Oncologie

Tech Science Press

DOI: 10.32604/oncologie.2022.020890

REVIEW

# The Integrated Histopathologic and Molecular Approach to Adult-Type Diffuse Astrocytomas: Status of the Art, Based on the 2021 WHO Classification of Central Nervous System Tumors

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Received: 17 December 2021 Accepted: 27 January 2022

**ABSTRACT**

The 2021 World Health Organization (WHO) Classification of Tumors of the Central Nervous System (CNS) improved our understanding of the brain neoplasm biology. In more details, differences between diffuse gliomas that primarily occur in adults and those that primarily occur in children have been identified by the terms "adult-type" and "pediatric-type" diffuse gliomas. More importantly, both diagnostic and grading criteria for adult-type diffuse astrocytomas have been modified, by adopting novel molecular markers: diffuse astrocytomas, *IDH*-mutant have been grouped into a single entity and graded as CNS WHO grades 2, 3, or 4, with the assignment of Grade 4 in the presence of *CDKN2A/B* homozygous deletion, regardless of the histology [1]. Additionally, at least one of the following genetic alterations has been considered as sufficient to confer to astrocytomas, *IDH* wild type, a CNS WHO grade 4: i) *TERT* promoter mutation, ii) *EGFR* gene amplification, iii) combined gain of whole chromosome 7 and loss of whole chromosome 10 [+7/−10]. However, histology remains the solid basis to support these new complementary molecular data, and an integrated diagnosis is highly recommended.

**KEYWORDS**

WHO classification; brain tumors; IDH; diagnosis; adult diffuse astrocytomas

## 1 Introduction

Central Nervous System (CNS) tumor classification has long been based on histological findings supported by ancillary studies like immunohistochemical stains, and ultrastructural changes but recent discoveries have deepened our understanding of the molecular features of CNS tumors. This advancement in molecular characterization of CNS tumors has reframed our understanding of its biology and the development of a new classification system.

The 2021 World Health Organization (WHO) Classification of Tumors of the CNS emphasizes the clinico-pathologic and molecular differences between diffuse gliomas that primarily occur in adults and that occur primarily in children, and accordingly termed as diffuse gliomas "adult-type" and "pediatric-type", respectively. Additionally, 2021 WHO classification of CNS adopted molecular markers into the

revised grading criteria for CNS tumors in general and astrocytoma in particular, consequently, all diffuse astrocytoma, *IDH*-mutant are considered as a single type and graded as CNS WHO grades 2, 3, or 4, with the assignment of grade 4 in the presence of *CDKN2A/B* homozygous deletion [1]. Furthermore, the presence of 1 or more of the three genetic alterations including *TERT* promoter mutation, *EGFR* gene amplification, and/or combined gain of entire chromosome 7 and loss of entire chromosome 10 [+7/−10]) are sufficient to assign WHO grade 4 for *IDH* wild type astrocytoma [2], however, the molecular testing to comply with these new WHO criteria is not always available, so an integrated diagnosis combining all available complementary data is still highly recommended [1]. In this report, we will review the integrated histopathologic and molecular approach of adult diffuse astrocytoma, based on 2021 WHO Classification of CNS Tumors, and discuss the new emerging genetic alterations that could be incorporated in the future for further sub-classification, risk stratification and most importantly, targeted therapy that could improve the overall survival of the patients.

## 2 Diffuse Astrocytoma, IDH-Mutant

The 2016 WHO Classification of Tumors of CNS (updated 4$^{th}$ edition) for the first time emphasizes the diagnostic relevance of *IDH1/2* mutations in diffuse gliomas, and provides a clinically meaningful classification for diffuse gliomas, particularly in adults [3]. Although immunohistochemistry is the routine method for the identification of the most frequent *IDH* mutations (*IDH1-R132H*), DNA sequencing of *IDH1/2* genes is required for identification of the less frequent non-canonical IDH1 and IDH2 mutations.

The 2016 WHO Classification of Tumors of the CNS classifies *IDH*-mutant gliomas into astrocytoma or oligodendroglioma based on the presence of *ATRX* and *TP53* mutations for former and 1p/19q co-deletion for latter [3]. The 2016 WHO grading system included *IDH*-mutant, diffuse astrocytoma (WHO grade 2) (Fig. 1), anaplastic astrocytoma (WHO grade 3) (Fig. 2), and glioblastoma (WHO grade 4) [4]. Consequently, testing for *IDH* mutations in adult diffuse astrocytoma is very important but may be insufficient, since other genetic alterations with prognostic significance maybemissed due to limited molecular testing.

The significant mitotic index, anaplastic nuclear features, and microvascular proliferation or necrosis have classically been the main criteria for performing the histological grading of adult diffuse astrocytomas. In WHO 2016 “significant” proliferative activity distinguishes WHO grade 3 (anaplastic) from WHO grade 2 astrocytoma [3]. In the years preceding the release of 2016 WHO, diffuse astrocytic tumors with ≥2 mitoses/10 HPFs were found to be associated with poorer outcomes and designated as WHO grade 3 [5–7]; moreover, taking into consideration the specimen size, one single mitosis has been also reported as sufficient for a WHO grade 3 diagnosis on a very small biopsy, while greater mitotic activity is necessary for the larger samples [3]. To date, there have been no studies for an alternative mitotic count nor the criteria of the proliferative index (e.g., based on Ki-67) that can reliably stratify risk among histologic grade 2 and 3, *IDH*-mutant astrocytomas [8].

Recent studies challenge the previously mentioned histopathologic criteria for WHO grading of *IDH*-mutant gliomas, highlight the importance of associated genetic alterations and emphasize that specific genetic alterations maybe more important than histopathologic features in predicting the prognosis and the outcomes. It has been shown that the risk for patients affected by grades 2 and 3, *IDH*-mutant astrocytoma cannot be stratified by histological grade alone [9–12] and indicated that the number of mitoses is not accurate for grading *IDH*-mutant astrocytomas [13]. Additional potential histopathologic and genetic biomarkers, that could act as predictors of more aggressive biological behavior and could be added to the grading system of these tumors, have been investigated [9–10,14–19]. Moreover, the presence of co-occurring, second genetic events that could be class-defining oncogenic drivers in *IDH*-mutated astrocytoma are important especially since infiltrating gliomas are difficult to treat and identifications of these targetable molecular alterations like pathogenic *BRAF*, *FGFR,* and *NTRK* can help

in targeted therapy and improving the overall survival of these patients [20–23], consequently, many molecular genetic alterations were investigated including *CDKN2A/B* homozygous deletion, alterations of *CDK4*, *RB1*, *PIK3CA*/*PIK3R1, PDGFRA*, *MYCN*, and chromosome 14 copy loss; these alterations could reliably stratify risk or identify tumors that would behave most aggressively among patients with *IDH*-mutant diffuse astrocytomas [24]. Very recently, pathogenic *BRAF, FGFR*, and *NTRK* were also investigated [25].

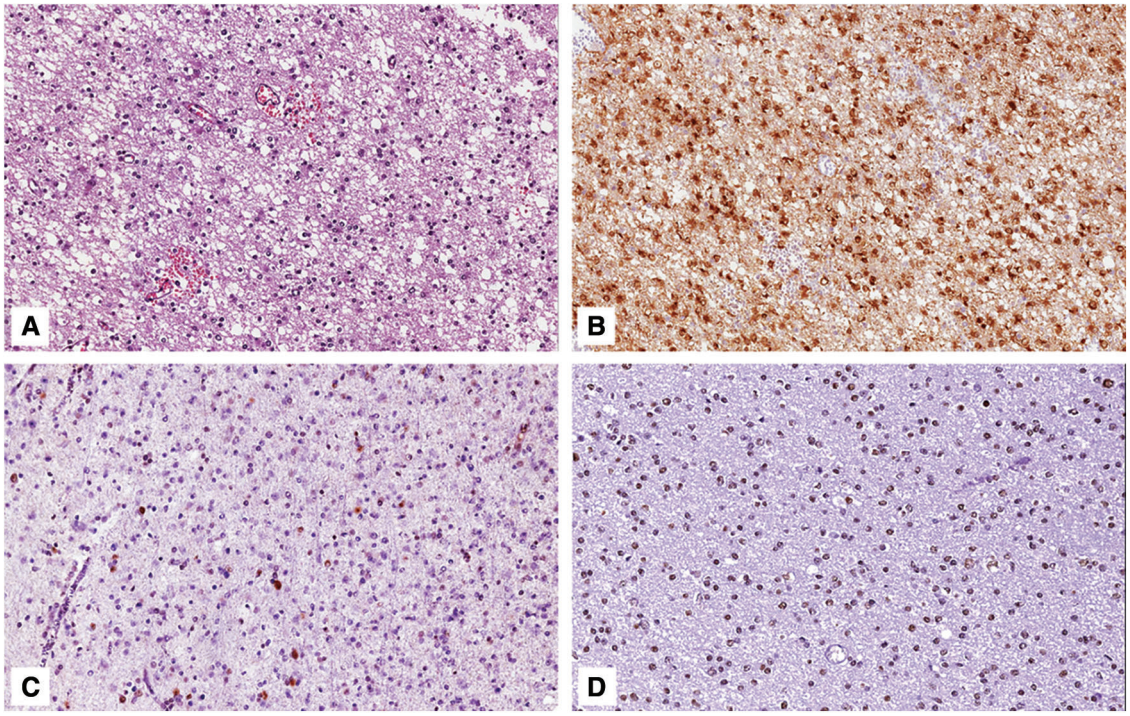


**Figure 1:** Grade 2 astrocytoma, *IDH*-mutant. A) Histological examination showing a lowly cellular astrocytic neoplasm composed of mitotically-inactive bland-looking cells with ovoid nuclei (hematoxylin and eosin; original magnification 150x); B) Neoplastic cells are diffusely stained with IDH1 (R132H) (immunoperoxidase; original magnification 150x); C) Tumor exhibits nuclear loss of ATRX; notice the entrapped ATRX-positive glial cells and endothelial cells that serve as internal positive control (immunoperoxidase; original magnification 150x); D) Neoplastic cells are diffusely stained with anti-p53 antibody (immunoperoxidase; original magnification 150x)

Homozygous deletion of *CDKN2A/B* and *CDK4* amplification was considered as a marker of poor prognosis and associated with decreased global DNA methylation levels in *IDH*-mutant astrocytomas [10,13–18,26], and the combination of *CDK4* amplification with chromosome 14 loss has been linked to poor prognosis and shorter overall survival in these astrocytomas [9,26]. Moreover, subsequent studies have shown that *CDKN2A/B* homozygous deletion is an independent marker for poor outcomes and shorter survival in all grades (WHO grades 2–4) of IDH-mutant astrocytomas [9,13,14,17,26] and this has been emphasized by a study that found that patients with histologic grade 3 IDH-mutant astrocytomas, and harboring *CDKN2A/B* homozygous deletions, showed biological behavior more similar to WHO grade 4 glioblastomas [27]. Other investigations have confirmed these findings [13,27,28], but the prognostic significance of *RB1* mutation or *CDK4* amplification that are functionally equivalent alterations to *CDKN2A/B* homozygous deletion, remains less well-defined, and are not yet recommended

for grading *IDH*-mutant astrocytomas [24]. Accordingly, The Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy (cIMPACT-NOW), assigns a grade 4 to astrocytomas, *IDH*-mutant, with homozygous deletion of *CDKN2A/B* [29]. As immunohistochemical tests for p16 protein have been demonstrated to have poor correlation with *CDKN2A/B* status, molecular investigations, such as FISH, quantitative real-time PCR, or next generation sequencing, are the most sensitive and specific tools for detecting *CDKN2A/B* homozygous deletion [17] cIMPACT-NOW Update 6 also suggested the discontinuation of the term "Glioblastoma, *IDH*-mutant", and the conversion to a single name "Astrocytoma, *IDH*-mutant" with Arabic numeral grades from 2–4; with the use of the previously mentioned histological features [29]. The distinction between histologic grade 2 and grade 3 tumors is based primarily on the detection of brisk mitotic activity, and the presence of necrosis and/or microvascular proliferation remains as the morphologic hallmarks for grade 4 tumors, regardless of *CDNK2A/B* status. In summary, the proposed grading system according to cIMPACT-NOW Updates 5 and 6 is to designate Astrocytoma, *IDH*-mutant, WHO grade 2 for tumors that lack significant mitotic activity, anaplastic features, microvascular proliferation, necrosis, and *CDKN2A/B* homozygous deletion (median overall survival >10 years) [14,15]; WHO grade 3 astrocytoma, *IDH*-mutant, is for those tumors that exhibit brisk mitotic activity and histologic anaplastic features in the absence of microvascular proliferation, necrosis, and *CDKN2A/B* homozygous deletion; finally, IDH-mutant astrocytic neoplasms that show at least one of microvascular proliferation, necrosis, and *CDKN2A/B* homozygous deletion, are designated as WHO grade 4 astrocytomas, *IDH*-mutant [1,24,29].

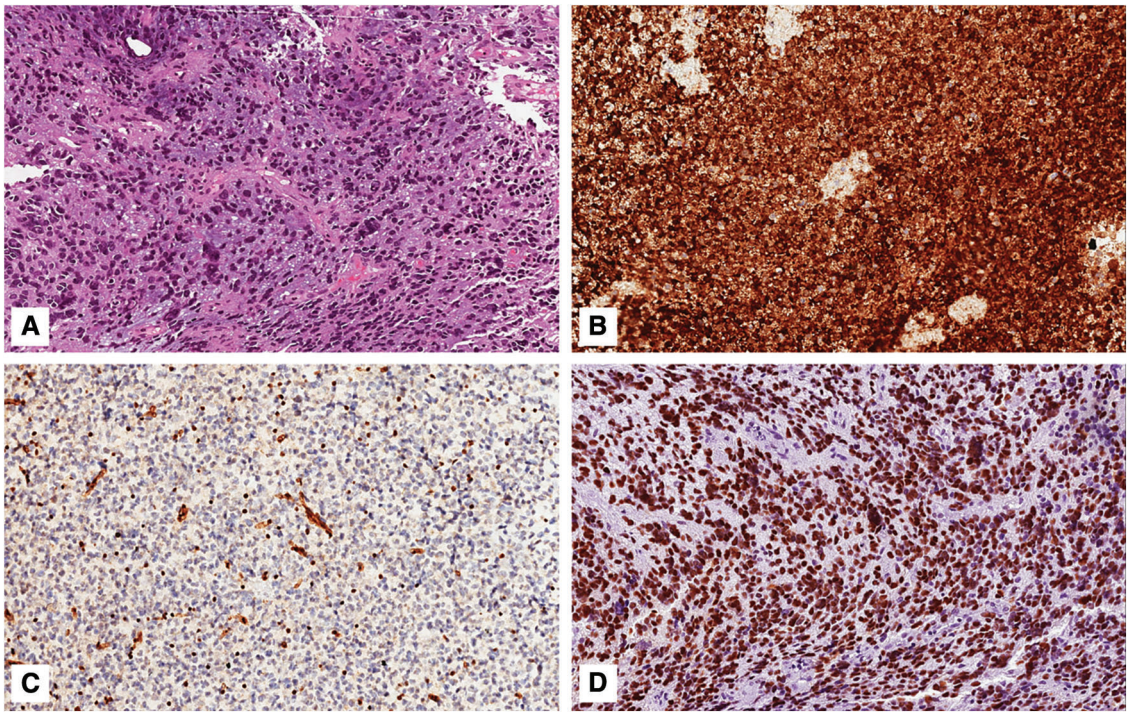


**Figure 2:** Grade 3 astrocytoma, *IDH*-mutant. A) Histological examination showing a highly cellular astrocytic tumor composed of mitotically-active cells with nuclear anaplasia; neither necrosis nor microvascular proliferation are seen (hematoxylin and eosin; original magnification 150x); B) Neoplastic cells are diffusely and strongly stained with IDH1 (R132H) (immunoperoxidase; original magnification 150x); C) Tumor exhibits nuclear loss of ATRX; notice the ATRX-positive endothelial cells that serve as internal positive control (immunoperoxidase; original magnification 150x); D) Neoplastic cells are diffusely and strongly stained with anti-p53 antibody (immunoperoxidase; original magnification 150x)

Other genetic alterations have also been associated with shorter overall survival, including *RB1* homozygous deletion, *PIK3CA* and PIK3R1 pathogenic mutations, PDGFRA amplification, and *MYCN* amplification [14–15,19]. Touat et al. also reported shorter survival times in a subset of hypermutated, mismatch repair-deficient gliomas, *IDH*-mutant [30]; Higher copy number variations (CNV) and somatic mutation levels were also correlated to poorer prognosis in WHO grades 2 and 3 *IDH*-mutant astrocytic tumors [14–15,31,32], however, there were limitations since the thresholds for high CNV and somatic mutation varied [33]. Additionally, one of the co-occurring, second genetic events that recently studied and could be class-defining oncogenic drivers in *IDH*-mutated astrocytoma is *BRAF*, which is known to be altered in different tumor types, including gliomas [34,35]: in more details, KIAA1549-BRAF fusions and *BRAF* V600E mutations have been identified as common genetic characteristics of pilocytic astrocytomas [36,37] and glial/glioneuronal tumors [38], respectively. Few studies have investigated the coexistence of *IDH* and *BRAF* molecular alterations within gliomas; two large studies tested a combined 252 glioma samples for *KIAA1549-BRAF* fusion, *BRAFV600E*, and *IDH1/2* mutations and concluded that *IDH* mutations and *BRAF* alterations were mutually exclusive [28,39]. Similarly, large-scale next generation sequencing studies of pediatrics and adults gliomas showed that *IDH* and *BRAF* alterations are mutually exclusive [4,40–42]. However, the co-existence of *IDH* mutations with *BRAF-KIAA1549* fusions or *BRAFV600E* mutations was reported in about 9% of diffuse gliomas of adult patients [43], but this high incidence is possibly due to a high false positive rate in their molecular methodology. Additionally, the authors of recent study [25] identified *IDH1/2* mutations and simultaneous *BRAF* alterations in 3/1879 glial tumors from their cohort. *FGFR* alterations also have been recognized as molecular drivers in pediatric and adult low and high-grade gliomas [44–46], with no prior reports of concomitant *IDH*-mutated gliomas and *FGFR* alterations, but a recent study by Ahrendsen et al. [25], reported seven gliomas with *FGFR* alterations with variable histology ranging from WHO grade 2 (diffuse astrocytoma and oligodendroglioma) to WHO grade 4.

*NTRK* gene rearrangements have also been found as an emerging oncogenic driver in a variety of tumors, including high- and low-grade, in both adults and pediatrics gliomas [47,48]. However, the presence of concomitant *IDH* mutation in *NTRK*-rearranged infiltrating gliomas has been described in a few cases with no indications of their significance [49,50]. But, in a recent study by Ahrendsen et al., seven *IDH*-mutated tumors with co-occurring *NTRK* fusion were discovered. Interestingly, all the cases that involved second-class defining alterations (*BRAF, FGFR*, and *NTRK*) by Ahrendsen et al., were of IDH1 (the majority *IDH1 R132H*). Additionally, in regard *to H3 K27M* mutation, many studies demonstrated mutual exclusivity of *IDH* and *H3 K27M* mutations in gliomas [25,27,42,50].

## 3 Diffuse Astrocytomas, IDH-Wild Type

Diffusely infiltrating astrocytomas, *IDH*-wild type, was considered by 2016 WHO Classification of CNS Tumors as a wide and heterogeneous spectrum of neoplasms, including WHO grade 4 glioblastoma, grade 2 diffuse astrocytoma, and grade 3 anaplastic astrocytoma [51]. The molecular landscape of diffuse astrocytomas, IDH-wild type was classically defined by the absence of *IDH1/2* mutations combined with the frequent lack of *ATRX* and *TP53* mutations [51]. In the 2016 edition of WHO Classification, grades 2 and 3 diffuse astrocytomas, *IDH* wild-type were considered as rare and provisional entities, whose biological behavior was more similar to that of glioblastoma, IDH-wild type than that of their *IDH*-mutant histologic counterparts [51]. As above-mentioned for IDH-mutant astrocytoma, morphology was the only criterion used to diagnose these entities, as mitoses and nuclear pleomorphism distinguished WHO grade 3 from WHO grade 2, and the presence of necrosis and/or microvascular proliferation defined WHO grade 4 (Fig. 3) [51]. Since then, several studies reported the extreme biological, clinical, and prognostic variability of these tumors, emphasizing the need for further sub-classification. In more details, it has been shown that a subset of adult diffuse astrocytoma, *IDH*-wildtype, corresponding to

histologic grade 2 or 3, had a very aggressive biological behavior, much more similar to that of glioblastoma, despite not exhibiting necrosis and/or microvascular proliferation [4,52–56]. In addition, a variety of glial and glioneuronal tumors, that could also occur in adults, such as pilocytic astrocytoma, pleomorphic xanthoastrocytoma, and other tumors, and lack of *IDH1/2* mutations could create differential diagnostic problems. As a result, the need to identify molecular features of adult diffuse adult astrocytoma, that could predict a poor outcome, arose.

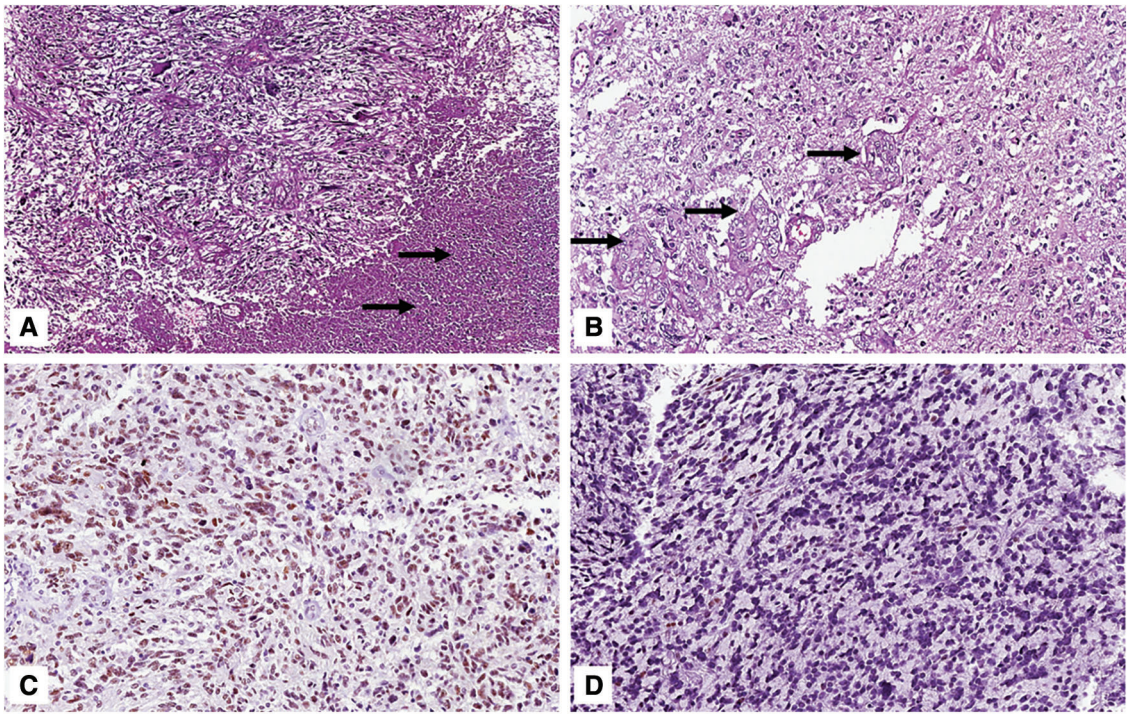


**Figure 3:** Grade 4 glioblastoma, IDH-wild type. A) Histological examination showing a hypercellular neoplasm composed of variably-shaped malignant cells with extensive foci of necrosis (arrows) (hematoxylin and eosin; original magnification 100x); B) Tumor also exhibits multiple foci of microvascular proliferation (arrows) (hematoxylin and eosin; original magnification 150x); C) Nuclear expression of ATRX is typically retained (immunoperoxidase; original magnification 150x); D) Wild type expression pattern of p53 in tumor cells (immunoperoxidase; original magnification 150x)

In 2018, the cIMPACT-NOW update 3 defined a minimal set of molecular characteristics that were able to reliably predict poor clinical outcomes among adult astrocytic tumors [57]. In this regard it was stated that an *IDH*-wild type adult diffuse astrocytoma with, at least, one of the following molecular features, would be better designated by the term "diffuse astrocytoma, *IDH*-wild type with molecular features of glioblastoma WHO grade 4", regardless of the histological grade [57]: i) *EGFR* amplification or ii) combined whole chromosome 7 gain and chromosome 10 loss or iii) *TERT* promoter mutation. It was noteworthy that *IDH*-wildtype diffuse astrocytoma lacking necrosis and/or microvascular proliferation, but exhibiting, at least, one of the above-mentioned genetic features, corresponding to WHO grade 4 [58,59]; notably, only 6 diffuse astrocytomas, *IDH*-wildtype, from the TCGA dataset of more than 500 histologic grade 2/3 diffuse gliomas had "molecular features of glioblastoma, WHO grade 4" [57].

In 2020, the cIMPACT-NOW update 6 [29] proposed to simplify the tumor nomenclature, by including the presence of, at least, one of the above-mentioned molecular features (*TERT* promoter mutation, *EGFR*

amplification, 7 gain/10 loss) among the diagnostic criteria of WHO grade 4 glioblastoma, *IDH*-wild type. Both *EGFR* amplification and chromosome 7 gain and chromosome 10 loss signature have high specificity for astrocytomas with poor outcomes [59]. Notably, *EGFR* amplification, which is defined as high-level EGFR copy number gains should be tested by validated molecular techniques; immunohistochemistry with anti-EGFR antibody should be avoided in diagnostic practice, as it lacks adequate reliability [60]. The prognostic value of *TERT* promoter mutations deserves a separate discussion, since different studies have yeilded partially contradictory results [4,52,55]. *TERT* promoter mutations were added to the list of minimal criteria for the diagnosis of "diffuse astrocytoma, IDH-wild type with molecular features of glioblastoma, WHO grade 4" in cIMPACT-NOW Update [57], especially that *TERT* promoter mutations were also found in almost all oligodendrogliomas and other IDH-wild type gliomas, that lacked both necrosis/microvascular proliferation and/or aggressive biology, such as pleomorphic xanthoastrocytomas, gangliogliomas, ependymomas and high-grade astrocytomas with piloid features [2,61–63]. However, as diffuse astrocytomas, *IDH*-wild type, frequently harbored *TERT* promoter mutations along with 7 gain/10 loss or *EGFR* amplification, the association between these features increased the specificity of *TERT* promoter mutation alone as a marker of molecular grade 4 astrocytoma [59]. Tesileanu et al. [2] retrospectively compared the overall survival time of 71 diffuse astrocytoma, with low-grade radiologic features, in which 22 of them exhibited only TERT promoter mutation, with those of 197 glioblastomas, *IDH*-wild type. The authors found that patients with *IDH*-wild type glioblastomas, and astrocytomas with only TERT promoter mutations, had similar overall survival [2]. A subsequent study by Berzero et al. [64], compared grade 2 to grade 3 diffuse astrocytomas, *IDH*-wildtype with molecular features of glioblastoma according to cIMPACT-NOW update 3. It has been found that in 62% of cases an isolated *TERT* promoter mutation was the only molecular alteration of WHO grade 4 glioblastoma, and it was not associated with aggressive biological behavior per se [64]. Based on these findings, Giannini et al. [65] recently questioned if the presence of *TERT* promoter mutation alone was sufficient to "call" grade 2 astrocytoma, *IDH*-wild type, as WHO grade 4 glioblastoma, emphasizing that the "old" histological grading still had prognostic utility in adult diffuse astrocytomas, *IDH*-wild type and that isolated *TERT* promoter mutation is insufficient to designate grade 2 diffuse astrocytomas, *IDH*-wild type as WHO grade 4 glioblastomas.

The current 2021 WHO Classification of Tumors of the CNS promoted the integrated molecular and histologic approach to these tumors, by adding *TERT* promoter mutation, *EGFR* amplification, 7 gain/10 loss as criteria for grading and, thus, prognostic biomarkers of adult diffuse astrocytomas, *IDH*-wildtype [1]. Accordingly, grade 2 diffuse astrocytoma, and grade 3 anaplastic astrocytoma, *IDH*-wild type are no longer included in the current edition of WHO Classification of CNS Tumors, as they have been incorporated into the WHO grade 4 glioblastoma group, which includes adult diffuse astrocytic tumors, *IDH*-wild type, with the histologic presence of necrosis and/or microvascular proliferation, or one (or more) of the above-mentioned molecular alterations [1].

*EGFR* amplification and 7 gain/10 loss phenotype are routinely tested by FISH, while DNA sequencing methods are needed to detect *TERT* promoter mutations.

Homozygous *CDKN2A/B* deletion, despite being more frequently occurring in astrocytomas, *IDH*-wild type with *EGFR* amplification, +7/−10 or *TERT* promoter mutations [57], is not a prognostic biomarker of WHO grade 4 behavior as what described before for diffuse astrocytoma, *IDH*-mutant. In addition, *IDH*-wild type glial tumors that are different from glioblastoma in many aspects including, histology, genetic profile, and clinical outcomes like pleomorphic xanthoastrocytoma and high-grade astrocytoma with piloid features, frequently harbor this deletion [63,66,67].

## 4 Utility of DNA Methylation Profiling for Brain Tumors Diagnosis and Classification

Genome-wide DNA methylation profiling (DMP) is an analytical technique that in recent years has been increasingly used for the identification and characterization of several CNS neoplasms [1]. Based on the differences in DNA methylation patterns between different tumor entities, DMP is able to reliably assign a neoplasm to one of the already known clusters of CNS tumors and to stratify patients into prognostically distinct subgroups. This technique tends to maintain high reproducibility both on fresh/frozen tissue and formalin-fixed and paraffin-embedded tumor specimens; furthermore, it is also effective when the biological material is represented by small biopsies on which the other molecular methods have limited applicability [1].

Methylome profiling can also detect copy number variations, including combined whole chromosome 7 gain and chromosome 10 loss, 1p/19q codeletion, gene amplifications and deletions. Although DMP is currently used for CNS tumor diagnosis, as an adjunctive tool to the "conventional" methods, such as histopathology, it is probably the most useful technique to classify neoplasms that exhibit unusual morphology and the only technique capable of reliably identifying novel and rare tumor entities [1]. However, when evaluating DMP results, neuropathologists and neuro-oncologists must pay close attention to the calibrated score values, keeping in mind that suggested diagnoses with scores <0.84 or 0.90 should be viewed with caution, while those with scores <0.50 should be probably rejected [1,68].

The 2021 WHO Classification of CNS Tumors included for the first time DMP results among the Definition and Essential and Desirable Diagnostic Criteria for some entities, including high-grade astrocytomas with piloid features, diffuse pediatric-type high-grade gliomas, H3-wild type, and IDH-wild type, diffuse glioneuronal tumors with oligodendroglioma-like features and nuclear clusters, diffuse leptomeningeal glioneuronal tumors and posterior fossa ependymomas [1].

## 5 Conclusion

The current review highlights the importance and the clinical significance of an integrated diagnostic approach to brain tumors, based on histology and genetic alterations, which is crucial to stratify prognosis, overall survival, and even grading in both *IDH*-mutant and *IDH*-wild type astrocytoma of adults [69–71]. The current 2021 WHO Classification reflects the provisional "state of the art" about the knowledge in the neuro-oncological field and it should be interpreted as a "further stage" in the evolution of the classification of brain tumors [1]. It should provide neuropathologists and other neuro-oncology experts with a practical and applicable guide for standardizing the diagnostic and the therapeutic approach of CNS neoplasms. However, the increasing need for advanced molecular techniques to correctly diagnose brain tumors, makes the global applicability of the 2021 Classification of CNS Tumors matter of debate, especially in low- and middle-income countries [72].

**Author Contributions:** Conceptualization, H.A. and G.B.; data curation, A.A. and G.B.; writing—original draft preparation, H.A. and G.B.; writing—review and editing, H.A., A.A., R.C. and G.B. All authors have read and agreed to the published version of the manuscript.

**Ethics Approval and Informed Consent Statement:** Not applicable.

**Availability of Data and Materials:** All data presented in this study are available from the corresponding author upon reasonable request.

**Funding Statement:** The authors received no specific funding for this study.

**Conflicts of Interest:** The authors declare that they have no conflicts of interest to report regarding the present study.

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