改訂2016WHO分類に基づいた神経膠腫連続400症例の 再分類

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Reclassification of 400 consecutive glioma cases based on the revised 2016WHO classification

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Abstract

In this study, we reclassified 400 consecutive glioma cases including pediatric cases, using the revised 2016 WHO classification with samples collected from the Kyushu University Brain Tumor Bank. The *IDH1/2*, *H3F3A*, key genetic markers in the 2016 classification, were analyzed using high-resolution melting, with DNA extracted from frozen tissues. The 1p/19q codeletions were evaluated using a microsatellite-based loss of heterozygosity analysis, with 18 markers, to detect loss of the entire chromosome arm. In the integrated diagnosis, 29 oligodendroglioma cases and 28 anaplastic oligodendroglioma cases were diagnosed as “*IDH*-mutant and 1p/19q-codeleted,” while 2 oligodendroglioma cases and 5 anaplastic oligodendroglioma cases were diagnosed as not otherwise specified (NOS). These “NOS” cases were either *IDH*-mutants or 1p/19q-codeleted, although characteristic oligodendroglial features were evident histologically. Better overall survival of patients with oligodendroglioma correlated with the molecular characteristic of “*IDH*-mutant and 1p/19q-codeleted,” rather than the WHO grade. Eleven “glioblastoma, *IDH*-wild-type” cases were classified as “1p/19q-codeleted” however, chromosome 10 loss was also detected in 10 out of 11 cases. The 2016 WHO criteria for

glioma classification leads to better diagnosis of patients. However, there are technical pitfalls and problems to be solved in the molecular analysis of routine diagnostics.

Introduction

In 2016, the revised World Health Organization (WHO) classification scheme of central nervous system tumors introduced molecular genetic diagnosis, such as *the isocitrate dehydrogenase (IDH)*, *H3F3A* mutation and the chromosome 1p/19q codeletion, in addition to the classical histopathological diagnosis[1-3]. The aim of this integrated diagnosis with phenotype and genotype characteristics is to detect more biologically homogenous tumor entities with better prognostic significance compared with the classical histology-oriented diagnostic criteria. Based on the status of the *IDH* mutation, diffuse glioma is divided into two groups. In addition, diffuse glioma with chromosome 1p/19q codeletion is one group referred to as “oligodendroglioma, *IDH*-mutant and 1p/19q codeleted”. Moreover, “diffuse midline glioma with a *H3F3A*-mutant” is a new diagnostic entity that was created to separate it from *IDH*-wildtype glioma due to the poor patient prognosis[4]. Accordingly, the diagnostic entity of oligoastrocytoma has been deleted in the revised 2016 classification since it has been confirmed that oligoastrocytoma can be separated into either astrocytoma or oligodendroglioma and diagnosed accordingly with prognostic significance[1].

This integrated diagnosis, however, has invoked confusion in the clinical setting in several ways. First, the methodology for the molecular diagnosis has not been mentioned in the revised classification. Therefore, technical differences and pitfalls are likely to be some concern in situations where molecular analysis is used for routine diagnosis. Secondly, there are still many concerns about how to diagnose the tumors with discordant results of their associated phenotype and genotype. For example, if a glioma with typical histological oligodendroglioma features presents with an *IDH* mutation but does not have the 1p/19q codeletion, the diagnosis is questionable. Given that there is a clear description that the genotype trumps the histological phenotype[1], this tumor should be designated as a “diffuse astrocytoma, *IDH*-mutant”. However, if this tumor has a *TERT* promoter mutation, the appropriate diagnosis becomes uncertain. Since reports suggest that *TERT* promoter mutation has been exclusively detected in oligodendroglioma with a *IDH*-mutant and 1p/19q codeleted[5, 6], the genotype does not match with the “diffuse astrocytoma, *IDH*-mutant”. In the reclassification of consecutive 400 glioma cases based on the revised 2016 WHO classification, we were confronted with these rare circumstances. In this study, we aim to verify the diagnostic significance

of the 2016 WHO classification and show the technical pitfalls and problems to be clarified in future studies.

METHODS

Tumor samples

Samples of glioma were obtained from patients during craniotomies at Kyushu University Hospital and other affiliated institutions and were registered in the brain tumor database of our department at our institute. Part of the tumor tissue was saved for histopathological examination, and the rest was snap-frozen in liquid nitrogen and stored at -80°C . Tumors were histologically diagnosed by established neuropathologists (SOS, TI) and graded according to WHO criteria (2007). In this study, we extracted consecutive tumor samples from patients diagnosed with glioma and glioneuronal tumors between 2002 and 2016, including 400 patients with the following gliomas (type, number of patients) : glioblastoma multiforme (GBM), 206; gliosarcoma, 6; anaplastic astrocytoma, 41; anaplastic oligodendroglioma, 33; anaplastic oligoastrocytoma, 7; diffuse astrocytoma, 49; oligodendroglioma, 28; oligoastrocytoma, 4; gliomatosis cerebri (GC), 4; astroblastoma, 3; pilomyxoid astrocytoma, 1; pilocytic astrocytoma 12; pleomorphic

xanthoastrocytoma, 2; ganglioglioma, 4; dysembryoplastic neuroepithelial tumor, 2; glioneuronal tumor, 3.. Patient ages ranged from 1 to 85 years (median 48 years). Forty patients were younger than 20 years of age. Regarding sex, 237 patients were males and 168 were females. This investigation was approved by the Ethics Committee of Kyushu University.

Genetic analysis for mutation detection

To screen for *IDH1/2*, *H3F3A* mutations, we performed high-resolution melting (HRM) analysis using DNA extracted from frozen tissue as described previously[7, 8]. Subsequently, we used direct sequencing to determine the base sequences of the mutations. The PCR products obtained from the HRM analysis were diluted 50-fold and purified using ExoSAP-IT (Affymetrix/USB, Santa Clara, CA, USA), after which cycle sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing kits (Applied Biosystems, Tokyo, Japan). Following purification with a BigDye XTerminator® Purification Kit (Applied Biosystems), electrophoresis and analysis were conducted using a PRISM® 310 Genetic Analyzer (Applied Biosystems). The *BRAF*

V600E mutations were analyzed in 359 samples and *TERT* promoter mutations were analyzed by sequencing for 325 samples as described previously[8].

Evaluation of 1p/19q codeletion and chromosome 10 loss

We analyzed chromosome 1p/19q codeletions by loss of heterozygosity (LOH) analysis as described previously[9]. The LOH on chromosomes 1p, 19q was evaluated using a polymerase chain reaction-based microsatellite analysis. Multiple polymorphic microsatellite markers were used to perform extensive analysis of these chromosomal regions. We used 11 markers in this analysis: D1S2667, D1S2647, D1S2734, D1S2797, D1S2766, D1S435, and D1S206 were used for 1p and D19S420, D19S219, D19S921, and D19S412 were used for 19q (Figure 1). Corresponding normal DNA was isolated from a blood sample of the same patient. We defined 1p/19q codeletion when all the markers on chromosome 1p and 19p showed LOH, indicating that the 1p/19q codeletion criteria were met. This was done to avoid detecting partial chromosome loss on chromosome 1p and 19p. In this study we also evaluated chromosome 10 loss using 10 markers: D10S249, D10S189, D10S1649, and D10S213 for 10p and D10S1652,

D10S537, D10S1765, D10S185, D10S587, and D10S216 for 10q (Figure 1).

Re-classification of the 2007 WHO glioma samples to the revised 2016 WHO classification and their clinical significance

Based on the histological evaluation and molecular analysis described above, we re-classified the 400 glioma samples diagnosed based the 2007 criteria to the revised 2016 WHO system. To evaluate the clinical significance of the revised 2016 WHO system, we analyzed and compared the overall survival of patients considering the diagnostic category.

RESULTS

Frequency of genetic mutation

Our results showed that the IDH 1/2 mutation was detected in 146 out of 405 cases (36%). The R132H mutation in IDH1 was the most frequent mutation that was detected in 139/146 cases (95%), followed by four cases of R172K in IDH2, two cases of R132S and one case of R132G in IDH1. The R172K mutation in IDH2 consisted of two oligodendrogliomas, one anaplastic oligodendroglioma, and one anaplastic astrocytoma.

The *H3F3A* mutation was detected in 17 cases out of 405 cases (4%), consisting of 13 K27M mutations and four G34R mutations. The K27M mutation was exclusively detected in glioblastoma, while the G34R mutation was observed in not only three glioblastomas but also in one astroblastoma, which has been reported previously[10].

BRAF V600E mutations and *TERT* promoter mutations were analyzed in some patients who had sufficient tissue samples for analysis. The HRM analyses revealed that among the patients with 359 samples out of 400 patients, seven patients

(2%) harbored *BRAF* V600E mutations. Direct sequencing showed that *TERT* promoter mutations were detected in 142 out of 325 cases (44%).

Frequency of 1p/19q codeletion and chromosome 10 loss

Allelic imbalance on each marker was evaluated by calculating the allelic ratio of the heterozygous alleles. The LOH was defined when the peak height ratio of alleles was statistically significantly different from that of normal as has been described previously[9]. The 1p/19q codeletion was defined when all the markers on chromosome 1p and 19p showed LOH. Our results revealed that 73 cases (18%) harbored the 1p/19q codeletion. Out of 73 cases with 1p/19q codeletions, 62 cases were oligodendrial tumors with an *IDH1/2* mutation, whereas 11 cases were glioblastoma without the *IDH* mutation. Partial chromosome 10 loss was detected in 87 cases (21%), and total chromosome 10 loss was detected in 126 cases (34%). None of the 1p/19q codeleted oligodendrial tumors with *IDH1/2* mutations showed chromosome 10 loss, while 10 out of 1 p/19q codeleted - glioblastomas without an *IDH* mutation showed chromosome 10 loss (either partial or total).

Reclassification of the samples

Based on the histopathology and molecular testing in our study, we performed an integrated diagnosis for each tumor and re-classified the 2007 WHO astrocytic and oligodendroglial tumors according to the 2016 WHO criteria (Figure 3). Although two oligodendrogliomas were placed into the NOS category, one was considered to be due to the sampling error because genetic alterations were not detected at all (Figure 3B). Among anaplastic oligodendrogliomas and oligoastrocytomas, three were placed into the NOS category (Figure 3B, 3C). Two tumors, both right frontal lobe tumors, did not have 1p/19q codeletions (one demonstrated no LOH at all, the other had partial LOH on 1p) with typical oligodendroglial histological features and *TERT* promoter mutations. We placed these two tumors into the NOS category and presented the neuroimaging, histopathological image and LOH analysis data in Figure 4. The two anaplastic oligoastrocytomas in a 20-year-old and a 25-year-old patient, respectively, were from the same patients who also had recurrent tumors. These tumors showed 1p/19q codeletions but no *IDH* mutation, leading to the diagnosis of anaplastic oligodendroglioma, NOS (Figure 3C).

Prognostic value of WHO 2016 classification

To investigate the clinical significance of the integrated diagnosis, we investigated the difference in overall survival using the integrated diagnosis in 45 oligodendrogial tumors (grade II and III) diagnosed based on WHO 2017 classification. The results revealed that oligodendrogliomas, *IDH*-mutant and 1p/19q codeletion showed better survival compared with anaplastic astrocytomas, *IDH*-mutant, and anaplastic astrocytomas, *IDH*-wildtype, indicating that the 2016 WHO revised criteria have better clinical significance (Figure 5A). Two oligodendrogliomas, with *IDH*-mutant and 1p/19q non-codeletion (Grade II and III) (Figure 4) showed f favorable survival in our study, although follow up periods were relatively short for these two cases. In addition, our results revealed that histological grading did not show survival difference in our study (Figure 5B).

Next, we determined the clinical significance of the 2016 WHO integrated diagnosis for astrocytic tumors (Figure 6). The results revealed that patients with diffuse astrocytomas with *IDH*-wildtype (grade II) showed a statistically significant survival

advantage than those with a diffuse astrocytoma *IDH*-mutant (Figure 6A). However, those with grade III and grade IV did not show a survival difference depending on the status of *IDH* mutation (Figure 6B, C). Regarding *H3F3A* mutation, K27M mutation and G34R mutation demonstrated similar prognostic outcomes as reported previously[10].

DISCUSSION

Clinical significance of WHO 2016 classification

Recent molecular genetic analyses have identified characteristic molecular alterations related to specific clinical features and prognosis within the same histological diagnostic criteria of the 2007 WHO classification system. The principal aim of introducing the integrated diagnosis that includes phenotypic and genetic information for the diagnosis of central nervous system tumors in the revised 2016 classification is to have available an integrated system that can help diagnosis and detect more homogeneous tumor entities with similar prognoses and treatment responses than in prior classification[1]. Consistent with this aspect, reclassification of oligodendroglial tumors by an integrated diagnosis in this study demonstrated prognostic differences (Figure 5). One of these differences indicated that the genotype of the IDH-mutant and 1p/19q codeletion has a prognostic significance in oligodendroglial tumors. This result was observed in our previous study[11] and other various publications[12-14]. For astrocytic tumors, the mutation status of the tumor had different prognostic significance depending on the tumor grade. In grade II diffuse astrocytomas, *IDH*-wildtype tumors demonstrated

longer overall survival, whereas those with an *IDH* mutation status did not differ in overall survival for grade III anaplastic astrocytomas and grade IV glioblastomas. This may be because grade II diffuse astrocytomas with the *IDH*-wildtype includes heterogenous tumor entities.

Methodology for molecular diagnosis

***IDH1/2* and *H3F3A* mutation**

Although the WHO introduced molecular diagnosis as a routine procedure in the clinic, WHO has not yet published information on the methodological requirements for the detection of molecular alterations, which has provoked confusion for using the integrated diagnosis approach in a clinical setting[15]. Given the emerging recognition of hot spot mutations such as *IDH* and *H3F3A*, specific antibodies targeting these hotspot mutations can be a useful molecular tool in the clinic. For the detection of *IDH* mutations, immunohistochemical (IHC) diagnosis using IDH1 R132 H mutant specific antibodies is easily applicable in the clinic. We have been introducing IHC as a routine part of clinical diagnosis; however, another *IDH1* minor mutation, other than the R132H mutation and

IDH2 mutation has been reported and identified in this study. Thus far, we find it important to detect the nucleotide change at the DNA level to avoid missing rare mutations. Although the WHO introduced a regulation which stipulates that the *IDH* mutation is rare in those over the age of fifty five, a precise detection of the *IDH* mutation can have more significance for assessing this age group in the near future when targeted therapy for *IDH* mutations is introduced in the clinic.

Detection of 1p/19q codeletion

Given that the 1p/19q codeletion has diagnostic and prognostic significance for the diagnosis of oligodendroglioma, the exact detection of 1p/19q codeletion is of upmost importance. However, the WHO guidelines do not mention the use of a molecular tool to detect the 1p/19q codeletion for routine molecular diagnosis in the clinic. The chromosome 1p/19q codeletion is generated by the balanced translocation between chromosome 1p and 19q, indicating that it is necessary to examine the allelic status of the entire 1p and 19q chromosome. To detect the 1p/19q codeletion, various technologies have been published including PCR-based LOH analysis and multiplexed ligation-

dependent probe amplification, fluorescent in situ hybridization (FISH), and array-based technology[16, 17]. In this study, we integrated the LOH analysis using multiple microsatellite markers on chromosome 1p and 19q to detect the 1p/19q codeletion. Since we define the 1p/19q codeletion when all the markers on the chromosome 1p and 19q show LOH, we believe that our method does not detect partial deletion on chromosome 1p and 19q. This is an important point because partial deletions on the chromosome 1p and 19q regions are reported to be deleted in high-grade astrocytomas[18, 19], and the prognostic significance of 1p and 19q partial deletions is different from that of a complete 1p/19q codeletion. This is the characteristic feature of oligodendrogliomas. It is critical to detect complete 1p/19q codeletions of glioma samples in the clinical setting. The drawback of LOH analysis is that we cannot get a copy number alteration, which means that deletion and low level of amplification cannot be differentiated in this method[16, 20]. In addition, control DNA is usually taken from leucocytes in the blood. Nevertheless, LOH analysis using multiple microsatellite markers is applicable to the clinic in terms of cost and availability.

A FISH analysis is a frequently used molecular tool to detect 1p/19q codeletions;

however, this method detects not only 1p and 19q whole chromosome loss but also partial 1p and 19q deletion because the commercially available FISH probe is usually designed to target the 1p36 and 19q13 regions. Therefore, the 1p/19q partial deletion cannot be differentiated from the 1p/19q entire chromosome codeletion by a FISH probe targeted to 1p36 and 19q13 region. High-throughput array technology such as the comparative genomic hybridization and SNP array is a straight-forward technology to detect chromosome deletion and amplification with copy number alteration; however, the expensive cost is not suitable for introducing as part of routine molecular diagnosis in the clinic.

Pitfalls and unsolved problem of molecular diagnosis

Although the 1p/19q codeletion is defined as extensive chromosome 1p and 19q loss, it rarely happens that the 1p/19q codeletion is detected as part of an extensive chromosomal abnormality in glioblastoma as previously reported[21]. Therefore, it is important to refer to the IDH status when evaluating the chromosome 1p/19q abnormality to differentiate authentic 1p/19q codeletions from false 1p/19q codeletions in PCR-based

LOH analysis.

In this study, we designated an oligodendroglioma and an anaplastic oligodendroglioma as NOS because these two cases were *IDH*-mutant, but did not show a 1p/19q codeletion (Figure 4). Although these two cases should be generally classified as diffuse astrocytoma and anaplastic astrocytoma, *IDH*-mutant, respectively, we classified these two cases as NOS within the category of oligodendroglioma because the histopathology of these two cases was compatible with typical oligodendroglial histological features. In addition, the tumor tissue for these two cases had *TERT* promoter mutations, which is rarely detected in astrocytomas with an *IDH* mutation. This is a topic that requires further investigation in the future.

We detected another discrepant case. This was a recurrent tumor diagnosed in a young boy who demonstrated the 1p/19q codeletion with *IDH*-wildtype. As pediatric-type oligodendrogliomas do not typically have *IDH* mutations or 1p/19q codeletions[22], our case was an unusual pediatric-type oligodendrogliomas.

Conclusion

The 2016 WHO criteria for glioma classification leads to a better diagnosis of patients. However, some technical issues do exist within the integrated molecular classification about how to detect 1p/19q codeletions precisely during routine clinical examinations because the biological significance of true 1p/19q codeletions is completely different from that of false 1p/19q codeletions.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Figure legends

Figure 1

The microsatellite loci for the detection of chromosome 1p19q codeletion and chromosome 10 loss (21 markers). These are the microsatellite loci used to detect LOHs on the G-banding ideograms of chromosomes 1p, 19q, and 10p/q.

Figure 2

Representative electrophoretic patterns of microsatellite loci.

Shown in the image are compatible electropherograms of blood (normal reference) and the tumor in a column which has the same x-axis scale. (A) Total loss at locus D1S2766. (B) Partial loss at locus D19S921. (C) The pattern without loss of heterozygosity at locus D1S2667.

Figure 3

Reclassification of 366 diffuse gliomas and oligodendroglial tumors based on the revised 2016 WHO classification. (A) astrocytic tumors (B) oligodendrogliomas (C)

oligoastrocytomas

Figure 4

Two cases classified into the NOS category.

(A) A 57-year old male with a right frontal lobe tumor. The tumor was calcified on CT (left), and histology showed diffuse proliferation of monomorphic tumor cells with round nuclei with perinuclear halo (center). Although the tumor had *IDH1* mutation and *TERT* promoter mutation, LOH was not detected at all (right). Thus, we designated this case as oligodendroglioma, NOS. (B) A 25-year old female with right frontal lobe tumor. The tumor was calcified on CT (left), and histological features show monomorphic tumor cells with perinuclear halo (center). Although the tumor had *IDH1* mutation and *TERT* promoter mutation, LOH was detected only on the 1p36 region (right). Thus, we designated this case as anaplastic oligodendroglioma, NOS. The LOH status on 17q region was also evaluated for these cases using D17S831, D17S1876, and D17S1791 markers.

Figure 5

Clinical significance of WHO 2016 classification in oligodendrogial tumors.

We extracted 45 oligodendrogial tumors (grade II and III) for which long time follow up information is available. (A) The results revealed that oligodendrogliomas, *IDH*-mutant and 1p/19q codeletion showed better survival compared with anaplastic astrocytoma, *IDH*-mutant, and anaplastic astrocytoma, *IDH*-wildtype. (B) Histological grading did not show survival difference in our study in 36 (anaplastic) oligodendroglioma, *IDH*-mutant and 1p/19q codeletion.

Figure 6

Clinical significance of WHO 2016 integrated diagnosis in astrocytic tumors. (A) The results revealed that diffuse astrocytoma with *IDH*-wildtype (grade II) showed statistically significant survival advantage than diffuse astrocytoma with *IDH*-mutant. Grade III (B) and grade IV (C) did not show survival difference depending on the status of *IDH* mutation.

Chromosome 1

Chromosome 19

Chromosome 10

A

152744-20848_R01_807.dna 152744-20848
D1S2744 152744-20848_T_041_813.dna 152744-20848 T
D1S2744

↑
Total allelic loss

B

293921-21928_R01_807.dna 293921-21928

293921-21928_T_041_807.dna 293921-21928 T

↑
Partial allelic loss

C

112647-10778_R01_807.dna 112647-10778

112647-10778_T_041_813.dna 112647-10778 T

↑
No LOH

Blood

Tumor

A

B

A

- Oligodendroglioma, IDH-mutant and 1p/19q codeleted (Grade II, III) (36)
- Oligodendroglioma, IDH-mutant and 1p/19q non-codeleted (Grade II, III) (2)
- Anaplastic astrocytoma, IDH-mutant (4)
- Anaplastic astrocytoma, IDH-wildtype (3)

B

A **B** **C**