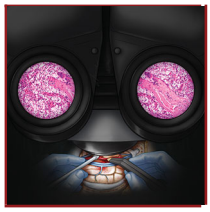

Adult Diffuse Astrocytic and Oligodendroglial Tumors

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Infiltrating gliomas comprise the most common group of primary intraparenchymal brain tumors and present a level of complexity which requires careful integration of histopathology and molecular diagnostics for optimal therapy. To this end, the fourth edition of the World Health Organization (WHO) Classification of Tumors of the Central Nervous System (CNS) has been followed by a series of publications by cIMPACT-NOW (the Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy) incorporating molecular signatures to propose updated diagnostic categories in anticipation of the upcoming fifth edition of CNS tumor classification. Integration of histopathology, immunophenotyping, and molecular findings is profoundly changing the practice of diagnostic surgical neuropathology and enabling a more personalized approach to treating patients with gliomas.

KEY WORDS: Astrocytoma, Diffuse midline glioma, Fluorescence in Situ hybridization, Glioblastoma, molecular diagnostics, Next-generation sequencing, Oligodendroglioma

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Infiltrating gliomas, or "diffuse astrocytomas and oligodendrogliomas," account for the majority of primary brain tumors, generally affect the cerebral hemispheres of adults, and are assigned grades 2 to 4 based on criteria set forth by the WHO Classification of Central Nervous System (CNS) Tumors.[1] (Note that following the recommendation put forth in cIMPACT-NOW [the Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy] update 5, Arabic, rather than Roman numerals, will be used to designate WHO grades.) Treatment is generally complete surgical excision, often followed by adjuvant radiation and/or chemotherapies.

ABBREVIATIONS: ATRX, α-thalassemia/mental-retardation-syndrome-X-linked; **CAR-T,** chimeric antigen receptor-t-cell therapy; **cIMPACT-NOW,** Consortium to Inform Molecular and Practical Approaches to Central Nervous System Tumor Taxonomy; **CNS,** Central Nervous System; **CNV,** copy number variation; **FFPE,** formalin-fixed, paraffin embedded; **FISH,** fluorescence in Situ hybridization; **FUBP1,** far-upstream binding protein 1; **GLASS,** Glioma Longitudinal AnalySiS Consortium; **IDH,** isocitrate dehydrogenase; **IHC,** immunohistochemistry; **MGMT,** O-6-methylguanine-DNA methyltransferase; **NEC,** not elsewhere classified; **NGS,** next-generation sequencing; **NOS,** not otherwise specified; **TERT,** telomerase reverse transcriptase; **TMZ,** temozolomide; **wt,** wild type

Despite enormous advances in molecular diagnostics, a recent review concluded that "while advanced molecular diagnostics like NGS are clearly enhancing the care of patients with CNS tumors..their maximal utility is in the context of detailed, expert histologic analysis, and will likely remain so for the foreseeable future."[2] Infiltrating astrocytomas are characterized by an astrocytic cellular morphology usually within dense fibrillary background, with either necrosis (Figure 1A) or microvascular proliferation (Figure 1B) establishing a diagnosis of glioblastoma, WHO grade 4. Although lower grade infiltrating gliomas have traditionally been graded using a variety of histopathologic features, distinguishing criteria, including mitotic and Ki-67 labeling indices, have not been agreed upon for either isocitrate dehydrogenase (IDH)-mutant astrocytomas or oligodendrogliomas.[3] Oligodendrogliomas are characterized by round nuclei and a paucity of cellular processes resulting in hypercellularity out of proportion to nuclear pleomorphism, as well as easy visualization of arcuate capillaries.

In daily practice, neuropathologists attempt to integrate clinical, radiographic, and

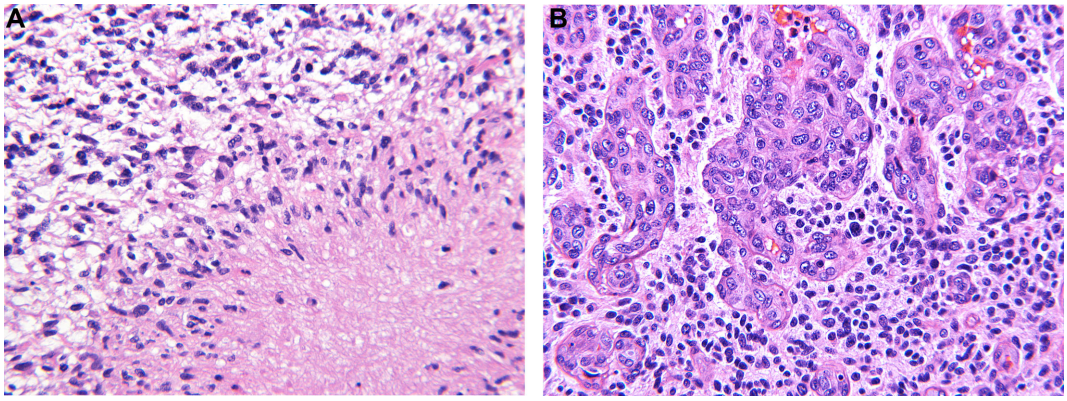

FIGURE 1. *Hematoxylin and eosin sections of a glioblastoma with pseudopalisading tumor necrosis* **A** *and microvascular proliferation* **B**.

histopathological features in order to answer a series of questions. Prior to evaluating the specimen, it is important to know if this is the first diagnostic intervention and whether or not the patient received previous chemotherapy and/or radiation. The location of the tumor is also helpful. For example, characteristic imaging features of diffuse glioma on T2/fluid-attenuated inversion-recovery (FLAIR) is hyperintensity extending from the white matter into the overlying gray matter ribbon and/or into deep gray matter in many cases; curvilinear/gyriform/chunky calcification is very suggestive of oligodendroglioma or the T2/FLAIR mismatch sign which has a strong predictive power for IDH-mutant diffuse astrocytoma in the context of an adult patient with a nonenhancing diffuse glioma.[4,5] Additionally, tumors can be either well demarcated or diffusely infiltrative, with single or multiple foci, affecting one or both hemispheres.

It is also important for the pathologist to know if the biopsy is targeted to the tumor core or to the margins of the tumor mass. In this regard, it is important to know that biopsies sometimes contain tissue outside of the radiologically identified lesion for a number of reasons including shifting of the brain due to edema after stereotactic coordinates have been taken preoperatively and what neurosurgeons consider the optimal target is not always the case. The diagnostic yield of intraoperative diagnoses is relatively high[6] and frozen section interpretation should be used in many cases to provide adequacy guidance with regard to acquisition of appropriate lesional tissue. Other factors to consider include whether the biopsy is being obtained primarily to discern the tumor's molecular signature or if it is a preamble to a planned gross total resection.

We first evaluate the morphology to establish the infiltrative nature of the tumor, and then attempt a preliminary classification of the infiltrating glioma as astrocytic or oligodendroglial. Based on our morphological assessment, as well as the clinical and radiographic features, we attempt immunophenotyping using antibodies to IDH1-R132H, ATRX, p53, and H3K27M, which have become practical surrogate markers for specific genetic alterations used for diagnosis, prognosis, and therapeutic guidance.[7]

IMMUNOHISTOCHEMICAL EVALUATION OF INFILTRATING GLIOMAS

Isocitrate Dehydrogenase

Identification of *IDH1* or 2 *IDH2* mutations is the essential first step in the evaluation of infiltrating gliomas (Figures 2 and 3). *IDH* mutations play a critical role in tumorigenesis and are identified in approximately 70% of diffuse gliomas, especially WHO grade 2 and 3 astrocytomas, oligodendrogliomas, and secondary glioblastomas.[7,8] Patients with *IDH* mutations tend to be younger and have a better outcome than patients without these mutations. *IDH1* mutation typically results in a substitution of arginine at codon R132,[9] which can be detected using a highly sensitive and specific monoclonal antibody to IDH1 R132H, which binds to the mutated protein, showing strong cytoplasmic staining and weak nuclear staining in mutated tumor cells (Figure 4A and 4B). Immunohistochemical (IHC) testing for IDH1-R132H expression should be performed on all infiltrating gliomas, regardless of patient age, as positive antibody staining obviates the need for further *IDH1/IDH2* mutational testing. Other point mutations in *IDH1*, as well as mutations in *IDH2*, are not detected by this antibody and are assessed using molecular methods if IHC is negative in patients less than 55 yr of age, as noncanonical *IDH* mutation is exceedingly rare in patients older than 55 yr (Figure 4C), which at current prices does not justify the cost of molecular testing.[10] *IDH2* mutations are infrequent (~3%) and are mostly encountered in oligodendrogliomas.

α-Thalassemia/Mental-Retardation-Syndrome-X-Linked Gene

The α-thalassemia/mental-retardation-syndrome-X-linked (*ATRX*) gene is involved in chromatin remodeling and is

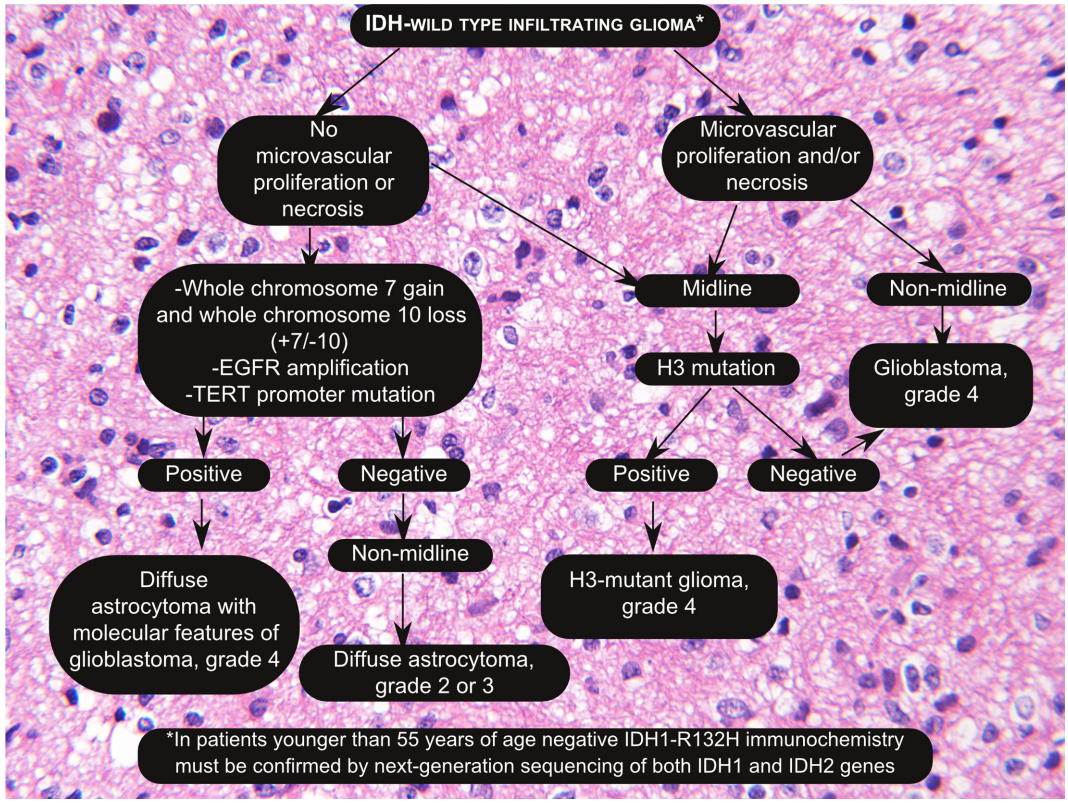

FIGURE 2. *Diagnostic algorithm for IDH wild-type infiltrating gliomas.*

expressed by many infiltrating gliomas (Figure 5A), as well as by non-neoplastic glial and endothelial cells.[11] Although not diagnostic, loss of nuclear ATRX expression is common in astrocytic tumors (Figure 5B) and is frequently (though not invariably) associated with *IDH1/2* or *H3F3A* mutations (Table 1).[12] Loss of expression typically results from either missense or truncating *ATRX* mutations. *ATRX* alterations are commonly associated with *TP53* mutations,[9] and are mutually exclusive with chromosome 1p/19q deletions, abrogating the need for chromosomal assessment in *ATRX* mutated gliomas. Retention of nuclear *ATRX* staining in *IDH1/2*-mutant tumors is strongly but not invariably associated with 1p/19q co-deletion and oligodendroglioma histology.

Tumor Protein 53

Tumor protein 53 (*TP53*) is a transcription factor and tumor suppressor gene located on chromosome 17p13.1. Mutations in *TP53* often occur concurrently with *IDH* and *ATRX* alterations, and are present in more than 50% of infiltrating gliomas. Unfortunately, the presence of *TP53* mutations in about 30% of *IDH* wild-type (wt) glioblastomas precludes the use of p53 immunoreactivity to screen for noncanonical IDH mutations. Strong nuclear p53 immunoreactivity in more than 10% of tumor cells usually, but not invariably, indicates a missense *TP53* mutation (Figure 6). False-negative immunostaining results may occur with nonsense *TP53* mutations or deletions that impair protein expression.

H3K27M

Missense mutations in *H3F3A* and *HIST1H3B*, which encode the H3.3 and H3.1 histones, respectively, were originally described in pediatric diffuse intrinsic pontine gliomas; however, they may also occur in adult midline gliomas.[13] All midline gliomas should be evaluated for H3 K27M mutations, as they carry an adverse prognosis sufficient to warrant a grade 4 designation regardless of the presence or absence of high-grade histologic features. Importantly, the presence of this mutation in nondiffuse, circumscribed gliomas does not warrant a diagnosis of diffuse midline glioma or a WHO grade 4 designation.[14] Currently, K27M mutations in both H3.3 and H3.1 can be detected by IHC using a H3K27M antibody[7] which does not react with tumor cells lacking this mutation (Figure 7A). Positive nuclear staining in tumor cells indicates a mutation; nontumor nuclei will be negative. Demonstration of adequate trimethylation using H3K27me3 antibodies (Figure 7B) may be used to confirm absence of the K27M mutation.

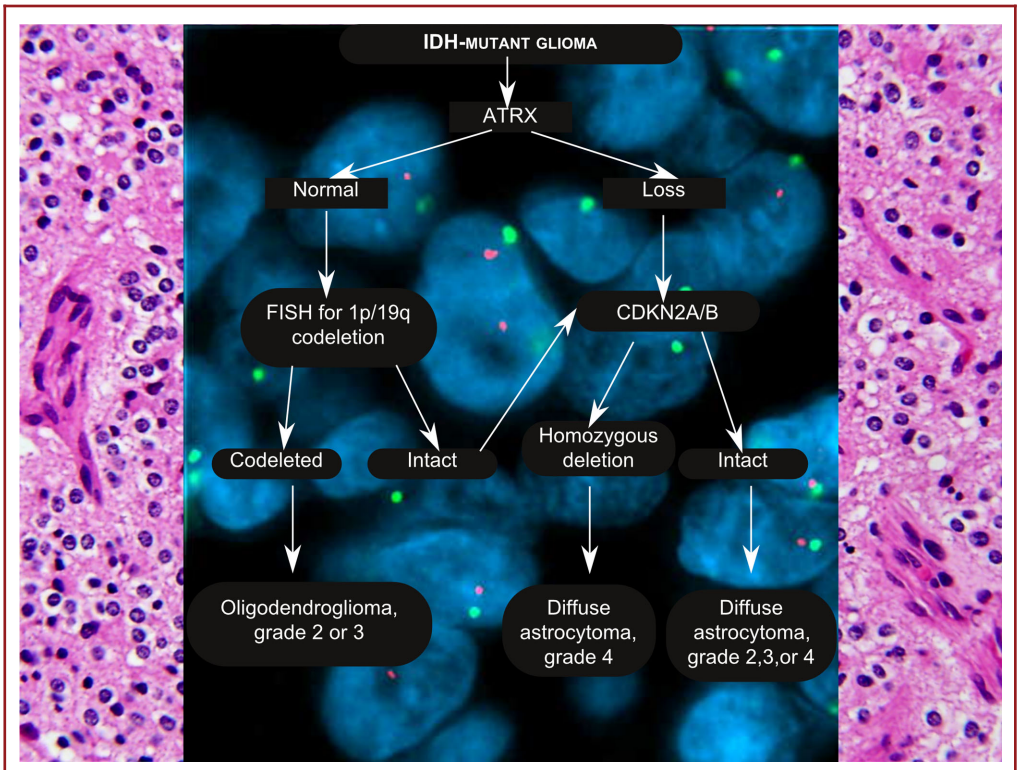

FIGURE 3. *Diagnostic algorithm for IDH mutant infiltrating gliomas.*

Molecular testing provides the third pillar for neuropathological diagnosis of infiltrating gliomas (Table 2). Although slower and considerably more expensive than histologic and IHC assessments, molecular diagnostics are critical to optimizing the care of glioma patients.

MOLECULAR TESTING OVERVIEW

Molecular biomarkers have been incorporated into the WHO classification of gliomas and are increasingly being used to guide therapeutic decision making. To accommodate the expanding list of relevant genes, many laboratories have constructed glioma focused next-generation sequencing (NGS) panels.[15] Most panels require fairly small amounts of formalin-fixed, paraffin embedded (FFPE) tissue, and have a relatively quick turnaround time (less than 10 d). While NGS analysis is not appropriate for many infiltrating gliomas, there are many situations in which it is imperative, such as in glioma patients under the age of 55 yr in which IDH1-R132H IHC is negative (as discussed above).

1p/19q

Fluorescence in Situ hybridization (FISH) should be performed for 1p/19q codeletion in all *IDH*-mutated *ATRX* wt infiltrating gliomas, regardless of the presence or absence of histological evidence of oligodendroglial differentiation.[16] The 1p/19q codeletion occurs due to an unbalanced translocation between chromosomes 1 and 19, resulting in whole arm losses of 1p and 19q. The combination of an *IDH1* or *IDH2* mutation and 1p/19q codeletion is sufficient for the diagnosis of oligodendroglioma.

FISH is a reliable, cost-effective method that allows detection of a chromosomal abnormality in minimal amounts of FFPE tissue sections.[17] Chromosomes 1 and 19 are assessed on separate slides by analyzing the distribution of test and control probes in 20 to 100 nonoverlapping nuclei. FISH results are expressed as a percentage of tumor cells with a deleted signal or as a ratio of test to control probes for each chromosome.[18]

As FISH cannot differentiate between whole chromosome arm deletions with centromeric breakpoints characteristic of 1p/19q codeleted oligodendrogliomas and smaller focal deletions (which are not associated with improved survival and response to chemotherapy), some laboratories might choose to utilize other molecular techniques including array comparative genomic hybridization, single nucleotide polymorphism array, or NGS-based methods. However, these techniques are currently more costly, labor intensive, and require greater technical expertise as well as highly cellular tumor samples.

Outcomes are best for oligodendrogliomas with 1p19q codeletion, although current clinical management is fairly consistent across the spectrum of 1p and 19q deletions.[19] Isolated

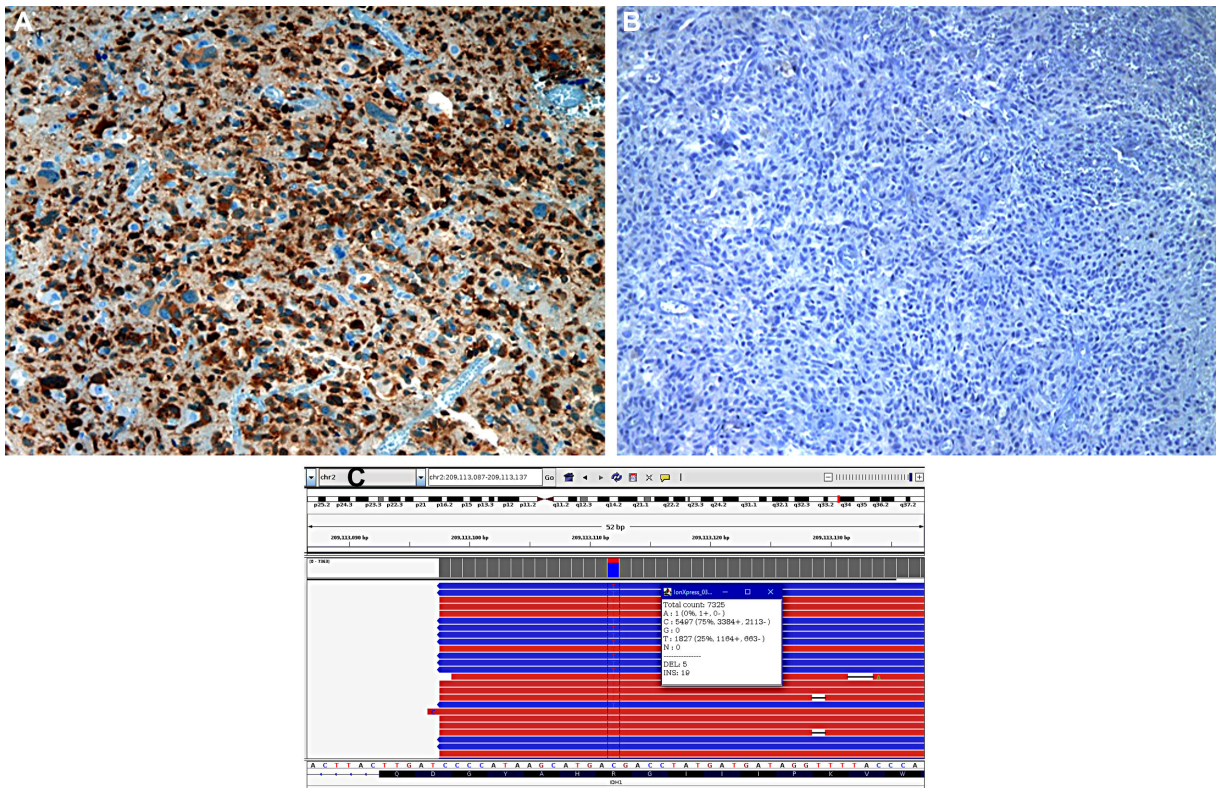

FIGURE 4. **A**, *Positive IDH1 R132H immunohistochemistry.* **B**, *Negative IDH1 R132H immunohistochemistry.* **C**, *Image from Integrative Genomics Viewer showing a mutation in IDH1 by NGS. At this position, the expected nucleotide is a C, but in this sample, there is a T instead (in 25% of the reads), so there is an IDH1 point mutation present.*

1p deletion seems to be associated with somewhat less favorable outcomes.

O-6-Methylguanine-DNA Methyltransferase

O-6-methylguanine-DNA methyltransferase (*MGMT*) promoter hypermethylation, often evaluated by NGS, was initially implemented in order to avoid deleterious side effects of chemotherapy in elderly patients with unmethylated tumors, but is now felt to be essential in the evaluation of all grade 3 and 4 infiltrating gliomas. The *MGMT* gene encodes a DNA repair enzyme which may be epigenetically silenced when the promoter region is hypermethylated, causing loss of DNA repair, which in turn renders tumor cells more responsive to alkylating agents. Temozolomide (TMZ), an alkylating agent that methylates DNA at the O6 position of guanine and can cross the blood-brain barrier, is the most common agent used to treat glioma patients. Recent literature also suggests that lomustine-TMZ combination therapy may increase overall survival in patients with *MGMT* hypermethylated malignant gliomas.[20] *MGMT* promoter methylation is predictive for response to alkylating agents and prognostic for longer overall survival in patients with glioblastomas and in patients with lower grade gliomas classified as "high risk" due to patient age, tumor size, invasion into corpus callosum, and/or preoperative neurological deficits.[21] Some TMZ-treated patients with low-grade infiltrating astrocytomas develop tumor recurrences characterized by a hypermutator phenotype with the TMZ-mutagenesis signature of G: C > A: T[22,23] which has led some investigators to question the utility of TMZ in the treatment of low-grade infiltrating gliomas.[24] On the other hand, it is currently not known whether hypermutation confers a better or worse prognosis or even what the implications are for salvage therapy in these patients.[25] Interestingly, the majority of TMZ-treated glioblastomas recur without hypermutation.[26]

Cyclin-Dependent Kinase Inhibitor 2A/B Homozygous Deletions

Testing for homozygous deletions of cyclin-dependent kinase inhibitor 2A/B (*CDKN2A/B*) should be performed in all IDH-mutant gliomas, as patients with *CDKN2A/B* deletions have a poor prognosis.[27] Homozygous deletion of the *CDKN2A* gene appears to indicate a worse overall survival for patients with

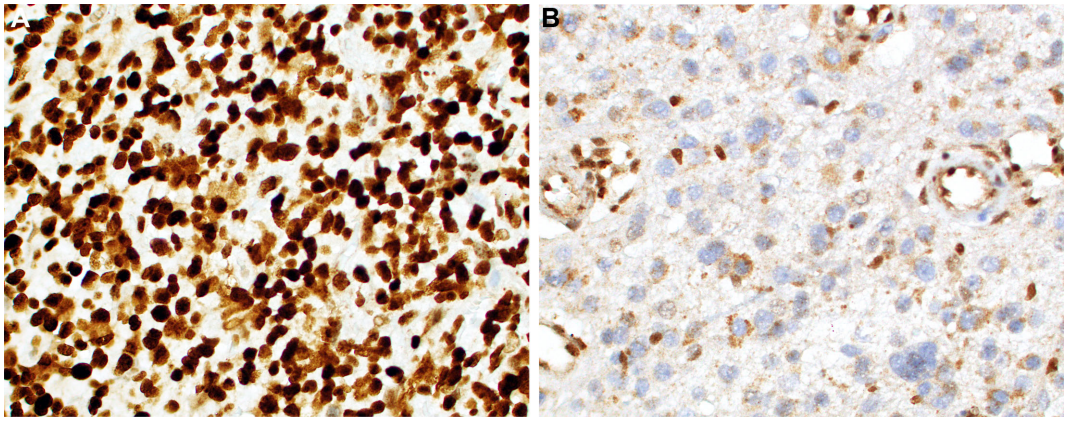

FIGURE 5. *ATRX immunohistochemistry demonstrating wild-type staining pattern* **A** *and ATRX mutant loss of staining* **B**.

TABLE 1. Biomarkers in Adult Astrocytic and Oligodendroglial Tumors

	Loss of *ATRX* expression	1p/19q codeletion	*CDKN2A/B* homozygous deletions	*EGFR* amplification	*TERT* promoter mutation	Gain of chromosome 7/monosomy of chromosome 10	*H3F3A* K27M mutation	*H3F3A* G34R/V mutation
Astrocytoma, IDH-mutant, WHO grade 2-3	++	−	−	−	−	−	−	−
Astrocytoma, IDH-mutant, WHO grade 4 (glioblastoma)	++	−	+/−	−	+/−	−	−	−
Oligodendroglioma/anaplastic oligodendroglioma, WHO grade 2-3	−	++	−	−	+/−	−	−	−
IDH wild-type astrocytoma, WHO grade 2-3	−	−	−	−	−	−	−	−
Astrocytoma, IDH wild-type, WHO grade 4 (glioblastoma)	−	−	−	+/−	+/−	+/−	−	−
Diffuse midline glioma, H3 K27M-mutant, WHO grade 4	+	−	−	−	−	−	++	−
Diffuse glioma H3.3 G34 mutant	+	−	−	−	+/−	−	−	++

ATRX, alpha thalassemia/mental retardation syndrome X-linked; *EGFR*, epidermal growth factor receptor; IDH, isocitrate dehydrogenase; *TERT*, telomerase reverse transcriptase; WHO, World Health Organization.

infiltrating gliomas, grades 2 to 4.[28] It seems that 1p/19q codeletion gliomas are not as affected by this finding as 1p/19q intact gliomas, although more studies are needed to corroborate this finding.

Other Alterations

IDH-wt glioblastomas may be characterized by other diagnostic genomic alterations, including a gain of chromosome 7 or monosomy of chromosome 10. *EGFR* amplifications are present in about 40% of patients.[28] Many of these amplified tumors also demonstrate aberrant expression of *EGFR* variant III (EGFRvIII), which lacks the extracellular ligand-binding region, but is constitutively active.[29] *EGFR* amplifications can be detected using NGS (Figure 8A) or FISH (Figure 8B and 8C). *EGFR* encodes for an ErbB family receptor tyrosine kinase.[30] Activation of *EGFR* prompts downstream signaling networks involved in cell division, migration, and cell survival. *EGFR* gene amplification, overexpression, and/or mutations occur in more than 60% of primary and less than 10% of secondary glioblastomas.[31] *EGFR* alterations in lower grade diffuse gliomas are much less common (<3%); thus, the presence of *EGFR* amplification can be used as a diagnostic marker to upgrade

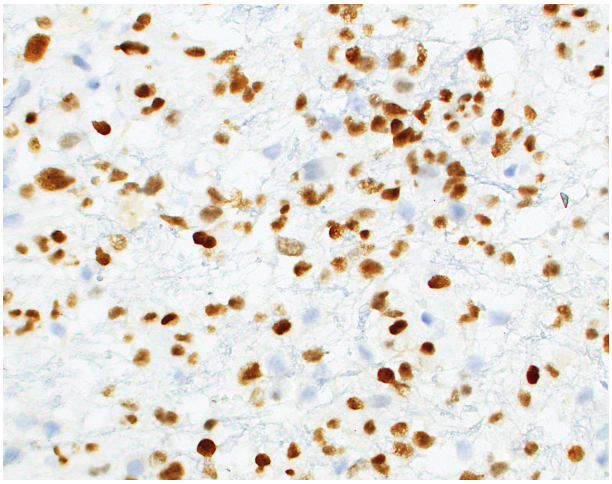

FIGURE 6. *p53 mutant immunohistochemistry demonstrating strong nuclear staining in tumor cells.*

gliomas with low-grade histology to glioblastomas.[32] Additional prognostic and therapeutic implications of *EGFR* alterations in glioblastomas are not yet clearly defined.

Telomerase reverse transcriptase (*TERT*) gene promoter mutation, a molecular marker for primary glioblastoma, is present in nearly 80% of *IDH*-wt tumors, and associated with poor outcomes.[33] In biopsies lacking histopathological features of glioblastoma, *TERT* promoter mutation is sufficient to classify the tumor as WHO grade 4.[34] Importantly, however, mutations are also identified in >95% of oligodendrogliomas,[28] and so cannot be used to upgrade *IDH*-mutant gliomas.

IMMUNOTHERAPY

Immunotherapies are a central component in the treatment of many solid tumors. Somewhat less clear, however, is both the efficacy of immunotherapeutic agents in the treatment of infiltrating gliomas and whether reliably predictive molecular determinants of immunoresponsiveness are available.

Immunotherapeutic approaches to gliomas range from active immunotherapy using viral vector-based vaccines[35,36] to passive immunotherapy, including lymphokine-activated killer cell immunotherapy or monoclonal antibodies against target antigens upregulated in tumor cells.[37] Vascular endothelial growth factor is often upregulated in high-grade gliomas and can be targeted by monoclonal antibodies such as bevacizumab. Despite favorable imaging responses suggesting tumor regression, there still is significant controversy regarding survival efficacy.[38] Similarly, immunotherapeutic targeting of *EGFR* and its mutant variant, EGFRvIII, has garnered reports of good survival outcomes and tumor regression,[38,39] while other clinical trials have failed to demonstrate positive outcomes.[39]

Great enthusiasm currently centers on PD-1-blocking antibodies, such as pembrolizumab, with encouraging responses reported in patients with hypermutant glioblastomas and tumors characterized by microsatellite instability or mismatch repair deficiency.[40-42] Importantly, however, the relationships among mismatch repair protein deficiencies, microsatellite instability, tumor mutation burden, and response to PD-1 blockade are complex, and most studies have been unable to demonstrate clear predictive value for any of these molecular assays within individual patients with malignant gliomas. Availability of adequate tumor tissue for analysis has been limited in some studies, which might preclude identification of potential responders.[43] Obtaining adequate tissue for analysis is particularly important now that TMZ-induced hypermutation of lower grade *IDH*-mutant gliomas has been well established.[23,44]

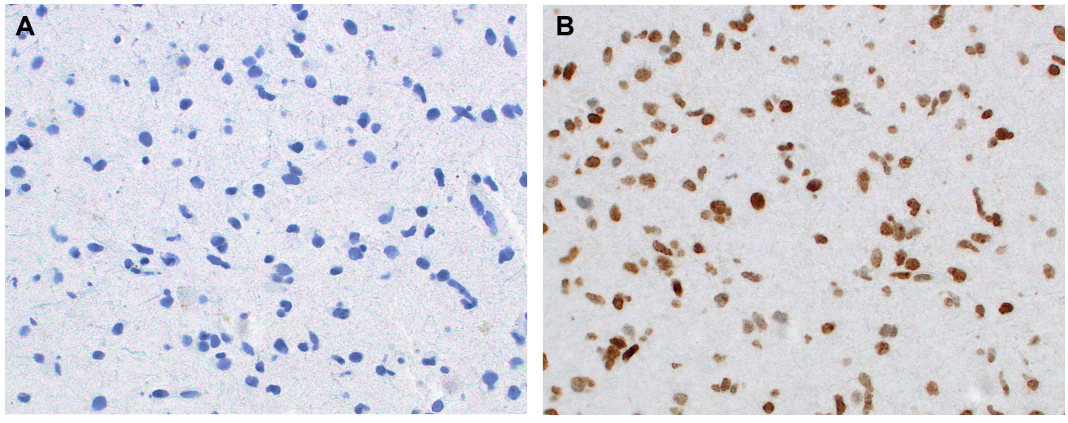

FIGURE 7. **A**, *H3K27M immunohistochemistry with negative staining indicating a wild-type pattern.* **B**, *H3K27me3 immunohistochemistry with strong nuclear staining indicating a wild-type pattern.*

TABLE 2. Common Genetic Alterations in Adult Diffuse Astrocytic and Oligodendroglial Tumors

Genetic alteration	Identification method(s)	Diagnostic relevance	Prognostic and therapeutic implications
IDH1/2[a]	IHC, NGS	Grade 2 and 3 astrocytomas, oligodendrogliomas, secondary GBM	Better prognosis
ATRX[b]	IHC, NGS	Seen in association with *IDH1/2*, *H3F3A*, and *TP53* alterations	
TP53	IHC, NGS	Seen in association with *ATRX* and *IDH1/2* alterations	
H3F3A K27M	IHC, NGS	Midline gliomas	Adverse prognosis
1p/19q codeletion[c]	FISH	Also have *IDH1/2* mutation; diagnostic for oligodendroglioma	
H3F3A G34R/V	NGS	Hemispheric location with primitive morphology	
MGMT promoter methylation	NGS, PCR	Should be performed in all grade 3 and 4 infiltrating gliomas; present in most IDH-mutant gliomas	Promoter methylation increases sensitivity to alkylating agents
CDKN2A/B homozygous deletions	NGS	Should be performed in all *IDH* mutant gliomas	Adverse prognosis
EGFR amplification	FISH, NGS	Diagnostic for GBM	EGFR inhibitors
TERT promoter	NGS	Diagnostic for GBM (in IDH wild type only)	
Gain of chromosome 7/monosomy of chromosome 10	FISH	Diagnostic for GBM	
G-CIMP	NGS	Seen in a subset of *IDH* mutant astrocytomas	Adverse prognosis (G-CIMP-low epigenetic signatures)
Other less common alterations: *MYB*, *MYBL1*, *FGFR1* TKD-duplicated, *FGFR1*, *BRAF* V600E, other MAPK pathway alterations	NGS	Diffuse glioma, *MYB*-altered Diffuse glioma, *MYBL1*-altered Diffuse glioma, *FGFR1* TKD-duplicated Diffuse glioma, *FGFR1*-mutant Diffuse glioma, *BRAF* V600E-mutant (but without *CDKN2A/B* deletion) Diffuse glioma, other MAPK pathway alteration	

FISH, fluorescence in Situ hybridization; GBM, glioblastoma multiforme; IHC, immunohistochemistry; NGS, next-generation sequencing; PCR, polymerase chain reaction.
[a]IDH-mutant astrocytomas with levels of copy number variations (CNVs) and somatic mutations have been associated with shorter overall survival.
[b]Infiltrating IDH-mutant gliomas with loss of *ATRX* nuclear expression and/or strong, diffuse p53 immunopositivity do not require 1p/19q testing. Infiltrating IDH-mutant gliomas with retained *ATRX* nuclear expression and/or faint/scattered p53 immunopositivity require 1p19q testing.
[c]Reference range: $\leq$0.8 is abnormal (if present in $\geq$50% of tumor nuclei); $>$0.8 is normal.

Chimeric antigen receptor-t-cell therapy (CAR-T) has shown therapeutic efficacy in lymphomas and some solid cancers[43,45] as well as encouraging in vitro results for malignant glial tumors.[45]

Preclinical studies targeting H3 K27M-mutant gliomas with epigenetic modifiers such as panobinostat, transcriptional regulators such as THZ1, CAR-T immunotherapy, and microenvironmental targets inhibition have shown promising results.[46-48] It is likely that a combined therapeutic approach addressing both the tumor microenvironment and immunotherapeutic targets will be necessary to control these aggressive tumors.[49]

In summary, although both active and passive immunotherapy studies have improved overall survival and progression free survival in patients with malignant gliomas,[50] additional studies are needed to assess whether immunotherapeutic responses can be predicted through pathological analysis of resected tumor tissue.

INTEGRATED DIAGNOSIS OF INFILTRATING GLIOMAS

The final step in the pathological assessment of infiltrating gliomas is synthesis of histopathologic, IHC, and molecular features into a final "integrated diagnosis."

Currently recognized infiltrating gliomas based on integrated diagnoses are as follows.

IDH-Mutant Gliomas (Defined by the Presence of *IDH1/IDH2* Gene Mutations)

IDH-Mutant Astrocytomas[14]

Astrocytoma, IDH-mutant, WHO grade 2 is defined by inconspicuous mitotic figures, no microvascular proliferation, necrosis, or *CDKN2A/B* homozygous deletions. Astrocytoma, IDH-mutant, WHO grade 3 is defined by significant mitotic

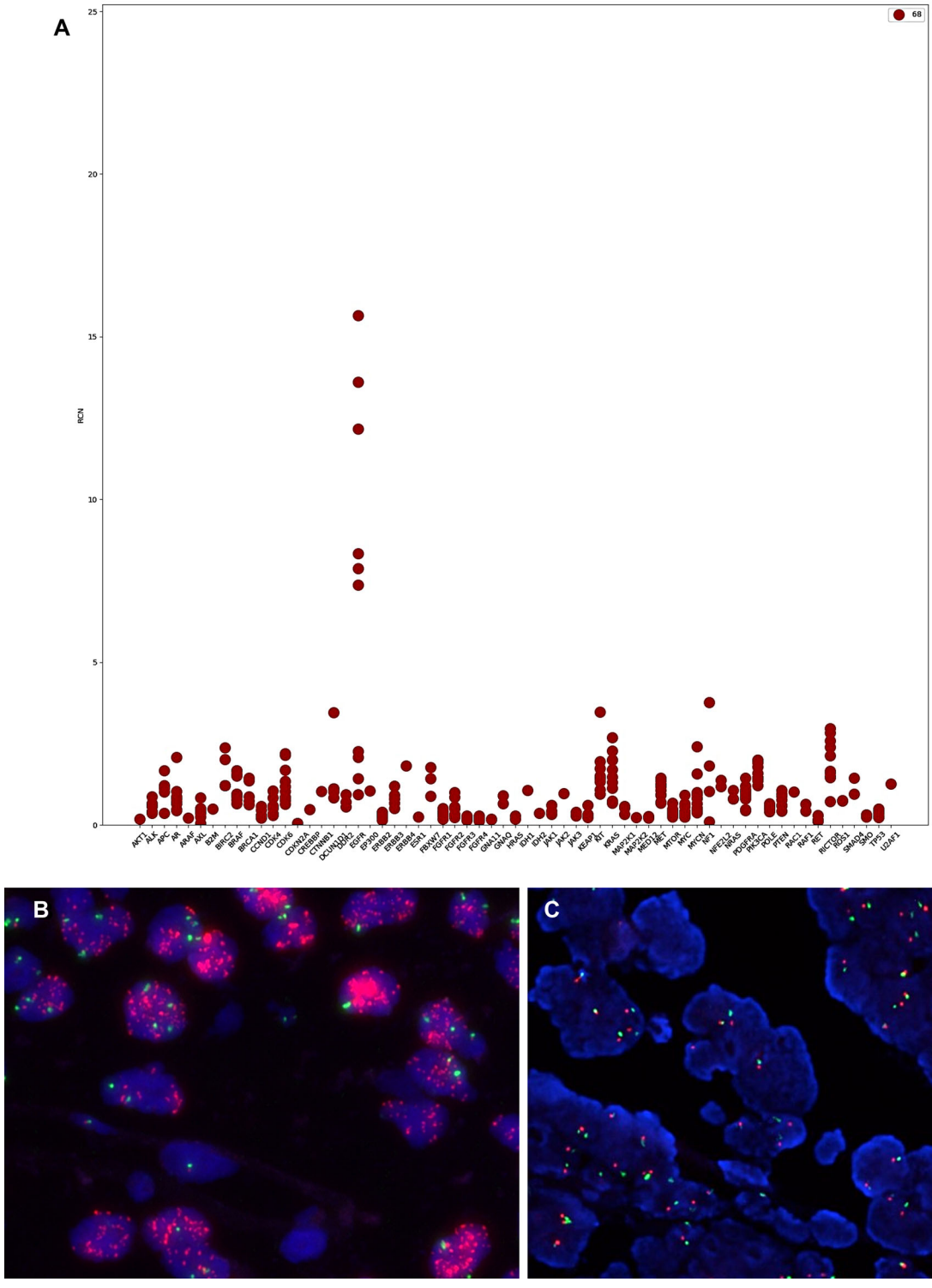

FIGURE 8. **A**, *CNV plot demonstrating an EGFR amplification identified by NGS. The dots represent the number of copies and demonstrate extra copies of EGFR compared to the other genes shown on the plot.* **B**, *Positive FISH for EGFR amplification. EGFR signal is red and the centromere signal is green.* **C**, *Negative FISH for EGFR amplification.*

activity, but without microvascular proliferation, necrosis, and *CDKN2A/B* homozygous deletions. Astrocytoma, IDH-mutant, WHO grade 4 is defined by microvascular proliferation and/or necrosis or *CDKN2A/B* homozygous deletions.

When loss of nuclear *ATRX* expression by IHC is identified in diffuse astrocytomas, it is strongly associated with either *IDH* or *H3F3A* mutations. Infiltrating IDH-mutant gliomas with loss of *ATRX* nuclear expression and/or strong, diffuse p53 immunopositivity often do not require 1p/19q testing. Infiltrating IDH-mutant gliomas with retained *ATRX* nuclear expression and/or faint/scattered p53 immunopositivity require 1p19q testing. *MGMT* promoter methylation is present in most IDH-mutant gliomas, and is associated with a favorable prognosis. A subset of IDH-mutant astrocytomas associated with a poor prognosis is characterized by a hypomethylation (G-CIMP-low epigenetic signature). IDH-mutant astrocytomas with *CDKN2A/B* homozygous deletions/*CDK4* amplification/*RB1* mutation/*PIK3CA* mutations/*PDGFRA* amplification/*MYCN* amplification are associated with poor prognosis. IDH-mutant astrocytomas with levels of copy number variations (CNVs) and somatic mutations have been associated with shorter overall survival. Ki-67 proliferative index is not predictive of biological behavior.

Oligodendrogliomas (defined by the presence of IDH1/IDH2 gene mutations in combination with chromosome 1p19q loss): The WHO requires both *IDH* mutation and 1p/19q codeletion for the diagnosis of oligodendrogliomas. The usual modus operandus is to proceed to FISH for 1p19q for all *IDH*-mutant gliomas with retained ATRX expression and weak p53 immunoreactivity with or without clear oligodendroglial cytology. It is important to recognize, however, that detection of 1p19q codeletion rarely be encountered in high-grade astrocytic tumors secondary to generalized genomic instability.

The canonical 1p/19q codeletion results from an unbalanced translocation t(1;19)(q10; p10) followed by whole arm losses of 1p and 19q. Different testing methods exist, including FISH, molecular inversion probe array, and NGS. The latter could reduce the risk of false positives but is not yet commonly available.

Mutations of unknown predictive value in oligodendroglial tumors involve tumor suppressor genes encoding far-upstream binding protein 1 (*FUBP1*) and human homolog of *Drosophila capicua* (*CIC*), on chromosomes 1p and 19q, respectively.[9] These mutations are mutually exclusive with *TP53* and *ATRX* mutations, and are rarely found in diffuse astrocytomas.

Most oligodendrogliomas also carry highly specific mutations in the *TERT* gene promoter (C228T or C250T). *TERT* promoter mutations and *ATRX* deficiency are mutually exclusive, which provides the biologic rationale for the non-necessity of FISH for chromosome 1p/19q deletions in *ATRX*-deficient gliomas. Oligodendrogliomas lacking *TERT* promoter mutations have significantly worse outcomes. A recent report posits that absence of H3K27me3 staining may be useful as a potential surrogate marker for oligodendroglioma diagnosis in IDH-mutant diffuse glioma with retention or nonconclusive nuclear ATRX staining, with a predicted probability of 0.9678 for 1p/19q codeletion.[51]

Other genetic alterations associated with shorter survival in patients with oligodendrogliomas involve *NOTCH1*, *PIK3CA*, *PIK3R1*, *ZBTB20*, and *ARID1A* genes. Testing for these genes is not current practice but may possibly be important as targeted therapeutic options become available.

IDH-wt, H3-wt Gliomas[3]

These comprise a heterogeneous group of tumors, from low grade to high grade. Absence of *IDH* mutation in a diffuse astrocytic glioma does not necessarily equate with aggressive clinical behavior, even though the majority of primary glioblastomas in adults are *IDH* wt. It is especially important to consider the possibility that the absence of *IDH* mutation may indicate that the tumor under consideration is not, in fact, an infiltrating glioma, but part of a glioneuronal tumor or other alternative tumor lineage, such as ependymoma.

IDH-wt infiltrating astrocytomas, supratentorial, WHO grade 2/3 glioblastoma, IGH-wt, WHO grade 4: *EGFR* amplification and/or whole chromosome 7 gain and whole chromosome 10 loss (+7/−10) and/or *TERT* promoter mutation. *TERT* promoter mutations and *ATRX* deficiency are mutually exclusive.

IDH-wt infiltrating astrocytomas, infratentorial, WHO grade 2/3: Beyond accessibility to surgical resection, tumor location has important implications with respect to molecular biomarkers. *IDH*-wt diffuse astrocytic gliomas arising in the adult cerebellum are less frequently associated with *EGFR* amplification, + 7/− 10, or *TERT* promoter mutations compared with their supratentorial counterparts.

Anaplastic astrocytomas with piloid features are characterized by frequent *CDKN2A/B* deletions, *BRAF* pathway gene alterations, and *ATRX* mutation, with marginally better outcomes than patients with *IDH*-wt glioblastoma, which might potentially be further improved with the use of targeted therapies.

IDH-wt glioblastoma, WHO grade 4: These occur predominantly in patients >50 yr of age with a short clinical history and without a pre-existent low-grade tumor. If IDH1-R132H immunoreactivity is not detected in patients under the age of 55 yr or in patients with evidence of pre-existing low-grade glioma, sequencing of *IDH1* and *IDH2* genes should be performed to rule out grade 4 *IDH*-mutant astrocytoma.[1] Approximately 40% of IDH-wt glioblastomas demonstrate *MGMT* hypermethylation with reduced *MGMT* expression. Results of phase III trials in patients >65 yr with glioblastomas showed that assessment of *MGMT* promoter methylation was critical in determining optimal adjuvant therapy.[52] This predictive value of *MGMT* promoter methylation for a TMZ responsiveness is restricted to IDH-wt gliomas.

Other less commonly recognized subtypes from cIMPACT-NOW update 4 include the following: diffuse glioma, *MYB*-altered, diffuse glioma, *MYBL1*-altered, diffuse glioma, *FGFR1* TKD-duplicated, diffuse glioma, *FGFR1*-mutant, diffuse glioma,

BRAF V600E-mutant (but without *CDKN2A/B* deletion), and diffuse glioma, other MAPK pathway alteration.[53]

In contrast to the adult gliomas discussed in this review, low-grade diffuse gliomas in children and adolescents are frequently IDH-wt/H3-wt, but harbor *BRAF* V600E mutations, *FGFR* alterations, or *MYB* or *MYBL1* rearrangements, which are associated with good clinical behavior and infrequent malignant progression.

IDH-wt, H3 K27M-Mutant Gliomas

H3 K27M-mutant diffuse midline gliomas are more common in children but are also seen in the brain stem, thalamus, cerebellum, and spinal cord of adults. *H3 K27M* mutations were originally identified in diffuse infiltrating gliomas with astrocytic morphology, but subsequently have been found in tumors with diverse differentiation including giant cells, rhabdoid cells, and embryonal or ependymal differentiation, which has led to the recommendation that all midline gliomas in patients of all ages and with all glial morphologies be tested for *H3 K27M*.[54] Both adult and pediatric patients with H3 K27M-mutant tumors show nearly identical dismal outcomes.[13,14]

Diffuse Glioma, H3.3 G34 Mutant

These diffuse *IDH*-wt gliomas involve the cerebral hemispheres of pediatric and young adult patients with a survival rate somewhat better than *IDH*-wt glioblastoma although some authors report dismal outcomes similar to *H3 K27M*-mutant diffuse midline gliomas.[3] The characteristic genetic feature is a missense mutation at position 34 of the mature histone H3.3 protein. *ATRX* and *TP53* mutations are frequent. Microscopically, these tumors often show a combination of astrocytic and primitive neuroectodermal type differentiation with high-grade features including mitotic activity, microvascular proliferation, and/or necrosis.

NOT OTHERWISE SPECIFIED AND NOT ELSEWHERE CLASSIFIED

Not otherwise specified (NOS): Diagnostic information (usually molecular) is not available to assign a specific WHO diagnosis.

Not elsewhere classified (NEC): All necessary diagnostic testing has been performed, but the results do not match established WHO defined entities.[55]

For example: Oligodendroglioma, NOS can be used for those histologically classic oligodendrogliomas without available 1p/19q codeletion results. Also, high-grade glioma, H3 G34 mutant, NEC is used because this is not currently included in the WHO classification.

CONCLUSIONS/PITFALLS

The Cancer Genome Atlas[56] has been crucial in deepening our understanding of adult diffuse gliomas and has enabled subclassification into clinically distinct entities incorporated into the 2016 WHO classification of CNS tumors.[56,57] The Glioma Longitudinal AnalySiS Consortium (GLASS) study has concluded that the strongest selective pressure in diffuse gliomas occurs during early gliomagenesis. Information on how diffuse gliomas evolve over time and in response to therapy will help in the design of more efficacious therapeutic strategies. Driver genes detected in resected gliomas appear to be retained in tumor recurrences, without evidence of recurrence-specific gene alterations.[58] In summary, several diagnostic considerations must be addressed during the evaluation of adult diffuse gliomas:

A panel of antibodies including IDH and ATRX (and H3K27M for those midline tumors) should be the starting point in the diagnosis of gliomas, with uncommon or unexpected combinations prompting further molecular testing. For example, a tumor with histopathological features of glioblastoma and loss of both IDH1-R132H and ATRX expression could be either a rare high-grade astrocytoma with a noncanonical IDH1/2 mutation or an IDH-wt glioblastoma with an uncommon ATRX mutation.

If IDH1 R132H IHC is negative in a patient younger than 55 yr of age, both *IDH1* and *IDH2* genes should be sequenced.

It is important to investigate the presence of *EGFR* amplification, + 7/–10, or *TERT* promoter mutations in all IDH-wt diffuse astrocytic gliomas, particularly if a high-grade tumor is suspected but not reflected in biopsy specimens.

CDKN2A/B homozygous deletions should be searched for in all IDH-mutant astrocytomas, as those with homozygous deletions should be designated grade 4 (*CDKN2A/B* deletion is not sufficient to designate IDH-wt astrocytic gliomas WHO grade 4).

Evaluate *MGMT* methylation status in grade 3 and 4 infiltrating gliomas.

IDH-mutant noncodeleted astrocytomas may develop a hypermutator phenotype after therapy with alkylating agents which could potentially dictate the use of alternative salvage therapies. To date, no significant differences in survival between recurrent hypermutant and nonhypermutant gliomas independent of age, phenotype, and *MGMT* promoter methylation status have been described.

NGS alone is not sufficient to reliably diagnose gliomas, as molecular alterations have differing implications in different gliomas subtypes, emphasizing the importance of an evidence-based diagnostic evaluation where histology, immunophenotyping, and molecular results are synthesized into a final integrated diagnosis. For example, both oligodendrogliomas and IDH-wt glioblastomas frequently carry TERTp mutations, which portend a more favorable prognosis in the former and a less favorable outcome in the latter.

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