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ORIGINAL ARTICLE

Clinical implications of molecular analysis in diffuse glioma stratification

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Abstract

The revised 4th edition of the 2016 World Health Organization Classification of Tumors of the Central Nervous System (2016 CNS WHO) has introduced the integrated diagnostic classification that combines molecular and histological diagnoses for diffuse gliomas. In this study, we evaluated the molecular alterations for consecutive 300 diffuse glioma cases (grade 2, 56; grade 3, 62; grade 4, 182) based on this classification. Mutations in the isocitrate dehydrogenase (IDH) genes were common in lower grade glioma (LGG: grade2–3), and when combined with 1p/19q status, LGGs could be stratified into three groups except for four cases (Astrocytoma, IDH-mutant: 44; Oligodendroglioma, IDH-mutant and 1p/19q codeleted: 37; Astrocytoma, *IDH*-wildtype: 33). 1p/19q-codeleted oligodendrogliomas were clinically the most favorable subgroup even with upfront chemotherapy. In contrast, *IDH*-wildtype astrocytomas had a relatively worse prognosis; however, this subgroup was more heterogeneous. Of this subgroup, 11 cases had *TERT* promoter (p*TERT*) mutation with shorter overall survival than 12 p*TERT*-wildtype cases. Additionally, a longitudinal analysis indicated p*TERT* mutation as early molecular event for gliomagenesis. Therefore, p*TERT* mutation is critical for the diagnosis of molecular glioblastoma (WHO grade 4), regardless of histological findings, and future treatment strategy should be considered based on the precise molecular analysis.

Keywords Glioma · IDH · 1p/19q codeletion · TERT promoter · WHO classification

Introduction

Recent revised WHO classification established integrated diagnosis for diffuse gliomas based on the combination of histological, molecular findings and clinical factors [1]. *IDH*1/2 mutation is considered one of the most crucial genetic alterations, which divide lower grade glioma (LGG) into two molecular trajectories during the early stage of gliomagenesis [2–4]. 1p/19q codeletion is another essential molecular alteration, which classified *IDH*-mutant LGGs into astrocytic and oligodendroglial tumors [5, 6]. *IDH*-wildtype LGG is considered to be a more aggressive genotype [2, 3]; however, it is a heterogeneous subgroup that should be further stratified [7]. Treatment strategy, including a surgical procedure, should be considered based on the integrated diagnosis [8–11] and optimal genetic analysis is recommended for the precise molecular classification. The Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy-Not Official WHO (cIMPACT-NOW) has provided novel information for clinical application of WHO classification via updates 1 through 7, published for the next WHO classification of CNS tumors [12–18]. The cIMPACT-NOW update 3 indicated that the tumor with molecular features of GBM, so-called "molecular GBM", exists within the *IDH*-wildtype LGG [14]. These cases also showed a clinical course similar to that of *IDH*-wildtype GBMs. Furthermore, the cIMPACT-NOW update 6 proposed that one of the three genetic alterations (*EGFR* amplification, Combined whole chromosome 7 gain and whole chromosome 10 loss (+7/−10), *TERT* promoter (p*TERT*) mutation) is sufficient to define lower grade astrocytoma as *IDH*-wildtype GBM, grade 4 [17]. *IDH*-wildtype astrocytoma with p*TERT* mutation exhibited a worse prognosis similar to *IDH*-wildtype GBM, even when

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these tumors did not show the typical radiological findings of histologically defined GBM [19]. The cIMPACT-NOW update 4 indicated that *IDH*-wildtype diffuse gliomas are more heterogeneous and complex, especially in pediatric and young adults [15, 20]. The cIMPACT-NOW update 5 improved the grading of *IDH*-mutant astrocytomas based on *CDKN2A/B* homozygous deletion [16]. Based on these updates, the grade of diffuse glioma can be determined by specific molecular alterations regardless of histological findings in some situations. Here we evaluated molecular alterations in 300 diffuse glioma cases and summarized these molecular characteristics to determine the future direction of practical molecular testing algorism for the next WHO classification.

Materials and methods

Tumor samples

Tumor samples were obtained from consecutive 300 patients diagnosed with diffuse glioma, who were initially treated at Kyushu University Hospital between 2002 and 2019. Tumor tissues were saved for histopathological examination, and also snap-frozen in liquid nitrogen and stored at − 80 °C. Tumors were histologically diagnosed by two expert neuropathologists (SOS, TI).

The tumor DNA and corresponding constitutional DNA from peripheral blood leukocytes were extracted using the QIAamp DNA Mini Kit and DNA Blood Kit (Qiagen Science, Germantown, MD, USA), respectively. This study was approved by the ethics committee of Kyushu University.

Evaluation of 1p/19q codeletion and chromosome10 loss

Loss of heterozygosity (LOH) on chromosomes 1p, 19q and 10 was detected by microsatellite analysis of blood and tumor DNA. We designed 20 microsatellite makers for covering the chromosome 1p, 10 and 19q13 regions as follow: D1S2667, D1S2647, D1S2734 (located on 1p36), D1S2797 (1p32), D1S2766, D1S435 (1p22); D10S537, D10S1649 (10p15), D10S213 (10p11), D10S196 (10q11), D10S1652 (10q21), D10S537 (10q22), D10S1765 (10q23, near PTEN region), D10S587, D10S216, D10S1655 (10q26); D19S420, D19S219, D19S921 (19q13.3), D19S418 (19q13.4). PCR and fluorescence labeling were performed according to previously described methods [21, 22]. Capillary electrophoresis was performed using 310 or 3730 Prism Genetic Analyzer (Applied Biosystems). Raw electrophoresis data were analyzed with GeneMapper analysis software (Applied Biosystems). Allelic status was assessed based on criteria established in a previous study [21].

Evaluation of IDH1/2, H3F3A, and pTERT mutation

The main driver genes (*IDH1/2*, *H3F3A*) were evaluated by high-resolution melting (HRM) analysis using DNA extracted from the frozen tissue as previously described [23]. *TERT* promoter mutations were retrospectively analyzed by direct sequencing, because it is difficult to detect these mutations due to the large cytosine-phosphate-guanine island promoter region [24].

Statistical analysis

Progression free survival (PFS) and overall survival (OS) were estimated by the Kaplan–Meier method. The log-rank test was used to compare the survival distribution of each molecular subgroup. The statistical analysis was performed using JMP 16.0 (SAS Institute, Cary, NC, USA).

Results

A total of 169 glioblastomas (GBM), 13 diffuse midline gliomas (DMG), H3 K27M-mutant and 118 LGGs (WHO grade 2–3) were diagnosed according to 2016 WHO CNS classification [1]. Amongst the 169 GBM, 153 *IDH*-wildtype GBM and 9 *IDH*-mutant GBM cases were identified, while 4 cases were not fully analyzed for the WHO CNS 2016 criteria and diagnosed as GBM-"not otherwise specified (NOS)". Among the 153 *IDH*-wildtype GBM cases, 3 pediatric cases showed *H3.3* G34R mutation. Of the 118 LGG, 33 cases showed *IDH* wildtype, including 13 diffuse astrocytomas and 20 anaplastic astrocytomas. Eighty-one cases showed *IDH* mutation including 22 diffuse astrocytoma, 22 anaplastic astrocytoma, 18 oligodendroglioma 1p19q codeleted, 19 anaplastic oligodendroglioma 1p19q codeleted, 2 oligodendroglioma NOS, 1 anaplastic oligodendroglioma NOS. Only one diffuse astrocytoma was diagnosed as "diffuse astrocytoma NOS" due to incomplete molecular testing. Among the 182 WHO grade 4 glioma patients, 14 were under 18 years old and 121 were over 55 years old. Among the 118 patients with LGG, 6 were under 18 years old and 27 were over 55 years old, while the remaining 85 cases ranged between 19 and 54 years old. The youngest patient with *IDH* mutation is 19 years old; thus all of the patients under the age of 18 were *IDH*-wildtype glioma. Among the 33 *IDH*-wildtype LGGs, 14 cases are over 55 years old. All *IDH*-mutant diffuse gliomas over 55 years old showed R132H *IDH1* mutation, which is consistent with a previous report [25]. Among the *IDH*-mutant diffuse gliomas, the age distribution of 1p/19q-codeleted oligodendroglioma is higher than that of astrocytoma regardless WHO grading. On the

other hand, *IDH*-wildtype anaplastic astrocytoma (grade 3) showed higher age distribution compared with *IDH*-wildtype diffuse astrocytoma (grade 2) (Table 1).

p*TERT* mutations were evaluated retrospectively for 278 of the cases. p*TERT* mutation was common molecular alteration among *IDH*-wildtype GBM and 1p/19q-codeleted oligodendrogliomas, 85/145 (58.6%) and 35/36 (97.2%) respectively. Within *IDH*-wildtype astrocytoma, four out of 13 diffuse astrocytomas and seven out of 18 anaplastic astrocytomas showed this mutation. Among *IDH*-mutant astrocytic tumors, one *IDH*-mutant GBM and one *IDH*-mutant anaplastic astrocytoma showed p*TERT* mutation. On the other hand, all of the 3 cases with oligodendroglioma/anaplastic oligodendroglioma-NOS showed p*TERT* mutation; however, the "not elsewhere classified (NEC)" diagnoses would apply according to cIMPACT-NOW update1 at present [12].

Survival analysis was performed for 101 adult LGGs including 35 1p/19q-codeleted oligodendroglioma, 43 *IDH*-mutant astrocytoma, 11 *IDH*-wildtype/pTERT-mutant astrocytoma and 12 *IDH*-wildtype/pTERT-wildtype astrocytoma. The median PFS for 1p/19q-codeleted oligodendroglioma, *IDH*-mutant astrocytoma, *IDH*-wildtype/p*TERT*-mutant astrocytoma and *IDH*-wildtype/p*TERT*-wildtype astrocytoma are 112, 36.6, 11.8, and 77.4 months, respectively. The median OS for *IDH*-mutant astrocytoma and *IDH*-wildtype/p*TERT*-mutant astrocytoma are 82 and 36.6 months, respectively. The median OS was not reached for the other two subtypes.

Survival analysis revealed that the most favorable outcome was with 1p/19q-codeleted oligodendrogliomas. Notably, both PFS and OS of *IDH*-wildtype LGGs were separated based on their p*TERT* mutation (Fig. 1).

Furthermore, three *IDH*-wildtype LGG cases underwent repeat surgery, and more than two samples of each case are analyzed longitudinally. Notably, the longitudinal analysis revealed that the *IDH*-wildtype LGG case with p*TERT* mutation gradually extended ch10 loss and finally showed total ch10 loss (Fig. 2). In contrast, the two cases without p*TERT* mutations never showed additional genetic alteration of the three parameters in repeat surgery.

Table 1 Age distribution for diffuse glioma subgroups

	Grade	Age median [range]	Age (0–18y)	Age (19–54y)	Age (55y–)	Total
GBM, IDHwt	4	65 [3–87]	3	35	115	153
GBM, IDHmut	4	38 [23–62]	0	6	3	9
H3 K27M	4	15 [5–46]	9	4	0	13
H3 G34V/R	4	10 [8–37]	2	1	0	3
DA, IDHmut	2	30.5 [20–57]	0	21	1	22
AA, IDHmut	3	39.5 [19–71]	0	21	1	22
DA, IDHwt	2	33 [2–70]	5	6	2	13
AA, IDHwt	3	59.5 [3–75]	1	7	12	20
OD, 1p19qcodel	2	44 [20–73]	0	14	4	18
AO, 1p19codel	3	47 [27–78]	0	13	6	19
NOS/NEC	2–4	55.5 [22–78]	0	4	4	8
Total		54 [2–87]	20	132	148	300

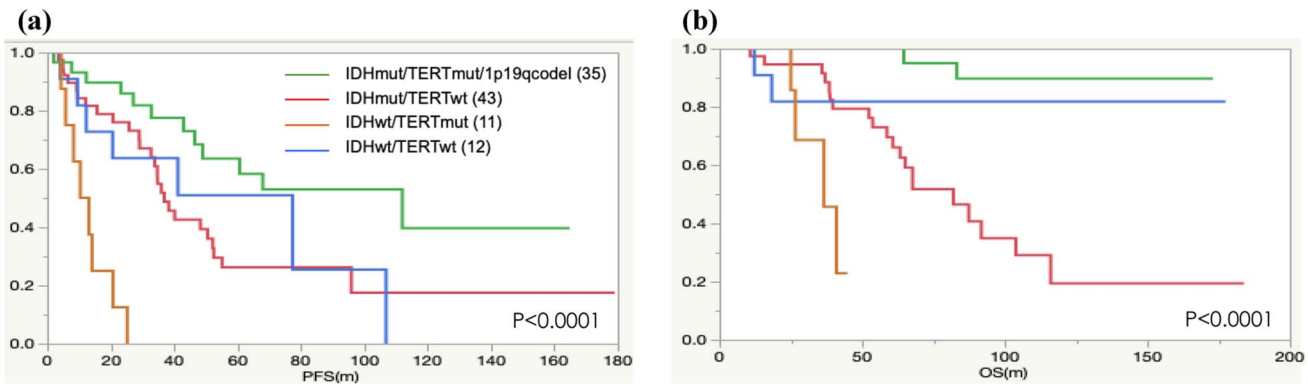

Fig. 1 Kaplan–Meier analysis of PFS (**a**) and OS (**b**) for lower grade gliomas stratified based on *IDH* and p*TERT* mutation. Oligodendroglioma, *IDH*mutant and 1p/19q codeleted show the most favorable outcome. Both PFS and OS of *IDH*-wildtype LGGs were stratified based on p*TERT* mutation

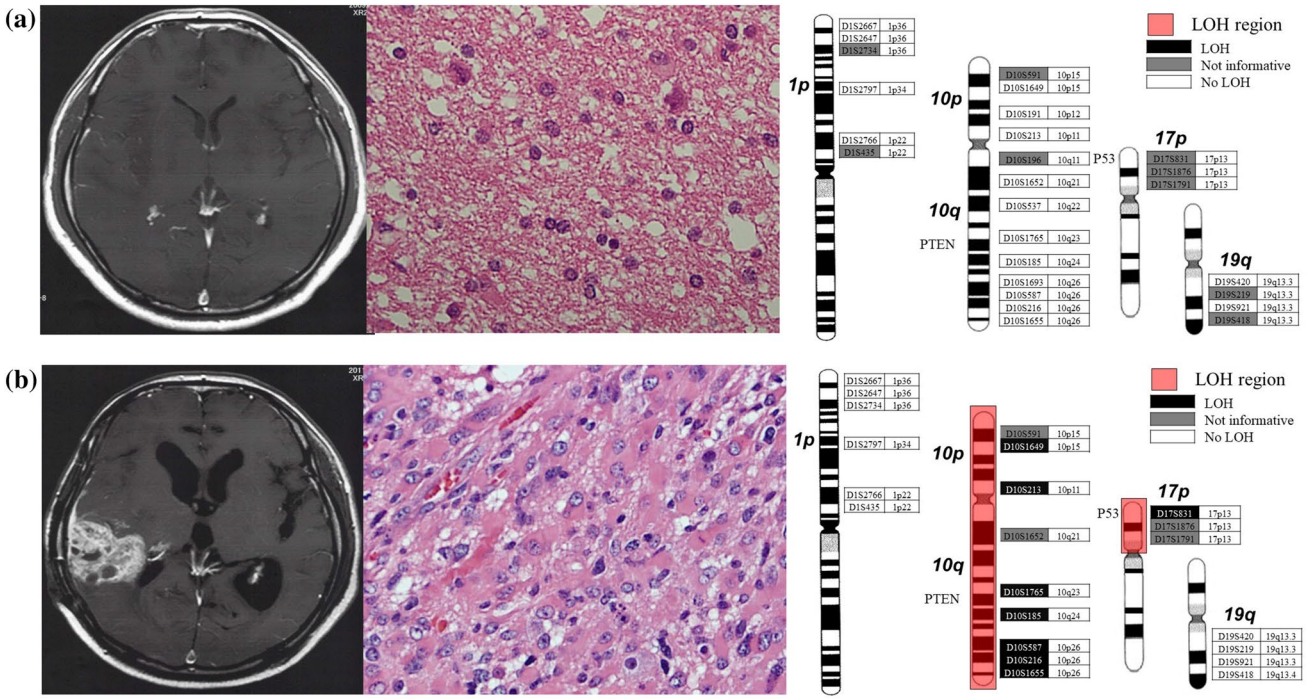

Fig. 2 A 44-year-old male diagnosed as Molecular GBM: The patient underwent four surgeries. MRI showed a non-enhanced mass in the right temporal lobe. First diagnosis is a diffuse astrocytoma, grade 2 without any LOH region (**a**). After repeat surgeries and adjuvant chemo-radiotherapy, MRI showed a heterogeneous enhanced mass. Final diagnosis is glioblastoma, grade 4 with ch10 loss (**b**). PFS and OS of this case are 4.0 and 26.6 months, respectively

Discussion

IDH1/2 mutation is now considered an early genetic event for gliomagenesis, and a critical genetic marker for diffuse glioma stratification [2, 3]. Combined with 1p/19q codeletion, diffuse glioma is classified into three major subgroups (*IDH*-mutant astrocytoma, *IDH*-mutant and 1p/19q-codeleted oligodendroglioma, and *IDH*-wildtype astrocytoma). *IDH*-wildtype GBM and 1p/19q-codeleted oligodendroglioma are common genotype well characterized by several previous clinical studies [8, 9, 26, 27].

Since 2002, we selected upfront chemotherapy and repeat surgeries for patients with 1p/19q-codeleted oligodendroglioma to prevent cognitive dysfunction [10, 11]. Precise detection of 1p/19q total loss caused by unbalanced translocation is crucial for selecting the less intensive treatments [28–30]. The cIMPACT-NOW update 2 proposed that histological astrocytic findings and alpha-thalassemia/mental retardation Syndrome X-linked helicase (ATRX)/p53 immunohistochemical results were sufficient for the diagnosis of "Astrocytoma, *IDH*-mutant" without 1p/19q molecular testing [13]. In our institution, however, the upfront chemotherapy has been selected only for the patients with 1p/19q codeletion confirmed by molecular analysis. Combined with the *IDH1/2* mutation, we can detect this molecular subgroup more precisely, because some *IDH*-wildtype GBM showed apparent 1p/19q codeletion as the part of chromosomal alterations [31]. p*TERT* mutation is another important molecular marker for this subgroup. In this study, all 1p/19q-codeleted oligodendroglioma except one case showed p*TERT* mutation and favorable clinical course. However, the patient with 1p/19q-codeleted oligodendroglioma without p*TERT* mutation showed a relatively worse prognosis, while three patients with 1p/19q-intact oligodendroglioma with p*TERT* mutation showed better prognosis. A recent report also emphasized the implication of p*TERT* mutation regardless of 1p/19q status in *IDH*-mutant LGGs [32]. Further molecular estimation is needed for the case showing a discrepancy between 1p/19q codeletion and p*TERT* mutation.

Within LGGs, *IDH*-wildtype astrocytoma is a relatively small subgroup that is considered to be a more aggressive genotype compared with *IDH*-mutant LGGs. Recent reports have revealed that *IDH*-wildtype LGG is a more heterogeneous subgroup, and all of these patients do not show a dismal prognosis [7, 27, 33, 34]. Further stratification is required based on genetic alterations for *IDH*-wildtype LGG because pediatric-type diffuse gliomas demonstrate complex molecular alterations [15, 35–37]. In particular, for tiny biopsy specimens, appropriate genetic testing is mandatory for accurate diagnosis.

At our institution, we routinely evaluate the genetic alterations of *IDH1/2*, *BRAF*, *H3F3A* and p*TERT*, adding to LOH

status of chromosomes 1p, 19q and 10 [22, 23, 31]. Using these molecular analyses, diffuse gliomas are diagnosed based on the 2016 CNS WHO classification. Combined with histological diagnosis, we identified 33 cases of *IDH*-wildtype LGG. According to the cIMPACT-NOW update 3, we detected 11 *IDH*-wildtype astrocytoma with molecular features of glioblastoma, WHO grade 4. Considering the molecular test algorithm for molecular GBM, the pivotal molecular parameter is p*TERT* mutation, which was the most sensitive for detecting this subtype [38]. More than 60% of molecularly diagnosed GBM cases can be identified from *IDH*-wildtype LGG by p*TERT* mutation analysis alone [19].

In this cohort, one case with WHO grade 2 diffuse astrocytoma suffered several recurrences, and finally became histologically diagnosed as GBM with whole ch10 loss (Fig. 2). Retrospective analysis revealed that p*TERT* mutation occurred in the initial tumor of this case. In contrast, two cases with *IDH*-wildtype LGG, which underwent repeat surgeries for recurrent lesion, did not show further genetic alterations of the three molecular markers (p*TERT* mutation, *EGFR* amplification, + 7/ − 10), when p*TERT* mutation was not identified in the initial operation. Furthermore, due to the high frequency of p*TERT* mutation in molecular GBM, p*TERT* mutation can be considered an earlier genetic event compared with the others (*EGFR*amplification, + 7/ − 10). Several reports also support that p*TERT* mutation precedes + 7/ − 10 in the molecular evolution of *IDH*-wildtype LGG [39–41]. In contrast, a recent report revealed that p*TERT* mutation was associated with the rapid tumor growth of *IDH*-wildtype GBM, while one or more chromosomal alterations (+ 7/ − 9p/ − 10) were required for the tumor initiation [42]. Nevertheless, p*TERT* mutation, similar to H3 K27M and G34R/V mutations, is considered to play an important role in the early stage of gliomagenesis [40, 43].

Diffuse glioma is well recognized as the tumor showing marked spatio-temporal heterogeneity. Recent longitudinal studies demonstrated the molecular evolution of diffuse glioma during disease progression [41, 42, 44]. Furthermore, the diversity of genetic/epigenetic states remains unclear due to the marked intratumoral heterogeneity [45, 46]. In particular, molecular heterogeneity is becoming more complicated for recurrent gliomas under therapy [47]. In the near future, heterogeneous molecular alterations will be accelerated under molecular target therapy such as tyrosine kinase inhibitors [41, 48]. More precise genetic/epigenetic characterization is required to overcome the marked spatio-temporal heterogeneity of diffuse glioma in the era of cancer genome medicine.

Recently the Japan Society of Brain Tumor Pathology proposed three levels of diagnoses for diffuse astrocytic and oligodendroglial tumors [49]. Especially for the subgroup of LGG, level 3A/B analysis (1p/19q codeletion and *IDH*1/2 mutation) is required for the precise diagnosis. In our institution, level 3A/B molecular analysis was applied as advanced medical care for diffuse glioma cases. After *IDH1/2* wildtype is defined, the tumors with *H3F3A* or *BRAF* mutant should be excluded for the further analysis of molecularly GBM. The next step was to evaluate p*TERT* mutation, the most sensitive molecular marker for molecular GBM. For *IDH*-wildtype LGG without p*TERT* mutation, evaluation of *EGFR* amplification is required for a precise diagnosis. *EGFR* amplification is the most specific parameter within these three markers; however, its sensitivity is relatively low [38]. We planned to apply multiplex ligation-dependent probe amplification (MLPA) kit P105 (MRC-Holland, Amsterdam, The Netherlands) for detecting *EGFR* amplification, thus, we can detect the copy number of this region of ch7p and *EGFR* variant III simultaneously [50]. Evaluation of whole ch7 gain is needed for the rare cases showing p*TERT* wildtype, *EGFR* gain and whole ch10 loss. Furthermore, this MLPA kit can also detect *CDKN2A* homozygous deletion, which is a critical molecular marker for "Astrocytoma, *IDH*-mutant, grade 4" [16]. This step-by-step diagnostic procedure is recommended for daily routine diagnostics of diffuse gliomas, not only for molecular GBM. Based on our results, the test algorism following the level 3A/B analysis is proposed in Fig. 3. Future treatment strategies should be considered based on precise molecular analysis.

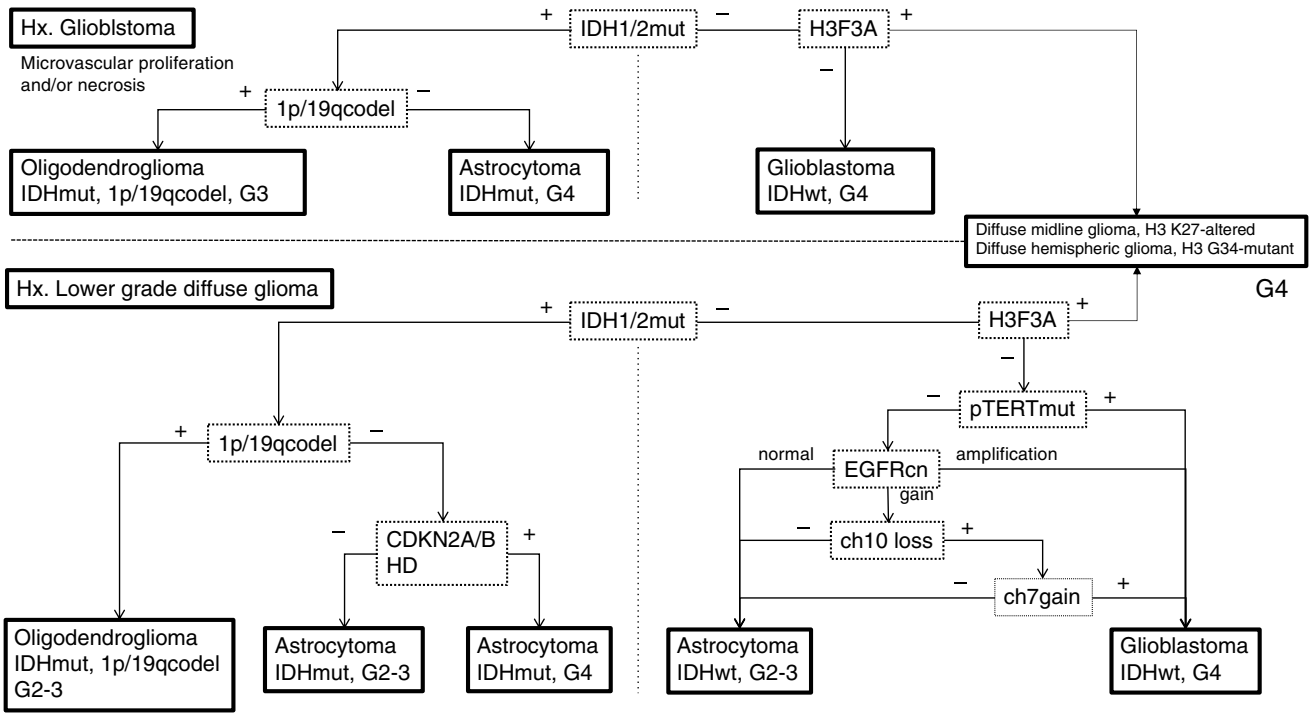

Fig. 3 Molecular testing algorism of diffuse gliomas for the next WHO CNS classification: The diagnosis of lower grade glioma becomes more complex. Optimal molecular analysis is necessary for precise diagnosis. *Hx* histological diagnosis; *EGFRcn* EGFR copy number

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Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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