Opinion & Special Article: Glioma Classification: How to Interpret Molecular Markers in a Diffuse Glioma Pathology Report

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Abstract
Diffuse infiltrating gliomas are the most common malignant brain tumors in adults. The 2021 World Health Organization classification for central nervous system tumors (CNS5 WHO) has significantly altered the rules for classification and grading of diffuse gliomas. Clinicians, including neurology residents and neurologists, will have to consider the changes that include the introduction of new tumor types, allotting established tumor types to other groups, and substituting previously essential morphological features for additional molecular markers. For example, in the current classification, glioblastoma is defined as isocitrate dehydrogenase (IDH)-wildtype, grade 4. Whereas, a grade 4 IDH-mutated astrocytic glioma is referred to as astrocytoma, IDH-mutated, grade 4. Additionally, potential targeted treatments, based on the underlying molecular alterations, have become therapeutic options for diffuse gliomas. For clinicians, it is important to know the rationale for why these options are only available for specific tumors. Due to the emphasis of molecular markers in the CNS5 WHO classification, interpretation of a pathology report and understanding of its clinical implications can be challenging. This review describes the most important molecular alterations in glioma, summarizes the recent changes in the CNS5 WHO classification for glioma, and presents a stepwise approach for trainees and neurologist to decipher a glioma pathology report.

Additional information can be found in eTable 1.

Introduction
In adult patients, diffuse infiltrating gliomas are the most common malignant primary brain tumors. After publication of the 2016 World Health Organization (WHO) classification of tumors of the central nervous system, the Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy-not officially WHO (cIMPACT-NOW) published summaries concerning evolving concepts in the biology of glioma and their prognostic implications. In 2021, these summaries served as the basis for the updated WHO CNS (CNS5) classification (Figure 1).

As molecular alterations became increasingly important in the classification and grading of brain tumors, the interpretation of a pathology report can be challenging for clinicians new to the field of neuro-oncology. Since neuro-oncology is not part of the curriculum for all neurology residents in the United States and Europe, we provide an overview of the most important molecular features in glioma including their diagnostic, prognostic, and potential therapeutic roles (Figure 1 and eTable 1).
Histology

Histological tissue evaluation is central and the first step in the diagnostic process (Figure 2). Much of the basis for classification and grading of diffuse glioma can be obtained by assessment of differentiation status, cell density, mitotic activity, presence or absence of necrosis and/or microvascular proliferation. In addition to classical histology, several immunohistochemical markers provide direct information on essential molecular parameters such as IDH1-R132H, H3 and ATRX status. However, classification and grading of many diffuse gliomas demand additional molecular analyses as a second step (Figure 2).

IDH

Hotspot mutations in isocitrate dehydrogenase (IDH) enzymes facilitate the production of 2-hydroxyglutarate which leads to competitive inhibition of alpha-ketoglutarate-dependent enzyme activity resulting in distinct epigenetic histone and DNA modification followed by metabolic re-programming. This effect can be mediated by mutations in both the IDH1 and IDH2 gene. IDH mutations are considered early driver-mutations and are now the basis of molecular glioma classification. The IDH status constitute a fundamental divide with IDH mutant astrocytoma and oligodendroglioma on one, and glioblastoma and some rare diffuse gliomas on the other side (Figure 1). A consequence of this central divide among diffuse gliomas has been renaming the formerly IDH-mutant glioblastoma to the current astrocytoma, IDH-mutant, WHO grade 4. The term glioblastoma, a glioma with the worst clinical prognosis, is reserved exclusively for IDH-wildtype grade 4 glioma.

Approximately 90% of the supratentorial IDH-mutations are of the IDH1-R132H type, and this can be detected with a mutation specific antibody (immunohistochemistry). Other IDH1 (R132C, R132L, R132S) or IDH2 (R172G, R172K, R172M)-mutations require DNA sequencing for detection. In patients over 55 years of age with a supratentorial tumor, IDH-mutations are rare. Therefore, when no IDH1-R132H-mutation is found, additional sequencing is required only for patients ≤55 years-old. Non-IDH1-R132H-mutations constitute roughly 80% of IDH alterations in infra-tentorial IDH-mutant astrocytoma.

1p/19q

Oligodendrogliomas have, by definition, an IDH-mutation and a second molecular alteration that defines the entity: the combined full losses of the short arm of chromosome 1 (1p) and the long arm of chromosome 19 (19q), better known as a 1p/19q co-deletion. The exact role of the 1p/19q loss in the
tumor biology is currently unclear. Of special importance here is that the 1p/19q status is decisive for the separation of astrocytoma from oligodendroglioma (both IDH-mutant) and that morphological aspects are second in relevance.

**ATRX**
The ATP-dependent X-linked helicase (ATRX) gene encodes for a protein involved with telomerase activity. Mutations in ATRX, immunohistochemically identified as loss of nuclear expression of this protein, are next to mutually exclusive with 1p/19p loss (oligodendrogliomas). Therefore, loss of nuclear ATRX expression can be used as surrogate marker for IDH-mutated astrocytoma when an IDH-mutation is present it eliminates the need for further 1p/19q testing.\(^3\)

**TP53**
TP53, a tumor suppressor gene, is mutated in the majority of grade 2, 3, and 4 IDH-mutated astrocytomas. Strong staining of p53 protein suggests a TP53 mutation, however, this has a lower specificity than loss of ATRX-expression for the diagnosis of an astrocytoma.

**CDKN2A/B**
In IDH-mutated astrocytoma, the cyclin-dependent kinase inhibitor 2A/B (CDKN2A/B) status is an important prognostic factor, since homozygous deletion of CDKN2A/B is associated with increased aggressiveness. Homozygous CDKN2A/B loss overrules the histological grade, so even without microvascular proliferation and/or necrosis the histological criteria for a grade 4 tumor, homozygous CDKN2A/B loss also warrants a grade 4 diagnosis in IDH-mutated astrocytomas.\(^9\) Due to the prognostic significant implications of CDKN2A/B, IDH-mutant astrocytoma without 1p/19q co-deletion should therefore always be tested for homozygous CDKN2A/B loss.

**EGFR amplification, +7/-10, and/or TERT-promotor mutation**
Three different molecular markers independently warrant the diagnosis of a grade 4 glioblastoma in IDH-wildtype diffuse gliomas, because they are associated with very poor clinical outcome:
- Epidermal growth factor receptor (EGFR)-amplification.
- The complete gain of chromosome 7 in combination with a complete loss of chromosome 10, often referred to as +7/-10. It is unclear whether tumors harboring variants of +7/-10 (e.g. +7p/-10 or +7/-10q) are as aggressive as tumors with the full +7/-10 loss.\(^{10}\)
- Mutations in the promoter of the telomerase reverse transcriptase (TERT) gene. TERT-promotor mutations lead to the reactivation of telomerase resulting in maintained telomere length and thus limitless cellular lifespan.

Thus, in an IDH-wildtype astrocytoma without microvascular proliferation and/or necrosis, the histological criteria for a grade 4 tumor, testing for these alterations is important because of the prognostic and possible therapeutic consequences. Overall, TERT mutations in this triad of markers are most sensitive but least specific, while both, EGFR mutation and +7/-10 signature have high specificity with lower sensitivity. Very frequently, these alterations occur together and the presence of any two of the three features is highly specific for glioblastoma. TERT-promotor mutations do also occur in tumors within the differential diagnosis of glioblastoma such as pleomorphic xanthoastrocytoma. Further, these mutations occur in the majority of oligodendrogliomas.

**H3 K27M and H3 G34**

Two diffuse glioma entities have histone 3 (H3) alterations. Both are rare, IDH-wildtype and have an aggressive course (grade 4). These tumors occur most often in children and young adults. *Diffuse midline gliomas, H3 K27-altered*, are gliomas with H3 K27-trimethylation alterations (H3F3A/B K27M mutation being most common), are located in midline CNS structures (i.e. brainstem, thalamus, or spinal cord), and have a diffuse infiltrative growth pattern. Presence of all criteria is important because H3 K27M can also be found in other entities which may have different pathogenesis and prognosis.

Diffuse gliomas which feature H3 G34-mutations are located most often in a supratentorial hemisphere and can have a non-enhancing appearance on MRI. These tumors are termed *diffuse hemispheric glioma, H3 G34-mutant*. H3 mutated gliomas have a prognosis similar to that of glioblastoma, IDH-wildtype.¹¹,¹²

**MGMT**

Hypermethylation of the O6-methylguanine-DNA methyl-transferase (MGMT) gene promoter leads to reduced expression of this DNA-repair protein. MGMT repairs the damage done by alkylating chemotherapeutic agents rendering the tumor more resistant to alkylating chemotherapy.¹³ *MGMT* promotor methylation status is a biomarker of both prognostic and therapeutic significance in glioblastoma, IDH-wildtype. Glioblastoma with MGMT promotor hypermethylation have a better prognosis and more favorable response to chemotherapy (eTable 1).¹⁴
**FGFR1/ NF1/ BRAF**

The RAS/ mitogen-activated protein kinase (MAPK) pathway plays an important role in cell regulation, including proliferation and differentiation. Although MAPK-pathway alterations occur most often in pediatric low-grade gliomas, they are also observed in adult diffuse gliomas. Several gene alterations can lead to an upregulation of this pathway. Genes involved with this pathway include fibroblast growth factor receptor 1 (FGFR1), neurofibromin 1 (NF1, affected in neurofibromatosis type 1), and BRAF. The most common MAPK-pathway alteration is a BRAF p.V600E mutation that occurs in approximately 2% of adult glioblastoma cases. The exact incidence of BRAF-fusions, FGFR and other MAPK-alterations, remains to be determined as novel techniques to detect them become more widely available.

For some MAPK-activating alterations antagonistic therapeutic options are available (eTable 1).

**Methylation brain tumor classifier**

In recent years methylation patterns generated by genome wide DNA methylation profiling have been increasingly employed to assist tumor diagnosis for their potential to predict specific tumor types. Methylation profiling requires a classification tool for tumor class prediction, a calibrated score for the validity of the prediction, and a copy number profile. The methylation-based approach has been employed for the identification of many novel brain tumor entities (e.g. high-grade astrocytoma with piloid features). Methylation analysis is a powerful emerging diagnostic tool and its use is encouraged for all diagnostically unresolved brain tumor samples.

**NOS/NEC**

In some cases, molecular testing is not performed or interpretable due to sparse resources or for intentional reasons (e.g. no therapeutic consequences). In those cases, the term “not otherwise specified (NOS)” should be added to the histological diagnosis: e.g. astrocytoma, WHO grade 2, NOS (note: no IDH-status listed).

In the event that molecular testing was performed and valid results were obtained, but the results did not lead to a CNS5-compatible diagnosis, the term “not elsewhere classified (NEC)” should be added to the diagnosis. For example, a non-diffuse hemispheric glioma with a K27M mutation results in non-diffuse non-midline K27-altered glioma, NEC.
Relevance to neurologists and trainees

Molecular alterations have become increasingly important in the diagnosis and classification of glioma. For many entities specific molecular alterations are required to render a WHO CNS5 compatible diagnosis.

In clinical practice, information regarding the studied material becomes available in a step-wise manner (Figure 2). This has resulted in a layered integrated diagnosis starting with a histopathological classification days after surgery. The histopathological findings are used to select subsequent molecular testing. For example, in histological low-grade diffuse glioma, it is important to determine the IDH-status and the presence of specific markers that determine the diagnosis (e.g. 1p/19q co-deletion for oligodendroglioma) or grade (homozygous CDKN2A/B loss for astrocytoma) (Figure 1 and eTable1). This additional information is added to the report, after which the integrated diagnosis is rendered. Alterations that can have potential therapeutic consequences (e.g. MGMT-promotor methylation) will be listed under the “molecular alterations” layer.

For example:

Integrated diagnosis: astrocytoma, IDH-mutant, WHO CNS5 grade 2
Histopathological classification: low-grade diffuse glioma
CNS5 WHO grade: 2
Molecular information: IDH1-R132H-mutated, ATRX-mutation, TP53-mutation, no CDKN2A/B loss, no 1p/19q co-deletion
Figure 1. Algorithm for the classification of diffuse gliomas in adults.

The first step is to check the histology of a tissue specimen if it is compatible with a diffuse tumor of glial origin. Next, is to assess by immunohistochemistry for the presence of an IDH mutation and/or loss of nuclear ATRX. A negative result for an IDH1-R132H-mutation warrants further testing by DNA sequencing to exclude non-canonical IDH mutations in patients under 55 years of age. An IDH-mutant astrocytoma with a homozygous deletion of CDKN2A/B automatically yields a grade 4 even if there is no necrosis and/or microvascular proliferation. Similarly, an IDH-wildtype astrocytoma without necrosis and/or microvascular proliferation warrants further testing for an EGFR amplification, +7/-10, and/or TERT promoter mutation as the presence of at least one of these molecular features yields a diagnosis of glioblastoma, IDH-wildtype, grade 4.
Figure 2. Stepwise approach and timeline of the diagnostic process.

In clinical practice, the information regarding the studied material becomes available in a stepwise manner. First, the material is studied using microscopic techniques. This includes the analysis of morphological features (e.g., cell atypia, mitotic activity, necrosis, and/or vascular proliferations) supplemented in most cases by immunohistochemical stains to investigate the presence of specific (mutated) proteins (e.g., ATRX, p53, and/or IDH-R132H). This first step of the investigation takes up to 1-3 working days to come up with a histological diagnosis that is then shared with the clinicians. In most cases a second step featuring molecular investigations is needed to arrive at the integrated diagnosis.

The method picked is determined by the histological diagnosis. In morphological glioblastoma cases in patients over 55 years of age with a negative IDH1-R132H immunohistochemical analysis, only requires testing for MGMT-promotor status. For other cases, next-generation sequencing (NGS) and/or methylation profiling using the brain tumor classifier will also be required to investigate specific molecular markers. Depending on local protocols and the technique used, information regarding the methylation class, chromosomal gains, losses, amplifications and/or specific mutations can be obtained.

With the required information on the presence or absence of an IDH-mutation, TERT-promotor mutation, 1p/19q co-deletion, EGFR-amplification, +7/-10 and a CDKN2A/B homozygous loss, the final integrated diagnosis based on the histological diagnosis and molecular alterations can be rendered.

Sometimes, the required techniques are initiated in parallel but when inconclusive results are obtained or when limited material is available, the techniques can also be initiated subsequently delaying the rendering of an integrated diagnosis. Note: the times listed are only an estimation and can differ based on local protocols. Additionally, since the analyses are performed in batches, further delays can be introduced in the case of low sample counts and/or infrequent analysis runs.

[Diagram showing stepwise approach and timeline of the diagnostic process.]

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