Microdissection Genotyping of Mixed Glial and Primitive Neuroectodermal Central Nervous System Neoplasm

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- A 22-year-old man with previous radiation treatment for childhood astrocytoma underwent resection of a right parietooccipital lesion. Histopathology revealed a malignant neoplasm with areas of astrocytic and primitive neuroectodermal components. To resolve the relationship and cellular origin, representative tissue was microdissected from several targets, obtaining a balanced mixture of each element. Nonneoplastic brain parenchyma was separately microdissected to determine polymorphic marker informativeness and to serve as an internal negative control. Despite the relatively small quantity of tissue removed for each microdissection target, sufficient material was available for reliable, balanced, polymerase chain reaction-format genotyping encompassing a panel of tumor suppressor genes and genetic loci associated with these forms of neoplasia. The findings revealed distinct discordant genotypic profiles for each of the neoplastic components. The efficacy of the approach used for molecular analysis of this complex neoplasm and the implication of the genotypic findings are discussed.

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The histopathologic diagnosis of central nervous system neoplasms is often rendered difficult by the relatively small sample size. Reactive astrocytic proliferations can closely resemble a true glial neoplasm, especially in a small biopsy specimen, which may not include adequate amounts of tissue to meet histologic criteria for specific abnormal growth patterns or enough cytologic atypia for the diagnosis of neoplasia. The diagnostic difficulty is further compounded by the intrinsic cellular heterogeneity of these neoplasms, which may display varying degrees of anaplasia in topographically distinct regions. Ancillary techniques suitable for use on small formalin-fixed tissue specimens and capable of providing objective discriminating information with respect to reactive hyperplasia versus neoplasia would be useful.

Histopathologic classification of brain neoplasms can be controversial and may result in discordant opinions even from expert pathologists concerning the cell of origin for a particular neoplasm. It is not uncommon for an individual neoplasm to present considerable variation in neoplastic cell type, particularly in the setting of increased anaplasia, where the cell of origin may be difficult to discern. By the same token, central nervous system neoplasms often present with biphasic or multiphasic patterns with transitional areas between glial, neural, and other cell types of origin. Immunohistochemical stains can be most effective in delineating the differing histogenesis of such neoplasms; however, on many occasions this distinction remains unresolved, despite the use of a broad panel of immunohistochemical markers. Under these circumstances, diagnostic tissue can be exhausted or found to be no longer representative of the cellular patterns observed in initial histology sections.

Advances in sequencing and increased knowledge of the human genome make it likely that a detailed mapping of cancer-related genes and their alterations will become available for clinical application in the near future. Translation of molecular information concerning the structure of the human genome requires appropriate methods to address critical clinical issues. Given the greater reliance on biopsy techniques to establish an initial histopathologic diagnosis, prognostication will depend on maximal extraction of information from small biopsy samples.

Molecular genotyping based on initial nucleic acid amplification by PCR affords a convenient means to generate information on the status of multiple different genes from a very small amount of starting tissue. This tissue, however, must be sampled in a highly representative manner and must be optimally pure with respect to the cellular elements it contains. Light microscopic methods have traditionally achieved this goal, enabling pathologists to directly link morphologic and/or staining features with corresponding biologic characteristics. Microdissected material then serves as the basis for genetic analysis, allowing a variety of specific genes and corresponding gene alterations to be identified.

Using techniques developed in our laboratory that were designed especially for use with small amounts of fixed tissue, we analyzed a complex central nervous system neoplasm notable for the presence of a varying admixture of...
astrocytic and neuronal growth patterns.\textsuperscript{11,12} Our specific objective was to address the issue of whether the variations in morphology were based on clonal differences in neoplasm cell populations or whether there were independent of cell type. This distinction is considered important, since in the former case, differences in biological behavior and treatment responsiveness might be anticipated, whereas in the latter a uniform clinical course can be expected, because the histopathologic variability might be considered unrelated to underlying changes directing neoplasm biology.

**REPORT OF A CASE**

A 22-year-old man who received 5000 rad (50 Gy) following the diagnosis of a cellular astrocytoma (grade III) in the right parietal lobe when he was 1 year old underwent resection of a right parietooccipital lesion. Histopathology revealed a malignant neoplasm with areas of astrocytic and primitive neuroectodermal components.

**MATERIALS AND METHODS**

Biopsy tissue was fixed in 10% neutral buffered formalin and embedded in paraffin. Four-micrometer-thick histologic sections were stained with hematoxylin-eosin, as well as with immunohistochemical stains for glial fibrillary acidic protein (GFAP; Dako Corporation, Carpinteria, Calif), neurofilament protein, S100 (Dako), synaptophysin (Ventana, Tucson, Ariz), Bcl-2 (Ventana), p53 protein (Dako), and Ki-67 (MB-1; Dako). Using the morphologic features derived from these stains, 5 discrete microdissections were formulated for mutational genotyping. Specific sites representing normal brain parenchyma, astrocytic areas,\textsuperscript{2} and unassociated with neoplasia, were selected for microdissection-based genotyping (Table). To control for allelic imbalance secondary to inadequate starting template DNA, the normal brain tissue sample was taken no larger than the remaining neoplasm samples, and all loss of heterozygosity determinations from this sample were required to be in allelic balance.

Nucleic acid amplification by PCR was performed according to the manufacturer’s instructions (GeneAmp kit, Perkin-Elmer-Cetus, Norwalk, Conn). P33-labeled deoxyadenosine triphosphate was used as the report molecule in the PCR reaction, which was carried out for 36 cycles (95°C denaturation, 55°C annealing, 72°C polymerization). Amplification primers were based on flanking sequences situated adjacent to the microsatellite of interest (Table). Sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/).

**PATHOLOGIC FINDINGS**

**Microscopic Findings**

Hematoxylin-eosin–stained cytology (Figure 1) and histologic sections (Figures 2 through 4) demonstrated hypercellular neural tissue within 2 regions. The first region contained astrocytes with moderate nuclear pleomorphism, scattered nuclear hyperchromasia, occasional multinucleated cells, and Rosenthal fibers (Figure 2). The second area was hypercellular and contained cells with nuclear pleomorphism, scattered nuclear hyperchromasia, and scattered multinucleated cells (Figure 3). In the latter, mitotic figures, vascular proliferation, endothelial cell hyperplasia, and islands of necrosis were identified. Immunohistochemical stains for GFAP revealed dense positivity in the first region and moderate positivity in the second region (Figure 6). A Ki-67 immunohistochemical stain showed varying degrees of positivity, ranging from 10% to 60% of cells (Figure 5); the highest degree of positive staining was found in the primitive neuroectodermal areas, and the mixed areas had a range of positive staining from 10% to 40%.

Immunoperoxidase techniques using GFAP strongly demonstrated positive astrocytic cells, interpreted as poorly differentiated astrocytes, rather than trapped “reactive” astrocytes (Figure 6). Within this network of the astrocytic cell processes, there was an another cellular population composed of small cells with scant cytoplasm and round nuclei, which were only weakly focally positive for GFAP (Figure 6). These cells are compatible with primitive neuroectodermal differentiation or primitive neuroectodermal origin. The Bcl-2 stain was strongly positive in these ne-
Figure 1. A touch preparation with 2 divergent cell types: small, pleomorphic, undifferentiated cells and glial cells with clear astrocytic differentiation (hematoxylin-eosin, original magnification ×200).

Figures 2 and 3. There are 2 histologic patterns of the neoplasm with divergent differentiation. The areas are composed of tightly packed, cohesive, small, undifferentiated cells in close contact with a more definitive, loosely packed glial component with clear astrocytic differentiation (Figure 2). Areas with atypical and pleomorphic astrocytes intermingling with thick and hyperplastic blood vessels are evident (Figure 3) (hematoxylin-eosin, original magnification ×100).

Figure 4. Strongly positive Bcl-2 expression in medulloblastoma-like cells (original magnification ×200).

Figure 5. Immunohistochemical staining for Ki-67 (MIB-1) reveals 10% positivity in the first region (A) and 60% positivity in the second region (B) (original magnification ×200).

Figure 6. Immunohistochemical stain for glial fibrillary acidic protein reveals dense positivity in the first regions (A) and moderate positivity in the second region (B) (original magnification ×200).
dulloblastoma-like areas (Figure 4), and the astrocytic cells were weakly positive. The p53 was uniformly negative.

Microdissection Genotyping

Despite the relatively small size of each microdissected tissue sample, adequate material was available to afford 12 different loss of heterozygosity determinations for a variety of gene targets (Table). Two genetic loci, 8q24 and 9p22, showed evidence of loss of heterozygosity exclusively in the primitive neuroectodermal component and not in the astrocytic areas of the neoplasm (Table); conversely, both astrocytic areas showed 9p21 and 10q23 allelic loss. The results were identical for the 2 separate astrocytic/primitive neuroectodermal samples, with one corresponding partial allelic loss pattern, in keeping with a mixed population of mutated and unmutated astrocytic mutation and nonmutated neoplastic cells. No evidence of p53 gene loss was present, consistent with the finding of absent p53 immunohistochemical staining.

COMMENT

Given the patient's history of radiation when he was 1 year old, it is possible that this malignant glioma was induced by radiation therapy, causing divergent cell differentiation showing areas of typical features of glioblastoma, astrocytoma, and medulloblastoma. We consider this neoplasm to be composed of undifferentiated primitive neuroectodermal cells and astrocytes. This neoplasm most likely represents a primitive neuroectodermal neoplasm with mixed glial and neuronal differentiation.

Primitive neuroectodermal neoplasms, despite their apparent morphologic uniformity, are composed of heterogeneous neoplastic cell populations. Maturation of these neoplastic cells and proliferation of cell pools is rapid with eventual preponderance of more anaplastic cell forms to be expected as a part of the neoplasm's normal evolution. It is not uncommon to find different morphologic patterns of growth within a neoplasm, suggesting to the observer that clonal expansion has taken place in a phenotypically distinct cellular component. This histologically and morphologically manifests as an increase in anaplasia, and we recommend that this be taken into account in the histologic grading of the neoplasm, which should reflect the highest degree of cellular pleomorphism. By the same token, morphologic variations may achieve a level of difference suggesting the presence of a multicomponent neoplasm with different cellular histogenesis. Such was the case in this patient, in whom the neoplasm displayed regions of prominent astrocytic growth alternating with areas of primitive neuroectodermal differentiation. Under these conditions, the relationship between these different components remains unclear. More importantly, the impact of these differing cellular elements on the biological behavior and treatment responsiveness remains undetermined.

Multifocal glial tumors have been reported in patients following radiation treatment. Furthermore, radiation in experimental animals may cause multifocal and multiple gliomas. The neoplasm discussed may represent more than 1 neoplasm induced by radiation. Immunohistochemical staining often provides valuable information on the cellular histogenesis of such multicomponent neoplasms and is a very useful diagnostic aid in neoplasm classification (Figures 4 through 6). Immunohistochemistry often fails, however, to provide meaningful predictive insights into the biological potential of such cellular changes. More effective prognostic information can be derived from an understanding of the constellation of genetic alterations present in these morphologically different areas, as gene damage is held to be causally responsible for neoplasm development and progression. This provided the basis for designing a system of multigene analysis that could be applied to discrete microdissection targets of highly representative sites within a given neoplasm (Figures 2 and 3; Table). Central nervous system neoplasms share with neoplasia in general the reliance on not just one but a variety of gene alterations in a specific malignancy. When certain patterns of gene damage are known to be associated with specific forms of human cancer, in general malignancies throughout the body tend to manifest a broad range of involvement of growth regulatory genes, oncogenes, neoplasm suppressor genes, and genes responsible for other forms of cell proliferation. Given that no single gene is universally altered in all cases of central nervous system cancer, the most effective approach to characterize an individual neoplasm on a molecular level is to apply a panel of gene tests and evaluate the aggregate information.

The alternative approach used here is to microdissect normal tissue samples no larger than that of the smallest neoplasm sample and then to maintain a high standard of allelic balance in all normal sample reactions. The availability of separate representative samples of each neoplastic component means that to ensure that microdissection genotyping is free from the effects of allelic dropout. Therefore, with the advantages and potential caveats in mind, morphology-based molecular genotyping offers a paradigm shift in the practice of pathology.

Dr Martinez died in December 2002 and we will be forever grateful for his contributions.

References

Microdissection-Based Genotyping—Mohan et al