Molecular Genetic Analysis in the Pathologic Evaluation of Solid Tumors: Theory and Practice

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INTRODUCTION

Microscopic interpretation of tissue alterations, otherwise known as anatomical pathology, to this day constitutes the standard, universally employed approach to diagnostic evaluation of human neoplasia. In fact, few tests performed on tissue samples (excluding microbiologic and virologic culture of pathogens) do not employ direct visual analysis of disease-associated microanatomic changes. As a technology, anatomical pathology is venerable (if not ancient), but as a practice it is highly experience and observer dependent. This in large part reflects the rich diversity of cellular and tissue level alterations (phenotype) inherent to disease states, particularly neoplasia. Thus, morphology has not only been successful in diagnosis of neoplasia, but in nuances of classification, staging and prognosis as well. Microscopic cellular patterns have even been employed to assess cells and tissues for risk of further progression to malignant neoplasia, as in the Papanicolaou smear. Finally, dynamic tissue alterations correlate so strikingly, and with such subtlety, to clinical phenomena that histology has long remained unchallenged as a model for which to assess the stages of solid tumor neoplastic progression.

The recent emergence of molecular genetic technologies (genotyping) has introduced a different, and possibly more refined, vision of neoplasia than classical tissue morphology (phenotype). Indeed, genetic analyses have defined the pathology of neoplasia at a different level—as a complex of structural DNA alterations that induce inappropriate gene expression leading to cellular and tissue alterations. To date, the major contributions evolving from genetic investigations of neoplasia have been centered on etiology and pathogenesis—areas largely unaddressed by the realm of anatomical pathology. In other words, histopathology constitutes an accurate reflection of how cell phenotype and tissue differentiation are abnormal but not necessarily why such alterations occurred. By contrast, molecular genetic technologies are gradually revealing how the structural pathology of cellular DNA causes abnormal proliferation and differentiation. Increasingly, though, technologies developed by the molecular and cytogeneticists are being applied to tissues historically

classified by anatomical pathologists. Herein, we shall briefly provide examples of how the newly established genetic technologies have already been employed on tissue samples in order to resolve specific clinicopathologic problems in evaluation of neoplasia. Then, we will take a closer look at two particular genes in order to highlight some of the theoretical and technical limitations of such studies.

GENETIC PATHOLOGY: UTILITY IN CLASSIFICATION OF SOLID TUMORS

The diagnostic utility of histology is largely dependent on the more or less reliable differences between tissue patterns imposed by neoplastic injury, versus reactive or inflammatory, disease states. Any surgical pathologist, however, would readily concede the existence of diseases with ambiguous microscopic features. A noteworthy example of this problem is the morphologic classification of lymphoid proliferations (reviewed elsewhere in the issue). Most malignant lymphomas are comprised of a monotonous population of cells, since they represent a clonal proliferation of cells sharing similar lineage and differentiation. Although constituting a relatively small minority of cases, some lymphomas present histologically with a deceptively heterogeneous microscopic appearance that may be difficult to reliably distinguish from reactive lymphoid hyperplasias. Molecular genetics has proved useful in resolving this problematic ambiguity in tissue morphology by establishing monoclonality among lymphoid populations through detection of “restricted” immunoglobulin or T-cell receptor gene rearrangements. A clonal population accounting for as few as 1% of the cells in the sample may be identified as an unusually large band of digested DNA in Southern blot assays. In such cases, the ability to detect a clonal population may provide useful diagnostic information. It should be noted, however, that hematopathologists emphasize the need to correlate such data in a judicious man-

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ner with clinical and morphologic findings since monoclonality alone is not fully sufficient for a diagnosis of lymphoma.

A significant degree of diversity may also lie obscured behind the apparent uniformity of a primitive, or "undifferentiated," phenotype. Thus, another noteworthy (albeit highly specialized) contribution of genetic testing to tumor classification has been to refine the differential diagnosis of "small round blue-cell neoplasms." This group of neoplasms, which typically occur in children, comprises a histogenetically diverse spectrum of entities including Ewing's sarcoma, primitive neuroectodermal tumor (PNET), certain forms of rhabdomyosarcoma, and lymphoma. The difficulty in pathological classification of such tumors derives from the similar histological appearance of this group of neoplasms, all of which consist of crowded cells, exhibiting high nuclear to cytoplasmic ratio and minimal stroma. Accurate classification, though, is clinically important owing to differential responses to therapeutic protocols. In spite of their morphologic overlap, an accumulating body of evidence would suggest that these primitive neoplasms may be distinguishable on the basis of genetic pathology or gene expression. Specifically, Ewing's sarcoma and a subset of PNETs are distinguished by the consistent presence (90% of cases) of a translocation involving chromosomes 11 and 22 (t[11;22] [q24;q12]) (1). This translocation splices the 5' end of a novel gene, called EWS, with the 3' end of an oncogene, FLI-1. Although the translocation was originally identified using karyotype analyses, metaphase spreads are impractical as a diagnostic modality even for reference laboratory facilities, since they are impossible to perform on fixed, embedded tissue samples. However, there are recent reports demonstrating identification of the fusion transcripts resulting from the translocation using RNA-polymerase chain reaction (PCR) on unfixed tumor aliquots (2). Obviously, the cost of such testing would be a function of probe availability as well as the number of tests performed by a given laboratory.

The presence of the t(11;22) translocation is not limited entirely to Ewing's sarcoma. Another neoplasm which occurs in young adults, called desmoplastic small round cell tumor, also demonstrates an 11;22 translocation (3). This translocation, however, occurs at p13;q12 and results in a fusion transcript between EWS and the Wilms' tumor gene, *WT-1*. The chimeric EWS-WT-1 gene transcript is believed to act as a transcription factor since it contains DNA binding domains. Studies such as these are typical of most current publications addressing the diagnostic relevance of genetic pathology in solid tumor neoplasia since they are concerned primarily with the issue of tumor histogenesis. The apparent genetic homology, for example, between Ewing's sarcoma and neuroepithelial tumors would appear to resolve the long debated histogenesis of Ewing's sarcoma, which is now regarded as being derived from a predifferentiated neuroepithelial cell. As previously implied, though, genetic technologies may also

be used in order to confirm myogenic differentiation in "small round blue cell tumors." This is performed by detection of MyoD1 gene expression using mRNA probes (4). Indeed, characteristic structural lesions involving chromosomes 11 and 13 have been shown to distinguish between two histologic variants of rhabdomyosarcoma—the embryonal and alveolar types.

These developments in "small round blue cell tumors" of childhood and youth raise the question of whether neoplasias may eventually be classified on the basis of distinctive genetic alterations. It certainly stands to reason that neoplastic cell morphology would, at least partially, reflect phenotypic features imposed by structural genetic perturbations through deranged expression of target genes. Histologically distinctive subsets of ovarian epithelial neoplasia, for example, are characterized by dramatically different frequencies of chromosome 17 deletion (serous, 49%; endometrioid, 15%; mucinous, 4%) (5). However, due to the obvious microscopic differences between these neoplasms, the clinical value of such genotypic distinctions remains marginal at best. Most "common" epithelial malignancies, moreover, such as lung, breast, or prostatic carcinomas, are genetically much more heterogeneous than hematopoietic or stromal tumors, such as Ewing's sarcoma. Among nonhematopoietic malignancies not previously mentioned, in fact, "diagnostic" cytogenetic lesions have only been described for myxoid liposarcoma (t[12;16] [q13;p11] clear cell sarcoma (t[12;2]), extraskeletal chondrosarcoma (t[9;22]), synovial sarcoma (t[x;18]), germ cell tumors (i[12p]), retinoblastoma (13q-), and Wilms' tumors (11p-) (6). Clear cell renal cell carcinomas demonstrate a 3p deletion in more than 95% of cases; however, this alteration may also be observed in other malignancies, such as small cell carcinoma.

Although such overlaps in structural genetic pathology would appear to limit the potential diagnostic relevance of a particular anomaly, it nonetheless stands to reason that the clinical diversity of common solid tumors is a reflection of genetic heterogeneity. However, investigators are only beginning to uncover the genetic basis of morphologic, clinical and epidemiologic diversity for colon, breast, and prostate carcinoma. More importantly, perhaps, is that the diagnostic relevance of genetic subclassification for these tumors is likely to depend on the extent to which clinical evaluation or therapeutic approach are altered by the findings. It has been reported, for example, that certain mutations of *k-ras* are associated with clinical aggressiveness in colorectal neoplasia. Unfortunately, treatment options for patients with this malignancy remain limited.

In breast carcinomas, though, the option of adjuvant therapy for stage I patients is determined largely by the apparent intrinsic aggressiveness of the primary lesion. Considerable debate has resulted over which "markers," or a set of "markers," would best identify patients with an aggressive subset of node-negative breast carcinomas, who may benefit from

adjuvant chemotherapy. In addition to traditional features such as differentiation and mitotic index, many investigators have attempted to stratify such cases by examining the prognostic relevance of oncogene overexpression or tumor suppressor gene mutation (see later). Ongoing molecular genetic investigations continue to uncover novel genes which may correlate with metastatic phenotype. One such report describes an association between loss of heterozygosity (LOH) on chromosome 12 and metastatic phenotype (7). Interestingly, LOH was present in only 4% (1/27) of the node-positive lesions, versus 41% (11/27) of node-negative cases. Although such studies suggest that molecular level genetic tests may at some point provide a basis for clinical decision making, this is clearly an evolving field which has yet to define the full extent and complexities of genetic pathology and their relationships to tumor behavior.

UTILITY OF GENETIC METHODOLOGY IN STAGING OF NEOPLASIA

In addition to the occasional diagnostic ambiguity of surgical pathology due to morphologic overlaps (either with other neoplasms or with reactive states), traditional tissue examination is also impaired by its relative inability to detect small numbers of malignant cells within otherwise benign tissues. This becomes clinically relevant when examining tissue samples for detection of "early" neoplasia or when searching for microscopic foci of local disease spread or metastasis. By virtue of its extreme sensitivity (detection of one cell in 10,000–100,000), PCR-based technologies offer considerable potential advantage in this area. To date, PCR-based submicroscopic detection of neoplasm-specific gene sequences has been attempted in three distinct clinical settings. First, it has been demonstrated to have greater sensitivity than immunohistology in detection of bone marrow micrometastases in patients with prostatic adenocarcinoma (8). The presence of so-called "occult" bone marrow micrometastases has been described for more than a decade in carcinomas of the breast, and they have been shown to be present (using immunohistochemical methods) in approximately one-fourth of patients with localized disease at initial diagnosis. Follow-up studies in breast cancer patients have demonstrated statistically significant correlations between presence of such "micrometastases" and treatment failure (9). Using RT-PCR methods, which are beginning to be marketed as kits, circulating tumor cells may be detected even in peripheral blood (10). Some investigators believe such tests may represent an objective method of establishing presence of extracapsular disease in prostatic adenocarcinomas. The problem with submicroscopic analysis of tissue samples, presuming acceptable costs and reproducibility, is how to interpret the results. It is well established, for example, that a large number of neoplastic circulate in the blood and some may even extravasate. However, the efficiency of circulating neoplastic cells in establishing

metastases is extremely low and estimated to occur from only one in 10^5 – 10^7 circulating cells. In other words, not all circulating tumor cells are destined to become secondary tumor growths. Thus, understanding the clinical relevance of tumor cell deposits found at an anatomic site with an intricate microcirculation, such as bone marrow, will require differentiating circulating cells from micrometastases. Given the preliminary nature of data presently available in published studies, a consensus has not yet been reached on the specificity and clinical value of such testing. Nevertheless it is likely that the ability to quantitate the number of cells at a given site or possibly to measure their expansion may, at some point, provide a guide to evolution of metastatic phenotype among circulating tumor cells.

Genetic analyses have also been employed to detect gene mutations at the edges of resected tumors, as a sort of molecular assessment of surgical margins (11). In squamous carcinomas of the head and neck, presence of such mutations may provide a marker of local relapse. Finally, it has been reported that mutated copies of ras encoding DNA may be identified in fecal samples (12), suggesting that PCR-based tests may be potentially efficacious in screening for neoplasia. Analogous data have been reported in urine samples for the p53 gene. The reader should bear in mind, however, that mutant gene sequences per se are by no means diagnostic for malignancy since they may be observed in adenomatous or dysplastic tissues.

PROGNOSTIC MARKERS: P53

By far the greatest volume of recent publications analyzing genetic pathology in neoplastic tissue addresses the value of specific alterations as prognostic markers. A substantial proportion of these studies investigate p53, which is perhaps fitting, since p53 mutations probably constitute the most frequent genetic alteration in human solid tissue neoplasia. Ongoing revelations that catalogue the diverse cellular ramifications of p53 activity appear to account for the seemingly universal importance of this gene to the biology of diverse tumor systems. It participates not only in regulating the progression through the cell cycle at the G1–S interface, but also in mediating apoptosis (13). The latter activity has generated considerable recent interest in view of the finding that p53-mediated apoptosis is induced under conditions of DNA damage (including therapeutic radiation). Lack of wild-type p53 activity, accordingly, correlates with genetic instability in addition to inappropriate cell cycle regulation (14). It is thus hardly surprising that p53 mutation is so strongly correlated with adverse prognosis for such a variety of human tumors (15).

The most commonly employed method of assessing p53 status in solid tumor specimens is immunostaining. Mutant forms of the protein are detectable by immunohistologic methods not only because they have a different conformation than wild-type protein, but also because they accumulate to higher

levels in neoplastic cell nuclei (16–18). Until recently it was presumed that p53 immunostaining with “mutant-specific” monoclonal reagents closely reflected the presence of alterations at the DNA level. This view, however, has been brought into question with the publication of data that reveal a significant incidence of staining without mutation or vice versa (19). Some discrepancies are no doubt accounted for by variable sensitivities and specificities of different commercially available monoclonal reagents (20–22). Closer examination, however, has demonstrated an even more complex situation. First, there is reasonably convincing evidence that wild-type protein accumulates to levels detectable by immunohistology within neoplastic cell nuclei of some tumors (23). Some have speculated that this may be a “physiologic” response to DNA damage within rapidly cycling, possibly ischemic, neoplastic populations. Wild-type protein may also exist in conformational states which overlap with mutant forms.

Second, p53 mutations are heterogeneous (24). Most occur in phylogenetically conserved exons that encode DNA binding sequences in the protein and result in base pair substitutions. Many of these point mutations, called *missense mutations*, result in a conformation-altering exchange of a single amino acid at a critical point in the protein sequence. Such examples would theoretically be positive by immunostaining. A significant number of mutations, however, represent base pair insertions, deletions, or substitutions, which result in *stop* codons. These so-called nonsense (or *frameshift*) mutations would in many cases result either in absence of protein or in a protein that is too truncated to be recognized by the monoclonal reagent. It is unclear at this point what proportion of p53 mutations are of the nonsense type. Preliminary investigations would suggest they are reasonably frequent in breast carcinomas, accounting for up to one-third of p53 mutations in some series (25). However, if previous experience with other genetic alterations holds true, then the pattern of mutational abnormalities will be dependent not only on primary site but also on a variety of epidemiologic factors, including ethnicity (26), geography, and degree of exposure to various mutagenic agents. Finally, some discrepancies between immunostaining and genotype may reflect presence of mutations outside of the conserved exons. The functional and clinical relevance of such mutations is unclear at this point. It is not necessarily the case, by the way, that all point mutations cause similar effects at the cellular level. Correlations between p53 status and elevated proliferative fraction have been shown to be dependent on which exon is involved by the mutation (27).

The p53 story implies that individual genes are characterized by considerable biological heterogeneity in alterations of structure and function within solid tumors, even for one particular primary site. These complexities impose a degree of uncertainty on the interpretation of genetic tests, particularly when performed on a single patient (i.e., as opposed to a carefully selected, statistically analyzed series). Moreover, in

addition to heterogeneous alterations within a given gene, such as p53, the prognostic relevance of a mutation, an amplification or a translocation is likely dependent on other factors that are unique to a particular neoplasm. In other words, neoplastic phenotype is a consequence of multiple genetic and environmental parameters. As such, it is unlikely that the status of any single gene alone is likely to provide a comprehensive picture of clinical behavior in all cases.

It has become widely accepted, in fact, that the aggressiveness of a particular neoplasm is a function of the phenotypic aberrations (i.e., loss of growth regulation or ability to invade and metastasize) induced by the sum, or aggregate, structural genetic pathology (28). That is, tumors of a given type having the fewest genetic lesions have a better prognosis. Given this paradigm, as well as the likelihood that genetic lesions are not apt to be acquired in a consistent sequence, the most informative genetic “test” would optimally provide a measure of the total, or cumulative, genetic deviation from normal. Given the extreme complexity and diversity of genetic pathology intrinsic to solid tumors, as well as the limited scope of any currently available genetic assays, the ability to improve outcome predictions over conventional staging and histopathologic data presently appears marginal at best. The College of American Pathologists, in fact, has recently analyzed the clinical value of “prognostic tests” (mostly “genetic markers”) for colorectal, breast, and prostatic carcinomas. Their Consensus Statements, assembled by leading researchers, have concluded that most such prognostic tests do not materially improve upon conventional clinicopathologic data (29).

FAMILIAL BREAST CARCINOMA AND BRCA1

The potential complexities of molecular genetic analyses applied to human neoplasia may also be illustrated with the so called breast cancer susceptibility gene, or BRCA1 (30). Familial clustering of breast carcinomas is well described and mendelian genetic predisposition is believed to account for 5–10% of cases. Patients with inherited breast cancer risk typically develop the disease, while premenopausal, at substantially earlier ages than sporadic cases. Many such families also have a predisposition to development of ovarian carcinomas. It had long been presumed that these disease patterns reflected the existence of an undiscovered tumor suppressor gene, akin to the retinoblastoma gene (31) (Rb), which harbored germline mutations in breast cancer families.

A high frequency of allelic losses on chromosome 17, both in familial and in sporadic breast cancers, led to intense examination of this chromosome, using blood and tissue samples of breast cancer patients (32,33). Near the end of 1994, Skolnick et al. (34) reported the presence of germline mutations at a 17q locus in 5 of 8 breast cancer kindreds. The corresponding protein contains two *zinc finger* regions, a structure that confers DNA binding capac-

ity. Thus, the putative BRCA1 locus is believed to encode a transcription factor.

Although tentative identification of a breast cancer susceptibility gene was met with great enthusiasm and media fanfare, it has also raised some disturbing technical, ethical, and biological issues. The estimated prevalence of BRCA1 in the female population—approximately 1 : 300 to 1 : 800—is expected to drive considerable demand for testing, not only in women with family histories of breast and ovarian cancer, but also in families of women who become afflicted with these diseases during their premenopausal years. Unfortunately, analysis of BRCA1 gene mutation, for several reasons, remains difficult and expensive. First, the gene is 100,000 kb in length, which is approximately 10 times larger than the average gene. More importantly perhaps is that the preponderance of mutations identified to date have been scattered more or less randomly through the gene sequence and may be of several types, as previously described with p53 (35). Approaches such as SSCP, a PCR-based technology, will therefore be costly and highly labor intensive. Interestingly, though, stop codons and regulatory mutations have accounted for about two-thirds of the initially recorded anomalies, raising the possibility that analysis of the cellular protein level may suffice to identify many carriers.

An unexpected recent development in the unfolding story of BRCA1 is that one particular alteration of BRCA1, 185delAG, has been identified in a set of high-risk families, all of whom were from an Ashkenazi Jewish background (36). The same mutation was also identified in blood samples from approximately 1% of Ashkenazi Jewish individuals whose family cancer history was unknown. The implication of such data is that there is now an identifiable population group who may benefit from objective genetic testing for breast cancer susceptibility as well as from genetic counseling based on the results of such tests.

Interestingly, acquired mutation of BRCA1 in sporadic breast carcinomas is apparently uncommon. This is very much unlike the other tumor suppressor genes, such as p53, Rb, or APC, where deactivation represents a consistent theme that unites sporadic and familial cases of a given form of neoplasia. Thus, the biological relevance of BRCA1 as a frequent common denominator of breast tumorigenesis and progression remains very much in doubt. This is ironic in the face of recent studies reporting frequent somatic BRCA1 mutations in prostatic carcinomas (37). Further, germline BRCA1 mutations are not believed to account for all familial breast cancer cases. Another gene, BRCA2, located on chromosome 13, is believed to be linked to breast cancer development in some familial breast cancer cases, further complicating detection of individuals at risk.

The potential for detection of cancer susceptibility in otherwise healthy individuals has important medical and ethical ramifications. Most experts believe that germline mutation of BRCA1 or BRCA2 imposes a 60–80% likelihood for de-

velopment of malignant neoplasia (38). How should this information be used, and who should have access to it? Although prophylactic mastectomy with reconstruction hardly seems unreasonable given this level of risk, the cost of such “therapy” is considerable. Who will pay for these procedures, not to mention the costs of “genetic screening” or genetic counseling? Unfortunately, other prophylactic strategies for breast or ovarian neoplasia remain largely untested and early detection technologies are marginally effective. We must thus face the unsettling possibility that, for some patients, the ability to collect ominous genetic information may exceed our capacity to fully resolve their dilemma.

Nevertheless, it is in the arena of “susceptibility testing” that genetic analyses may first find widespread clinical application in the solid tumor neoplasia field. Up to 10% of colon cancer patients, for example, have an inherited mutation of a gene called MSH2, which imposes an 80% likelihood of developing malignancy, not only of the colon but of other organs as well (39). Moreover, approximately 1% of the white population in the U.S. are heterozygotes for the ataxia-telangiectasia gene (40). These individuals have a three- to fivefold increased risk of malignant neoplasia relative to the population at large. Ongoing investigations will almost certainly reveal other genetic “markers” of neoplasia susceptibility, either germline or somatic, and possibly having particular application to gender, ethnic, or racially distinct populations.

The demand for such information, not only among the population at large, but also among insurers, will be potentially enormous. In addition to the medical and financial issues noted above, then, there will almost certainly be ethical and political consequences to the emergence of genetic testing for neoplasia risk. The regulatory burden on testing facilities which evolves in the wake of such problems will no doubt be considerable.

CONCLUSION

Surgical pathologists have spent decades correlating neoplastic tissue patterns with clinical disease parameters. Anatomical pathology and clinical oncology, in a sense, have co-evolved and become co-dependent. The broad utility of tissue morphology is also a function of the enormous amount of biological information intrinsic not only to individual cellular characteristics but also to spatial relationships between diverse cell societies. The emerging molecular genetic technologies present an entirely new and heretofore uncultivated perspective for the pathologic evaluation of disease states. The relevance of such technologies to neoplasia has become manifest, particularly in view of the increasing realization that the primary pathologic alterations leading to malignancy are, in fact, structural lesions involving specific DNA sequences. However, we are only at the beginning of the arduous process of correlating the information provided by such

genetic analyses with histopathology and clinical disease parameters. This task is further complicated by the accelerating pace at which such technologies become available and refined as well as by the explosion of information relevant to neoplasia generated by the cell biologists. Some pathologists have referred to this trend as the "gene of the month club." Nevertheless, genetic technologies are already in demand in order to assist clinical decision making, albeit at points where traditional information gathering has proven ambiguous. These clinical dilemmas include such examples as (a) identification of patients (genetic pedigrees) predisposed to develop cancer or a specific cancer; (b) identification of early neoplastic transformation, especially in situations where the malignant cells are in the minority; (c) better classification of "undifferentiated" malignant tumors to allow a more specific treatment; and (d) prognostic genetic changes in morphologically similar tumors that predict for survival, response to therapy, etc. These applications seem appropriate, but given the highly resolved, yet limited, spectrum of data obtained from the status of a single gene remain at a preliminary stage. In the final analysis, genetic methods are not unlike innumerable previous applications of scientific technology to the art of medicine, the long term value of which was determined by their ability explain clinical phenomena and predict outcome in a practical and reliable manner, as judged by the crucible of experience.

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