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PATHOBIOLOGY IN FOCUS

Why microscopy will remain a cornerstone of surgical pathology

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Recent years have seen increasing predictions of the demise of conventional microscopy in patient care and investigative medicine. However, these predictions fail to recognize the power of morphologic analysis by a skilled observer. The amount of information that can be obtained from a simple H&E slide represents a windfall in terms of data quality, quantity and cost when compared to any other available technique. Moreover, the value of such interpretation is irreplaceable as we develop newer and more sophisticated technologies. Overall, it appears that reports of the death of microscopy have been greatly exaggerated.

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The discussion of the role that microscopy plays and in all likelihood will continue to play in 'the molecular age' of medicine can be divided into two separate categories: diagnostic pathology and investigative pathology. Regarding the former, it is my opinion that there is no available technique that provides so much information so abundantly, so quickly and as inexpensively as conventional microscopy, in the form colloquially known as the H&E technique. Thus, it stands to reason that morphologic analysis by skilled observers, that is, well-trained surgical pathologists, will be with us for many years to come.

MORPHOLOGY AS THE GOLD STANDARD

Before too many eyebrows are raised, let me provide two examples of the incredible power of morphology. The first is a biopsy of a cervical lymph node in a 25-year-old woman that shows a papillary carcinoma featuring nuclear pseudoinclusions and psammoma bodies (Figure 1a). The pathologist examining the H&E section will be able to tell the clinician that the patient has a metastatic papillary carcinoma from the unilateral lobe of the thyroid gland, probably accompanied by multicentric disease. Other lymph nodes are likely to be affected, but radioactive iodine will be effective therapy and the patient has a nearly 100% chance of survival.

The second example is a biopsy from a rapidly growing large mass centered in the thyroid gland of a 72-year-old man showing a malignant spindle cell tumor featuring numerous mitoses, extensive necrosis, and blood vessel invasion (Figure 1b). After looking at just an H&E section of this tumor, the pathologist will know that despite its sarcoma-like appearance, the tumor is likely to be an anaplastic thyroid carcinoma arising from a pre-existing well-differentiated papillary or follicular carcinoma, invaded most of the gland, have metastasized to nodes and distant sites, be present at the surgical margins of resection and that the chances for survival are close to zero.

Any new technique must provide information of prognostic or therapeutic significance beyond that provided by the current gold standard

The amount of information that the examination of these samples has provided is staggering. This should not be too surprising. After all, the microscopic appearance of a tumor as seen in an H&E slide represents the grand synthesis of thousands of genes working in concert and sometimes in opposition. In addition, there is probably no single gene that plays an important role in the neoplastic process whose expression is not manifest in one way or another as a morphologic finding that can be detected by those with the proper training and ability. Therefore, from a practical clinical standpoint, the challenge of any new technique is to demonstrate that it can provide information of prognostic or therapeutic significance beyond that provided by the current gold standard. It is my impression that this does not happen as often as some claim. Of the hundreds of 'markers' that

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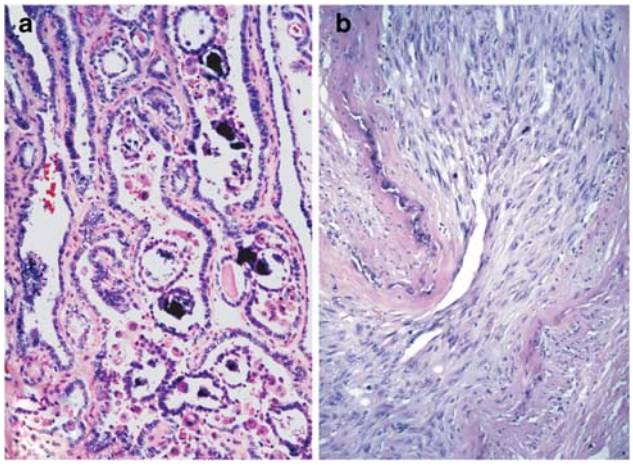

Figure 1 Examples of the power of H&E analysis. (**a**) Papillary thyroid carcinoma. (**b**) Anaplastic thyroid carcinoma.

have been described as being statistically associated with a clinically important feature of solid tumors (such as survival), only a very small minority retain independent predictive value in multivariate analyses when clinical and microscopic parameters are considered.[1] This sobering fact rarely comes across in articles about prognostic markers. The most benevolent explanation is that the authors did not think about it, perhaps because there was no anatomic pathologist involved with the study that could have raised the issue and interpreted the findings. A less sympathetic interpretation might be that an effort, conscious or unconscious, was made to suppress that aspect of the study.

Viewed from this angle, microscopy remains the gold standard against which any claim based on new technology needs to be measured.[2,3] Gonzalez-Crussi[4] expressed this feeling very elegantly when stating that, "as pathologists, we subscribe to a belief that the time-honored interpretation of histopathology is pre-eminent in tumor diagnosis. Yes: however sophisticated and 'modern', a novel diagnostic technique ought to be suspect if it does violence to a universally agreed upon diagnosis arrived at by more traditional means."

MORPHOLOGY AS A MEANS OF DATA INTEGRATION

From the point of view of etiology and pathogenesis, morphology is in a position to play a significant role, although unfortunately many people do not understand this. Many examples can be quoted in support of this claim, the history of desmoplastic small cell tumor being a good (and close to the heart) example.[5] The entity was first identified through morphology; a process that led to the discovery of a specific chromosomal translocation and a specific gene fusion (Figure 2). How long would it have taken for the molecular techniques to lead, on their own, to the discovery that the changes are unique and, from there, to the description of a new tumor? Actually, if one thinks about the major advances that have been made in the field in recent years, one realizes that most of them (at least in the field of solid tumors) have occurred as a result of the symbiosis of pathologists and molecular biologists, rather than each working in isolation.

Some years ago, the National Cancer Institute sought to develop a new classification of tumors based on molecular parameters that would replace the existing morphology-based classification and provide more clinically meaningful information. A US $100 million effort to sequence the genomes of lung, brain, and ovarian cancers, as part of a the US $1.5 billion Cancer Genome Atlas Project, to find all mutations in human cancers was begun.[6] The level of complexity stunned the researchers; on average each tumor harbored 100 gene mutations. "From my perspective, it's hopeless," said cancer biologist Lawrence Loeb: "It is a wonderful study, but there is no core of genes associated with a particular cancer. It started with a wonderful optimistic view, but now it presents enormous complexity."[6] For example, more than 21 000 mutations have been found for the protein p53 alone. Another sobering thought is that the number of mutations relevant to cancer represented only 0.2% of the mutations initially detected.[6]

These observations render it painfully obvious that unraveling the molecular features of cancer will take many years, probably decades, and that the crucial role microscopy will continue to play during this period will provide irreplaceable data if properly integrated with newer techniques. The key word here is 'integration', a concept which follows a long line of tradition among anatomic pathologists, in the sense that the basic information obtained with the H&E slide has always been integrated with the newer information obtained from ancillary techniques such as 'special stains', enzyme histochemistry, tissue culture, electron microscopy, and immunohistochemistry. Each of these methods has contributed to the information obtained from the routine method, and wherever useful, has been incorporated into the diagnostic armamentarium.

The same expansion in new technology is now occurring in the realm of molecular biology, except that the impact is far greater than that of electron microscopy and immunohistochemistry and is almost of cataclysmic proportions. One major difference between molecular approaches and ancillary methods employed previously is that molecular biology techniques have, with few exceptions, done away with the microscopic 'handle' that provided the link needed to relate the new findings to morphologic observations. To be sure, there is still a microscopic component in fluorescent and chromogenic *in situ* hybridization (FISH/CISH) and conventional cytogenetics, but hardly any in RT-PCR, comparative genomic hybridization (CGH), spectral karyotype imaging, the ubiquitous microarray chips ('expression profiling'), and proteomics. In other words, we have entered a brave new world, and not an easy one for surgical pathologists to inhabit. Yet the chasm is not as wide as it would seem. Rather, there is a conceptual continuity among these methods that transcends their superficial technological differences. All

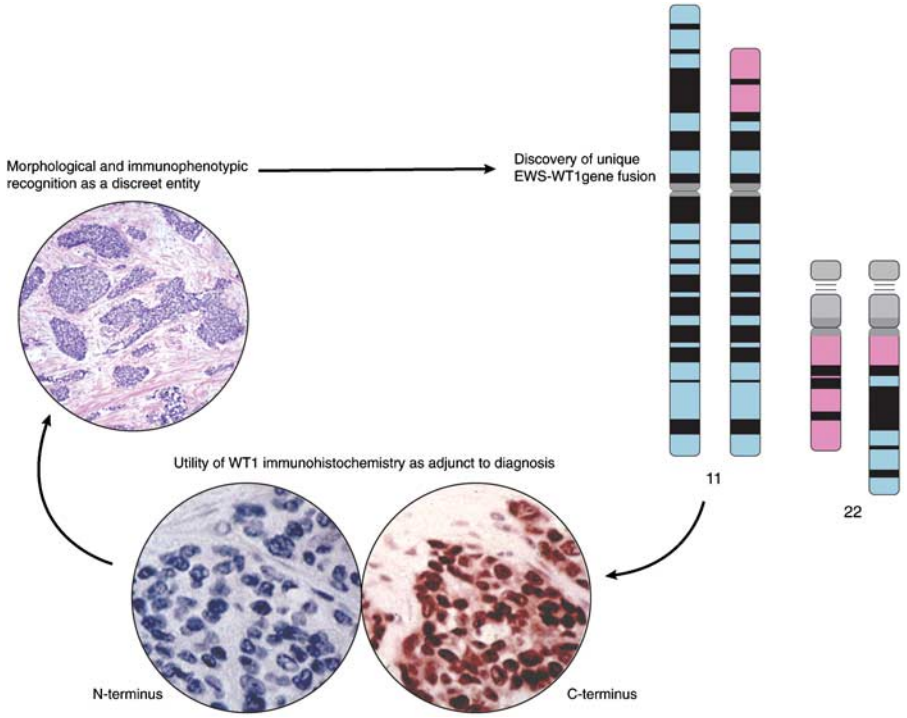

Figure 2 Role of morphology in the discovery of desmoplastic small cell tumor. Initial recognition of the lesion as an unique entity based on morphology and immunohistochemistry allowed molecular study that identified the EWS-WT1 gene fusion as the molecular hallmark of this tumor. This information has allowed immunohistochemical documentation of expression of the C-terminal half of WT1 to be used as an adjunct to diagnosis.

of these technologies can be simply viewed as different ways to explore the various levels of complexity within the tree of life: DNA–RNA–protein–cell–tissue–organism, starting with the genotype and ending with the phenotype…[7] Indeed, the key challenge will be to correlate morphologic phenotypes with the genotypic signatures on which the morphologic phenotype is based.

Because of my background, I have emphasized the contributions of surgical pathology to oncologic diseases, but it should be obvious that they apply just as well to the evaluation of medical diseases. As an example, one could note the discriminatory power of morphology in differentiating between inflammatory bowel disease and acute self-limited colitis in a patient presenting with acute colitis after a trip to an area in which enteric infections are endemic[8,9] Surveillance for dysplasia in ulcerative colitis is another area in which histologic diagnoses and molecular analysis could work in a synergistic fashion rather than in isolation, the patient benefiting medically and financially.[10–12]

THE MARRIAGE OF MORPHOLOGY WITH MOLECULAR DIAGNOSIS

In his book 'Bridging the genotype-phenotype gap', Omholt[13] lucidly states: "Biology is finally in position to start revealing the causal links between genotype and phenotype in the wide sense. In this century, biological research will become almost synonymous with the efforts to understand the functional expression of genes within the context of integrated biological systems. The task is among the most complex scientific endeavors ever, and it will force substantial numbers of biologists to become much more theoretically oriented and interdisciplinary." The same concept was expressed by Fisher[14] in his essay appropriately titled 'The evolution of tumor biology: seeking a balance between gene expression profiling and morphologic studies.' The effective correlation of morphology with molecular biology is already playing an important role in oncology, which for the purpose of this discussion can be divided into three aspects: diagnosis, prognosis, and prediction of therapeutic response.

Diagnosis

An important effort during the forthcoming years will be the continuing search for correlations between specific cytogenetic and molecular alterations (translocations, point mutations, amplifications, deletions, etc.), morphologic phenotype, and biologic behavior. Many such correlations have already been discovered, particularly in hematopoietic diseases, soft tissue sarcomas, pediatric small cell tumors, renal tumors, and thyroid tumors. As a matter of fact, it could be said that diagnostic molecular pathology was born with the article by Cleary *et al*[15] describing immunoglobulin gene rearrangement as a diagnostic criterion in B-cell lymphoma.

The evolving story of the soft tissue sarcomas is also fascinating. Who would have guessed only 20 years ago that a significant number of those tumors, whose categorization was often viewed as an intellectual challenge of limited diagnostic import, would be associated with specific translocations resulting in unique gene fusions? In a conceptual inversion, these translocations now serve as powerful diagnostic adjuncts that independently validate subtle and often controversial

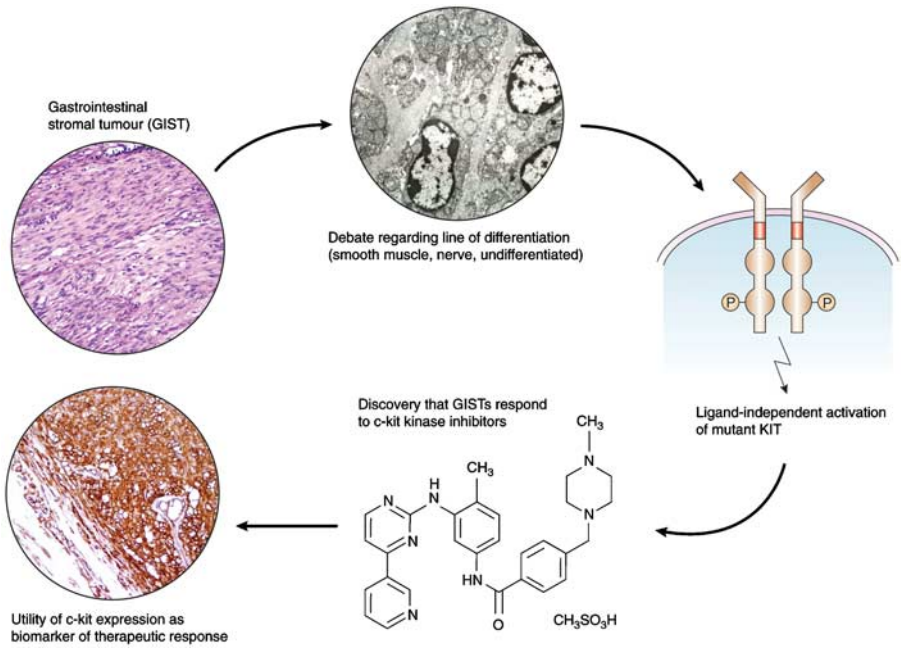

Figure 3 Convergence of morphology and molecular biology in development of specific therapy for gastrointestinal stromal tumor (GIST). The recognition of GISTs as different from other spindle cell proliferations made studies of GIST differentiation popular and controversial. This was resolved by the recognition that the majority of GISTs harbor mutations in the c-kit receptor that allow ligand-independent kinase activation. This was followed rapidly by the discovery that GISTs respond to specific kinase inhibitors and routine use of c-kit immunohistochemistry to predict therapeutic response.

morphologic distinctions that were, in previous decades, made only by expert pathologists.[16,17]Another important role of the pathologist is the identification of the phenotype and behavior of tumors arising in hereditary syndromes owing to germ line mutations thus serving as a conduit for the detection of those syndromes. Examples abound. Among them are medullary thyroid carcinoma as a possible indicator of multiple endocrine neoplasia (MEN), the cribriform-morular-variant of papillary thyroid carcinoma as a clue to familial colonic polyposis,[18] medullary carcinoma of the breast in a young woman as a possible indicator of a BRC-1 germ line mutation,[19] and sebaceous skin lesions as a possible sign of hereditary non-polyposis colorectal carcinoma.[20] While the goal of a personalized genome sequence for US $1000 is laudable,[21] much of the clinical guidance derived may also be obtained through methods as simple as a detailed clinical history, a careful physical examination, and interpretation of a biopsy by a skilled surgical pathologist.

Prognosis

With some outstanding exceptions, most of the molecular genetic abnormalities found so far have not correlated as closely with the biologic behavior and prognosis of those tumors as has microscopic morphology. Thus, the microscopic grade of a synovial sarcoma or the mere presence of rhabdoid cells (a solely morphologic observation) bears a closer relationship to prognosis than the type of gene fusion that the tumor cells carry.[22–24] At present the main prognostic criteria for most solid tumors remain those of a morphologic nature: tumor size, depth of invasion, mitotic activity, necrosis, vascular invasion, rhabdoid morphology, and the like. The contribution made to the prognostic evaluation of tumors by molecular genetic techniques remains modest and sometimes controversial, but will undoubtedly grow in the coming years. Examples already validated and incorporated into the clinical armamentarium are microsatellite instability (MSI) determination in colorectal carcinoma[25] and 1p/19q loss in oligodendroglioma.[26] Conversely, the independent prognostic value of specific gene fusions in sarcomas, the 'upstaging' of sentinel lymph node status based on molecular diagnostic studies, and the much touted 'gene expression profiles' (microarrays) still need to be accurately and dispassionately assessed.

Prediction of Therapeutic Response

This subject has become the hottest topic in the field. To be sure, a certain degree of therapeutic response prediction was already provided in selected tumors (malignant lymphoma, germ cell tumors, small cell lung carcinoma) by morphology for decades, but the discovery of molecules that can be specifically targeted opens a whole new dimension. The classical examples are Her2-neu amplification in breast carcinoma as a predictor of response to herceptin, c-kit overexpression in GIST as a predictor of response to imatinib (Figure 3), and EGFR mutation in lung carcinoma as a predictor of response to gefitinib. The implications for pathologists of the advent of targeted therapeutics have been well enumerated by Hess[27] as follows: (1) providing primary diagnosis; (2) determining whether a specific molecular target is present before initiation of therapy; (3) evaluating the efficacy and possible side effects of new therapies; and (4) harvesting and analyzing tissues in therapeutic failures.

The greatest hope lies in molecular profiling and the promise that these tests may lead to personalized management.[28] The results are certainly promising, but further optimization and standardization of techniques and properly

designed clinical trials are required before tools such as microarrays should be used as tools for clinical decision making.[29] In fact, some authors note that, at present, 'good old' clinical markers have similar power in breast cancer prognosis as do gene expression profiles.[30] As O'Shaughnessy[31] states: "At present, it is not clear that the quantification of the level of expression of dozens or hundreds of genes provides more information about the potential of a cancer for metastasis, virulence, and response to therapy for an individual patient than does an optimal analysis of the standard and readily available histopathological prognostic factors."

It should be self-evident that pathologists ought to be actively involved in the development and evaluation of these tests. As Giordano[32] states, "Prediction of therapeutic response by molecular profiling is the logical and natural extension of the work of the surgical pathologist." Ideally, he should be an active participant in all these phases of the process: (1) initial design of the investigative microarray; (2) tissue acquisition; (3) critical evaluation of the results; (4) comparison with traditional methods; and (5) definitive design of the diagnostic gene or tissue microarray. Regarding the second item, it is self-evident that to establish and implement rigorous criteria for the acquisition and selection of tissue used for gene expression profiling the participation of an experienced surgical pathologist is necessary.

With regard to evaluation of results from molecular methodology, the pathologist is in an ideal position to monitor the claims (sometimes exaggerated) that are made by overenthusiastic writers. For instance, in a seminal paper on 'Molecular portraits of human breast tumors', Perou *et al*[33] made the claim that a striking feature of the intrinsic gene subset cluster analysis led to a novel division of the breast cancers into two subgroups: basal (myoepithelial) and luminal. This would be remarkable save for the fact that Murad[34] made almost identical divisions more than 35 years ago on the basis of histochemical and electron microscopic criteria when he stated that the features found suggested a dual origin for the two main variants of mammary duct carcinoma, one closely resembling epithelium and the other related to myoepithelium. Despite this 'reinvention of the wheel' with more sophisticated technologies, there are certainly problems likely to benefit greatly from gene expression analysis in conjunction with morphology. For example, the search for the unknown primary tumor is almost certain to be aided by the myriad of tissue-specific markers, both known and to be discovered.[28]

In closing, it needs to be said that a key requirement for integration is a close cooperation among surgical pathologists and molecular biologists. It is a great pity that this all-too-obvious goal is so difficult to achieve in real life. All that it requires (and it is not easy) is mutual respect and the willingness to view things from different angles. As Fisher[14] wisely stated: "It would be a mistake to discourage cancer researchers from seeking the insight of seasoned morpho logically-oriented pathologists, or to discourage young pathologists from asking what morphology tells about the biology of cancer". From the vantage point of the pathologist, the challenge is great. To quote Giordano[32] once more: "If we are unable to find a way to implement molecular profiling into our practices, surgical pathologists will be excluded from one of the most exciting and transformational developments to come around in a long time. And that, in my opinion as an academic and molecular pathologist, would be a real shame."

Note: Some of the opinions in this article have been expressed previously.[35–37]

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