The Critical Role of Histology in an Era of Genomics and Proteomics: A Commentary and Reflection

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Abstract: The role of histologic examination in lymphoma diagnosis has been called into question by proponents of new technologies, such as genomics and proteomics. We review the history and salient features of morphologic evaluation in lymphoid diseases, and discuss the general and specific limitations of mature ancillary techniques, such as immunohistochemistry, flow cytometry, and molecular studies. We then speculate on the future relationship between morphology and the new genomic and proteomic technologies as they become integrated into clinical practice.

Key Words: lymphoma, histology, morphology

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''Morphology is always important, and some diseases are primarily defined by morphology with immunophenotype as backup in difficult cases.''—Nancy Harris, et al.[1]

PREFACE

Making a histologic diagnosis is an art and not a science, and given the subjective nature of this art, it is inherently difficult to reproduce. To skillfully practice this art, it has to be carefully taught by experienced subspecialists, remembering that the learning process is slow, methodical, and painstaking. To obtain in-depth experience requires daily exposure to a large volume of case material, so that imprinting (etching) of images occurs in the mind for instant future recall in diagnostic practice.[2,3] For imprinting of images to occur in the mind, they have to be studied carefully, critically, and repeatedly. Moreover, because most histologic features have a wide spectrum of presentations, and different diseases have a selection of diagnostic criteria, the complexity and difficulty of this imprinting process is greatly magnified.[2,3]

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The maturation process from being ignorant to being an apprentice, to being an independent pathologist who gains further experience over the years, can also be summarized in a different way: we do not know what we do not know. In other words, we recognize what we know, and we do not recognize what we do not know. And the more we know, the more we recognize.[3]

Malignant lymphomas and benign lymphoid diseases have been diagnosed on the basis of histologic/architectural patterns and cytologic features for over 150 years. The advantages and limitations of this morphologic approach are well known, and have been summarized elsewhere.[2,3]

This commentary and reflection will focus on the fundamental role that morphologic examination plays in an integrated approach to the diagnostic process. We will also examine the advantages and limitations of newer diagnostic technologies as they relate to such an integrated approach to diagnosis. For the purposes of this discussion, owing to limitations of space, we will quote review articles in most cases, rather than the original scientific papers in which specific observations were published.

New techniques that became available in the 1970s and 1980s, such as immunophenotype analysis by flow cytometry, immunohistochemistry, and molecular testing, were touted by some as the new benchmark for diagnosis and classification of lymphomas. Many predicted that these new methodologies would soon be the ''gold standard'' for diagnosis. Moreover, new knowledge about the diagnostic and clinical relevance of cytogenetic abnormalities and molecular rearrangements led many to believe that the role of morphologic tissue examination would soon become obsolete. However, in the ensuing years, it was established that these new techniques had many limitations as well, such as issues with inadequate sampling, lack of sensitivity and specificity of immunophenotype analysis by flow cytometry and immunohistochemistry, and significant false-results with gene rearrangement studies.[4]

Despite these limitations, these new techniques have contributed vastly to our understanding of neoplastic and benign lymphoid diseases. The advent of immunohistochemistry and cytogenetic characterization of lymphoproliferative disorders was a great innovation in the field of hematopathology. Even now, 30 years later, we are still riding the crest of the wave of new information that became available using these new techniques. The rise of

immunophenotype analysis by flow cytometry, high-resolution molecular cytogenetics, fluorescent in situ hybridization (FISH), microdissection, and most recently, the development of polymerase chain reaction (PCR) and other molecular amplification techniques have initiated great surges of discovery that have increased our knowledge of the genotypic and phenotypic aspects of lymphoid disease by leaps and bounds.[5–8]

Currently, in the years since the human genome was completely sequenced, there is tremendous, unbridled excitement over the prospect that genomic and proteomic signatures will serve as the primary method to establish the diagnosis, prognosis, and treatment of lymphoma patients.[9–16] Within the past several years, many studies have shown that the majority of lymphomas, predominantly the overt and advanced, have characteristic gene expression profiles (signatures) that provide more clinical relevance than the morphologic and immunohistochemical evaluation of tissue sections.[17–28] Therefore, the major goals of investigators conducting basic and clinical research in this field has been to develop a classification of lymphomas based on molecular genetic and proteomic signatures, as well as to further develop the bioinformatic and technologic tools that allow these signatures to be isolated and used for true, individualized patient therapy.[29–36]

It is clear that new diagnostic, prognostic, and therapeutic information will be generated by the introduction of lymphoma and leukemia genomic gene expression arrays, or "chips," and by proteomic expression analysis. Much advanced work has been done in this regard, especially as relates to leukemias, given the ease of obtaining relatively pure samples of neoplastic cells from blood or bone marrow, in liquid form with virtually no intervening stroma. It is becoming apparent that such technology is likely to revolutionize leukemia classification, diagnosis, and staging in the coming years.

In contrast, lymphomas usually require a surgical biopsy, and contain considerable stromal tissue and benign lymphoid cells. For these reasons, it is expected that it will be a more difficult matter to develop and introduce a classification system of lymphomas based on genomic chip technology that will be useful in routine diagnostic practice.

This technology will soon become available, and that it will come to be used, at the bare minimum as an adjunct to morphology, seems a foregone conclusion. However, its precise role in diagnostic and therapeutic hematopathology remains to be defined. Will this new technology replace the more mature technologies of cytogenetics, immunohistochemistry, flow cytometry, and conventional molecular testing by PCR, or will it become integrated into a systematic approach to diagnosis along with the older technologies?

In this brave, new world, what will be the precise role of histologic tissue examination? To answer this question, we must look to the past and the role that morphology has played during the development of immunologic, cytogenetic, and molecular technologies over the last 30 years. Furthermore, we must evaluate the role of morphology in the present tense, and see how these genomic and proteomic technologies have already affected change in current histopathologic practice. To address these questions, the goals of our review are as follows:

1. To place the art of histologic tissue examination in its historical context, to review the inherent role of histologic examination, and to discuss the relative value of morphology as a diagnostic tool in this era of new technologies.
2. To summarize common morphologic patterns observed in various benign and malignant lymphoid proliferations, and to outline a systematic approach to accurate diagnosis based on histologic examination.
3. To outline the known limitations of immunophenotyping by flow cytometry and immunohistochemistry, molecular studies, and to speculate on the theoretical limitations of the use of genomics (lymphoma chips) in routine diagnostic practice.
4. To outline the most important histologic features of those lymphomas that are distinct disease entities, as defined in the WHO classification.[1]
5. To briefly outline the immunophenotypic and molecular profiles of the most common lymphoid malignancies, with an emphasis on the value of morphology when the typical genotype/phenotype is absent or an aberrant genotype/phenotype is present.

THE INHERENT ROLE OF HISTOLOGY

The histologic/clinicopathologic approach for recognition and classification of lymphoid diseases long preceded the use of flow cytometry and immunohistochemistry in routine diagnostic practice. For example, Hodgkin disease was described first in the 18th and 19th centuries. In 1937, the Jackson and Parker classification of Hodgkin disease was published[37,38] with 3 categories, which was modified by Lukes[39] in 1963 in 6 categories, by Lukes and Butler[40] in 1966, and was then slightly modified in the current World Health Organization (WHO) classification[1] to yield 5 categories.

Mediastinal lymphoblastic lymphoma was first reported by Sternberg[41] in 1908. In 1932, Cooke[42] reported 9 boys with mediastinal lymphoblastic lymphoma, and additionally summarized the world literature on 74 similar cases. Histiocytic medullary reticulosis was described in 1938 by Robb-Smith,[43] and now falls mostly under the category of systemic anaplastic large cell lymphoma (ALCL).

Follicular lymphoma (FL) was first described in 1942[44] and Burkitt lymphoma (BL) in 1958.[45] In 1973, immunoblastic lymphadenopathy was described as a clinicopathologic entity,[46,47] and in 1974 angioimmunoblatic lymphadenopathy was reported.[48,49] In 1975, the intermediate lymphocytic and centrocytic lymphomas were reported.[50,51] In 1983, the mucosa-associated lymphoid tissue (MALT) lymphoma was described as a distinct entity.[52]

Like malignant lymphomas, historically, many benign lymphoid proliferations were recognized as distinctive diseases based on clinical features and histologic findings without the use of immunophenotyping. The histologic descriptions of distinctive benign diseases, such as syphilis, tuberculosis, leprosy, sarcoidosis, brucellosis, toxoplasmosis, cat-scratch disease, infections mononucleosis, cytomegalovirus, systemic lupus erythematosus, giant lymph node hyperplasia, sinus histiocytosis with massive lymphadenopathy, Kikuchi-Fujimoto disease, histiocytosis X, and dermatopathic lymphadenitis, preceded immunophenotyping by flow cytometry and immunohistochemistry.

Over the last 30 years, immunohistochemistry, cytogenetics, and molecular studies have confirmed or refined the above diseases into distinct entities, and also identified many new unique diseases, which were codified in the Revised European-American Lymphoma classification of lymphoid neoplasms,[53] and later in the WHO classification.[1]

The Global Importance of Morphology, and Its Value as Part of a Systematic Approach to Diagnosis

The historical contribution, however, does not answer the question of how morphology meets the needs of current pathology practice. Taking into consideration the number of small community hospitals, mid-sized and large referral centers, and also outpatient clinics, doctors' offices in various surgical and medical specialties (dermatology, gastroenterology, gynecology, etc), and other outpatient surgical centers owned by a variety of entities, the number of new biopsies that are performed each year in the United Sates is vast and they are examined histologically. Moreover, in all hospitals that are accredited in the United States by the Joint Commission on Accreditation of Healthcare Organizations, the hospital policies require that, in any new patient on whom a biopsy has been done, for proper patient care, the biopsy specimen must be sent to the histopathology laboratory for review. Before we discuss the responsibilities of the histopathologist, we must briefly summarize the inherent complexities, and also the wealth of information, contained within the biopsy section, and the histologic approach to formulating a differential diagnosis.

We have previously described a methodical approach relating to the systematic histologic review of lymph nodes elsewhere,[2,3] so we will restrict our present discussion to a few comments regarding the examination of extranodal tissues. In 2006, the American Cancer Society reported 66,670 new lymphomas in the United States, of which 7800 were Hodgkin lymphoma and 58,870 were non-Hodgkin lymphomas.[54] Primary extranodal lymphomas comprise about 40% of malignant lymphomas, and virtually all are non-Hodgkin lymphomas.

The lymphoid proliferations that occur at extranodal sites can be clearly benign or overt lymphomas of different types. However, many lymphomas, especially MALT lymphomas, which were in the past misdiagnosed as pseudolymphomas, have to be distinguished from those benign lymphoid diseases that have histologic features that resemble malignancy. Over the years, it has been recognized that infections, such as *Helicobacter pylori*, *Borrelia burgdorferii*, *Chlamydia psittaci*, and Hepatitis C are associated with an increased risk of developing a MALT lymphoma in a benign, inflammatory histologic background. Also, autoimmune diseases, such as Hashimoto thyroiditis and Sjogren syndrome, have a 70-fold and 44-fold increased risk of developing MALT lymphoma, respectively.[1]

To emphasize the difficulties in navigating through the histologic sections, sifting and extracting the critical patterns and cytologic features that are present and are necessary to form a rational differential diagnosis, we present here a few histologic scenarios at extranodal sites that will hopefully help the reader to appreciate the inherent complexity evident within each specimen.

For example, at an extranodal site, the lymphoid process could be focal, multifocal, or diffuse. It may consist of one or more cell types with no or with varying degrees of cellular "atypia," and with absent to many mitoses. The extranodal proliferation may consist solely of small lymphoid cells, or it may be admixed with few to many plasma cells. In some fragments or specimens, there may be the presence of few to many follicles, with or without surrounding mantle zones, and some may be focally or multifocally, partially or completely surrounded by marginal zone B cells, with or without clear cytoplasm. These cells may also occur as isolated clusters. In between the follicles, besides lymphocytes and/or plasma cells, there may be large lymphoid cells, neutrophils, and possibly other cell types. Lymphoepithelial lesions, if present, may be small and subtle, and may be focal or multifocal. Furthermore, there could be subtle or overt, focal or multifocal follicular colonization produced by marginal zone B cells.

In the above extranodal scenarios, we have introduced a level of complexity that may not be apparent to the untrained eye. We can state unequivocally from our experience that it is very difficult for those who are not subspecialized pathologists to recognize: (1) all the different types of pathologic processes present, (2) the distinction between the normal inflammatory component and the abnormal component, and (3) the relationship between the two. In fact, sifting through the slide and correctly identifying the amount and location of the marginal zone B-cell component, the presence of follicular colonization, and the presence of lymphoepithelial lesions is complex and often painstaking task. After this exercise, a proper, narrow, histologic differential diagnosis can be formed, and the decision can be made whether or not any additional immunostains are needed to reach a final diagnosis. Toward this end, we offer a few words of caution. It must be kept in mind that small specimens often may be focally crushed, cauterized, or necrotic. This problem is compounded by the common practice of ordering deeper levels, which will reduce the informative tissue present in the paraffin block to the

TABLE 1. Global Importance of Morphology as a First Step Toward Establishing a Correct Diagnosis

1. Responsibility of the histopathologist
   a. Histologic evaluation of biopsy specimen
      i. Adequacy of biopsy (amount and type of tissue, pathologic process present or absent)
      ii. Quality assurance (small samples, crush artifacts, and the presence of normal or nonrepresentative tissue)
      iii. Formation of a differential diagnosis
   b. Correlation and integration of the differential diagnosis with clinical information and ancillary studies resulting in a narrow differential diagnosis or final diagnosis
   c. Prognostic and therapeutic information may be offered

Note: This integrative approach requires a thorough understanding of the limitations of each test to achieve this end

2. Value of the histopathology report
   a. A common language for communication among pathologists and consultants
   b. The microscopic description documents what the pathologist saw, and how he/she arrived at a diagnosis, thus it can be valuable for legal purposes, clinical care, teaching and research
   c. Histologic diagnostic qualifiers, such as "worrisome for," "suggestive of," "suspicious for," or "consistent with" indicate limitations in morphologic criteria and/or sample quality, serving as triggers for additional biopsies/laboratory studies
   d. Guardian of quality control
      i. The histopathology report and diagnosis often serves as a benchmark against which results of newer tests are compared and correlated, highlighting values and limitations of both histology and ancillary tests
      ii. This iterative process consequently improves
         1. Histologic criteria and diagnosis
         2. The quality of newer tests
         3. Teaching
         4. Promotes and permits focused clinical and basic research

3. Value of archived reports, slides and paraffin blocks
   a. Histologic comparisons with subsequent biopsies to evaluate regression, progression, or transformation of diseases
   b. Valuable for research studies
   c. Valuable for teaching and training of future pathologists

point where the proper number of immunostains cannot be ordered, and a meaningful diagnosis cannot be reached.

The responsibilities of the practicing histopathologist are summarized in Table 1. The biopsy specimen has to be examined grossly and microscopically, and a report has to be issued. The paraffin block, slides, and reports must be stored for future retrieval and use.

In the current systematic approach to the diagnosis of lymphoid diseases, histologic examination is the first step in the evaluation of a tissue sample, which can establish whether or not the biopsy specimen is both adequate for interpretation and a representative sampling of the lesion. The responsibility of the diagnostic histopathologist is to record and report the important morphologic features of a specimen, which then form the basis for a differential diagnosis. The differential diagnosis dictates additional studies that the histopathologist has to carry out to further characterize and precisely classify a disease process. It is the responsibility of the histopathologist to correlate all available data with the clinical information, and based on these data, to arrive at an accurate final diagnosis that may offer prognostic and therapeutic information. This integrative approach requires a thorough understanding of the limitations of each test employed to achieve this goal.

Archival storage of tissue blocks and slides is of critical value for histologic comparisons with subsequent biopsies to ascertain whether or not there is regression, progression, or transformation of disease. Also, the archived material is valuable for the teaching and training of future pathologists, and for all types of research.

Histologic diagnosis is an important benchmark against which results of newer tests are validated. On the basis of such comparisons, the limitations, and values of morphology, as well as those of other tests that are being compared and evaluated, can be better determined and defined. This practice has yielded improved morphologic criteria and diagnoses, better quality of new tests, thoroughness in teaching, and more focused research studies, which have consequently led to superior patient care.

WHO Classification Emphasized the Fundamental Value of Morphology

The WHO classification of malignant lymphomas is used worldwide and is a consensus classification that carries with it great authority, respect, and credibility. The introduction by Harris et al states that "morphology is always important, and some diseases are primarily defined by morphology, with immunophenotyping as a backup in difficult cases.[1]"

Specific Examples

According to the WHO classification, the nomenclature of each lymphoma depends on the most useful information necessary to reach a final accurate diagnosis.

B-cell Lymphomas Named After the Compartments From which They Arise or on the Basis of Histologic Patterns

In the WHO classification, most B-cell lymphomas are homogeneous disease entities, whereas a minority is not. The homogeneous B-cell lymphomas are follicular, mantle cell, marginal zone, and small lymphocytic. In the United States, the incidence of FL is about 35%, marginal zone B-cell lymphoma (MZBCL) is about 10%, and mantle cell and small lymphocytic lymphomas (SLL) are about 8% each.[55]

These lymphomas have distinctive and characteristic histologic patterns, which greatly facilitate their accurate identification. FL can have follicular, follicular and diffuse, inverse follicular, and marginal zone patterns. The MZBCL exhibit, in more than 90% of the cases, one or more of the following distinctive patterns: marginal zone, inverse follicular, follicular colonization, interfollicular, and sinus patterns. Mantle cell lymphomas (MCL), in at least 70% of the cases, exhibit one or more of the following distinctive patterns: mantle zone, mantle cell nodular, and follicular colonization. SLL exhibit, in 99% of cases, a pseudofollicular pattern (proliferation centers).

Diffuse large B-cell lymphomas (DLBCL) are morphologically not homogeneous, but heterogeneous,[1,53,55] and consist of several variants and subtypes. As the name implies, they typically have a diffuse pattern.

Recognition of Follicular, Marginal Zone, Mantle Cell, and Small Lymphocytic Lymphomas on the Basis of Morphology

The international non-Hodgkin lymphoma classification project[55] had undertaken a study to ascertain the clinical validity of the Revised European-American Lymphoma classification.[53] In this study, 5 expert hematopathologists reviewed about 1400 cases of non-Hodgkin lymphomas and reached a consensus diagnosis. The design of the study required that in each case, 3 diagnoses should be rendered by each pathologist. The first diagnosis would be made on the basis of morphologic features alone, which was coded as the morphologic diagnosis. The pathologist then reviewed the immunostained slides and could change the diagnosis, if it was considered necessary. The third diagnosis was the final diagnosis, which was made in light of the clinical information.[55]

Table 2 shows a comparison of the consensus diagnosis with the diagnosis made by each pathologist on the basis of morphologic features alone, then with the subsequent review and determination of the immunophenotype. From this table, it becomes apparent that in FL, after immunophenotyping, the pathologists changed their diagnosis in 1% of the cases; in MALT lymphomas, the pathologists changed their diagnosis after immunophenotyping in 2% of the cases; in SLL, in 3% of the cases; and in MCL, in 10% of the cases.[55] These results established that expert lymphoma pathologists can accurately diagnose those B-cell lymphomas that are homogeneous and distinct disease entities on the basis of morphologic features in a very high proportion of cases, and that immunophenotyping increased the accuracy in a very small proportion of cases.

Accurate Diagnosis of Diffuse Large B-cell Lymphomas and T-cell Lymphomas Required Immunophenotyping

In DLBCL, in the WHO classification, is a heterogeneous category and not a homogeneous entity, the expert pathologists changed their diagnosis in 14% of the cases after immunophenotyping.

With respect to T-cell lymphomas, which often do not possess characteristic histologic patterns, and also because they are relatively uncommon (about 12% of non-Hodgkin lymphomas), the pathologists changed their diagnoses far more frequently after immunophenotyping (35% to 45%) than for the B-cell lymphomas.[55] These results show that for those lymphomas that do not have distinctive patterns, immunophenotyping is necessary to establish a correct diagnosis.

Lymphomas Named According to Cytologic Features

The rationale for naming some lymphomas according to specific cytologic features is that they guide the pathologist by emphasizing the most important features that are necessary and most useful to make an accurate diagnosis. However, many of these lymphomas also show histologic patterns in tissue sections that help considerably to guide the pathologist in making an accurate diagnosis. For example, lymphoplasmacytic lymphoma often exhibits patent sinuses. In hairy cell leukemia, a sinusoidal pattern, vascular lakes, and pseudosinus formation is found. In ALCL, Langerhans cell histiocytosis, and systemic mastocytosis, sinus, and interfollicular patterns are seen in most cases. Angioimmunoblastic T-cell lymphomas (AILT) often show intravascular and extravascular clusters of clear cells, marked vascular proliferation, and burned-out follicles. Also, in mixed cellularity Hodgkin lymphoma, scattered reactive follicles, benign mantle cell nodules, and clusters of epithelioid cells are often seen in the interfollicular areas.

Distinctive Patterns Associated With Most Hodgkin Lymphomas

For roughly every 8 new cases of non-Hodgkin lymphoma, there is 1 Hodgkin lymphoma.[54] Overall then, Hodgkin lymphomas are the most common lymphoma after MZBCL. Therefore, recognition of Hodgkin lymphoma is very important in routine diagnostic practice. Hodgkin lymphoma often exhibits distinctive patterns, which help in an accurate identification of this lymphoma. By the WHO classification[1] definition, 3 types of Hodgkin lymphoma have a nodular pattern: (1) nodular lymphocyte predominant Hodgkin lymphoma (NLPHL), (2) nodular lymphocyte-rich classic Hodgkin lymphoma (NLRCHL), and (3) nodular sclerosing classic Hodgkin lymphoma (CHL). These 3 types collectively comprise about 60% of the Hodgkin lymphomas.

TABLE 2. Consensus Diagnoses of B-cell Lymphomas Using Morphology With and Without Immunophenotyping

	Diagnosis of Lymphomas With Distinctive Patterns[56]		
Consensus Diagnosis of Lymphoma	**Morphology Alone (%)**	**Morphology With Immunophenotyping (%)**	**Contribution of Immunophenotyping (%)**
Follicular	93	94	1
Marginal zone B-cell of MALT type	84	86	2
Small lymphocytic	84	87	3
Mantle cell	77	87	10
Diffuse large B cell	73	87	14

Moreover in mixed cellularity Hodgkin lymphoma, an interfollicular pattern is often seen, and commonly, there are benign mantle cell nodules.

Because the malignant cells in Hodgkin lymphoma are in a small minority and the benign component is the predominant finding, this diagnosis can be readily missed, resulting in the lymphoma being mistaken for a benign disease. Hence the word nodular is emphasized in the terminology.

The Importance of Morphology in the Diagnosis of Benign Lymphoid Diseases

Because benign lymphoid diseases frequently do not have specific immunohistochemical and flow cytometric profiles, histologic examination is indispensable for their precise diagnosis. Moreover, the fundamental value of morphology is further underscored by the lack of sensitivity and specificity of the various antibodies used in diagnostic practice. Thus, it is often more difficult to distinguish benign lymphoid proliferations from malignant ones, than it is to subclassify a specific lymphoma.

These benign diseases produce distinctive changes in one or more compartments of the lymph node. Because they involve these compartments focally or multifocally, it is difficult to recognize these diseases. In contrast, malignant lymphomas are relatively easier to recognize because they usually involve the entire lymph node, and the malignant population is often the sole finding.

Benign diseases produce distinctive patterns, which can closely resemble those seen in malignant lymphomas that involve one or more compartments. For example, in both FL and florid follicular hyperplasia, a follicular pattern is seen; in MCL and mantle cell hyperplasia, a mantle zone pattern is seen; and MZBCL were in the past often misidentified as pseudolymphomas.

As stated previously, the malignant cells in Hodgkin lymphoma are in a small minority and the benign component is the predominant finding; hence, they resemble many benign diseases and can be readily confused with them. Conversely, florid viral infections, especially florid infectious mononucleosis, can be easily mistaken for Hodgkin lymphoma or large cell lymphoma. Similarly, the proliferative phase of Kikuchi-Fujimoto disease, which is characterized by an absence of overt necrosis and exuberant proliferation of cells of different types, is often mistaken for Hodgkin lymphoma or a large cell lymphoma.

Correlation Between Morphologic Patterns, Immunohistochemistry, and Cytogenetics

That the distinctive histologic patterns described above are real and valuable in diagnostic practice is underscored by the fact that immunohistochemical staining establishes the specific patterns seen in histologic sections. Moreover, there is also an excellent biologic correlation between the specific patterns and the specific underlying recurrent balanced translocations [eg, t(14;18) in FL, t(11;14) in MCL, and t(11;18) in MALT lymphoma], further underscoring the fundamental value of accurately recognizing patterns.[17,57,58]

A Summary of Our Systematic Approach to Accurate Histologic Diagnosis

In this era of cost containment, patients, physicians, and insurers demand both cost-effective and accurate diagnoses. It is impossible to perform all tests on all patients. Therefore, a logical, well-defined, standardized, and cost-effective approach to diagnosis becomes essential, to avoid an inefficient competition between advocates of morphology, immunophenotyping, genomics, proteomics, and future technologies. Each of these modalities may provide diagnostic, therapeutic, and prognostic information; however, the relative utility of each must be weighed carefully in choosing which tests to pursue to generate the most relevant clinical information.

Morphology is still the first and most important step toward establishing an accurate diagnosis, and it must be used appropriately and precisely. In the hands of well-trained histopathologists, careful morphologic evaluations as part of an integrative diagnostic approach can help reduce diagnostic errors. Our systematic approach to morphologic diagnosis is described elsewhere in detail.[3,59–61] Herein, we briefly summarize, with minor modifications,[2,3] our approach to making an accurate morphologic diagnosis[2,3]:

1. Methodical diagnostic approach
   a. Technical prerequisites for making an accurate diagnosis
      i. Optimal specimen
      ii. Adequate microscope (scanning lens, continuous rheostat)
      iii. Adequate time allotted for detailed review
   b. Knowledge prerequisites
      i. Understanding of migration of cells between lymph node compartments
      ii. Familiarity with histologic spectrum (normal compartments, benign diseases, and lymphomas)
      iii. Specific diagnostic criteria for each disease

2. Recognition of pathologic processes
   a. Low magnification appearance of pathologic processes
   b. Methods for identifying pathologic processes
      i. Sequential examination of each compartment
      ii. Comparison of similar compartments with each other
      iii. Comparison of adjacent compartments with each other
      iv. Recognition of cell types in each compartment based on colors, for example, plasma cells (purple), small lymphocytes (dark blue), and eosinophils (brick red), etc
      v. Assessment of cellularity in various compartments or proliferations (hypercellular, normocellular, and hypocellular)

c. Methods for accurate pattern recognition
   i. Diagnostic/prognostic importance of patterns
   ii. Methods for distinction of true patterns from nonspecific areas
   iii. Methods for identification of similar, multiple, and subtle patterns
   iv. Categorization of types of patterns
      A. Spherical structures: *follicular pattern*, *mantle zone pattern*, *inverse follicular pattern*, *marginal zone pattern*, *pseudofollicular pattern*, *mantle cell nodules*, *marginal zone B-cell nodules*, *follicular colonization*, *paracortical nodular T-zone hyperplasia*, *progressive transformation of germinal centers*, *lymphocytic and histiocytic* (*L&H*) *nodules*, and *fibrous nodular pattern*
      B. Other patterns (nonspherical structures): *sinus*, *interfollicular*, *mottling*, *Lennert's*, *starry-sky*, *vascular*, *necrosis*, *diffuse*, *mixed*, and *miscellaneous*

3. Formulation of a differential diagnosis
4. Resolution of a differential diagnosis
   a. Clinicopathologic correlation
   b. Immunohistochemistry
   c. Flow cytometry
   d. Cytogenetic/molecular techniques

Frequently, morphologic, clinical, immunologic, and molecular information obtained may be in disagreement. To resolve any discordance, a pathologist must use in-depth knowledge and experience in the field to weigh competing criteria to arrive at the most accurate diagnosis.

Our purpose here is to emphasize that morphology is very important and that histologic review should be very critical and careful, which will permit formation of a proper, narrow differential diagnosis, which in turn, would guide the pathologist to order only those ancillary studies that will resolve that specific differential diagnosis. "Shotgun" ordering of ancillary tests is to be avoided because of the lack of specificity and sensitivity of these tests as will be discussed in detail below.

THE ROLE OF MORPHOLOGY, WHEN IMMUNOPHENOTYPE AND MOLECULAR STUDIES ARE NEGATIVE, ABERRANT, OR EQUIVOCAL

Histologic patterns, typical or subtle, are present in most B-cell and Hodgkin lymphomas. Additionally, some T-cell lymphomas, such as AILT and ALCL also have distinctive patterns. The remaining cases of B-cell and T-cell lymphomas exhibit a fairly wide histologic spectrum, which leads to a differential diagnosis that may include other specific types of lymphoma and/or benign diseases. In such cases, immunophenotyping and molecular studies can be essential secondary tests in confirming a diagnosis or resolving a specific differential diagnosis that has been formulated based on the results of morphologic examination. The results of these ancillary studies must always be interpreted in the context of morphology and clinical information.

Because of the widespread use of immunohistochemistry, flow cytometry, and molecular studies, we will attempt to briefly discuss below some of the general limitations and pitfalls encountered with their use. Subsequently, we will discuss their known, specific limitations in the most common lymphomas. In the specific instances presented later in this chapter, histology often becomes very useful for a correct interpretation of the ancillary testing results and helps avoid misinterpretation and ultimately an incorrect diagnosis.

Limitations of Immunohistochemistry, General

Before we discuss the limitations of immunohistochemistry, we must state at the outset that there is no question that immunohistochemistry is an indispensable and necessary diagnostic aid, which is most useful when used in a context of resolving a specific histologic differential diagnosis, and in establishing histogenesis.

After recognizing histologic patterns in the hematoxylin and eosin (H&E)-stained sections, the differential diagnostic process is formed. Immunohistochemical staining's most important advantage is its ability to confirm, highlight, or clarify histologic patterns seen in the H&E-stained slides. In addition, immunohistochemical staining of individual cells in the different compartments (within structures or patterns) facilitates the identification of their origin, differentiation, and transformation. By such correlations, the pathologist becomes a functional morphologist, who can now understand and explain the mechanism of diseases.

The latest evidence on the limitations of immunohistochemistry is highlighted in a 2006 multi-institutional, collaborative study conducted by leading lymphoma laboratories in Europe (Germany, France, and Netherlands) and Canada.[62] These investigators studied 36 cases of DLBCL that originated from 6 different laboratories and were stained in 8 different laboratories using 8 common antibodies (CD20, CD10, bcl-6, bcl-2, MUM-1, MIB-1, CD5, and HLA-DR).[62] The authors performed a very careful review and critical analysis of their results, and reported the following findings[62]:

1. "Different laboratory staining techniques resulted in unexpectedly highly variable results and very poor reproducibility in scoring for almost all markers.
2. Importantly, no single laboratory stood out as uniformly poor or uniformly excellent.
3. Very poor agreement was found for bcl-6 and MIB-1.
4. With elimination of laboratory variations, high agreement was found for CD20, HLA-DR, and CD10."

On the basis of these results,[62] the authors concluded that "published results should be regarded critically and with caution."

One can extrapolate, therefore, that the results of the above study would have been significantly worse had the authors studied a larger sample (eg, 300 consecutive nonselected cases of Hodgkin and non-Hodgkin

lymphomas of B-cell and T-cell types, rather than only 36 cases of the most common lymphoma, namely DLBCL). Importantly, a larger study of lymphomas of different types would have necessitated use of at least 20 antibodies, rather than the 8 that they used, hence more adverse results.

Interpretation of immunohistochemical stains requires experience because of the lack of specificity and sensitivity associated with each antibody. The use of subjective classifiers such as "most cases," "usually positive or negative," "may be positive or negative," and "rarely positive or negative" to describe immunostaining patterns of specific lymphomas further confounds the interpretation of results and makes an accurate final diagnosis more difficult.

Interpretation of individual immunostains should be based on thorough knowledge of the staining pattern, that is, membranous, nuclear, cytoplasmic or Golgi zone, and also recognition and interpretation of both internal and external positive and negative controls. Importantly, because lymphoid cells of different types migrate from one compartment to another, and mixtures of different cell types are present in most compartments, it is necessary that the cellular details of positive and negative cells in each immunostain are clearly visible to accurately recognize and characterize these different cell types, regardless of whether or not they are malignant. To achieve this goal, adequate amounts of a counterstain (eg, hematoxylin) should be used in conjunction with the primary immunostain. This allows accurate interpretation of the cell size, shape, chromatin, and amount of cytoplasm in the positive and negative cells.

Also, artifacts commonly encountered during processing and antigen retrieval procedures may cause nuclear bubbling or lifting up of cells or an increase in intercellular spaces, and thus lead to misinterpretation of the findings. Other technical factors that may significantly influence immunostaining results and interpretation by introduction of artifacts are the fixatives used (B5, formalin, zinc formalin, others), duration of fixation, and prior freezing, processing, cutting, and staining. The common end-results of these variables are false-negative, false-positive, and/or nonspecific staining. A well-trained and experienced pathologist will take these artifacts into consideration and use experience and familiarity with the individual laboratory to exclude such changes, or to request a repeat of the stain, and/or additional stains.

Limitations of Flow Cytometry, General

Our experience has shown that flow cytometric analysis is very valuable, very sensitive, and superior to immunohistochemistry for the detection of light chain restriction and coexpression of CD5 by B cells. It is particularly useful in picking up very small clonal populations of B cells and revealing their phenotype. There are many other advantages to flow cytometry, but because of space limitations, we will not dwell upon them, other than to acknowledge that flow cytometry can be a valuable adjunct to histology in the differential diagnosis of lymphoid diseases in tissues.

To underscore the major limitations of flow cytometry as a stand-alone diagnostic tool, we summarize the flow cytometry results of selected literature published in 2006 and 2007 by senior, highly experienced, and recognized hematopathologists in the United States[63–66]

TABLE 3. Review of 2006 and 2007 Literature of Flow Cytometry on Lymphomas: Frequency of Errors if Diagnosis is Based on Flow Results Alone

Specific Disease Entity	Absence of Specific Marker or Absence of Typical Phenotype/Presence of Aberrant Phenotype or Phenotype Negated by FISH Results	Frequency of Error (Approximately) (%)	Reference Number
Precursor-T-lymphoblastic lymphoma/leukemia	TdT negative	18	66
	34 negative	68	
Precursor-B-lymphoblastic lymphoma/leukemia	TdT negative	4	
	34 negative	24	
MCL	Typical phenotype but t(11;14) negative by FISH with cytogenetic abnormalities of SLL/CLL present	32	64
	t(11;14) negative by FISH and no other cytogenetic abnormalities present	11	
	Aberrant phenotype (CD5 positive, CD23 positive, partial)	30	63
FL	CD10 negative	40	63
	Absence of light chain restriction in positive bone marrow biopsy	42	65
Lymphoplasmacytic lymphoma	CD5 positive	50	63
	CD23 positive	6	
	CD10 positive	6	
MALT lymphoma	CD5 positive	50	63
MZBCL	CD5 positive	32	63
	CD103 and CD11c/CD22 positive	3	63
Small lymphocytic lymphoma/ CLL	CD23 positive, partial or bright surface immunoglobulin	25	63

(Table 3). In all 4 studies[63–66] referenced in this table, flow cytometric results obtained from tissue sections, blood, and/or bone marrow were correlated with diagnoses based on histologic sections with or without immunophenotyping and/or FISH results. We concluded from the data we summarized in Table 3 that, if results of flow cytometry were used alone as a gold standard to make a final diagnosis, the overall error rate would be up to 68%, depending on the lymphoma type. The error rate for the more common B-cell lymphoma disease entities was 42% to 50% for FL, 25% for SLL, and 30% to 41% for MCL. MZBCL, MALT lymphoma, and lymphoplasmacytic lymphoma did not have a specific immunophenotype.[63–66]

In our diagnostic practice of lymph node pathology, which spans a period of 33 years and has involved the study to date of approximately 75,000 cases, we have found that when we compared morphology with or without immunohistochemistry to the flow cytometry results, there was discordance in at least 25% of cases (personal observation). In almost all instances (approximately 98%), we had to overrule the flow cytometry results and make the diagnosis on the basis of H&E morphology with or without the aid of immunohistochemistry.

General technical factors that influence flow cytometric analysis are in large part similar to those that affect immunohistochemistry. Specific critical issues are operator training and experience, correlation with clinical information, and review of specimen slides and touch imprints that dictate gating strategies. Because sampling and viability are also critical, correlation with morphologic examination of the tissue must be performed to ascertain that the pathologic process is adequately represented in the tissue submitted. If this correlation is not undertaken, it will lead to an increase in interpretative errors. This can be particularly important in benign diseases or Hodgkin lymphomas, wherein the flow cytometry results are typically negative.

To further emphasize the critical importance of this limitation, all flow cytometry reports contain a disclaimer, which states that the results obtained should be correlated with morphology, clinical information, and other ancillary tests performed.

Limitations of Cytogenetic/Molecular Studies, General

Molecular analysis of lymphomas is a powerful tool when used as part of an integrated, systematic approach, in correlation with morphology, immunohistochemistry, and clinical information.[3,6,7] The molecular results can confirm the diagnosis, can help in the precise classification of an entity, and can offer guidance in terms of treatment and prognosis.

However, molecular testing has many inherent limitations (Table 4). A positive result does not always equate with malignancy, just as a negative result does not always exclude it. False-positive results may be seen when there is contamination of the specimen, "pseudoclonality" in the case of small biopsies or few malignant cells, inflammatory or reactive processes (ie, viral infections, autoimmune disease), immune reconstitution after bone marrow transplant, immune response to malignancy, "clonal" dermatidites, etc. False-negative results, which can be seen in up to a sizeable proportion of B-cell lymphomas, may occur owing to sampling issues, tissue degradation or poor fixation, technical issues related to the molecular methodology, and even the biology of the neoplasm itself.[56] Thus, an abnormality detected by PCR may not correspond to an abnormal process detected by histologic examination and immunohistochemistry.

TABLE 4. Limitations of Molecular Testing in Lymphomas

1. False-positive gene rearrangement
   - Small biopsy samples having very few lymphoid cells
   - Infections
     - Bacterial—*H. pylori*
     - Viral—hepatitis
   - Autoimmune
     - Rheumatiod arthritis
     - Sjogren syndrome
   - Other—"clonal dermatidities," host clonal T-cell response to lymphoma
     - Postbone marrow transplantation repopulation
     - Qualitative PCR methods; canonical T-cell receptor gamma chain rearrangements involving Vγ9 segments
   - Highly sensitive qualitative PCR methods detects very low levels of translocations in normal individuals:
     - t(14;18) seen in FL
     - t(2;5) seen in ALCL
     - inv(2) seen in ALCL
2. False-negative gene rearrangement
   - Degradation of DNA or inadequate DNA extractions from paraffin sections
   - Technical issues using very few primers (single V-regions, CDR III only upstream primers)
   - "Malignant cells," very few admixed with many polyclonal B cells
3. Inherent limitations of the PCR Method
   - Poor primer selection
4. Variability of specific recurrent balanced translocations in specific lymphomas entities with distinctive patterns
   - t(14;18) negative in 88% of pediatric FL [67]
   - t(14;18) negative in 5-30% of adult FL [68,69]
   - t(11;14) negative in 22% of MCL [70]
   - t(11;18) negative in 84% of MALT lymphoma[71]
   - t(14;18) negative in 82% of MALT lymphoma[72]
   - t(1;14) negative in 95% of MALT lymphoma[73]
   - t(3;14) negative in 91% of MALT lymphoma[74]

The presence of the t(14;18) translocation is a hallmark of FL occurring in adults (Table 5). Some investigators believe that the absence of this translocation argues against a diagnosis of FL, or that it represents a different type of FL. Many of the FL in adults that are t(14;18) negative exhibit bcl-6 gene rearrangements. In routine diagnostic practice, we make diagnoses of FL on the basis of histologic features and in conjunction with immunohistochemistry, without requiring FISH studies to confirm the presence of the t(14;18). Similarly, we also make the diagnosis of FL even though the t(14;18) is absent by FISH. As shown in Table 5, in children, the t(14;18) translocation is absent by FISH in 88% of the cases.

In MALT lymphomas, the 4 commonly reported, recurrent, balanced translocations are absent in anywhere from 82% to 95% of the cases (Table 5). Thus, most

TABLE 5. Pivotal Role of Morphology When Immunophenotype Analysis and Molecular Studies are Negative, Aberrant, or Equivocal

Diagnosis	Immunophenotype Analysis Results		Molecular/FISH Results
	Flow Cytometry	Immunohistochemistry	
1. Benign disease	Nonspecific	Nonspecific	Negative
2. Follicular			
Children	Not always specific	Negative bcl-2 in 70% of cases[67]	Negative t(14;18) in 88% of cases[67]
Adult	Not always specific	Negative bcl-2 in 3%-26% of cases[75]	Negative t(14;18) in 5%-30% of cases[68,69]
3. Mantle cell	Negative CD5 in < 5% of cases[76]	Negative CD5 in 10% of cases[76,77]	Negative t(11;14) in 22% of cases[70]
		Negative cyclin d1 in 5% of cases[78–80]	
		Negative CD23 in 90% of cases[81,82]	
4. MZL/MALT	Nonspecific	Nonspecific markers	Negative t(11;18) in 84% of cases[71]
		Negative light chain restriction in malignant marginal zone cells with monocytoid features in 85%-90% of cases[83]	Negative t(14;18) in 82% of cases[72] Negative t(1;14) in 95% of cases[73] Negative t(3;14) in 91% of cases[74]
5. SLL/CLL	CD23 rarely negative	Negative CD5 in 20% of cases[84]	
6. Hodgkin			
Nodular LP	Negative	CD30 focally positive in 20% of cases[85–87] CD15 positive in few cells in 3% of cases[86] EMA is positive in 50% of cases[1,85]	Negative
Classic	Negative	Negative CD15 in up to 30% of cases[88,89] Positive CD30 in benign large cells and other lymphomas	Negative

MALT lymphomas do not show any of these 4 translocations. We still make a diagnosis of MALT lymphoma on the basis of morphologic features alone or in conjunction with immunohistochemistry, even though this lymphoma does not have a specific immunophenotype.

In 2007, 2 abstracts presented at the annual meeting of the United States and Canadian Academy of Pathology/International Academy of Pathology reported 12 new translocations in MALT lymphoma, bringing the number of known, recurrent translocations to 16, of which 14 are shown in Table 6.[90–92] Do these 16 reported translocations, then, represent 16 different types of MALT lymphomas?

An additional abstract presented at this meeting reported numerous chromosomal gains and losses in ocular adnexal MALT lymphomas using array-based comparative genomic hybridization technique.[90] These results suggest that modern medicine has only just scratched the surface of the very large iceberg of tumor biology.

Furthermore, amplification of specific recurrent balanced translocations is only useful in a minority of the distinct disease entities in the WHO classification (Table 5). Indeed, rare cells containing recurrent balanced translocations that have pathologic significance may be found in normal, healthy individuals by highly sensitive techniques such as PCR.[67,75,93,94] In these cases, it is of paramount importance to interpret the results in the context of morphology, immunophenotype, and clinical information to arrive at a correct diagnosis.

The limitations of these ancillary techniques are underscored by the fact that lymphomas are occasionally discovered as incidental, unexpected findings in lymph nodes removed at the time of surgery for other malignancies (eg, carcinoma, etc) or other benign diseases or conditions (eg, carotid endarterectomy). In such cases, fresh tissue for flow cytometry, genomics, and proteomics will not have been triaged, and so will not be available. In all the above instances, a diagnosis or a differential diagnosis must be formulated on the basis of histologic features, which subsequently dictate the additional tests and diagnostic work-up that should be done.

The disclaimer attached to flow cytometry reports similarly accompanies molecular study reports, to ensure correlation of the results in all cases with the histologic

TABLE 6. Genetic Heterogeneity of MALT Lymphoma

Gains*	Losses*	Translocations†
1p21.1	2p16.2	t(14;18)/IGH-MALT1
1p12	2q34	t(11;18)(q21;q21)
3q13.31	2q37.3	t(3;14)(p14.1;q32)
3q26.31	4q12	t(1;14)(p22;q32)
6p	4q21.23	t(3;4)(q21;p16)
6p25.3	4q28.1	t(5;17)(p11;p11)
8p23.2	4q31.1	t(1;3)(q32;p14.2)
8p21.2	8q23.3	t(2;19)(p15;q13.4)
18q	6q25.3	t(1;22)(q11;p11)
19q13.2	7p14.3	t(X;6)(q22;q13)
19q13.31	7p21.1	t(5;14)/IGH-OD22
22q11.21	7q36.3	t(1;14)/IGH-CNN3
	10q11.21	t(9;14)/IGH-JMJD2C
	11q13.1	Translocations involving 6p
	11q24.2	
	13q34	
	16q12.1	
	17p13.3	
	19p13.2	

*Ref. 90.
†Refs. 91, 92.

and immunophenotypic findings. The results of such a test should be interpreted in the context of all clinical and pathologic findings and should not be used as the sole criterion for diagnosing malignancy.

Theoretical Limitations of the Use of Genomics and Proteomics in Routine Diagnostic Practice

Before new diagnostic criteria for lymphoid diseases based on genomics and proteomics can be routinely used in diagnostic practice, it will be necessary to undertake large, retrospective, and prospective diagnostic trials that would examine thousands of consecutive patient biopsy samples from all types of lymphomas and benign lymphoid diseases, occurring at nodal and all extranodal sites. These studies will determine whether or not the new technologies can improve upon the morphologic classification systems, which have been independently established and verified by the mature technologies of immunohistochemistry, cytogenetics, and conventional molecular techniques. The results of these studies will answer the question of whether or not histologic examination will retain its current diagnostic role, or will morphology become merely a triage tool to determine what genomic and proteomic tests to undertake to reach a final diagnosis.

An important question that often arises is what the absence of a specific genetic signature means. We know that lymphomas and benign lymphoid diseases can show focal or multifocal pathology. Also, in some lymphomas, such as Hodgkin lymphoma, the malignant cells are in a minority. How will the genetic signature of these neoplastic cells be appreciated in the background of reactive lymphocytes? Furthermore, what are the genetic signatures of the distinctive benign diseases that typically show multiple pathologies?[95–97] In all such cases, histologic evaluation and diagnosis would be mandatory.

Currently, there is no commercially available "lymphoma chip" that is adequate to be used as a diagnostic tool. However, genomic arrays have been used as research tools for many years, and have provided specific genetic signatures for many distinct lymphoma entities. Some of the subtypes and variants of lymphomas do not have specific genetic signatures that have been characterized to date. For example, the WHO classification[1] lists 2 subtypes of DLBCL (intravascular and primary mediastinal), and 6 morphologic variants: centroblastic, immunoblastic, T-cell/histiocytes-rich, anaplastic, plasmablastic, and full-length ALK-positive. Gene arrays, although providing significant advances in prognostic information, only resolve the heterogeneous category of DLBCL into 2 subtypes: germinal center B-cell–like type and activated (postgerminal center) B-cell–like type. The former roughly corresponds to the centroblastic variant, with an improved prognosis, whereas the latter encompasses the other 5 histologic variants. The primary mediastinal DLBCL have a genetic signature different than that of the germinal center and postgerminal center cell types.

Many lymphomas and benign diseases exhibit a wide histologic spectrum, and they frequently have atypical features. Whether or not such cases have the typical genetic signatures found in the garden-variety examples of many lymphomas remains to be determined. Moreover, we do not know how these genetic signatures will present in small biopsies, crushed samples, necrotic samples, focal lymphomas, incidentally discovered lymphomas, benign diseases, and all the myriad suboptimal biopsies that are evaluated every day. There is no question that morphology will remain an integral part of the diagnostic process. At the bare minimum, morphology will be required to dictate the subsequent diagnostic testing that will be necessary to establish the most accurate diagnosis.

The Value of Histologic Diagnosis in the Face of Equivocal Immunohistochemical and Molecular Studies, as Relates to Specific Lymphomas

Follicular Lymphoma

Typical Histology

In 80% of FL, initial inspection at low magnification will reveal poorly defined follicles are present throughout, are closely packed and may show a "back-to-back" arrangement.[60] Most frequently, benign mantle zones are conspicuously absent, but when they are present, they are thin and form incomplete rings around the follicles.

Cytologically, at high magnification, the follicles show few mitotic figures and starry-sky phagocytes and they lack prominent polarity. Also, the follicle center cells are monomorphic with respect to their size, shape, and chromatin structure, although they may show a wide pleomorphism in nuclear clefting and angulation. Small centrocytes constitute more than 95% of the cells within the follicles in about 50% of FL.

The 5 expert pathologists who participated in the International non-Hodgkin Lymphoma Study[55] were able to correctly identify FL in 93% of cases based solely on histologic features. After reviewing the immunophenotype on these cases, their agreement with the consensus diagnosis increased by only 1% (Table 2).[55] This information highlights the marked importance of morphology, and shows that for the diagnosis of FL, immunophenotype was of little additional value. As FL is the most common (or second most common, depending on the geographic area) lymphoma in the Western world, these results further underscore the importance of histology, not only for the formulation of a differential diagnosis, but also for resolution of a definitive diagnosis.

Typical Immunophenotype

FL cells are positive with CD20, CD10, bcl-6, and bcl-2, and show follicular dendritic reticulum cells (FDRCs) in the CD21 stain. However, this typical profile is absent in up to 40% to 50% of cases.[98,99]

Frequency of the Absence of a Typical Immunophenotype or Presence of an Aberrant Immunophenotype in Follicular Lymphoma

Absence of a typical immunophenotype or the presence of an aberrant one in FL is common, and this is emphasized throughout the discussion. In such instances, the morphology can play a critical role in helping make a correct diagnosis. This is particularly true for FL in children, where the bcl-2 protein overexpression is absent in 70% (30% are positive by immunostaining) of cases and the t(14;18) translocation is absent in 88% of pediatric cases.[100] Moreover, when the FL morphologically resembles one or more of SLL/chronic lymphocytic leukemia (CLL), MCL, MZBCL, or follicular hyperplasia, one or more of the phenotypic markers (CD10, bcl-6, CD5, CD23, and bcl-2) may be aberrantly negative or positive, as discussed below.

κ and λ *light chain*: although κ and λ stains are most useful in detecting monotypic populations, they are difficult to interpret because of difficulties in distinguishing background staining and nonspecific uptake from true positivity. In most laboratories, monotypic light chain expression is detected in only up to 30% of cases.

bcl-2: in adult FL grade 3, bcl-2 is negative in up to 26% of cases, in grade 2 in up to 17% of cases, and in grade 1 in up to 3% of cases.[101] Follicular hyperplasia is typically bcl-2 negative. In contrast, in children the bcl-2 protein expression is absent in up to 70% of cases of FL (positive in only 30% of cases).[100] Benign T cells are bcl-2 positive and are always found within follicles and sometimes they can be numerous. Also, benign mantle cells and benign marginal zone cells are bcl-2 positive and they can also be found within the follicles, and therefore they can be misidentified as malignant centrocytes. Other lymphomas and nonhematopoietic neoplasms are also frequently bcl-2 positive.

CD10: CD10 is not very sensitive for FL and it may be negative in up to 50% of the cases.[98,99] Benign follicular center cells are also CD10 negative in up to 68% of the cases.[102] Moreover, CD10 is positive in almost all B-lymphoblastic and often T-lymphoblastic lymphoma, diffuse follicular center cell lymphomas grade 1 and 2, DLBCL, AILT, occasionally in peripheral T-cell lymphoma, and other tumors, including nonhematologic malignancies.[103]

bcl-6: bcl-6 is negative in 25% of cases of FL and follicular hyperplasia.[99] It is also positive in some DLBCL and transformed MZBCL.[104] Moreover it is positive in T-lymphoblastic, often in AILT, and occasionally in peripheral T-cell lymphoma and also other tumors.[68,69,105,106]

CD21: FDRCs can be CD21 negative in less than 5% of cases within follicles, regardless of whether or not they are malignant. Some membranous CD21 expression is also seen in the malignant cells in less than 50% of cases.[99]

CD23: similar to CD21, the malignant cells may show membranous staining in up to 88% of cases.[77] FDRCs have elongated cytoplasmic processes that are positive using CD23 in up to 70% of the cases. A subset of benign mantle cells is also positive for CD23.[76]

CD5: rare cases of FL may show aberrant CD5 positivity.[107]

CD43: CD43 is positive in 1% to 6% of FL.

Limitations of Cytogenetic/Molecular Studies in Follicular Lymphoma

The t(14;18)(q32;q21) translocation or the bcl-2 gene rearrangement may occur in early B cells in a small percentage of normal, generally elderly, individuals and can be detected in very few cells in the blood.[93,94,108]

In adults, the t(14;18)(q32;q21) bcl-2 gene rearrangement is absent in anywhere from 5% to 30% of the cases,[109,110] whereas in children it is absent in up to 88% of the cases.[100]

The problems regarding false-positivity and false-negativity of molecular studies and the frequency of cytogenetic abnormalities in all the specific entities described are presented in Tables 4 and 5.

Mantle Cell Lymphomas

Typical Histology

Seventy percent of MCL will typically exhibit one or more of the following patterns: mantle zone, mantle cell nodular, follicular colonization (benign germinal centers partially or completely replaced by malignant mantle cells), fusion of adjacent mantle zones, starry-sky pattern produced by epithelioid cells, and hyalinized vascular pattern. A completely diffuse pattern is present in only 20% of cases. In most cases, more than 1 pattern is present.

A *mantle zone* pattern is produced when the mantle zones become thickened and expanded as a result of mantle cell proliferation. If this expansion continues outwards, the mantle zones of adjacent follicles may undergo fusion, resulting in focal or multifocal diffuse areas.

During the process of mantle cell proliferation and mantle zone expansion, islands of mantle cells become separated from the mantle zones and form distinct mantle cell nodules, resulting in a *mantle cell nodular* pattern. If these enlarge and coalesce, they may form focal or multifocal diffuse areas.

When the mantle cell proliferation extends inwards, the benign follicular centers will become partially or completely (massively) infiltrated by malignant mantle cells, resulting in a *follicular colonization* pattern. In this situation, the benign follicular centers will contain benign centroblasts and centrocytes and malignant mantle cells in varying proportions. Partially colonized follicles may resemble those of FL grades 1 or 2, depending on the number of residual benign centroblasts present. Completely colonized follicles, however, may be hard to differentiate from mantle cell nodules, because both will appear as 1-layered spherical structures. In addition, FL grade 1 with absent benign mantle zones will appear as 1-layered spherical structures. CD21 immunostain is helpful in these cases to highlight FDRCs, and CD10,

bcl-6, bcl-2, CD5, and bcl-1 will aid in establishing a definitive diagnosis.

Approximately 20% of MCL will show a subtle "starry-sky" pattern produced by scattered large epithelioid cells with eosinophilic cytoplasm containing minimal cytoplasmic debris. This pattern should be differentiated from the starry-sky pattern seen in Burkitt-, Burkitt-like– and lymphoblastic lymphomas, which is produced by benign histiocytes with abundant pale to clear cytoplasm containing prominent debris. Cases of blastic MCL will most often be diffuse and may have a prominent starry-sky pattern.

Approximately 20% of MCL will show markedly hyalinized small vessels and capillaries, most of which show obliterated lumens, producing a *hyalinized vascular* pattern.

A minority of MCL will show a completely *diffuse* pattern, characterized by the absence of any other identifiable, residual patterns. In this situation, immunostaining will be able to highlight the presence or absence of residual distinctive patterns as described above.

Cytologically, the malignant mantle cells are typically of small to medium size with slight to moderate nuclear irregularities, open nuclear chromatin structure, small, inconspicuous nucleoli, and scant cytoplasm, resulting in a hypercellular appearance at low magnification. Mitotic figures are frequent. Numerous mitotic figures and/or blastic morphology are associated with a more aggressive behavior and poor outcome. Proliferative activity highlighted in the Ki-67 (MIB-1) stain correlates well with prognosis: a low proliferative activity is associated with a good prognosis, whereas a high proliferative activity is associated with a poor prognosis.[81]

Typical Immunophenotype

The malignant mantle cells are positive with CD20, CD5, bcl-1 (cyclin d1), bcl-2, CD43, IgM, and IgD but negative with CD3, CD23, CD10, and bcl-6.[1]

The benign germinal centers are highlighted well in the Ki-67 (MIB-1) stain, showing high proliferative activity. They are positive with CD10 and bcl-6 and negative with bcl-2. The CD21 stain is positive in the cytoplasmic processes of benign FDRCs in the benign follicles and follicles that are partially or completely colonized by malignant mantle cells. The FDRC meshwork is distorted in the colonized follicles in contrast to the normal FDRC meshwork in the benign, noncolonized follicles. Together, these stains are valuable in establishing the diagnosis of a MCL.

Frequency of the Absence of Typical Immunophenotype or Aberrant Immunophenotype in Mantle Cell Lymphomas

CD5 can be negative in paraffin sections in up to 10% of the cases of MCL.[78,82] CD5 negative cases of MCL may be difficult to distinguish from FL, because the CD10 can be negative in up to 50% and the bcl-6 can be negative in up to 25% of FL.[79,102] CD5 is also positive in SLL and can be positive rarely in FL and/or marginal zone lymphoma.[80,107,111,112] Thus, relying on immunostains alone in such cases may lead to diagnostic errors.

CD23 is usually negative in MCL, but can be positive in a small percentage of cells.[113,114]

CD10 is rarely positive in MCL.[107]

Cyclin d1 using older polyclonal antibodies is negative in up to 30% of MCL. New monoclonal antibodies are much more sensitive, and cyclin d1 negativity is seen in approximately 5% of cases.[70,115,116] Rarely, MZBCL can be CD5 and/or cyclin d1 positive.[80,117] Cyclin d1 positivity has also been reported in multiple myeloma, hairy cell leukemia, and SLL/CLL.[83,118,119]

Limitations of Cytogenetic/Molecular Studies

Cyclin d1 was absent in up to 22% of MCL using quantitative, real-time PCR in 1 study, and these cases were also t(11;14) negative by FISH.[120] Moreover, cyclin d1 overexpression has been found to be absent in up to 15% of MCL diagnosed by morphologic criteria.[121] Gene expression profiling has also confirmed that 6% of MCL do not express cyclin d1 mRNA, and lack the characteristic t(11;14) translocation by FISH. These cyclin d1-negative MCL have identical genetic expression signatures to those that are cyclin d1 and t(11;14) positive,[122] and have similar prognosis.

Marginal Zone B-cell Lymphoma

Typical Histology

MZBCL is the third most common non-Hodgkin lymphoma (9%).[123] In typical examples of various other non-Hodgkin lymphomas, the malignant component predominates. In contrast, however, the morphology of a typical MZBCL is distinctive and unlike all other non-Hodgkin lymphoma, because of the presence of a prominent benign component, which often predominates. In fact, extranodal MZBCL of MALT type have often been classified as pseudolymphomas because the benign component usually overshadows the malignant component.

Benign Component

The benign component may consist of naked germinal centers, benign follicles surrounded by well-defined benign mantle zones, benign monomorphic mantle cell nodules, plasma cells, neutrophils, and epithelioid cell clusters. The presence or absence and the prominence of the above-mentioned structures and the cell types vary from one case to another.

Specifically, benign follicles may consist of small and/or large, naked germinal centers, that may be well to poorly defined and may represent the predominant finding. When they are closely packed together, they may resemble the malignant follicles of a FL.

When the benign follicles are surrounded by well-defined mantle zones, these may be thick. In addition, as the mantle zones become thicker and grow outwards, they become confluent and form monomorphic mantle cell nodules that vary in number.

Benign plasma cells can be numerous and occasionally form clusters and islands in the lamina propria of mucosal sites, or in nodal and extranodal sites.

Few to numerous neutrophils may be found within clusters of marginal zone B cells. Neutrophils tend to be more common in the nodal cases, especially when the marginal zone B cells have monocytoid features and resemble benign monocytoid B cells.

Epithelioid cell clusters are occasionally seen and may rarely be prominent.

Thus, being aware of the spectrum of the above-mentioned benign component helps in distinguishing it from the malignant component.

Malignant Component

The malignant component varies from focal to prominent and always consists of marginal zone B cells with less than 20% admixed transformed cells that are scattered and do not form islands or clusters. Approximately one third of the cases may show malignant plasmacytoid forms.[1] In a minority of cases, the neoplastic component predominates. A completely diffuse pattern is seen in only 10% of cases of MZBCL.

The marginal zone B cells are 1.5 to 3 times the size of small lymphocytes. On one end of the cytologic spectrum they have round to slightly irregular nuclei with scant cytoplasm, and thus resemble mantle cells or centrocytes. At the other end of the spectrum, as the neoplastic cells acquire more cytoplasm and form clusters and islands of clear cells, they appear as pale staining areas and thus are easy to recognize at low magnification. Also, on the basis of the pale color of the clear cells, the presence of these cells in the different compartments can be readily identified at low magnification.

The malignant marginal zone B cells organize themselves to form one or more distinctive patterns.[3] When they surround naked germinal centers as an outer second layer, they will produce an *inverse follicular* pattern. Similarly, when they surround monomorphic mantle cell nodules as a second outer layer, they will also produce an *inverse follicular* pattern. When the marginal zone B cells surround normal follicles with benign mantle zones, as a third, outer layer, they will produce a *marginal zone* pattern. As the marginal zone B cells extend outwards into the interfollicular areas, they form clusters and islands, which often become confluent and result in an *interfollicular* pattern. The marginal zone B cells often distend the subcapsular and trabecular sinuses, and produce a *sinus* pattern.

Besides growing outwards, the marginal zone B cells may grow inwards into the follicles and produce either partial or complete *follicular colonization*.[124–126] Again, the pale staining abundant cytoplasm resulting in a clear cell appearance facilitates their identification within the follicles and all other compartments where they may be present. When the colonization of follicles is complete and the follicles do not have any mantle zones surrounding them and are closely packed together, they resemble the neoplastic follicles of a FL.

Plasmacytoid Differentiation

Plasmacytoid differentiation manifests itself in multiple ways. For example, the marginal zone B cells with monocytoid features may have moderate to abundant deeply staining cytoplasm, and thus appear plasmacytoid. In addition, there may be admixed lymphoplasmacytic cells and plasma cells. Moreover, Dutcher bodies and Russell bodies may be evident. The neoplastic, monotypic plasma cells are usually seen as clusters and/or sheets admixed with marginal zone B cells or at their periphery. Interestingly, these monotypic plasma cells can be seen within the follicles, occasionally forming clusters.

Typical Immunophenotype

Unlike other B-cell non-Hodgkin lymphomas, there is *no specific phenotype* that characterizes MZBCL. Typically, malignant cells are positive with CD20 and negative with CD10, CD5, and CD23, but this profile is not definitive. There is also considerable overlap among MALT, nodal and splenic MZBCL.[1]

Frequency of the Absence of a Typical Immunophenotype or Presence of an Aberrant Immunophenotype in Marginal Zone B-cell Lymphoma

κ and λ are positive in only 10% to 15% of cases when the malignant marginal zone B cells show scant or abundant pale staining cytoplasm.[126] In contrast, when malignant cells show plasmacytoid differentiation, light chain restriction is almost always demonstrated.

CD43 coexpression by malignant cells is seen in up to 40% of MALT-type and nodal MZBCL, but is usually absent in the splenic variant.[127–129] CD43 positivity is nonspecific, and can be present in small lymphocytic, mantle cell, and rarely FL as well as in neoplastic marginal zone B cells with plasmacytoid features. Furthermore, CD43 can be positive in other B-cell lymphomas, most T-cell lymphomas, myeloid leukemias, and benign T cells. Interestingly, benign marginal zone cells in patients with chronic sialadenitis or Sjogren syndrome can also be CD43 positive.[127,128,130]

IgD is expressed by the malignant cells of adult splenic MZBCL and in 27% of adult nodal MZBCL, but is typically absent in MALT-type MZBCL.[1,129,131] However, in children and young adults, IgD was expressed in 26% of nodal MZBCL and in 20% of MALT lymphoma.[74,100,132]

bcl-6 protein can be rarely positive in MALT lymphomas that have transformed into a DLBCL,[72] but is negative in low-grade cases.[79]

CD21 and CD35 have been reported to be positive in the neoplastic cells in rare cases of MZBCL.[1]

Limitations of Cytogenetic/Molecular Studies

Several recurrent balanced translocations have been described for cases of MZBCL.[71,73,90–92,133–135] The t(11;18) translocation is negative in up to 84% of cases depending on the site of involvement,[136] the t(14;18)(q32;q21) is negative in up to 82% of cases,[135] the t(1;14) is negative in up to 95% of cases,[137] and the

t(3;14) is negative in up to 91% of cases.[134] Prevalence of the 12 newly reported, recurrent translocations[90–92] has not yet been established in any large series.

Small Lymphocytic Lymphomas/Chronic Lymphocytic Leukemia

Typical Histology

In a typical case of SLL/CLL, the most important feature is the presence of pseudofollicles creating a pseudofollicular pattern that is readily identifiable at low magnification. In the WHO classification,[1] the pseudofollicles have been termed "proliferation centers." The lymph node sinuses in typical cases are obliterated, reactive follicles are absent, and there is pericapsular lymphomatous infiltration.

Proliferation centers (pseudofollicular pattern) are seen, in our experience, in 99% of SLL/CLL and in 25% of SLL with plasmacytoid features (lymphoplasmacytoid lymphoma). Proliferation centers have a distinctive pattern and cytologic features that are not seen in any other benign lymphoid disease or lymphoma. The proliferation centers are pale staining, small to medium sized, one-layered, poorly defined, spherical ball-like structures that are separated from each other. The pale staining color of the proliferation centers is due to loosely packed malignant cells that are separated by clear spaces.

Cytologically, within most proliferation centers, there are predominantly small cells with a few medium and large ones admixed. All small lymphoid cells have round nuclei and generally scant cytoplasm. The medium and large cells have an open chromatin structure. The pathognomonic cytologic feature of the malignant cells within the proliferation centers is the presence of 1 prominent nucleolus in some of the small, medium, and large lymphoid cells, which are known as prolymphocytes by us or paraimmunoblasts by others.

In addition, some medium and large lymphoid cells with multiple small nucleoli are admixed within the proliferation centers. Importantly, mitoses are absent to rare within the proliferation center, starry-sky phagocytes are not present, and FDRCs are rare. All of these features distinguish the proliferation centers from benign and malignant follicles.

In between the proliferation centers, there is a monomorphic proliferation of small, round, densely packed malignant lymphocytes with scant cytoplasm, and dark blue color that contrasts with the pale color of the proliferation centers. These small cells are derived from the same clone as those present within the proliferation centers.

Similar to other malignant lymphomas, SLL/CLL shows a spectrum of architectural patterns and cytologic features. For example, the proliferation centers may be closely packed together (back-to-back arrangement) and thus mimic a FL. In the early phases of SLL/CLL, the sinuses are patent and variable numbers of reactive follicles are seen. Thus, the proliferation centers (pseudofollicles) are seen in the interfollicular areas, or they may surround reactive follicles and produce inverse and/or marginal zone patterns, and thus resemble a MZBCL. When nuclear irregularities are prominent in the malignant cells, a differential diagnosis that includes FL and/or MCL is entertained. However, in all of these cases, a subtle or overt *pseudofollicular* pattern is still present, and malignant prolymphoblasts/paraimmunoblasts (pathognomonic cells of SLL/CLL) are seen within the proliferation centers, pointing to the correct diagnosis.

Typical Immunophenotype

The malignant lymphocytes in SLL/CLL are typically positive with CD20, CD5, CD43, bcl-2, and CD23, and may exhibit light chain restriction. They are negative with CD10 and cyclin d1. Some cases that have unmutated immunoglobulin variable region genes may be positive with CD38.[84,138,139] Zap-70 positivity is also associated with an unmutated immunoglobulin heavy chain variable region gene and more aggressive disease. Zap-70 may be a better predictor of the need for treatment.[84,140–142]

Frequency of the Absence of a Typical Immunophenotype or Presence of an Aberrant Immunophenotype in Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphomas

CD23 is negative or partially positive in up to 15% of cases.[98,114] Also, some cases of MCL partially express CD23, thus assessment of cyclin d1 expression is essential in $CD5^+$ and $CD23^-$ cases.[113]

CD5 is negative by paraffin immunohistochemistry in up to 20% of cases.[143]

κ and λ are negative by immunohistochemistry in up to 95% of cases of CLL/SLL.[1]

Cyclin d1 is positive in up to 14% of cases.[144,145]

CD43 coexpression is absent in up to 10% of cases; however, CD43 coexpression is not specific to this entity, and is positive in MCL and up to 6% of FL.

bcl-2 is negative in up to 5% of cases.

Zap-70 is difficult to reproduce in tissue immunohistochemistry and there are many unresolved methodologic issues by flow cytometry.[146]

Limitations of Cytogenetic/Molecular Studies in Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphomas

Up to 20% of cases can be negative when examined by FISH analysis.[1] Trisomy 12 is seen in up to 16% of cases and correlates with atypical histology and a more aggressive course[147,148] Also, deletions at 13q14 are seen in up to 54% of cases, deletions at 11q22-23 in up to 23%, and deletions at 6q21 and 17p13 (p53 locus) in 5% and 12% of cases, respectively.[148–150] The marked heterogeneity of cytogenetic abnormalities and the high number of cases with normal karyotypes may confound diagnostic interpretation in the absence of morphologic correlation.

Anaplastic Large Cell Lymphoma of T-cell and Null Cell Type

Typical Histology

The malignant cells in ALCL can be found in various compartments. When they are present in the interfollicular areas, the malignant cells can form multiple, large, compact, and occasionally confluent clusters, islands, and/or sheets, producing an *interfollicular* pattern. The malignant cells often form a prominent *perivascular* pattern and they can also be found distending the subcapsular and trabecular sinuses, producing a *sinusoidal* pattern. In a typical case, a few residual reactive follicles are found scattered throughout the node. However, some cases may show a *diffuse* pattern.

Cytologically, although the malignant cells show a spectrum of appearances, typical "hallmark cells" are found in most cases. These cells are large and have reniform, horseshoe-shaped or doughnut-shaped nuclei that contain one to multiple nucleoli. The cytoplasm is abundant and eosinophilic, basophilic, or clear. Often a prominent paranuclear Golgi zone is seen. Sometimes tumor giant cells are present, which contain multiple nuclei arranged in a wreathlike pattern.

At least 3 common morphologic variants of ALCL have been described[1]:

a. The common variant comprises 70% of ALCL, and contains the cells described above, but sometimes malignant cells with round nuclei are admixed.
b. The lymphohistiocytic variant comprises 10% of ALCL and has rare malignant cells that may be overlooked among numerous benign histiocytes.
c. The small cell variant comprises 5% to 10% of ALCL and has malignant cells of medium size with irregular nuclei, but a few, large, typical, hallmark cells are often present.

Typical Immunophenotype

The malignant cells are positive with CD30. Most cases are positive with epithelial membrane antigen (EMA), CD2, CD43, CD4, CD25, clusterin, TIA-1, granzyme B, and perforin. The ALK-1 protein is positive in 60% to 85% of cases.[1] CD3, CD5, CD7, and CD8 are usually negative. Epstein-Barr virus encoded small RNA (EBER) in situ hybridization is always negative. The CD45 may be positive. CD15 is rarely positive, and if positive only a few malignant cells are positive. ALK-negative cases are not a distinctive clinicopathologic entity.

Frequency of the Absence of a Typical Immunophenotype or Presence of an Aberrant Immunophenotype in Anaplastic Large Cell Lymphoma

CD30 must be positive in the membrane and in the Golgi zone. Diffuse cytoplasmic staining must be interpreted as nonspecific. Smaller tumor cells often stain very weakly or may be negative with CD30. Also, there are rare morphologically characteristic cases of ALCL that are CD30 negative.

ALK is negative in 15% to 40% of cases.[1] Because these cases are CD30 positive, a differential diagnosis with CD30-positive peripheral T-cell lymphomas arises. The ALK-negative cases do not represent a prognostically distinct disease entity. Inflammatory myofibroblastic tumors are often ALK-1 positive.

EMA is positive in the majority of cases of ALCL, with a staining pattern similar to that of CD30. Frequently, only a small subset of malignant cells is positive with EMA, and some cases may be negative.

CD3 is negative in up to 75% of cases,[1,151] thus recognition of hallmark cells becomes of paramount importance in guiding the diagnostic process (ie, ordering a CD30 stain). Very frequently cases of ALCL will be negative with all T-cell markers, and T-cell lineage must be demonstrated at the genetic level.

CD43 is negative in up to 33% of cases, and is not a lineage-specific antigen.[1]

Limitations of Cytogenetic/Molecular Studies

Up to 10% of cases of ALCL can be negative for clonal rearrangement of the T-cell receptor genes, regardless of T-cell antigen expression.[152]

The most frequent recurrent balanced translocation seen is t(2;5)(p23;35), present in 70% to 80% of cases.[153] Variant translocations involving ALK and partner genes on chromosomes 1 (10% to 20%), 2 (2% to 5%), 3 (2% to 5%), and 17 (2% to 5%) have also been reported.[154]

In situ hybridization for the t(2;5) fusion alone will be negative in up to 33% of cases.[155,156] The use of ALK break-apart probes, or specific fusion probes that are designed to detect the variant translocations and the classic t(2;5)(p23;35), will significantly decrease the frequency of a false-negative test result.[157]

Precursor B and T-lymphoblastic Lymphoma/Acute Lymphoblastic Leukemia

Typical Histology

Morphologically, B and T-lymphoblastic lymphomas [pre-B and pre-T acute lymphoblastic leukemia (ALL)] cannot be distinguished from each other. Lymphoblastic lymphomas typically show a diffuse pattern. The proliferation of lymphoblasts is hypercellular and frequently shows extensive infiltration of pericapsular fat and/or blood vessel walls. The blood vessels may also show tumor emboli. A "single file" arrangement of malignant cells may be seen focally. When a few scattered reactive follicles are seen, an interfollicular pattern is present. A focal to prominent starry-sky pattern, owing to numerous tingible-body macrophages, may be seen in up to 25% of cases.

Cytologically, the blasts are monotonous in size, chromatin structure, and amount of cytoplasm. The lymphoblasts are typically of medium size, with round to convoluted nuclei, fine to delicate nuclear chromatin structure, absent to inconspicuous nucleoli, and scant cytoplasm. Coarse azurophilic granules are present in some lymphoblasts in approximately 10% of cases.[1] Usually there is a very high mitotic activity.

Because lymphoblastic lymphoma/leukemia has a blastic, fine chromatin structure, a differential diagnosis arises that includes blastic MCL, acute myeloid leukemia, and blastic NK-cell lymphoma.

Typical Immunohistophenotype

B-lymphoblasts are positive with CD22, CD10, and CD79a. CD10 is usually negative when the t(4;11) translocation is present.[1] Lineage nonspecific antigens that are positive in B-lymphoblasts are TdT, CD34, CD38, and CD45. B-lymphoblasts can also be positive with myeloid associated antigens such as CD13 and CD33 but are frequently negative with CD20 and CD45.

T-lymphoblasts are positive with TdT and in most cases with CD7 and cytoplasmic CD3. T-cell markers such as CD1a, CD2, CD4, and CD8 are variably expressed. As in B-lymphoblastic leukemia/lymphoma, CD79a, CD10, CD13, and CD33 may be positive.

Frequency of the Absence of a Typical Immunophenotype or Presence of an Aberrant Immunophenotype in Precursor B or T-lymphoblastic Lymphoma/Acute Lymphoblastic Leukemia

TdT is negative in up to 6% of cases of pre-B and 18% of pre-T ALL.[64] When positive, TdT can be used to differentiate ALL from lymphoproliferative disorders of mature lymphocytes.[158,159] TdT is also expressed by acute myeloid leukemias[160,161] and normal B lymphocyte precursors (hematogones).[162] The latter property often creates diagnostic confusion, especially in young children. TdT is also positive in the benign cortical cells of the thymus and in benign T cells found in thymomas.

CD34 is negative in up to 74% of precursor-B ALL.[163]

CD20 is negative in up to 46% of cases of precursor-B ALL.[164]

CD79a and *CD22* are negative only in rare cases of precursor-B ALL. Initially thought to be lineage specific, CD79a and CD22 expression can be found in some cases of acute myelogenous leukemia and pre-T ALL.

CD10 may be negative in up to 50% of pre-B ALL in infants and children,[165] and up to 24% in adults.[164] Negative cases will typically have an underlying genetic abnormality consisting of t(4;11)(q21;q23).[1] Up to 37% of pre-T ALL cases are negative with CD10.[166]

CD45 may be absent in some cases of pre-B ALL.[1]

Limitations of Cytogenetic/Molecular Studies

Clonal immunoglobulin heavy chain gene rearrangements are negative in up to 10% of cases of precursor-B-lymphoblastic lymphomas by PCR. Conversely, T-cell receptor gene rearrangements are positive in up to 93% of cases of pre-B ALL in children.[167] Clonal T-cell receptor gene rearrangements were seen in up to 77% of precursor-T-lymphoblastic lymphomas,[168] and up to 22% of pre-T ALL may show immunoglobulin heavy chain rearrangements.[169] Thus, gene rearrangement studies should not be relied upon in diagnosing either variant of this entity.

Burkitt Lymphoma

Typical Histology

A diffuse and prominent starry-sky pattern is seen at low magnification in all cases BL. The *diffuse* pattern results from proliferation of medium sized, monomorphic malignant cells that form sheets. BL has the shortest doubling time of all lymphomas, and consequently there is a very high turnover of malignant cells, many of which undergo cell death. The prominent starry-sky pattern seen within the sheets of malignant cells results from recruitment of many scattered benign histiocytic phagocytes (''starry-sky macrophages'') that scavenge dead cells, as evidenced by prominent debris within their abundant clear cytoplasm.

Cytologically, the malignant cells are monotonous with respect to their size, shape, nuclear chromatin structure, and cytoplasmic features. The neoplastic cells are of medium size, round, have multiple distinct nucleoli and moderate quantities of deeply basophilic cytoplasm. At high magnification, numerous mitotic figures are readily identified among the malignant cells. In touch imprint smears, the cytoplasm exhibits multiple lipid vacuoles.

BL has 2 cytologic variants:

a. BL with plasmacytoid differentiation.
b. Atypical Burkitt/Burkitt-like lymphoma. This variant often raises a differential diagnosis of DLBCL.

Typical Immunophenotype

BL cells are positive with pan-B-cell markers (CD20, CD22, and CD79a), have surface IgM, show light chain restriction, and must be CD10 and bcl-6 positive, but negative for bcl-2 expression.[1] The malignant cells are often CD43 positive.[128] The Ki-67 (MIB-1) stain shows a proliferative activity of $> 99\%$. Wild-type EBV latency is found in up to 30% of sporadic BL,[1,170] with expression of EBNA1 but not of EBNA2-4 or LMP-1, although this pattern of expression may be altered by incorporation of defective EBV viral genomes.[170,171]

Frequency of the Absence of a Typical Immunophenotype or Presence of an Aberrant Immunophenotype in Burkitt Lymphoma

bcl-2 is positive in approximately 5% of cases of BL exhibiting a characteristic translocation [ie, t(8;14), t(2;8), or t(8;22)] and typical morphology.[172,173]

Ki-67 is positive in more than 99% of the malignant cells in BL, but may also achieve this level of positivity in up to 5% of DLBCL. These cases of DLBCL will also show the characteristic immunophenotype of BL, and will occasionally harbor a *c-myc* translocation.[173,174] Thus, morphology is very useful in such cases to reach a correct diagnosis.

EBV genomes can be found in nearly all cases of endemic BL, but are absent in up to 75% of cases of immunodeficiency-associated and up to 70% of sporadic BL cases.[1]

c-myc immunostaining of paraffin-embedded tissue is technically very difficult and varies from one laboratory to another.

Limitations of Molecular Studies

Most cases show the recurrent balanced translocation t(8;14), involving the *c-myc* and the immunoglobulin heavy chain gene, with a lesser proportion, demonstrating translocations t(2;8) or t(8;22), involving *c-myc* and one of the immunoglobulin light chain genes.[1] The abnormal fusion protein expression can be demonstrated in paraffin sections by "long-distance" PCR[175–177] or FISH,[178,179] rather than by immunohistochemistry. However, the *c-myc* translocations have also been reported in secondary pre-B ALL after FL and in DLBCL.[173,180,181]

Angioimmunoblastic T-cell Lymphomas

Typical Histology

The lymph node in AILT shows architectural features that include specific abnormalities in different compartments and in small blood vessels. For example, a prominent *pericapsular* infiltration is usually found. Also, some of the subcapsular sinuses are patent and dilated, and may occasionally contain benign monocytoid B cells.

Within the nodal parenchyma, the architecture is partially effaced and the cortex may show a few regressed, scattered, small, benign follicles. These follicles have atrophic, hypocellular germinal centers that contain few follicular center cells, but many FDRCs. Some cases show many benign hyperplastic follicles that appear abnormal because they are large, poorly defined, and often have absent mantle zones. An interstitial, eosinophilic precipitate is often seen within and between the follicles.

In the interfollicular areas, there is a striking proliferation of high endothelial venules, which show arborization and have hyalinized vascular walls lined by plump endothelial cells that appear to obliterate their lumens. Also present in the interfollicular areas are readily identifiable transformed lymphoid cells of medium and large size, many plasma cells, plasmacytoid forms, eosinophils, small lymphocytes, scattered follicular dendritic cells, and occasional epithelioid cell clusters. Normal and abnormal plasma cells and plasmacytoid forms are characteristically distributed around the hyalinized blood vessels, producing a *perivascular* pattern. Variably sized, well to poorly defined intravascular and extravascular clusters of malignant lymphoid cells with clear cytoplasm are seen in most cases.

Cytologically, the malignant cells are of small, medium, and large size, with moderate to abundant pale to clear cytoplasm ("clear cells") in most cases. Because the clear cells have a pale color, they can be readily recognized at low magnification in different compartments, including marginal zones (*marginal zone* pattern) and within benign germinal centers (*follicular colonization*). Occasional Sternberg-Reed (S-R) like cells and other large B and/or T cells with abundant, deeply staining cytoplasm are often admixed. Mitoses are frequent.

Typical Immunophenotype

The clear cells are positive with CD2, CD3, CD4, CD10, and often with bcl-6.[1,105,182–184] In the Ki-67 (MIB-1) stain, the proliferative activity is high in the clear cells. The large cells with deeply staining cytoplasm frequently are CD20 positive, polytypic and often EBER positive by in situ hybridization.[185,186] The CD21 stain shows more FDRCs within and outside follicles, and they may also form a characteristic meshwork around the high endothelial venules.[187] The normal and abnormal plasma cells are often numerous and polytypic.

Frequency of the Absence of a Typical Immunophenotype or Presence of an Aberrant Immunophenotype in Angioimmunoblastic T-cell Lymphomas

CD10 is negative in the malignant cells in 10% to 30% of cases, depending on the laboratory and the methodology used.[182]

bcl-6 is negative in the malignant cells in at least 50% of cases.[105]

CXCL13 is positive in 89% to 100% of cases.[188,189]

The immunostaining profile of AILT is not entirely specific and does not help distinguish this entity from other T-cell lymphomas when the CD10 and bcl-6 are negative in the malignant cells. Thus, it is essential to interpret the immunophenotype in the light of morphology and especially the presence or absence of the characteristic clinical syndrome to reach an accurate diagnosis. In the absence of the typical clinical findings (rash, fever, polyclonal gammopathy, etc), a diagnosis of AILT should not be made.

Limitations of Cytogenetic/Molecular Studies

T-cell receptor gene rearrangements are negative in up to 30% of cases.[85,86,190] Conversely, immunoglobulin gene rearrangement can be found in as many as 10% of cases of AILT, and most likely represents expanded EBV-positive B-cell clones.[1,190]

Nodular Lymphocyte Predominant Hodgkin Lymphoma

Typical Histology

In the WHO classification,[1] NLPHL requires by definition the presence of at least one L&H "nodule" to distinguish it from other lymphomas and to correctly diagnose it. The name of this entity emphasizes the word "nodular," underscoring the fundamental importance of the L&H nodular pattern in arriving at the diagnosis of NLPHL.

The L&H nodules are usually large, poorly defined, closely packed together, and often exhibit 2 distinctive colors at low magnification:

a. A predominating dark blue color of many benign small mantle cells and few small benign T cells that form small and large confluent clusters.
b. Lightly (pale) staining clusters that are small, irregularly shaped, and interspersed among the darkly staining clusters mentioned above, producing a "moth-eaten" pattern at low magnification. In these

pale staining areas, one or more of the following cell types are present:

i. Transformed benign T cells that are approximately 2-3 times the size of small lymphocytes. These cells have round nuclei with open chromatin structure, small nucleoli, and moderate quantities of clear cytoplasm. Because of these features, these cells may be misinterpreted as malignant.
ii. Few to many benign epithelioid cells/histiocytes.
iii. Few single malignant cells, termed L&H variants of S-R cells. These L&H cells are also referred to as "popcorn cells," because they have hyperlobated nuclei that give them the appearance of popped corn.

In one study, small benign germinal centers were focally seen within and at the periphery of the L&H nodules in up to 30% of the cases and prominent sclerosis was present in 20% of the cases.[191] Some cases also show small monomorphic benign mantle cell nodules.

The L&H nodules most likely represent abnormal follicles, and resemble the end-stage of progressively transformed germinal centers in many ways. In L&H nodules, a distinct follicular center cell compartment is usually absent, but FDRCs are present and most benign small lymphocytes in the nodules are benign mantle cells that have extensively infiltrated the benign germinal center, admixed with few to moderate numbers of benign T cells. L&H cells are scattered, usually very few in number, and are found in a variable number of nodules. Eosinophils and plasma cells are rare to absent. The mitotic activity is typically low.

In the largest series of NLPHL and several other closely related entities, a completely or partially *L&H nodular* pattern was reportedly present in 97% (212 of 219 cases) of cases classified as NLPHL.[192] A second large study involving 118 cases of NLPHL required the presence of an *L&H nodular* pattern as a criterion for diagnosis and inclusion into the study.[191]

At low magnification, a lymph node involved by NLPHL typically shows many large nodules throughout, resulting in the *L&H nodular* pattern. Frequently, the neoplastic L&H nodular growth compresses the residual benign lymphoid tissue with patent sinuses to the periphery, resulting in a pushing border, also known as the "mass effect[193]" pattern. Other patterns commonly seen in NLPHL are *mantle zone*, *mantle cell nodular*, and *prominent sclerosis* patterns.

Fan et al[191] used morphology and immunohistochemistry in 118 cases of NLPHL to define 6 distinct nodular pattern variants that correlated well with progression of disease to a diffuse pattern and frequency of relapse: *classic B-cell–rich nodular*, *serpiginous/interconnected*, *prominent extranodular L&H cells*, *T-cell–rich nodular*, *diffuse* (*T-cell–rich B-cell lymphomalike*), and *diffuse* moth-eaten *B-cell–rich*.

The morphologic variant showing a focal nodular pattern and diffuse areas rich in T cells (*T-cell–rich B-cell lymphomalike* pattern) was shown by Fan et al[191] to be an independent predictor of recurrent disease. The same study also suggested that the presence of numerous popcorn cells outside the nodules (*prominent extranodular L&H* pattern) might represent early evolution to a *diffuse* pattern. In a study by Boudova et al,[194] cases of NLPHL which were rich in small T cells presented with stage III or IV disease in 89% of cases, but had excellent prognosis similar to that seen in typical NLPHL.

Cytologically, the L&H cells (popcorn cells) are very large (larger than large centroblasts). The nucleus of the popcorn cells is polypoid and/or multilobated, usually has small nucleoli, and occupies 70% to 95% of the cell surface area. Multinucleation is uncommon, but may be seen. The cytoplasm is scant. This is in contrast to the "lacunar cells" of the nodular sclerosing variant of CHL that have copious quantities of pale staining to clear cytoplasm.

Benign FDRCs present within the L&H nodules can rarely become very large and/or multilobated, and they can be mistaken for popcorn cells.

Because most cases of NLPHL show very few L&H cells, they are often overlooked and thus recognizing the *L&H nodular* pattern and other characteristic patterns aids in making a correct diagnosis, predicting progression of disease, and staging of disease.

Typical Immunophenotype

Within the L&H nodules, by definition, the CD21 stain highlights distorted networks of FDRCs. The FDRC fibers that are thin, long, loosely packed, and widely separated. The diffuse areas, if present, lack FDRCs.

Most small benign lymphocytes within the nodules are round mantle cells that are CD20-positive, IgD-positive, and IgM-positive. In addition, there are scattered, round, benign, small T cells that are CD3-positive, CD4-positive, and often CD57-positive and that frequently produce rosettes around some of the L&H cells. The CD3-positive cells are often arranged in small clusters.[1]

The benign transformed T-cells 2-3 times larger than small lymphocytes are CD3-positive and often CD4-positive and usually form small clusters within L&H nodules adjacent to the L&H cells. Also, within the clusters of these transformed T cells, L&H cells may be seen.

The L&H cells are positive with CD20 (98%), CD79a (80%), J-chain (91.5%), PAX-5, Oct-2 (nuclear), Bob-1 (nuclear and cytoplasmic), CD22, CD45, Ki-67, bcl-6, and often with EMA (54%),[1,192] but are negative with EBV-LMP, CD30, CD15, fascin, and EBER in situ hybridization.[1,87,192] In one study, however, CD30 was focally positive in up to 20% and CD15 was focally positive in up to 3% of L&H cells.[191]

Frequency of the Absence of a Typical Immunophenotype or Presence of an Aberrant Immunophenotype in Nodular Lymphocyte Predominant Hodgkin Lymphoma

CD20 can be negative in some malignant cells.

CD45 is frequently difficult to interpret because the numerous mantle cells and benign T cells are strongly

CD45-positive and are tightly packed between and around the L&H cells. This arrangement makes it difficult to state whether the L&H cells or the surrounding small cells are CD45 positive.

CD30 is typically negative, but can be positive in 20% of cases in a minority of L&H cells.[191,192,195] Often, scattered, benign, large transformed lymphoid cells are present that are also CD30-positive, and it may be difficult to establish whether these are benign transformed cells or L&H cells.[88] Almost all cases reported to date indicate that these CD30-positive cases are CD15 negative; however, CD15 can also be negative in up to 34% of CHL cases.[89,196]

Limitations of Cytogenetic/Molecular Studies

There are no specific recurrent balanced translocations associated with this disease described to date.

Monoclonal immunoglobulin gene rearrangements can be present. These rearrangements, however, are usually not detectable in whole tissue DNA, but only in DNA isolated from single L&H cells using microdissection, owing to the paucity of these malignant cells within the lesion.[1]

Classic Hodgkin Lymphoma

Nodular, Lymphocyte Rich Variant of Classic Hodgkin Lymphoma

Typical histology: NLRCHL is a specific nodular variant of CHL that shares some common features with NLPHL and CHL.

Similarly to NLPHL, a *nodular* pattern is required by definition. Also, a *nodular and diffuse* pattern may be seen occasionally. Other similar features are the abundance of small lymphocytes in the background, the presence of numerous benign mantle cells and epithelioid cells in the nodules, and the or paucity of plasma cells, neutrophils, and eosinophils.

Benign germinal centers are small, regressed, and typically eccentrically placed within the nodules. Thin bands of fibroconnective tissue are also seen in NLRCHL, with a higher frequency than seen in NLPHL.

Cytologically, NLRCHL shows few to moderate numbers of readily identifiable mononuclear and classic S-R cells in most cases (90%), and few L&H variants of S-R cells (popcorn cells). These morphologic features often correlate well with immunophenotypic findings.

Typical immunophenotype: S-R cells of NLRCHL are typically positive with CD30 (92.5%), CD15 (81%),[192] and negative with CD45 and J-chain.[192,197,198] They may also be positive with CD20 (32.5%), CD79a (8.7%), and EBER in situ hybridization (41%).[192] A CD21 stain outlines the FDRC meshwork of the benign germinal centers.

These findings are in contrast to NLPHL, which is positive with CD20 (98%), CD79a (80%), J-chain (91.5%), rarely positive with CD15 (3%), but negative with CD30 (80%) and EBER in situ hybridization.[89,191,192,195]

Frequency of the Absence of a Typical Immunophenotype or Presence of an Aberrant Immunophenotype in Nodular Lymphocyte-rich Classic Hodgkin Lymphoma

CD30 is negative in up to 7.5% of cases of NLRCHL.[192]

CD15 is negative in up to 19% of cases of NLRCHL.[192]

CD20 can be positive in up to 32.5% of cases, making it difficult to distinguish CD20-positive cases of NLRCHL from NLPHL.[192]

CD79a can be positive in 8.7% of cases.[192]

EBER in situ hybridization is negative in up to 59% of cases.[192]

Nodular Sclerosis Variant of Classic Hodgkin Lymphoma

Typical histology: NSCHL is the most common type of Hodgkin lymphoma in the Western world. The presence of atleast one nodule (pattern) produced by fibro-collagenous bands is required by definition for the diagnosis of nodular sclerosis CHL (NSCHL).

Pericapsular infiltration is common in NSCHL, but uncommon in other types of Hodgkin lymphoma. The lymph node capsule is typically thickened and extends into the nodal parenchyma as wide, fibro-collagenous bands that produce compartmentalization (*fibrous nodular* pattern). These fibrous bands vary in thickness, number, and they may show focal or occasionally diffuse hyalinization. Within the nodules produced by these bands, there are variable numbers of scattered lacunar cells and other variants of S-R cells (collectively called Hodgkin cells). One or more small areas of necrosis may be present at the edge of which Hodgkin cells and apoptotic, ''mummified'' S-R cells are often found. Lacunar cells may form compact clusters. In grade 2 NSCHL, some nodules have an extensive, monomorphic proliferation of lacunar cells, and these large clusters can be sometimes mistaken for carcinoma cells.

The residual nodal parenchyma typically shows a few scattered reactive follicles, and a marked expansion of the interfollicular areas with many vessels that may show marked hyalinization of their walls. The background lymphocytes are small, round to irregular in shape, and have scant cytoplasm. Eosinophils, neutrophils, and occasional plasma cells are usually present. Eosinophilic and/or neutrophilic microabscesses can occasionally be seen. Epithelioid cell clusters and granulomatous changes are uncommon in this type of Hodgkin lymphoma, in contrast to the mixed-cellularity variant.

Cytologically, lacunar cells are much larger than centroblasts and have to copious quantities of pale to clear cytoplasm. The nucleus is often polypoid and multilobated, with contains small nucleoli and occasional nuclear grooves. It is important to differentiate true lacunar cells from mononuclear and other variants of S-R cells that can sometimes be found in clear, ''lacunar''-like spaces, thus mimicking lacunar cells. Other variants of S-R cells are present in variable numbers.

Typical immunophenotype: the lacunar cells are positive with CD30, CD15, fascin, Ki-67, but negative

with CD3, ALK, and CD43. Few lacunar cells can be positive with CD20. The background lymphocytes are predominantly CD3-positive T cells. Occasional, small, benign, CD20, and IgD-positive mantle cell nodules containing lacunar cells may be seen.

Frequency of the Absence of a Typical Immunophenotype or Presence of an Aberrant Immunophenotype in Nodular Sclerosis Classic Hodgkin lymphoma

CD15 has been reported to be negative in up to 30% of cases of NSCHL.[89,196]

CD30 is negative in 3% of lacunar cells of NSCHL.[89,196]

Fascin is positive in lacunar cells and also benign histiocytes and interdigitating dendritic reticulum cells. In cases that have small numbers of malignant cells, it may be difficult to distinguish between the various fascin-positive cells. Furthermore, differential diagnosis may include the neoplastic cells of ALCL, which are also fascin-positive.[199,200]

CD20 is positive in malignant cells in 28% of cases.[89,196]

EMA is positive in a minority of cases, but it is positive in the L&H variants of S-R cells in 54% of cases of NLPHL[192] and in most cases of ALCL of the T/null cell type.[1] Inflammatory pseudotumors are ALK-positive, and sometimes NSCHL exhibits a very prominent fibrotic proliferation with inflammatory cells and few lacunar cells, thus compounding the diagnostic difficulty.

Limitations of Cytogenetic/Molecular Studies

In almost all cases, the S-R cells contain monoclonal immunoglobulin gene rearrangements, and in rare cases they contain clonal T-cell receptor gene rearrangements.[6,201] However, these rearrangements are usually not detectable in whole tissue DNA, but only in DNA obtained from single S-R cells isolated by microdissection, owing to the paucity of these cells within the neoplasm.

There are no specific recurrent balanced translocations associated with Hodgkin lymphoma described to date, and flow cytometry is not useful for the diagnosis of Hodgkin lymphoma because it cannot detect the presence of malignant cells in a background of immunophenotypically benign cells.

CONCLUSIONS

In light of these many issues, we have tried to elucidate the role of the histopathologist and histologic examination in general, in this era of genomics and proteomics. As in the WHO, we advocate a combination of morphologic examination and ancillary diagnostic studies as the most appropriate approach to diagnosis.

In any newly biopsied patient, there is no question that morphology will remain an integral part of the diagnostic process. At the bare minimum, morphology will be required to dictate the subsequent diagnostic testing that will be necessary to establish the most accurate diagnosis.

KEY POINTS

1. The new and evolving technologies of genomics and proteomics have ushered in a revolution in our approach to diagnosis, prognosis, and therapy, and may ultimately replace some of the more mature technologies such as cytogenetics, flow cytometry, immunohistochemistry, and conventional molecular testing in selected disorders.
2. Current technologies, such as immunohistochemistry, flow cytometry, cytogenetics, FISH, and conventional molecular testing by PCR have well-defined and well-studied limitations to their use in the establishment of a final diagnosis, determination of prognosis, and guidance of therapy.
3. Because of the limitations of the nascent genomic and proteomic technologies, these techniques should not be used as the primary diagnostic modalities in a patient biopsied for the first time.
4. None of the technologies described above have definitive and exclusive diagnostic superiorities over all the others, therefore an integrated approach to diagnosis should be attempted, using all appropriate and available techniques of investigation to reach an accurate final diagnosis, to provide patient-specific prognostic information, and to recommend targeted, patient-specific therapy, if available.
5. Careful and critical histologic examination of tissue sections remains the most important first diagnostic step in the evaluation of the pathologic specimen and the determination of what ancillary tests are required to reach a final diagnosis.

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