Establishing the Diagnosis of Lymphoma: From Initial Biopsy to Clinical Staging

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Successful therapy for most of the non-Hodgkin's lymphomas requires an accurate pathologic diagnosis. Routine morphologic examination of excisional biopsies from nodal or extranodal sites provides the cornerstone for establishing a definitive diagnosis. The list of ancillary studies, however, used to complement these routine approaches is increasing both in number and complexity. Proper use of these diagnostic tools can be of great help in arriving at the correct diagnosis in difficult cases. Fine-needle aspiration and needle-core biopsies have a role in lymphoma staging and in the assessment of recurrent disease, but are limited as primary diagnostic tests. This review will focus on the standard approaches used to establish a diagnosis of malignant lymphoma, and the clinical utility of immunophenotypic, molecular genetic, and cytogenetic studies in providing useful data for diagnosis. The standard practice of synthesizing all of the data from multiparameter analysis to arrive at a diagnosis in difficult cases will be emphasized. [ONCOLOGY 12(Suppl 8):11-16, 1998]

Introduction

During the past decade, a significant amount of clarity has been brought to our understanding of the pathogenesis of non-Hodgkin's lymphoma. Many of the insights gleaned from our knowledge of the molecular mechanisms that underlie lymphomagenesis have translated into improved classification schemes. The Working Formulation is a clinically driven scheme that has long since outlived its usefulness.[1] It has largely been replaced by the Revised European and American Lymphoma classification, now known as the REAL proposal.[2]

This scheme recognizes "real" disease entities that have a clinical, morphologic, immunophenotypic, cytogenetic, and molecular genetic correlate. Within each lymphoma category, a spectrum of clinical behavior may be seen, and this diversity is important to our understanding of non-Hodgkin's lymphoma.[3]

Pathologists welcome this classification, as it focuses on the biology of these disorders rather than lumping diseases together based on a common clinical outcome. The information imparted by an accurate diagnosis, together with the relevant clinical data, can now be used to make definitive treatment decisions.

Classification Techniques

The newly developed and more sophisticated techniques for analysis of lymphoma cells have provided us with the tools necessary for precise classification of non-Hodgkin's lymphoma. Nonetheless, routine histologic studies remain the gold standard for diagnosis. This review will focus on the approaches used both for diagnosis and staging and will attempt to provide some guidelines as to how and when these tests should be employed.

Excisional Biopsy

A well-processed hematoxylin and eosin (H&E) stained section of an excised lymph node is the mainstay of pathologic diagnosis.[4] Most often, the diagnosis of difficult lesions relies heavily on a careful assessment of the underlying architecture. Lymphoma diagnoses are much less about cytologic detail and far more about altered architecture. For example, follicular small-cleaved cell lymphoma (FSC) is characterized by an abundance of neoplastic lymphoid follicles containing monomorphous small-cleaved lymphocytes. The individual cells themselves, however, are otherwise typical small-cleaved lymphocytes seen in the benign follicles of reactive lymph nodes.
The loss of normal nodal architecture that accompanies an infiltrate is of paramount importance in making a diagnosis. An incisional lymph node provides only a glimpse of the architecture, making interpretation difficult. Our surgical colleagues must be instructed to biopsy the most clinically significant site, and whenever possible, to remove an intact lymph node for pathological processing. The tissue should be delivered fresh to pathology at an appropriate time of the day in order to maximize the material for lymphoma protocol studies.

Many hematopathologists prefer to triage the material using imprint preparations, whereby a fresh cut surface of the node is touched onto glass slides for Romanowsky staining. Experienced pathologists are able to make a good approximation of the disease process based on the touch prep morphology, thus resulting in the efficient ordering of additional tests.

When the size of the tissue is limiting, the first priority must be to process the material routinely for fixation and H&E sections. Properly fixed specimens can be used for regular histologic examination, paraffin section immunoperoxidase staining, and depending on the fixative, for gene rearrangement studies by polymerase chain reaction (PCR).[5] Although B5 is the optimal fixative for routine lymphoid histology and is preferred for immunoperoxidase studies, it precludes PCR studies in most laboratories. Formalin fixation is preferred when the biopsy is small because all of the above studies, including PCR, can be performed.

**Diagnosing Disease at Extranodal Sites**

Approximately 30% to 35% of cases of non-Hodgkin’s lymphoma in adults present primarily at extranodal sites. Much less is known about the molecular mechanisms involved in these disorders in comparison to node-based disease. Therefore, it is important to remember to process extranodal biopsy material for lymphoma protocol studies whenever there is a suspicion of a hematolymphoid neoplasm.

Molecular genetic and cytogenetic data from gastric and pulmonary resection specimens have enormous potential to provide insights into the pathogenesis of mucosal-associated lymphoid tissue (MALT) lymphomas but, unfortunately, lymphoma protocol is frequently overlooked in this setting.[7,8] Nonetheless, examination of a well-processed H&E section from an excisional biopsy by an experienced hematopathologist will be sufficient to establish a diagnosis in the majority of cases.

**Needle-Core Biopsy**

Needle-core biopsies have a role in lymphoma pathology, although it remains limited.[9] The use of 14 to 22 gauge needles under ultrasound or radiological guidance to establish a diagnosis of non-Hodgkin’s lymphoma is problematic because of technical difficulties with biopsy crush artifact, inadequate sampling, and the usual vagaries of lymphoma pathology. Although this technique has advantages over fine-needle aspiration (FNA), it should be used judiciously as a diagnostic tool for patients with suspected non-Hodgkin’s lymphoma. Needle-core biopsies do allow a minimal assessment of architecture in addition to immunostaining procedures, but interpretation can be problematic in cases of T-cell rich B-cell lymphoma, angioimmunoblastic-type peripheral T-cell lymphoma, or MALT lymphoma where much of the lymphoid infiltrate is reactive.

A careful review of most excisional lymph node biopsies demonstrates marked cytologic and architectural variation throughout the section, underscoring the complexity of non-Hodgkin’s lymphoma diagnoses in what would otherwise be considered routine circumstances. Needle-core biopsies are unable to detect this variability, leading to the possibility of incorrect diagnoses in many cases. Although recent studies have recommended increased use of these techniques, patient selection and failure to provide convincing evidence that the “right treatment” decision was made in the majority of cases hamper their interpretation.[10,11] Also, many of these studies included patients with an established diagnosis of either non-Hodgkin’s lymphoma or Hodgkin’s disease—an approach that differs significantly from a diagnostic procedure.[9]

In managing ill patients or those with significant comorbid disease who are unable to tolerate an invasive surgical procedure, needle-core biopsies offer a better alternative to FNA for the diagnosis of intra-abdominal or thoracic disease. Ideally, two or three cores should be obtained with one core routinely processed for histology and the remainder used for lineage and clonality studies. In this...
setting, cautious interpretation of the biopsy by an experienced hematopathologist and integration of the results of the ancillary studies should allow a reasonable treatment decision to be made in most cases.

**Fine-Needle Aspiration**

The use of FNA as a diagnostic tool in lymph node-based disease is surrounded in controversy. In general, this technique has no place as the sole diagnostic strategy in patients with suspected lymphoma. The main reasons are twofold. First, the technique suffers from the problem of small sample size and, unlike needle-core biopsies, provides no information about architecture. Second, most non-Hodgkin’s lymphomas are cytologically normal and thus, there are significant numbers of false-negative results in specimens obtained via this technique.

Fine-needle aspiration, however, is useful in the assessment of lymph nodes suspected to show involvement by a metastatic neoplasm, but the technique has little utility as an initial screen for patients suspected to have lymphoma. If a patient is thought to have benign disease, many argue that FNA offers an easy alternative to open biopsy, with a negative result used to reassure the patient. Alternatively, a less expensive approach would rely on a combination of good clinical acumen and close follow-up, foregoing FNA. If the lymph node completely disappears, nothing more need be done. If the lymph node persists and/or enlarges, and a hematological neoplasm is suspected, then one can proceed directly to open biopsy and save the added cost of two procedures.

Recent studies have suggested a role for FNA as a diagnostic approach when routine morphology is combined with flow cytometric immunophenotyping and possible molecular genetic studies. Unfortunately, this combined approach still leaves one without a clear appreciation of architecture and relies on certain assumptions about the diagnostic accuracy of signature phenotypes or the presence of clonality.

**Limits of FNA**

Fine-needle aspiration is not able to detect subtle variability within the node, or the presence of clinically significant histologic findings such as focal transformation, vague follicle formation, or a composite histology. It cannot discern the presence of a diffuse sheet of large B-cells within a background of follicular lymphoma, nor can FNA predict the growth pattern in mantle cell lymphoma (e.g., mantle-zone vs nodular vs diffuse).

There is nothing more disturbing than reviewing an open biopsy in a young patient with a bulky mass and an obvious lymphoid tumor whose prior history reveals numerous nondiagnostic FNA studies. The reliance on FNA techniques to establish a primary diagnosis of non-Hodgkin’s lymphoma should be abandoned, as virtually every FNA report concludes that an open biopsy is required for an accurate histologic assessment.

Under certain circumstances, FNA does have a role in the staging and follow-up of patients with non-Hodgkin’s lymphoma. In cases with an established diagnosis of lymphoma, FNA can be used to assess clinically worrisome distant sites as part of the staging work-up. However, the meaning of a negative FNA requires careful interpretation. For example, FNA of a contralateral neck node in a patient with a salivary gland MALT non-Hodgkin’s lymphoma would be virtually impossible to interpret. As much of the infiltrate in a MALT lymphoma consists of reactive lymphoid follicles, FNA would not be helpful in distinguishing between a reactive node vs MALT lymphoma.

For patients with diffuse large-cell lymphoma, FNA does offer a practical approach for sampling possible metastatic sites. As a follow-up procedure for patients with a questionable relapse, FNA may also provide useful information about the nature of the infiltrate. Finally, for patients in extremis and without clinically accessible sites of adenopathy, FNA can be used as a practical means to guide therapy. Needle-core biopsy would be the preferred approach but FNA, combined with immunophenotypic analysis, may suffice in some cases.

**Immunophenotypic Analysis**

As an ancillary study, immunophenotyping has been of great benefit in the diagnosis of non-Hodgkin’s lymphoma. It provides two essential pieces of information—lineage and clonality—that are complementary to the histology in a given case. A variety of techniques are
available today for performing these studies. The growing list of monoclonal antibodies with activity against fixation-resistant epitopes has enhanced our ability to do paraffin-section immunoperoxidase stains. These studies provide important information about lineage in diffuse large-cell lymphoma, which in many centers will affect treatment decisions.[21] They also provide useful information about immuno-architecture of great benefit in the differential diagnosis of diffuse small B-cell non-Hodgkin's lymphoma.[22]

Subtle follicularity can be easily appreciated following staining of follicular dendritic cells with anti-CD21. The antibody to bcl-2 has a major role in lymphoma diagnosis and in particular in distinguishing between reactive follicular hyperplasia vs follicular lymphoma.[23] bcl-2 expression has also been shown to provide important prognostic information in diffuse large B-cell lymphoma.

Coexpression of CD43 or CD5 on small B cells is extremely helpful in establishing a lymphoma diagnosis and provides significant clues for subclassification.[22] CD30 expression is important for the recognition of anaplastic large-cell lymphoma and together with expression of p80/ALK protein may have great therapeutic significance.[26] The antibody to bcl-1 is perhaps the single most useful reagent for establishing a diagnosis of mantle cell lymphoma.[27] All of these examples are amenable to paraffin-section immunostaining techniques, and therefore can be routinely applied to archived tissue samples.

Clonality can be determined in many cases of B-cell non-Hodgkin's lymphoma using the principles of light-chain restriction and staining for kappa and lambda. This is particularly helpful in distinguishing atypical lymphoid infiltrates from non-Hodgkin's lymphoma and subclassifying certain lymphoma subtypes such as lymphoplasmacytoid lymphoma and MALT lymphoma.[28]

Frozen-section immunoperoxidase techniques or flow cytometric studies require more sophistication, but allow one to assess the entire complement of available monoclonal or polyclonal antibody reagents used in the diagnosis of non-Hodgkin's lymphoma. The former approach yields details about architecture, whereas flow cytometry improves precision because of the large number of cells analyzed.

These two techniques are most useful in the assessment of diffuse small B-cell non-Hodgkin's lymphoma, wherein detailed phenotypic information is critical to subclassification. For example, information about CD5, CD10, CD11c, CD23, CD43, and FMC-7 expression by the neoplastic cells is extremely helpful for distinguishing between small lymphocytic, lymphoplasmacytoid, mantle cell, and marginal-zone B-cell lymphoma.[20] In most centers, distinguishing between these entities has both therapeutic and prognostic impact.

### Molecular Genetic Studies

Lineage assignment and the determination of clonality can also be performed using gene rearrangement studies.[29] Mature lymphoid cells that give rise to non-Hodgkin's lymphoma are differentiated beyond the point where they have undergone an orchestrated rearrangement of their antigen receptor genes, providing a unique molecular fingerprint for each cell. In a clonal population, all of the cells will share the same rearrangement and, therefore, standard techniques such as Southern blot and PCR can be used to determine the presence of clonality.

Unlike many of the immature acute leukemias, non-Hodgkin's lymphomas do not typically demonstrate lineage infidelity with rearrangement of both the immunoglobulin heavy chain (IgH) and T-cell receptor (TCR) genes in the same cell. Therefore, B-cell lymphoma will usually have a clonal IgH rearrangement, and non-Hodgkin's lymphoma of T-cell lineage, a clonal TCR rearrangement. These signature molecular findings can serve as useful markers of both lineage and clonality.

The Southern blot technique remains the gold standard for the molecular determination of clonality. However, it requires large amounts of fresh or frozen high-quality DNA, takes several days to perform, is labor-intensive, is not particularly sensitive in the detection of small clonal populations, and requires radioactive isotope exposure.
Polymerase chain reaction, on the other hand, can be performed without radioisotopes, requires minimal starting template DNA, takes 2 to 3 working days to complete, is of sufficient sensitivity to detect small clonal populations, and can be performed using DNA extracted from paraffin-embedded, formalin-fixed tissue. It does, however, suffer from the problem of false-negative results.

Nonetheless, it is the preferred method for lineage and clonality assessment when immunophenotypic data are either not available or noncontributory. Polymerase chain reaction is most helpful in difficult cases for distinguishing between atypical lymphoid hyperplasia vs non-Hodgkin’s lymphoma.[30] Although clonality and malignancy cannot be equated, these data do provide additional support in favor of non-Hodgkin’s lymphoma for cases lacking definitive histologic evidence. For example, PCR studies are particularly helpful in the assessment of small endoscopic gastric biopsies and punch biopsies of skin.[31]

In addition to molecular studies for lineage and clonality, oncogene translocations serve as useful markers for molecular analysis and are particularly helpful for the subclassification of non-Hodgkin’s lymphoma. Rearrangement of the bcl-2 oncogene is the molecular equivalent of the t(14;18) or its variants. It indicates commitment to the B-cell lineage, provides good evidence for a follicle center derivation and is a stable clonal marker. bcl-2 polymerase chain reaction studies can also be used in the assessment of minimal residual disease, and to check the purity of ex-vivo samples such as peripheral blood stem cell or bone marrow harvests used for bone marrow transplantation.[32]

Other oncogene rearrangements amenable to molecular studies include the bcl-1 translocation characteristic of mantle cell lymphoma, bcl-3 found in occasional cases of chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL), bcl-6 seen in some follicular lymphomas and many diffuse large B-cell lymphomas, and the NPM-ALK rearrangement characteristic of anaplastic large-cell lymphoma.[33-36] The latter oncogene is the molecular equivalent of the t(2;5) translocation found in anaplastic large-cell lymphoma, and requires RNA extraction and reverse transcriptase PCR for its detection.

**Cytogenetic Studies**

Classical cytogenetic studies have contributed enormously to the understanding of the biology and the determination of prognosis in acute leukemia. Similarly, they have provided very important information in non-Hodgkin’s lymphoma, and have been critical to the discovery of many of the oncogenes now known to be intimately involved in the pathogenesis of these disorders. The description of the t(14;18)(q32;q21) of follicular lymphoma in 1979 led shortly thereafter to the discovery of the bcl-2 oncogene located at the breakpoint on chromosome 18q21.[37,38] This opened up the field of programmed cell death or apoptosis, and shifted the focus of cancer biology towards cell death and away from cell division as a major mechanism of neoplastic growth.[39] The implications of this discovery go far beyond the field of lymphoma biology and have significance for many other cancers.

Classic cytogenetic studies are expensive, labor-intensive, take 1 to 2 weeks to perform, require technical expertise, and must be performed using fresh biopsy specimens. Therefore, these studies need to be considered at the time of biopsy when their importance to a particular case cannot be appreciated. One cost-effective approach is to culture the cells and harvest the metaphases, but suspend the analysis until deemed necessary following review of the histology and other ancillary studies. Using such a strategy, one can focus the cytogenetic studies on the difficult cases including the follicular lymphomas that fail to express bcl-2 protein and the extranodal lymphomas in which little cytogenetic data are available.

Other examples of recurrent cytogenetic findings associated with specific lymphoma subtypes include:

1. the t(11;14)(q13;q32) of mantle cell lymphoma;
2. the t(14;19)(q32;q13) of CLL/SLL;

3. the t(3;14)(q27;q32) or variants of diffuse large B-cell lymphoma and many t(14;18)-negative follicular lymphomas;

4. the t(8;14)(q24;q32) or variants associated with Burkitt’s lymphoma;

5. the t(2;5)(p23;q35) of anaplastic large-cell lymphoma;

6. the t(9;14)(p13;q32) of lympho-plasmacytoid lymphoma;[40] and

7. the t(11;18)(q21;q21) of MALT lymphoma.[7,8]

The discovery of most of the lymphoma oncogenes has been the direct result of classical cytogenetic studies. The practical value of this testing again pertains to those difficult cases with atypical histology, noncontributory immunophenotypic and molecular genetic data whereby the presence of a cytogenetic clone and the specifics of the translocation(s) may be important to unraveling the diagnosis.

**Non-Hodgkin’s Lymphoma Staging**

Once a diagnosis of non-Hodgkin’s lymphoma is rendered, clinical and pathologic staging is necessary to determine the extent of disease. This information is required for treatment planning and prognostication. Possible metastatic sites may require pathological assessment if clinical examination and imaging studies are inconclusive, as the results of these tests may alter the final determination of stage. Depending on the histology of the diagnostic biopsy, open biopsy of the suspicious site may be required. Generally, needle-core biopsy or a FNA will suffice if combined with immunophenotypic analysis and/or molecular genetic studies. Bone marrow examination is needed for staging of non-Hodgkin’s lymphoma, but the requirement for unilateral vs bilateral core biopsies differs in many centers.

Typically, a unilateral bone marrow aspirate and biopsy are sufficient, and may be combined with flow cytometric immunophenotyping and/or gene rearrangement studies.[41] The contribution of these latter two studies toward improving the sensitivity of non-Hodgkin’s lymphoma staging is controversial.[42] Similarly, the clinical significance of minimal residual disease testing is unclear. Independent of the bone marrow findings, the peripheral blood smear should be carefully reviewed for the presence of circulating lymphoma cells. Suspicious cases should be analyzed using flow cytometric immunophenotyping or possibly molecular genetic studies. The presence of any leukemic component in certain non-Hodgkin’s lymphomas may alter therapy and contribute to prognosis.[43] In many centers, peripheral blood involvement at the time of diagnosis does not preclude a bone marrow examination, although the need for the test in this setting remains unclear.

**Summary**

In conclusion, **Table 1** outlines a practical approach to the methodology used in the study of non-Hodgkin’s lymphoma. Effective therapy requires that an accurate diagnosis be made and that proper staging is able to determine all sites of disease. Although routine histologic approaches remain the gold standard for diagnosis of non-Hodgkin’s lymphoma, immunophenotypic analysis, molecular genetic studies, and classical cytogenetics may be required to resolve the diagnosis in difficult cases. A close working relationship between the medical oncologist and the hematopathologist is needed to assure the proper interpretation of these complex tests and their appropriate clinical application in this era of sophisticated diagnosis and tailored therapy.
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