Molecular Diagnostics in Pathology

Time for a Next-Generation Pathologist?

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Context.—Comprehensive molecular investigations of mainstream carcinogenic processes have led to the use of effective molecular targeted agents in most cases of solid tumors in clinical settings.

Objective.—To update readers regarding the evolving role of the pathologist in the therapeutic decision-making process and the introduction of next-generation technologies into pathology practice.

Data Sources.—Current literature on the topic, primarily sourced from the PubMed (National Center for Biotechnology Information, Bethesda, Maryland) database, were reviewed.

Conclusions.—Adequate evaluation of cytologic-based and tissue-based predictive diagnostic biomarkers largely depends on both proper pathologic characterization and customized processing of biospecimens. Moreover, increased requests for molecular testing have paralleled the recent, sharp decrease in tumor material to be analyzed—material that currently comprises cytology specimens or, at minimum, small biopsies in most cases of metastatic/advanced disease. Traditional diagnostic pathology has been completely revolutionized by the introduction of next-generation technologies, which provide multigene, targeted mutational profiling, even in the most complex of clinical cases. Combining traditional and molecular knowledge, pathologists integrate the morphological, clinical, and molecular dimensions of a disease, leading to a proper diagnosis and, therefore, the most-appropriate tailored therapy.


Three main paradigm shifts1 have radically changed the pathology world. In 1761, publishing De Sedibus et Causis Morborum per Anatomiam Indagatis [On the Seats and Causes of Diseases],2 Giovanni Battista Morgagni first raised pathologic anatomy to an experimental science and demonstrated that most illnesses have their origins in specific organs or tissues. Approximately a century later, in 1858, Rudolf Virchow published Die Cellularpathologie in ihrer Begründung auf Physiologische and Pathologische Gewebestudien [Cellular Pathology as Based Upon Physiological and Pathological Histology],3 in which Virchow demonstrated that illnesses originate in the cells, promoting the introduction of histopathology. Another century later, the discovery of the DNA helix by Watson and Crick (1953)4 led to the birth of molecular pathology.

Throughout the past 3 centuries, the rigorous scientific method, first employed by Morgagni and later introduced into clinical practice, was first enriched by Virchow’s cyto/histologic dogma and subsequently further developed by the histochimical, immunohistochemical, and electron microscopy technologies.

However, many years passed from the interpretation of the genomic code to the clinical translation of the molecular data, engendering the birth of “personalized medicine.” For many decades, there was no significant change in the daily practices of surgical pathologists and cytopathologists, who performed primarily microscopic evaluation in addition to clinicopathologic correlations. Indeed, only in recent years has a complete morphomolecular approach had a central role in the therapeutic decision trail of most solid tumors, sharply affecting pathologic diagnostic practices and laboratory workflows.

The actual challenges are (1) not only to ensure that pathologists remain conscious of the clinical application of molecular profiling but also to reassert that the value of traditional pathology has only been enriched by molecular profiling (similar to that of incorporating immunohistochemistry), (2) to integrate sophisticated molecular technologies into traditional pathology laboratories by introducing new workflows, and (3) to disseminate knowledge of molecular pathology among pathologists by investing in postgraduate medical education programs for trained pathologists and by improving pathology residency training programs.

THE NOVEL CENTRAL ROLE OF PATHOLOGISTS IN THE THERAPEUTIC DECISION-MAKING PROCESS

Before the “molecular revolution,” the pathologist was broadly considered an “oracle,” far from daily clinical practice, who would confirm clinical and/or radiologic...
suspicions. However, that notion is clearly no longer the case for the following reasons:

1. A pathologist is a clinician who provides an interpretation of the morphological (and molecular) features consistent with the clinical, radiologic, therapeutic, and laboratory-collected data. A typical example is the evaluation of a thyroid aspirate purely composed of Hurthle cells; in that setting, the decision to follow a patient, rather than to perform a lobectomy, is based on clinical, sonographic, and laboratory correlations.

2. A pathologic diagnosis relies on a subjective evaluation. Indeed, pathologic features often overlap among various diseases, and tissue sampling may be insufficient. All these factors may preclude a definitive diagnosis and may, therefore, encourage the direct involvement of the pathologist in multidisciplinary meetings.

The Introduction of Biomarkers into Clinical Practice

The “solitude of the pathologist” ended with the introduction into clinical practice of a new generation of drugs targeting specific genes and molecular pathways, combined with cellular/tissue biomarkers, which would allow caregivers to foresee the patient’s response to those expensive drugs. The introduction of new genetic therapies has demanded a reevaluation of pathologic diagnoses as the backbone of therapeutic decision-making and not merely confirmation of clinical hypotheses.

For example, advanced patients with colorectal cancer, who are eligible for anti-epidermal growth factor receptor (anti-EGFR) therapy, should undergo “extended” RAS mutational testing. Moreover, DNA mismatch repair status has been recommended for all patients with colorectal cancer to investigate for possible Lynch syndrome. The pathologic diagnostic report should provide all such information.

In a similar manner, the EGFR/ALK (anaplastic lymphoma kinase)/ROSI (and in some instances, MET and KRAS) status may be assessed in lung adenocarcinomas at diagnosis of an advanced disease, at recurrence, or in patients with an early stage (ie, stages I-III) disease who undergo surgical resection. The BRAF (NRAS and KIT) status may be tested in metastatic malignant melanoma, the KIT/PDGFRA mutations in gastrointestinal stromal tumors, the somatic BRCAl/2 status in ovarian cancers, and the 1p/19q codeletion, MGMT promoter methylation, and mutation in IDH1/2 genes in brain tumors. Furthermore, the targeting of immune checkpoints has led to the use of novel biomarkers (primarily the programmed death ligand-1 [PD-L1] immunohistochemical assessment) in many different tumors.

Although traditional pathologists remain wary of this complex molecular background, several years ago, morphologic classification was completely replaced by molecular classification in breast cancers. Following initial difficulties in standardizing the assessment of the new biomarkers, the proliferation index MIBI, estrogen/progesterone receptor status, and HER2 (human epidermal growth factor receptor 2) became mandatory in a breast cancer pathologic report.

In short, the molecular revolution has barely begun, and the necessity of morphomolecular diagnoses will continue to increase.

Notably, many promising biomarkers have not been implemented into routine pathology because of inaccurate assessment and preservation of the cytologic/histologic biospecimens, significantly affecting their clinical indication. This experience has pinpointed the compelling prerequisite for the increasing involvement of pathologists in biomarker research and development. In fact, the morphological and molecular evaluations of a biospecimen are not mutually exclusive, but complementary, and pathologists are the only people capable of piecing together the morphological, molecular, and clinical features of each case of disease.

This notion is also true for the introduction of liquid biopsies into clinical practice: it is impossible to contemplate treating a patient without first determining a cytologic/histologic diagnosis.

The Selection of the Most Adequate Sample

One typical example of the dichotomy between the pathologist and the primary care clinician is the single request of a mutational profile of a biospecimen with no (or incomplete) morphologic assessment because the clinician may think the latter is not worth pursuing (eg, pleural effusions obtained in stage IV lung adenocarcinoma patients or biopsy samples obtained to test eligibility for innovative clinical trials). In any case, it is common in pathology that some of those biospecimens will not contain sufficient material to conduct an adequate molecular survey.

Thus, a morphologic assessment should be performed on every biospecimen for the following reasons: (1) to verify that the sample is the best representation of the disease (ie, the presence of tumor necrosis, sampling errors, strong inflammatory component), (2) to confirm the histotype of the lesion, (3) to select the most proper area for the molecular survey, (4) to choose the correct analysis according to the technical sensitivity of the molecular device used, (5) to identify artifacts because of inaccurate preanalytic steps that can dramatically affect molecular profiling, and (6) to assess the tumor’s morphological heterogeneity, which can introduce analytic biases (Figure 1).

Moreover, the pathologist’s simultaneous evaluation of the morphology of the sample and of the molecular profiling to be performed is the most effective option of ensuring avoidance of useless (and expensive) molecular surveys.

Pathologists should proceed according to the proper choice of the required molecular survey, according to published molecular testing guidelines, and to the application of the molecular test for every patient who needs it (even if that could potentially clash with some public health-spending review plans).

Notably, reflex testing by pathologists has previously been conducted in numerous pathologic fields (ie, thyroid nodules, lymphoproliferative disorders, and soft-tissue sarcomas, in particular) in which molecular information is an essential diagnostic tool. For example, the assessment of BRAF status may play a central role in distinguishing the diagnosis of hairy cell leukemia from other B-cell lymphoproliferative neoplasms exhibiting similar clinical and morphological features. The importance of the molecular survey in routine diagnosis is highlighted by the introduction of molecular profiling in the classification of many tumors (particularly in distinguishing different malignant central nervous system tumors).

Conversely, only referencing molecular information may often be misleading if that information is not supported by the histopathologic features, emphasizing the importance of a matched morphomolecular diagnosis. This scenario occurs in cases of Ig or TCR gene clonality, which can not be
considered proof of B-cell or T-cell malignancy without jointly assessing the histopathologic, flow cytometry, and immunophenotypic features.40,41

NEXT-GENERATION TECHNOLOGIES IN OLD-GENERATION LABORATORIES

High-quality microscopy is the primary prerequisite for remarkable molecular pathology.23 However, most of the technologies employed in cytologic and histopathologic evaluations were designed more than a century ago and rely on morphology. Therefore, “traditional” cyto/histopathology technologies could have a dramatic effect on molecular pathology.42 In addition, the preanalytic phase, which includes the management and processing of biospecimens, can affect the quality of proteins and nucleic acids present in the samples (Figure 2, A through E).29,43,44

Contributing to the conceptual confusion in the diagnostic routine, the concept of “a single biomarker for a specific drug” has recently become outdated, engendering a multi-marker diagnostic routine that necessitates the use of a set of different markers (for example, next-generation sequencing [NGS] and multiplex genotyping platforms).45–48 Furthermore, improvement in molecular technologies has been largely encouraged to respond to both the growing demand for molecular surveys in clinical practice and the simultaneous decrease in the amount of neoplastic samples in nearly every metastatic/advanced case.49,50

Preanalytic Phase in Cytology

Cytopathologic specimens have the primary role in the molecular profiling of several neoplastic lesions.51–56 For example, in lung cancers, cytology may represent the only way to obtain diagnostic samples in patients with advanced diseases or with low performance status who are unable to undergo an open biopsy.57,58 However, the DNA/RNA supplied by most cytologic specimens is limited (even though enriched in neoplastic cells) and is thus inadequate for sequential, single-gene diagnostic tests.59,60 This problem has recently been overcome by the introduction of NGS applications into clinical practice.59,60

The combination of rapid on-site evaluation,61 the almost complete absence of formalin-based fixation, and the potential use of smears immediately in combination with cell blocks represent the best situation in the extraction of
high-quality DNA/RNA, which is nearly impossible in traditional histology.62 Furthermore, cytology is a rapid, low-cost, minimally invasive, and a well-tolerated procedure that is widely employed to achieve a definitive diagnosis in many laboratories.63 However, a more-customized approach is required for the management of specimens when compared with histology. Indeed, all the international recommendations for good practices in lung cancer clearly state the crucial need for an accurate acquisition and preservation of all residual cytologic material for molecular investigations.29,64,65 Consequently, all the remaining aspirates and needle rinses are generally recovered in preservatives or in cell blocks for further analyses in current clinical practices.66,67

Figure 2. Examples of cross-contamination during sample processing and section preparation. A, A messy pathology hood after the resection of a fresh specimen. Gauze was placed under the specimen to demonstrate how the amount of blood and microscopic fragments of the specimens can accumulate after a single resection. This image demonstrates that the waste of the hood can significantly affect the following molecular surveys. B and C, Preparation of histology slices. The knife should be replaced regularly, and the microtome must be cleaned with every change of formalin-fixed, paraffin-embedded sample. This process is true for both histopathology and cell block specimens. D and E, Typical example of a “shared” water bath showing the increase in small debris because of the accumulation of previous cut samples.
and limit the detection to relevant genes using known reference standards. A specific problem in cytology is establishing the minimum cellular cutoff required for NGS analyses.

Adequate preanalytic management of cytologic specimens has become the leading component of the subsequent molecular assessment of lesions. Preanalytic variability strongly depends on applied cytologic preparations (eg, direct smears, cytospin preparations, cell blocks, and liquid-based cytology samples), which include typical processing techniques, fixatives, and stains.

Among several cytologic preparations, the cell block is similar to typical surgical pathologic processing. However, similar to histology, the process of paraffin embedding generally comprises several formalin-fixation steps, and use of the same histologic processors, even though samples are collected in nonformalin-based solution. Moreover, cell block preparation methods can vary widely at different institutions.

A remarkable advantage of using FFPE cell block samples is the opportunity to have a better selection of the material to be molecularly analyzed; an inadequate qualification of the sample can influence the results more than the technical inconsistencies from the brittleness of the FFPE specimen. Conversely, an unwarranted cell block preanalytic step is the section preparation of the molecular analysis, which may be the primary source of cross-contamination (Figure 2, B). This risk must be considered after the implementation of sensitive molecular technologies that are capable of analyzing biospecimens containing less than 1% mutated alleles. Technicians may prevent contamination with (1) the regular replacement of the knife and the use of disposable plastic ware, and (2) by preventing the use of "shared" water baths.

An easier option than the cell block for extracting high-quality DNA is the use of precise, small amounts of cytologic sample in suspension, which has been shown well in the molecular characterization of human papillomavirus testing and thyroid nodules. Moreover, the NGS approaches can also be applied successfully in DNA retrieved from fine-needle aspiration (FNA) needle rinses and effusion fluids, whereas the use of diagnostic smears (and their definite loss) is reserved for cases in which no other material is available.

Specimens must be examined to verify the amount of neoplastic cells before molecular profiling, and doing so, occasionally, requires great effort to achieve the proper microdissection to remove the nonneoplastic portion (mainly inflammatory cells and necrotic debris) and enrich the tumor cell portion. Moreover, it should be clear that if the cytology is a good representation of the lesion, the cytologic samples are also affected by intratumor molecular heterogeneity. In addition, FNAs frequently have a higher neoplastic to healthy-cell ratio compared with the core needle biopsies.

Ideally, in the case of indeterminate samples, a (cyto)pathologist skilled in molecular pathology who is aware of the analytic sensitivities of the different molecular devices should always offer a point of view toward choosing the most appropriate technology. In addition, it is common for traditional (cyto)pathologists to overstate neoplastic cellularity because they often tend to analyze the extension of the neoplastic areas, rather than the neoplastic to healthy-cell ratio in the same area (a large, diploid cancer cell nucleus has nearly the same genomic content as the small nuclei of tiny healthy lymphocytes), which is the correct assessment before conducting molecular profiling.

A specific problem in cytology is establishing the minimum cellular cutoff required for NGS analyses. Most published studies chose samples with at least 20% neoplastic cells, which may not reflect daily diagnostic practices. "The magic number" depends on both the target capture and the various platform types, which range between 100 and 15 000 cells. Moreover, even the DNA input can significantly range from 10 ng for the Ion Torrent (Thermo Fisher Scientific, Waltham, Massachusetts) sequencing of PCR products to the 170 ng required for the Illumina NGS hybridization capture (Illumina, San Diego, California). In summary, these results indicate that an adequate molecular survey requires an acceptable morphological analysis derived from a neoplastic cell–rich FNA biospecimen.

The Preanalytic Phase in Histopathology

Every pathology department processes biospecimens from various health clinics and surgeries and, occasionally, from different hospitals. Therefore, DNA/RNA degradation may result because of a delay in transporting those specimens and/or incorrect sample preservation. To solve that problem, biospecimens should be stored using a chemical fixative or be vacuum packaged. However, an important preanalytic variable is the poor fixation of a biospecimen, particularly in surgical samples. However, tissue formalin-fixation causes nucleic acid fragmentation. Fixation in neutral-buffered formalin for 12 to 24 hours (8 hours for small tissue specimens) is the best option for preserving nucleic acids and maintaining good morphology, whereas overfixation may considerably hamper the next molecular analysis. Moreover, something equally remarkable is that most pathology laboratories do not operate every day of the week. Therefore, overfixation of biospecimens is a daily occurrence because of laboratory closures.

Cross-contamination is another important preanalytic step to be checked before molecular profiling. As for cell block samples, section preparation may represent a significant source of cross-contamination. Another important cause is represented by inadequate sampling room cleanliness, particularly in high-throughput laboratories. In the larger sections of molecular laboratories, investigations are also conducted daily on specimens sent from external laboratories, which makes it impossible to provide evidence of cross-contamination that may have occurred before paraffin inclusion.

Despite the preanalytic steps, insufficient or inadequate samples can lead to misguided selection of the neoplastic area. Indeed, most biospecimens lack an adequate tumor cell component, impairing the accuracy of the molecular test (ie, tumor component under the technical sensitivity of the molecular method), therefore, in most cases, a macro/microdissection or an eventual 1-mm core sampling must be performed to enrich the neoplastic cellularity. The laser-captured microdissection is generally reserved for research.

Since the first reports regarding the accuracy of the FFPE-derived DNA for NGS–based analyses were published, several (primarily) amplicon-based NGS assays have been approved clinically, even if most of them employed a set of the most-frequent clinically actionable genomic alterations, shortening the turnaround time required to provide molecular profiling. Moreover, following the "FFPE-friendly" approach, NGS is now easier to perform,

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including on both clinically and pathologically well-profiled FFPE blocks retrieved from pathology archives. However, one of the most important challenges for the molecular pathologist is the standardization of NGS assays because NGS combines several devices, reagents, and bioinformatics pipelines.50

Bioinformatics has been poorly applied in pathology because of its complexity in analytic methods.52 In addition, bioinformaticians must consider that both the preanalytic processing and the preservation of FFPE biospecimens can introduce artifacts into DNA, such as the deamination of cytosine bases, resulting in C:G $\rightarrow$ T:A substitutions during amplification.86 These types of artifacts are because of both formalin fixation and the degradation of the biospecimen that occurs naturally (which is intrinsically associated with hydrolytic deamination).87 Notably, pretreatment of the DNA sample with uracil-DNA glycosylase has been shown to sharply decrease the artifactual deamination, with no changes to the DNA helix itself.87

The Next-Generation Working Group

For many years, the organization of pathology laboratories was based on the close collaboration among technicians, biologists, and pathologists, working together on both the improvement of cytologic/histologic processing and the achievement of the most-sophisticated morphological diagnoses.

During the past few years, because of new impetus, the modern pathology laboratory has been enriched by the novel expertise of molecular biologists/biotechnologists and laboratory technicians with a molecular background who have a basic knowledge of cytology and surgical pathology.88

This multidisciplinary team generally involves trained staff in complex bioinformatics data interpretation.60,89 Indeed, pathology informatics has become increasingly relevant not only in the NGS context but also in some basic elements of traditional pathology, such as immunohistochemistry and in situ hybridization techniques. These 2 methods continue to have a central role in both diagnosing illness—because of their excellent reproducibility and accuracy—and in detecting a genetic alteration in FFPE specimens. However, these analyses could be affected by interpathologists’ and intrapathologists’ interpretative biases, which are not consistent with the concept of personalized medicine.90

The recent introduction of digital pathology through the employment of automated scoring of the immunohistochemical expression and the automated count of hybridization signals has led to a more-quantitative interpretation of results.91,92 As has been observed for molecular profiling, the great challenge lies in having to address the large amount of data that requires interpretation with a comprehensive algorithm capable of translating digital information into a simple quantitative score.

Therefore, modern pathology laboratories should invest more in informatics because analyses of these data will become the bottleneck of our future diagnostic workflows.

(CYTO)PATHOLOGIST, MOLECULAR (CYTO)PATHOLOGIST, OR NEXT-GENERATION (CYTO)PATHOLOGIST?

In the “next-generation sequencing era,” many pathology laboratories have implemented NGS or multigene high-throughput technologies in their daily practices. Although, today, we take next-generation technology for granted, can we, therefore, assume that there is already a next-generation pathologist?

The molecular revolution that has occurred in pathology diagnostics has not been joined by the updating of training programs, allowing access to high-throughput technology information to only a few people. This impasse and its implications for both the diagnostic and academic/research dimensions have been recognized by the pathology community.93–96 and integration of traditional pathology training by molecular diagnostics is widely suggested.

This suggestion does not mean that all pathologists should develop a particular expertise in genomic medicine and genome-based research. However, all pathologists (in addition to their imperative training in morphology) should be able to address molecular information, know how to select the most adequate sample for analysis, understand the molecular performance of such tests, and incorporate the morphological and molecular data into an integrated diagnostic report.

For example, a general cytopathologist should be aware of how to manage a thyroid cytology specimen for both an adequate cytology diagnosis and RAS/BRAF testing.37 Moreover, the general cytopathologist must know how to interpret the molecular data compared with phenotypic findings and to provide an appropriate diagnostic message to clinicians/surgeons, even though the pathologist may not be required to have an in-depth knowledge of “coverage,” bioinformatics analysis, and qualification of the obtained DNAs/RNAs. However, every pathology department should have subspecialized pathologists in molecular diagnostics who should be familiar with high-throughput technologies and will be the cultural bridge between “traditional” pathologists and the molecular pathology laboratory. Those people, however, should also be involved in routine cytopathology/histopathology diagnostics to maintain close contact with the specimens and, therefore, the clinic.

Traditional pathology core curriculum must be implemented with basic molecular notions and the interpretation of the molecular data within the clinical context.97 However, molecular pathologists should recognize that a BRAFV600E mutation is not a diagnosis, but merely a portion of one.

This goal of integrating traditional pathology training with molecular diagnostics can be reached by modifying residency education programs in pathology98,99 and by creating appropriate continuing medical education programs for trained pathologists. This latter point is central: we cannot afford to wait to integrate new generations of pathologists into our institutions. Moreover, the standardization and implementation of training programs is far from being a forthcoming result. The molecular revolution is now, and trained traditional pathologists are already principal actors in that paradigm. Pathologists should incorporate a clinically fitting molecular background rapidly, and they must change the manner in which they practice cellular and surgical pathology diagnostics.

CONCLUSIONS

Modern pathology practice has been suddenly and completely changed by the increased complexity of modern medicine, primarily regarding oncology, which this concise review has attempted to describe. Consequently, the frameworks of our laboratories and pathology training...
programs are experiencing a radical change in accordance with a pressing request for innovation. Currently, the pathologist has a central role in the age of next-generation medicine, providing the most properly matched morpho-molecular assessment of biospecimens for the clinician.

What will our future look like? We may glimpse our future by recalling our past. Indeed, we should be mindful of Morgagni’s teaching: “to admire [...] and to follow not the ancient, not the modern, not the traditional, but always and only the truth”.18 In shifting from “old” teaching to “next-generation pathology,” we are able to realize that having only one manner of providing a diagnosis is insufficient (ie, cytology, histology, or molecular profiling); the diagnosis itself must be provided. This is our greatest effort today: the personalization of patient therapy.

References