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**"CRITICAL" IMMUNOHISTOCHEMICAL AND GENETIC MARKERS IN GLIAL  
NEOPLASMS**

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## Introduction:

Pathologists have been inundated by published reports of new and potentially interesting diagnostic, prognostic, and putative predictive “markers” whose expression (or loss) hold great promise for more enlightened diagnoses and ultimately better patient care. The first findings of the NCI's Cancer Genome Atlas project (1) suggest that although an understanding of therapeutically (and possibly diagnostically) relevant pathways of glioblastoma may be at hand, significant challenges remain (2). While some immunohistochemical and genetic tests have proved to be useful in day to day practice, the utility of many others awaits further study and validation. Yip and colleagues (3) have stressed the importance of critical review of the literature and careful consideration of practical issues such as test standardization, compliance, cost-effectiveness and availability.

Histological study of appropriately sampled tissue remains the gold standard for glioma diagnosis (4). The current WHO Classification of nervous system tumors (5) is based on histopathological diagnosis of defined entities and variants with confirmation by ancillary testing. A guiding principle of this presentation is that immunohistochemical and molecular studies supplement or complement conventional H&E histology. Ancillary studies are particularly useful in small samples from stereotactic biopsies that are used in surgical neuropathology. Information such as patient age, duration of clinical symptoms, and imaging findings showing the location (intra-axial vs. extra-axial) and appearance of the lesion typically provide a working differential diagnosis for subsequent histopathological analysis. An awareness of clinicopathologic conditions in surgical neuropathology and known variations or variants of histopathologic entities should guide the judicious use of ancillary studies (rather than the other way around).

Another reason to advocate a practical approach for ancillary testing in glioma diagnosis is that many patients will opt for an investigational therapeutic protocol. Most protocols today require tissue studies using the paraffin blocks. A requirement for as much as 200 mg of viable tumor will be necessary in some cases for the patient's inclusion in a clinical trial. Therefore, when the diagnosis of a glioblastoma (for example) is readily apparent by routine histology, it may be more prudent to ensure that sufficient tissue is available for such protocols rather than exhausting the block with many unnecessary or poorly validated immunostains.

## Putting the “Multiforme” back into Glioblastoma (WHO Grade IV)

Since the 2000 WHO classification of nervous system tumors, the term “multiforme” has been excluded from glioblastoma multiforme (GBM), the most common malignant glioma of adults (6). However, GBM (the ‘M’ being retained for present purposes) is defined by significant variations in histology, both within individual tumors and among different patients. Molecular heterogeneity is well recognized in GBM (7) and some molecular genetic characteristics have been associated with certain histologic features (8-11). Molecular testing is now required for patient stratification in clinical trials for GBM but such has not yet reached ‘mainstream’ pathology. In this section, the utility of ancillary studies for the diagnosis of some important GBMs representing two ends of a histologic spectrum is discussed.

The presence of occasional multinucleated tumor giant cells is common in typical examples of glioblastoma multiforme. However, the predominance of giant cells along with cohesion, distinct cell borders, and a reticulin-rich stroma are histologic features of the **giant cell variant of GBM (GCGBM)** (5). This variant accounts for up to 5% of all glioblastomas. The average age of presentation is 42 years, which is less than for GBM overall (55 years), and the former has a wider age range. Historically referred to as “monstrocellular sarcoma”, the advent of immunohistochemistry and strong reactivity of the tumor cells for glial fibrillary acidic protein (GFAP) defined the glial lineage of this neoplasm. GCGBM may mimic a metastasis because both tumors typically show gross circumscription with associated peritumoral edema, a subcortical location, and highly atypical tumor cells with cohesive cell clusters and distinct cell borders. However, presence of a single lesion on imaging studies in a relatively young patient would favor a primary CNS tumor. The finding of GFAP reactivity in GCGBM is especially useful because of the known cross-reactivity of some cytokeratin antibody cocktails (CAM5.2, MAK6) in glioma cells and even reactive astrocytes (12,13). This potential pitfall should be kept in mind when the differential diagnosis is between glioma and metastasis.

Molecular and genetic analysis has revealed that GCGBM occupies an intermediate position between primary (*de novo*) GBM and those that are believed to arise from sequential anaplastic transformation of lower grade tumors (so called "secondary GBM")(5,10,14,15). Like the primary GBM, GCGBMs tend to arise *de novo*, have a short clinical history, and have mutations of the *PTEN* gene in about one third of cases. However, *p53* mutations have been demonstrated in 75-90% of GCGBM while *EGFR* amplification is rare. The opposite is true of 'primary' GBM (5). Frequent *p53* mutations and the lower age at diagnosis are features shared by GCGBM and secondary GBM. While this variant of glioblastoma has been reported to have a better prognosis than more typical GBM, this may be related to the higher proportion of younger patients with the giant cell variant.

At the other end of the size spectrum, small neoplastic cells are also common in glioblastomas. However, malignant astrocytic gliomas composed primarily of small neoplastic cells have been recently defined (8,9). The "**small cell glioblastoma**" (WHO Grade IV) is important to recognize because of its histologic resemblance to the anaplastic oligodendroglioma (WHO Grade III). Histologically, glioblastomas showing predominantly small cell architecture are highly cellular and cytologically monotonous (8). They are primarily composed of small astrocytic cells with oval, mildly hyperchromatic, and deceptively bland nuclei that contain occasional small nucleoli (9). Mitoses are typically frequent and cytoplasmic borders are inconspicuous. While vascular endothelial proliferation and necrosis are present in the small cell GBM, as they are in other forms of GBM, Perry and colleagues emphasized that gliomas falling within the histologic spectrum of WHO grade III anaplastic astrocytomas could also show small cell histology (9). Such tumors can mimic the radiologic and histopathologic features of anaplastic oligodendrogliomas, but have a much worse prognosis (11 months median survival). In addition to nuclear and cellular uniformity, chicken-wire vasculature, clear haloes, perineuronal satellitosis, and microcalcifications were observed, thus further simulating oligodendroglial tumors. The findings of oval or elongated nuclei and inconspicuous cytoplasm in small cell astrocytic tumors are perhaps most useful in distinguishing them from anaplastic oligodendrogliomas, which are typically composed of cells with uniformly round nuclei and well-defined clear to amphophilic cytoplasm.

Astrocytic features of small cell glioblastomas and anaplastic astrocytomas are readily demonstrated by immunohistochemistry for GFAP, which reveals strong reactivity of thin cytoplasmic processes. A high MIB-1 labeling index is consistent with the frequent mitotic figures that characterize these tumors. Molecular characteristics help to further define the small cell glioblastoma. A high proportion of GBMs with small cell phenotype show amplification of the epidermal growth factor receptor (EGFR) (8,9,11). About 50% of tumors are immunoreactive for EGFR variant III, while about 80% are at least focally positive for wild type EGFR. Additional molecular changes include gains or polysomy of chromosome 7 and chromosome 10q deletions. In marked contrast to oligodendroglial neoplasms, none of the small cell astrocytic tumors showed 1p deletions and only one case was deleted for chromosome 19q. These findings lead Perry et al. to conclude that, in addition to 1p and 19q testing, analysis of EGFR and 10q status may improve diagnostic sensitivity in difficult cases (9).

### **Infiltrating vs. Focal Processes**

Diffuse gliomas encompassing WHO Grades II-IV all invade the central nervous system. Pleomorphism, mitoses, vascular endothelial proliferation/hyperplasia, and necrosis are used for grading gliomas but all will have infiltration (4,5). Thus, **diffuse astrocytomas (WHO Grade II)** are composed of GFAP-immunoreactive, differentiated fibrillary or gemistocytic, astrocyte-like cells that infiltrate gray matter producing 'perineuronal satellitosis' with subpial and perivascular tumor cell accumulations (i.e., "Scherer's secondary structures"). Infiltration of white matter by tumor may occasionally be more subtle but can be demonstrated by immunohistochemistry using antibodies that recognize phosphorylation-dependant epitopes of neurofilament protein (NFP) to visualize axons. This may be useful for small biopsies or in situations where more focal forms of neoplasia are being considered. However, even "circumscribed" tumors such as pilocytic astrocytoma or ganglioglioma may at least focally infiltrate adjacent brain tissue.

Bizarre giant neoplastic cells, nuclear pseudo-inclusions, prominent intratumoral collections of lymphocytes, and cytoplasmic lipidization are features of the pleomorphic xanthoastrocytoma (PXA). This

tumor primarily affects younger individuals with superficially located solid or cystic brain lesions and a long history of seizures. PXA can be distinguished from the giant cell glioblastoma by the paucity of mitotic figures and relative lack of necrosis. Bizarre-appearing giant cells are also a feature of the subependymal giant cell astrocytoma (SEGA) associated with tuberous sclerosis. However, the clinical history, periventricular location, and absence of mitotic activity or spontaneous necrosis of the SEGA distinguish it from the GCGBM. The PXA and SEGA share in common a tendency for individual neoplastic cells to express neuronal lineage antigens such as synaptophysin and neurofilament protein subtypes. Glioneuronal tumors will not be discussed formally here. However, the availability of several quite dependable antibodies that recognize neuronal lineage antigens will be useful in the evaluation of such tumors and their distinction from the more common gliomas.

### **Cell Proliferation in the Diffuse Gliomas**

A critical decision point in glioma diagnosis is the distinction between a WHO Grade II diffuse astrocytoma and a grade III **anaplastic astrocytoma**. The finding of mitoses by H&E is the most defining diagnostic criterion (4,5,16-18). However, technical considerations, subjectivity, and the experience and diligence of the pathologist are all limiting factors in accurate mitotic counting. The immunohistochemical detection of phospho-histone H3, which is expressed during chromatin condensation during mitosis, was recently shown to be an independent predictor of survival in the diffuse astrocytomas and shows great promise for increased accuracy in detecting mitotic figures (19). Ki67 is a non-histone, cell-cycle-associated antigen that is expressed during G1, S, and G2/M phases. Immunohistochemistry using the Ki67/MIB-1 monoclonal antibody is the most reliable and technically feasible method to measure cell proliferation or tumor growth fraction and has been extensively investigated in the gliomas (4,20-26). Several studies have documented a significant positive correlation between Ki67 labeling indices and tumor grade, and an inverse correlation with survival. In practice, Ki67 is often useful in limited biopsy samples where the differential diagnosis involves diffuse low grade astrocytoma vs. anaplastic astrocytoma. Here, an estimation of proliferative rate can confirm or support the diagnosis when clinical, radiological, and histologic findings have been considered. Measurement of Ki67/MIB-1 is not included in the WHO grading criteria, although Ki67 labeling indices for diffuse astrocytomas (WHO Grade II) are "usually less than 4%" (WHO 2007)(5). Issues such as individual laboratory technique (manual vs. automated counting), interobserver variation, and regional heterogeneity within diffuse gliomas have precluded the establishment of definitive cutoffs between low and high grade gliomas at this time.

### **Molecular Diagnosis in Gliomas: A Work in Progress:**

Current recommendations for molecular testing for the malignant gliomas have been addressed thoroughly in several outstanding reviews (3,4,27). Only a brief update will be provided here. Temozolamide is the first-line chemotherapeutic agent of choice for malignant gliomas that damages DNA by adding methyl groups to the O<sub>6</sub> position of guanine. This effect can be reversed by the endogenous DNA repair enzyme **MGMT** (O<sub>6</sub>-methylguanine-DNA methyltransferase), thus providing a mechanism of chemoresistance to alkylating agent chemotherapies. Several studies have suggested that epigenetic 'silencing' of MGMT by methylation of the gene's promoter region, which occurs in 40-50% of glioblastomas, is associated with improved survival and, by implication, a better response to temozolamide and other alkylating agents (28,29). Assessments of MGMT promoter methylation are currently being performed in clinical trials, and strategies to modulate MGMT activity to make tumors more responsive to therapy are under investigation (29).

However, routine laboratory testing for MGMT promoter methylation or expression is not recommended at the present time (3). First of all, assays for measuring MGMT promoter methylation are nontrivial and each method presents different advantages and disadvantages. There has not been good correlation between MGMT promoter methylation status and protein expression as determined by immunohistochemistry (30-32), and it was recently reported that MGMT promoter methylation status may change in serial glioblastoma samples (33). Furthermore, there are currently no good alternatives to temozolamide and radiation therapy in the treatment of glioblastoma, and even patients with non-methylated MGMT promoter regions demonstrate a survival benefit with this therapy.

Identifying molecular genetic pathways leading to glioma tumorigenesis holds great promise for diagnostic, prognostic, and predictive testing. Involvement of specific genetic alterations was suggested by the common cytogenetic findings of chromosome 10 loss and chromosome 7 amplification in glioblastoma. Subsequently, mutation or deletion of **PTEN** (chromosome 10) was identified as a mechanism for activation of the **PI3K/AKT pathway**, which is associated with increased invasion, proliferation, and tumor cell survival (27). Chromosome 7 gains correlate with amplification of the epidermal growth factor receptor (**EGFR**) that occurs in about 40% of GBMs (4). A subset of patients with glioblastomas that express a truncated, constitutively activated form of this receptor (**EGFRvIII**) and co-express PTEN (thus inhibiting the PI3K/AKT pathway) were shown to respond better to therapy with small molecule inhibitors (gefitinib, erlotinib) (34). However, limited availability of an EGFRvIII antibody and disappointing results of subsequent trials with small molecule inhibitors have argued against routine laboratory testing at this time (3). Continued study of EGFR and the PTEN/PI3K/AKT pathway may better patient stratification for therapy based on tissue analysis (35).

### **Oligodendroglial tumors: Mind your (1)P's and (19)Q's!**

The peak incidence of oligodendroglioma (O, WHO Grade II) and anaplastic oligodendroglioma (AO, WHO Grade III) occurs during the fifth decade (5). One of the most significant discoveries in glioma diagnosis and treatment is the strong association between co-deletions of chromosomes 1p and 19q and oligodendroglioma histology and improved prognosis in AO (36-40). A specific 1p/19q translocation was recently described suggesting a possible mechanism of co-deletion in some oligodendrogliomas (41,42). The association of this genetic change with the 'oligodendroglial' phenotype is strongest when strict histopathological criteria are used (36,37,40). Namely, classic oligodendroglioma is defined by a highly uniform population of tumor cells with round/regular nuclei, and little cell variability. Non-specific but useful features include extensive involvement of the cerebral cortex, perinuclear cytoplasmic clearing ('haloes'), presence of nodules, microcalcifications, microcysts, and delicate branching (chicken-wire-like) capillaries (4,5,40). The presence of strongly GFAP-reactive 'minigemistocytes' and 'gliofibrillary oligodendrocytes' in some oligodendrogliomas does not permit a histopathological distinction between astrocytic and oligodendrocytic tumors on this basis.

Testing of diffuse gliomas with oligodendroglioma-like features for 1p/19q deletions is currently performed by fluorescence in situ hybridization (FISH), loss of heterozygosity assays, or array comparative genomic hybridization. Although there are advantages and disadvantages of each method, testing is robust and is recommended at this time for diagnosis (to complement histopathology) and prognosis (4,5). Such testing may be quite useful in the 'mixed' gliomas, a gray area in neuropathology where other than a lack of histological uniformity, a definitive diagnosis may be difficult (3-5). The utility of this test in distinguishing small cell astrocytic gliomas from oligodendroglial neoplasms was discussed above. Also, several oligodendroglioma mimics such as central neurocytoma, dysembryoplastic neuroepithelial tumor, extraventricular neurocytoma, and clear cell ependymoma do not show the 1p/19q deletion. Finally, oligodendrogliomas are rare in children and typically do not show 1p/19q deletions in this population.

### **Important mimics: Active demyelination and lymphoma:**

Immunohistochemical studies along with standard special histochemical stains may be useful in evaluating non-glial tumors and tumefactive non-neoplastic processes. **Demyelinating disease** is the most important of these since therapy will be much different from that of a malignant glioma. Identifying macrophages on intra-operative consultation is critical to distinguish a neoplastic process from a demyelinating (or other destructive non-neoplastic) disorder. The discohesive character of macrophages is best demonstrated on touch or squash preparations. Immunohistochemical staining for CD68 may be used for confirmation. Although axonal stains (neurofilament IHC or Bodian) may show relative sparing of axons typical of a demyelinating disease, axons degenerate in advanced lesions. In suspected cases of progressive multifocal leukoencephalopathy (PML), characteristic oligodendroglial viral inclusions, recognition of the reactive nature of bizarre astrocytes, and immunohistochemical stains for polyomavirus large T antigen supports the diagnosis. One important caveat is that p53, which is immunoreactive in

about 50% of gliomas (especially astrocytic types), is also reactive in the bizarre astrocytes of PML (43-45).

While the histopathological appearance of atypical lymphoid cells showing an angiocentric growth pattern may suggest a **primary CNS lymphoma**, immunohistochemical studies for B- (CD20) and T-lymphocytes (CD3) will usually be required for definitive diagnosis.

### **Ependymomas and 'ependymal-like' tumors**

Classic ependymomas with their uniform histology, perivascular pseudorosettes, and tapering GFAP-immunoreactive processes may not present a significant diagnostic challenge for experienced pathologists. However, several recently described gliomas show histological, immunophenotypic, and/or ultrastructural characteristics that suggest ependymal-like differentiation (46). The **tanycytic variant of ependymoma** primarily arises in the spinal cord but occasionally presents as a third ventricular or hypothalamic mass. They are typically well demarcated from the surrounding neural tissue. The highly spindled microscopic appearance of bipolar tumor cell processes creates an astrocytic appearance, which is further suggested by poorly defined or absent perivascular pseudorosettes. However, tumor cell nuclei are quite uniform, round to oval, and have salt and pepper-like chromatin similar to other types of ependymomas. Tumor cell processes are typically strongly immunoreactive for GFAP, S-100, vimentin, and CD99. Ultrastructural features including intercellular junctions, numerous slender surface microvilli, and even microvilli-lined lumina are distinctly ependymal. The **clear cell ependymoma** may mimic an oligodendroglioma in its cytologic uniformity. While a circumscribed (rather than infiltrative) border distinguishes this tumor from an oligodendroglioma, the demonstration of 'ependymal' features by EM may be required for definitive diagnosis in some cases.

**Astroblastoma** is a rare glial neoplasm primarily affects children and young adults and has the unifying histological feature of perivascular pseudorosette-like arrangements composed of broad, non-tapering cell processes (47,48). There is no formal WHO Grade at this time. By definition, astroblastomas lack the histologic features of other infiltrating astrocytomas, gemistocytic astrocytomas, and typical ependymomas. The processes of astroblastoma are shorter and less spindled than those of ependymoma and sclerosis of vascular elements may be striking. Immunohistochemical and ultrastructural findings suggest both astrocytic and ependymal features. Tumor cell processes are typically reactive for vimentin and S-100, while GFAP shows variable positivity. Focal membranous pattern of EMA reactivity is well described while neuronal lineage is not typically seen. Ultrastructural features of basal body polarization, apical cytoplasmic blebs with microvilli, and lamellar cytoplasmic interdigitations have suggested a tanycytic histogenesis. A relationship between Ki-67 labeling has not been established as an independent prognostic feature but astroblastomas with high grade histology have higher labeling indices. Gross total resection is associated with a favorable outcome even in high grade tumors although the prognosis is better in patients with low grade histology.

Clinicopathologic features of the **angiocentric glioma** (WHO Grade I) ("monomorphous angiocentric glioma", "angiocentric neuroepithelial tumor") were independently described by two groups in 2005 (49,50). Most of these tumors have arisen in children and young adults with a history of chronic intractable epilepsy. Common sites include superficial cortical regions of the frontoparietal lobe and the temporal lobes including the hippocampi and parahippocampal gyri. Imaging studies and low magnification histology suggest an infiltrating glioma with striking perivascular and subpial spread. However, higher power reveals the tumor to be composed of monomorphous, bipolar cells with elongated nuclei having distinctly speckled, "crisp" chromatin (49). The striking angiocentric pattern is produced by the circumferential or longitudinal orientation of tumor cells along large and small blood vessels. Radial perivascular arrangements of tumor cell processes may focally resemble that of conventional ependymoma or even astroblastoma. Mitoses are rare or absent and there is typically no vascular endothelial proliferation or necrosis. Tumor cells are immunoreactive for GFAP, S-100, and vimentin but are negative for antigens of neuronal lineage (49-51). A distinctive dot-like, microlumen pattern of immunoreactivity for epithelial membrane antigen is typical. MIB-1/Ki67 labeling indices are low (1-5%) although one anaplastic recurrence had a Ki-67 labeling index of 10% (49). A histogenetic origin from bipolar radial glia has been suggested. The prognosis is generally good with gross total excision. An anaplastic recurrence was noted in one subtotally resected tumor (49).

The **chordoid glioma of the third ventricle** was first defined by Brat and colleagues in 1998 (52) and this rare brain tumor is formally recognized as a codified entity by the WHO. This distinctive neoplasm typically arises in the anterior third ventricle in the region of the lamina terminalis (53). Adults are most often affected with a mean age of 46 years and an approximately 2:1 female predominance. Patients may present with hydrocephalus, headaches, and nausea due to ventricular obstruction. Endocrine dysfunction and visual disturbances may also occur due to compressive effects on the hypothalamus and optic chiasm, respectively. Imaging reveals a contrast enhancing, well circumscribed mass of the third ventricle. Classic histologic findings include cohesive cords and clusters of epithelial-like tumor cells that are dispersed within a myxoid stroma that contains a striking lymphoplasmacytic infiltrate. While these features might suggest a chordoma or chordoid meningioma, the finding of strong GFAP immunoreactivity of the tumor cells will confirm the diagnosis. Mitoses are rare or absent and MIB-1 labeling indices are typically low. Numerous Russell bodies can be observed within the stromal lymphoplasmacytic infiltrates. Tumor cells are also immunoreactive for vimentin and variably positive for S-100. Ultrastructurally, tumor cells contain intermediate filaments, intercellular lumina with apical microvilli, hemidesmosomes, basal laminae, and possibly secretory vacuoles. Derivation from specialized ependyma that covers the laminae terminalis and circumventricular organs has been suggested. The attachment of this WHO Grade II tumor to adjacent hypothalamic and suprasellar structures may not allow for complete excision and subsequent recurrence (5).

### **Future Directions in Glioma Diagnosis:**

Gene expression profiling using high-throughput oligonucleotide array platforms holds great promise for improved diagnostic, prognostic and predictive testing, and for the discovery of novel therapeutic targets. Recent studies have indicated that such profiling is as good as histology in subclassifying diffuse gliomas and better than histology in predicting outcomes of mixed or otherwise ambiguous cases (27,54-57). Also, it was recently discovered that gains (tandem duplications) on chromosome 7q34 that involve the kinase domain of the BRAF oncogene occur in pilocytic astrocytomas (58,59) but not in other gliomas. These studies provided evidence for activation of a RAS-BRAF-ERK signaling pathway that could be targeted in the treatment of non-resectable cases. Finally, the identification and study of tumor initiating stem cells is yet another exciting area that may provide a better understanding of glioma tumorigenesis and the development of resistance during therapy (60,61).

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