaposi's sarcoma herpesvirus/human herpesvirus-8 (KSHV/HHV-8),¹⁰⁶ but this association is highly controversial. This virus is also detected in primary effusion lymphomas and cases of multicentric Castleman's disease.¹⁰⁷ KSHV/HHV-8 is usually detected by direct PCR. Recently described antibodies directed against the latent nuclear antigen of KSHV, reportedly suitable for use in paraffin sections, may offer an alternative to the PCR test.¹⁰⁸

Hepatitis C is reported to be associated with a variety of types of B cell lymphomas, although most of the reported cases occur in patients with mixed cryoglobulinemia, a disease with a known association with lymphoplasmacytic lymphoma.^{109,110} Because most studies of this virus in lymphoma use serological or PCR methodologies, definite infection of the lymphoma cells with the virus has not been clearly demonstrated for most cases. Future studies with other detection methodologies should help to clarify the role of this virus in malignant lymphoma.¹¹¹

Diagnostic Approach

Though many of the lymphoma-associated translocations are not routinely offered in most molecular diagnostic laboratories, not all tests are needed for most diagnoses. The majority of lymphoma cases are diagnosed reliably by morphology and immunophenotyping studies. Specific translocations may be studied to aid in the classification of some lymphomas or to help confirm clonality of the lesion. Most molecular genetic testing in lymphoma is performed to confirm clonality in cases in which the differential diagnosis is between a reactive *versus* neoplastic proliferation.

Figure 7 provides an algorithm used in the author's laboratory for the approach to most cases. Immunophe-

Figure 7. A diagnostic algorithm for clonality molecular testing in lymphoid proliferations. Additional studies could be performed to detect disease specific cytogenetic translocations.

notyping studies are useful in determining the starting point of testing for most cases. If a B cell neoplasm is suspected, IgH PCR studies are performed. This test is preferably performed with primers directed against more than one framework region of the immunoglobulin heavy chain variable genes. Because of the high rate of false negative results with this test in follicular and plasma cell disorders, testing for *JH/BCL-2* and/or for Igκ gene rearrangements follows a negative result. Understanding that 10 to 15% of clonal B cell proliferations will still be negative for all of these tests, negative samples are then tested by Southern blot analysis for B cell gene rearrangements. In cases with insufficient fresh tissue for Southern blot analysis or those with only paraffin-embedded tissue, a comment should be placed in the report in regards to the false negative rate for the methodology used.

If T cell neoplasia is suspected, PCR analysis for TCRγ is performed. Some laboratories chose to perform Southern blot analysis of the PCR-negative cases, but the number of cases detected with this approach is very low if the PCR test used for TCRγ covers all of the V and J regions of the TCRγ gene. This simple algorithm provides a logical and cost-effective approach to the molecular evaluation of most malignant lymphomas.

More focused testing can address specific questions that arise in the evaluation of lymphomas. When the specific question is between follicular lymphoma and follicular hyperplasia, immunohistochemistry for BCL-2 is an appropriate initial test because the majority of follicular lymphomas will express this protein, in contrast to the lack of expression in reactive follicle center cells.¹¹² In the 15% of follicular lymphoma that are BCL-2 protein-negative, molecular studies may be useful. Because of the relatively high frequency of false negatives for IgH by PCR in follicular lymphoma, going directly to PCR testing for the *JH/BCL-2* translocations may be appropriate, but the use of combination of IgH primers will detect a clonal population in many follicular lymphoma cases. This combined immunohistochemical and molecular diagnostic approach should resolve the vast majority of cases.

Some cases of mantle cell lymphoma will have a nodular pattern that may be confused with follicular lymphoma. In this setting immunohistochemical studies are

again appropriate in the initial evaluation. Detection of CD5 and/or BCL-1 protein expression in the neoplastic B cell population would strongly support a diagnosis of mantle cell lymphoma, whereas CD10 expression by the cells would support a diagnosis of follicular lymphoma. In cases with inconclusive immunophenotyping, molecular studies for *JH/BCL-2* and *JH/BCL-1* would be useful, but the relatively high frequency of *JH/BCL-1*-negative mantle cell lymphomas, using the routine PCR method, must be understood. IgH PCR would be of little value in the differential diagnosis between nodular mantle cell lymphoma and follicular lymphoma, since both are clonal B cell neoplasms.

The differential diagnosis of diffuse B cell lymphomas of small lymphocytes includes mantle cell lymphoma, small lymphocytic lymphoma, and marginal zone lymphoma. Distinguishing mantle cell lymphoma from the others is extremely important because of the aggressive nature of that disease.¹¹³ This differential diagnosis is also of importance on small gastric biopsies that contain diffuse B cell infiltrates, but may be too small for the traditional pattern evaluations used in most lymphoma evaluations. Although many of these cases represent extranodal marginal zone lymphomas, the other lymphomas mentioned may involve this site, and proper classification is necessary for appropriate treatment. The use of immunophenotyping studies, as mentioned above, is often useful in this differential diagnosis, particularly the detection of BCL-1 protein in mantle cell lymphoma. Testing for *JH/BCL-1* of mantle cell lymphoma and the addition of future tests for the *API2/MLT* of many extranodal marginal zone lymphomas may aid in this differential diagnosis.

In the differential diagnosis of anaplastic large cell lymphoma, these tests are often useful. Anaplastic large cell lymphoma has morphological features that are easily confused with other malignancies, including poorly differentiated carcinoma and malignant melanoma. In addition, many cases of anaplastic large cell lymphoma will not immunoreact with T- or B-cell-associated antibodies, and CD30 expression may be detected in tumors other than anaplastic large cell lymphoma.¹¹⁴ Detection of a T cell receptor gene rearrangement, t(2;5), or ALK protein in these cases is often useful in resolving this differential diagnosis. Also, as mentioned earlier, ALK protein expression identifies cases of anaplastic large cell lymphoma that have an improved prognosis, and this study should be performed on all cases.

The use of molecular testing in the evaluation of post-therapy specimens for minimal residual disease is becoming more common with quantitative "real-time" instruments available,¹¹⁵⁻¹¹⁸ and the clinical significance of this type of testing is well studied in the lymphoblastic malignancies.¹¹⁹⁻¹²¹ Such testing is often PCR-based, and any of the translocations mentioned above can be used for this evaluation. Because of the relatively low detection rate of some of the PCR and RT-PCR tests for these translocations, such as *JH/BCL-1* and *NPM/ALK*, the ability to detect the abnormality in the original tumor should be confirmed before using the test for minimal residual disease testing. Testing for residual disease af-

ter chemotherapy or bone marrow transplantation in follicular lymphoma is one of the most common of these tests. Such testing requires a highly sensitive test without false positives. To increase sensitivity, some laboratories transfer the $J_H/BCL-2$ PCR product to a membrane and blot with radioactive- or fluorescent-labeled probes directed against a region of the expected MBR or MCR product. Such methods allow for the detection of one translocated cell in 100,000 cells. Appropriate dilution controls must be included to confirm this level of sensitivity, if minimal residual disease testing is being performed.

The previously mentioned reports of the detection of t(14;18) by PCR analysis in healthy adults suggest that false positive results may occur in the PCR analysis of minimal residual disease in patients with previous follicular lymphomas. The finding of this translocation in non-neoplastic specimens may be reduced with non-nested procedures or with the use of 45 or fewer PCR amplification cycles on 500 ng to 1 μ g of genomic DNA, using a standard metal block thermocycler.²⁸

Consensus primers of IgH are less useful for detection of minimal residual disease because of their low sensitivity. For this reason, some studies have used patient specific primers for residual disease detection of immunoglobulin or T cell receptor gene rearrangements.^{122,123} This is a time-consuming process in which the original tumor clone is amplified using consensus primers, and the PCR product is sequenced. The patient specific primers are made based on the actual patient sequence. Because the patient specific primers are exact matches to tumor clone, they can detect much lower levels of clone than traditional consensus primers. However, if the patient has biclonal disease, recurrence of the second clone not covered by the patient specific primers will not be detected. This methodology is now being used in a number of clinical trials to test its clinical utility, and may become a more routine test in the future.

There are a variety of molecular diagnostic tools available for the evaluation of malignant lymphoma. The tests currently offered in most laboratories are most useful in the evaluation of clonality and in the classification of the lymphomas of small B lymphocytes. The ordering physician must understand the significance and limitations of the available tests, and the methodology used should be considered in the context of the question being asked. The discovery of new abnormalities in malignant lymphoma and the validation of their clinical significance will certainly increase the number of tests offered in the future.

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