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REVIEW ARTICLE

IDH1/2 mutation detection in gliomas

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Abstract Somatic mutations of isocitrate dehydrogenase 1 and 2 (*IDH1/2*) are strongly associated with pathological subtypes, genetic profiles, and clinical features in gliomas. The *IDH1/2* status is currently regarded as one of the most important molecular markers in gliomas and should be assessed accurately and robustly. However, the methods used for *IDH1/2* testing are not fully standardized. The purpose of this paper is to review the clinical significance of *IDH1/2* mutations and the methods used for *IDH1/2* testing. The optimal method for *IDH1/2* testing varies depending on a number of factors, including the purpose, sample types, sample number, or laboratory equipment. It is therefore important to acknowledge the advantages and disadvantages of each method.

Keywords Glioma · *IDH1* · *IDH2*

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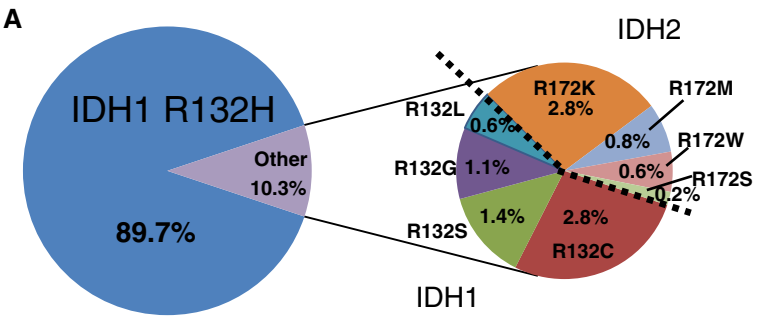
Introduction

Somatic mutations of isocitrate dehydrogenase 1 (*IDH1*, 2q34) were first identified in a small subset of glioblastomas [1, 2]. A large number of studies soon followed, reporting that *IDH1* mutations actually occur in the majority of lower grade gliomas [2–6]. Later, mutations of isocitrate dehydrogenase 2 (*IDH2*, 15q26) were also reported in a small number of gliomas [3, 4]. *IDH1/2* mutations are currently regarded as the earliest event in gliomagenesis and one of the most significant genetic alterations in glioma biology [4]. *IDH1/2* mutations also seem to be clinically significant based on their relationship with pathology, other genetic changes, and clinical presentation. A rapidly growing number of published studies constantly provide new information about *IDH1/2*, and *IDH1/2* status is almost routinely evaluated in laboratories. However, the methods for *IDH1/2* testing are not fully standardized. The purpose of this report is to review the clinical significance of *IDH1/2* mutation and the methods used for testing these mutations.

Genetic aspect of *IDH1/2* mutations

Mutational pattern of *IDH1/2* in gliomas (Fig. 1)

All reported *IDH1/2* mutations are missense mutations. They are almost always hemizygous and mutually exclusive to each other. IDH1 and IDH2 are homologous enzymes localized in the cytosol and mitochondria, respectively. IDH1/2 catalyze the conversion of isocitrate to α-ketoglutarate using $NADP^+$ as a cofactor. The *IDH1/2* mutations in gliomas result in the reduced ability to produce α-ketoglutarate and the acquisition of abnormal



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IDH1

Codon 132		
CGT		WT
CAT	c.394C>A	R132H
TGT	c.394C>T	R132C
GGT	c.394C>G	R132G
AGT	c.394C>A	R132S
CTT	c.395G>T	R132L
GTT	c.394C>G, c.395G>T	R132V

IDH2

Codon 172		
AGG		WT
AAG	c.515G>A	R172K
ATG	c.515G>T	R172M
TGG	c.514A>T	R172W
AGC	c.516G>C	R172S

Fig. 1 **a** Frequency of each mutation in *IDH1/2*. The ratio was calculated from the pooled data from eight independent studies, including the data on a total of 3,029 glioma cases [3, 6, 9–14]. The *left* circular chart represents the frequency of *IDH1* R132H mutation and non-R132H *IDH1/2* mutations. The *right chart* represents the frequency of each type of non-R132H *IDH1/2* mutation. **b** The nucleotide transitions in *IDH1/2*. All the mutations except R132V are point mutations

function to convert α-ketoglutarate into (R)-2-hydroxyglutarate ((R)-2HG) by using NADPH as a cofactor [7, 8]. (R)-2HG is considered to be a major oncometabolite, causing various biological effects on *IDH1/2* mutated tumors. *IDH1/2* mutations are therefore considered to be gain-of-function alterations [7].

The frequency of each mutation type varies according to the reports. The frequency of each mutation in a pooled data from 8 independent studies, describing the mutation types are shown in Fig. 1a [3, 6, 9–14]. The most common *IDH1/2* mutation in gliomas is c.395G>A transition in *IDH1*, which replaces the arginine with a histidine at codon 132 (R132H) (Fig. 1b). Other less common mutations also occur at codon 132 in *IDH1* or codon 172 in *IDH2*. The second most frequent mutations are R132C in *IDH1* and R172K in *IDH2* (2.8 % of all mutations, respectively). Other mutations include R132S, R132G, and R132L in *IDH1* and R172M, R172W, and R172S in *IDH2*. Extremely rare mutations include R132P, R132V, or those affecting R49, G97, and R100 in *IDH1* and R172T in *IDH2* ([15, 16] and references therein). The mutations affecting R140 in *IDH2* are among the most common mutations in myeloproliferative neoplasms; however, these are not observed in gliomas [8, 17].

Thus, the great majority of *IDH1/2* mutations involve codon 132 in *IDH1* or codon 172 in *IDH2*, and approximately 90 % of them are R132H in *IDH1*.

The relationship with other genetic alterations

IDH1/2 mutations show a distinct pattern with other genetic alteration characteristics in diffuse gliomas.

Genetic changes

IDH1/2 mutations are frequently observed in grade II–III gliomas, as described above, and are mostly associated with either *TP53* mutations or total 1p19q loss [4] (Fig. 2). *TP53* mutations are typically observed in grade II–III astrocytomas with *IDH1/2* mutations, and *ATRX* mutations are commonly observed in these populations [18]. Total 1p19q loss almost always coexists with *IDH1/2* mutations and *TERT* promoter mutations. This combination is typically observed in oligodendroglial tumors [9, 19]. *TP53* mutations and total 1p19q loss are mutually exclusive [4]. Similarly, *ATRX* and *TERT* promoter mutations also show mutually exclusive patterns [12, 19]. Thus, the combination of *TP53-ATRX* mutations or total 1p19q loss-*TERT* mutations is the hallmark of astrocytic or oligodendroglial tumors harboring *IDH1/2* mutations, respectively.

Epigenetic changes

Another important feature in *IDH1/2* mutated tumors is the epigenetic changes, which are some of the most

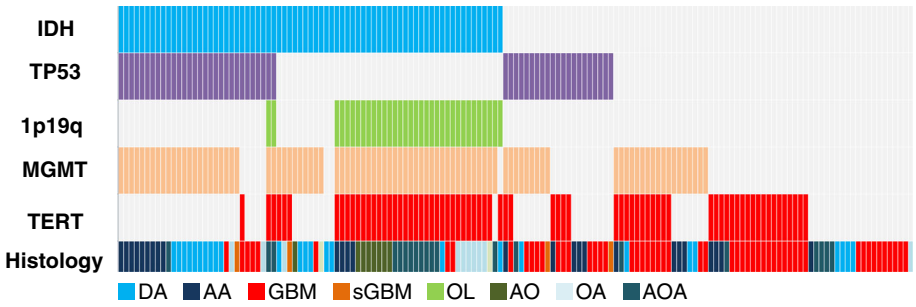

Fig. 2 Relationships among *IDH1/2* mutations, other genetic alterations, and histological subtypes. *Each column* represents individual tumors. *IDH1/2* mutations are strongly associated with *TP53* mutation or total 1p19q loss. Most *IDH1/2* mutated tumors show *MGMT* methylation. The data for the cases from National Cancer Center (Tokyo, Japan) are extracted and modified from the study by Arita et al. [9]. *DA* diffuse astrocytoma, *AA* anaplastic astrocytoma, *GBM* primary glioblastoma, *sGBM* secondary glioblastoma, *OL* oligodendroglioma, *AO* anaplastic astrocytoma, *OA* oligoastrocytoma, *AOA* anaplastic astrocytoma

fundamental alterations induced by these mutations. *IDH1/2* mutations have strongly been associated with the glioma-CpG island methylator phenotype (G-CIMP) and aberrant histone methylation [20, 21]. The mechanisms involved in the changes in DNA methylation and histone methylation status caused by *IDH1/2* mutations have been extensively discussed in several reviews [16, 22]. Thus, we only briefly summarized them in this review.

G-CIMP

G-CIMP was identified as a phenomenon in which DNA methylation in the CpG islands is increased genome-wide in a subset of gliomas [20]. It is well documented that G-CIMP is tightly associated with the presence of *IDH1/2* mutations and relative absence of typical copy-number alterations normally observed in glioblastomas, including *EGFR* amplification and chromosomal arm 10q loss. The tumors with G-CIMP present gene expression profiles of the proneural type [20]. DNA hypermethylation in these loci is considered to downregulate the expression of target genes, some of which may act as a tumor suppressor.

Aberrant (R)-2HG production in *IDH1/2* mutated tumors may inhibit α-ketoglutarate-dependent dioxygenase family, including ten–eleven-translocation (TET) [23]. The TET family catalyzes the conversion of 5-methylcytosine (5mC) to 5-hydroxymethycytosine (5hmC), which is a critical step in demethylating methylcytosine in the CpG dinucleotides, the main target of DNA methylation [24]. Inhibition of TET2 by (R)-2HG may possibly contribute to global DNA methylation in G-CIMP [21].

Histone modification

In *IDH1/2* mutated tumors, trimethylation at lysine residues of histone H3, including H3K9, H3K27, and H3K79 are increased [23]. Histone methylation is regulated by histone methyltransferase and demethylases and may affect gene transcription by altering chromatin structures. A Fe(II) and α-ketoglutarate-dependent subset of histone demethylase (e.g., lysine (K)-specific demethylase 6A (KDM6A)) is inhibited by (R)-2HG in *IDH1/2* mutated tumors, resulting in global alterations of histone demethylation and gene expression [23].

MGMT

CpG island methylation in O^6-methylguanine-DNA methyltransferase (*MGMT*) has also been associated with *IDH1/2* mutations [25, 26]. MGMT is a DNA-repair protein that removes alkyl adducts from O^6 position of guanine. Increased activity of MGMT reduces the chemosensitivity of alkylating agents, including temozolomide because O^6 position of guanine is the main target of DNA alkylation. *MGMT* methylation results in reduced MGMT expression and may lead to better response to temozolomide in glioblastoma [27]. *MGMT* methylation is invariably observed in *IDH1/2* mutated tumors [26]. On the other hand, about half of glioblastomas harbor *MGMT* methylation, regardless of the *IDH1/2* status (Fig. 2).

Clinical value

The relationship with pathology

In the pooled data from the eight reports (see above), *IDH1/2* mutations were observed frequently in grade II–III astrocytomas and oligodendrogliomas (53–83 %) as well as in secondary glioblastomas (54 %), but rarely in primary glioblastomas (6.3 %). *IDH1/2* mutations are highly specific to diffuse gliomas among CNS tumors. Other neuroepithelial tumors, including pilocytic astrocytoma, ependymoma, and ganglioglioma, rarely harbor *IDH1/2* mutations [15]. Non-neoplastic lesions mimicking gliomas never present these mutations [28]. The presence of *IDH1/*

2 mutations is a strong evidence of diffuse gliomas even in such cases (discussed further below).

Prognostic value

Several studies have suggested that patients with *IDH1/2* mutated tumor show longer survival than those with *IDH1/2* wild-type tumor, in most entities of gliomas [29].

The favorable prognosis of *IDH1/2* mutated glioblastomas was first reported in the pioneering study about *IDH1* [1], which was then followed by several other studies [3, 4, 14, 25, 30, 31]. *IDH1/2* mutations are generally regarded as a positive prognostic factor in glioblastomas. The overall survival in *IDH1/2* mutated cases is about twice longer than that of *IDH1/2* wild-type cases (24–31 vs. 9.9–15 months) [3, 25, 30]. Using a multivariate analysis, some studies have shown that *IDH1/2* status is an independent prognostic factor in glioblastomas [25], while others failed to reproduce this finding [31]. The small population of *IDH1/2* mutated glioblastomas might cause this controversy.

Several studies have reported that *IDH1/2* status is also a prognostic factor in grade III gliomas [14, 25, 32]. Some reports even demonstrated the positive prognostic value of *IDH1/2* mutations in each subtype: anaplastic astrocytomas [3] or anaplastic oligodendroglial tumors [33].

The prognostic value of *IDH1/2* status in grade II gliomas remains under debate. Some reports associated the presence of *IDH1/2* mutations with better prognosis [25, 34–37], while others did not [14, 38]. Sun et al. [39] investigated the prognostic value of *IDH1/2* status through a meta-analysis of ten previous studies and found that *IDH1/2* mutation was associated with longer survival in grade II gliomas. They pointed out several problems in the interpretation of currently available data, which include mixed cohorts of astrocytomas and oligodendroglial tumors, the close relationship with other prognostic factors, different methodology to evaluate *IDH1/2* status, and most importantly the lack of standard treatment in grade II gliomas. There are also other studies investigating the prognostic value in each subtype (i.e., astrocytomas or oligodendrogliomas); however, they remain inconclusive [14, 34, 35].

Overall, the independent value of *IDH1/2* as a biomarker remains somewhat controversial. This can be attributed to confounding factors or the study design as pointed out by Sun et al. [39] in their analysis of grade II gliomas. *IDH1/2* mutations are closely related to other prognostic/predictive factors, including patient age, *MGMT* status, or 1p19q copy number. Limited cohort size, different treatment, and the heterogeneity of tumor subtypes in each study may also result in conflicting results. Nonetheless, the *IDH1/2* status adds valuable information to the WHO grades in predicting the clinical course, and it should be considered as a stratification factor in clinical trials of gliomas [40].

Molecular classification and *IDH1/2*

One of the aims of the current WHO classification is to predict the clinical outcome of the patients harboring the tumor [41]. Nonetheless, the current diagnostic system poses the limitation that one type of tumor can include biologically and clinically different subsets of tumors. The molecular classification is expected to refine the current diagnostic system [42]. *IDH1/2* mutations present a strong association with the histological types and clinical outcome as described above. Therefore, these mutations are among the most promising markers.

The existence of *IDH1/2* mutations strongly supports the diagnosis of grade II–III gliomas. Intriguingly, Hartmann and colleagues reported that patients with *IDH1* wild-type anaplastic astrocytomas exhibited shorter survival than those with *IDH1*-mutated glioblastomas [43]. Underestimation of the tumor grades in histological diagnosis may lead to this observation along with the prognostic impact of the *IDH1* mutation itself. Malignant gliomas are histologically heterogeneous, and missampling can lead to undergrading [43]. Their findings argue for the significant value of *IDH1/2* mutation in the molecular classification combined with the current histological classification.

Combination of *IDH1/2* and other genetic status may aid in further predicting the subtypes of gliomas, because *IDH1/2* mutations show strong relationships with either the combination of *TP53-ATRX* mutations or total 1p19q loss-*TERT* mutation in astrocytic or oligodendroglial tumors, as described above.

The molecular diagnosis seems promising; however, it poses some significant limitations. The evaluation for molecular markers often needs DNA analysis, which requires expensive equipment and reduces the feasibility of the method in clinical use. Another issue is that standardization of the testing for each marker is needed. For example, *MGMT* methylation status is evaluated using various methods, including methylation-specific PCR (MSP) or pyrosequencing. However, these tests have not yet been standardized. Availability of molecular markers requires the accessibility of detection methods and their standardization.

IDH1/2 detection

IDH1/2 mutations undoubtedly divide diffuse gliomas into two groups, which have distinct biological and clinical features, as described above. *IDH1/2* mutations also have a

Table 1 The methods for *IDH1/2* detection

	Advantage	Limitation
Sanger sequencing	Gold standard	Modestly sensitive (>20 % of mutant allele is required)
	Detects all types of mutations	
Immunohistochemistry	Sensitive and robust	Detects only the mutation specific to the antibody used
	Available for FFPE samples without additional treatment	
Pyrosequencing	Sensitive	Needs special equipment
	Quantitative	The robustness depends on the assay design
Melting curve analysis	Rapid	Needs special equipment
	Detects all types of mutations	Modestly sensitive (improved by combining with COLD-PCR)
MRS	Noninvasive	Not validated and standardized for clinical use

MRS magnetic resonance spectroscopy, *FFPE* formalin-fixed paraffin-embedded, *COLD-PCR* co-amplification at lower denaturation temperature-polymerase chain reaction

significant impact on glioma diagnosis. Their highly specific distribution in grade II–III diffuse gliomas among CNS tumors also indicates that the presence of *IDH1/2* mutation is almost sufficient for the diagnosis of diffuse glioma, although the absence of these mutations does not exclude the diagnosis of gliomas [28]. Equivocal microscopic diagnosis can be derived from various factors, including small sample size (i.e., obtained by needle biopsy), sampling site (i.e., from infiltrative zone apart from the tumor core), or sample quality. The *IDH1/2* status may provide clinically important information in such cases. *IDH1/2* status therefore needs to be evaluated accurately.

IDH1/2 testing includes various methods targeting DNA sequence, mutant protein, or aberrant increase in (R)-2HG levels. However, *IDH1/2* testing should be easy to be incorporated into daily diagnostic practice. Sanger sequencing and immunohistochemistry (IHC) are conventionally applied for the assessment of *IDH1/2* status. Each assay has advantages and limitations (Table 1). Some of the currently available methods for *IDH1/2* testing are reviewed below.

Sanger sequencing

Sanger sequencing is the gold standard for detecting *IDH1/2* mutations, and most of the published data are based on this method [1–3]. This technique can detect all types of *IDH1/2* mutations and, if the mutation is detected, the result is reliable.

However, this technique requires sophisticated equipment and trained personnel [44]. Complicated procedures including DNA extraction, polymerase chain reaction (PCR), or purification of PCR products need to be optimized. The difference in the equipment or procedure may yield inconsistent results between laboratories [45].

Another caveat is that the results of this method largely depend on the tumor cell content of the samples. The source of non-neoplastic DNA includes adjacent normal brain, infiltrating lymphocytes, and microglia or endothelial cells, which may dilute mutant alleles and cause false-negative results [15]. At least 20 % of the mutant allele is required for detection by Sanger sequencing in our analysis, evaluating the sensitivity of *IDH1/2* detection [46] (Fig. 3a). Diagnosis for small samples obtained from tumor margin is challenging, but clinically important. The critical limitation of this method is its relatively low sensitivity, which might lead to missing out *IDH1/2* mutations.

Immunohistochemistry (IHC)

IHC is universally performed in clinical practice, and the IHC-based mutation detection is one of the most accessible technologies.

The specific antibodies for IDH1 R132H mutation, monoclonal antibody (mAb) H09 and Imab-1, are well characterized and commonly used [47, 48]. The cytoplasm of tumor cells with IDH1 R132H mutation is strongly stained, while tumor cells without this mutation are not stained (Fig. 3b). These antibodies are highly specific to the mutant protein, and residual brain tissues, including reactive glia, endothelial cells, or blood cells are not stained [47–49]. A weak diffuse background staining and a strong granular cytoplasmic staining of macrophages are also observed in IDH1 wild-type tumors. Meningiomas and schwannomas can show nonspecific positive-stained fibers. These patterns can be easily recognized and distinguished from the true-positive staining [49]. Based on these criteria, the specificity for IDH1 R132H mutation is considered to be nearly 100 % [50]. A cross-reactivity for R132L mutant has been reported [44]; however, this would not cause misdiagnosis.

This method is highly accurate. It can detect tumor cells with *IDH1/2* mutation in even tissues containing 6–9 % mutant allele [46, 51]. It has been claimed that the antibody can stain single cells reliably even in the infiltration zone

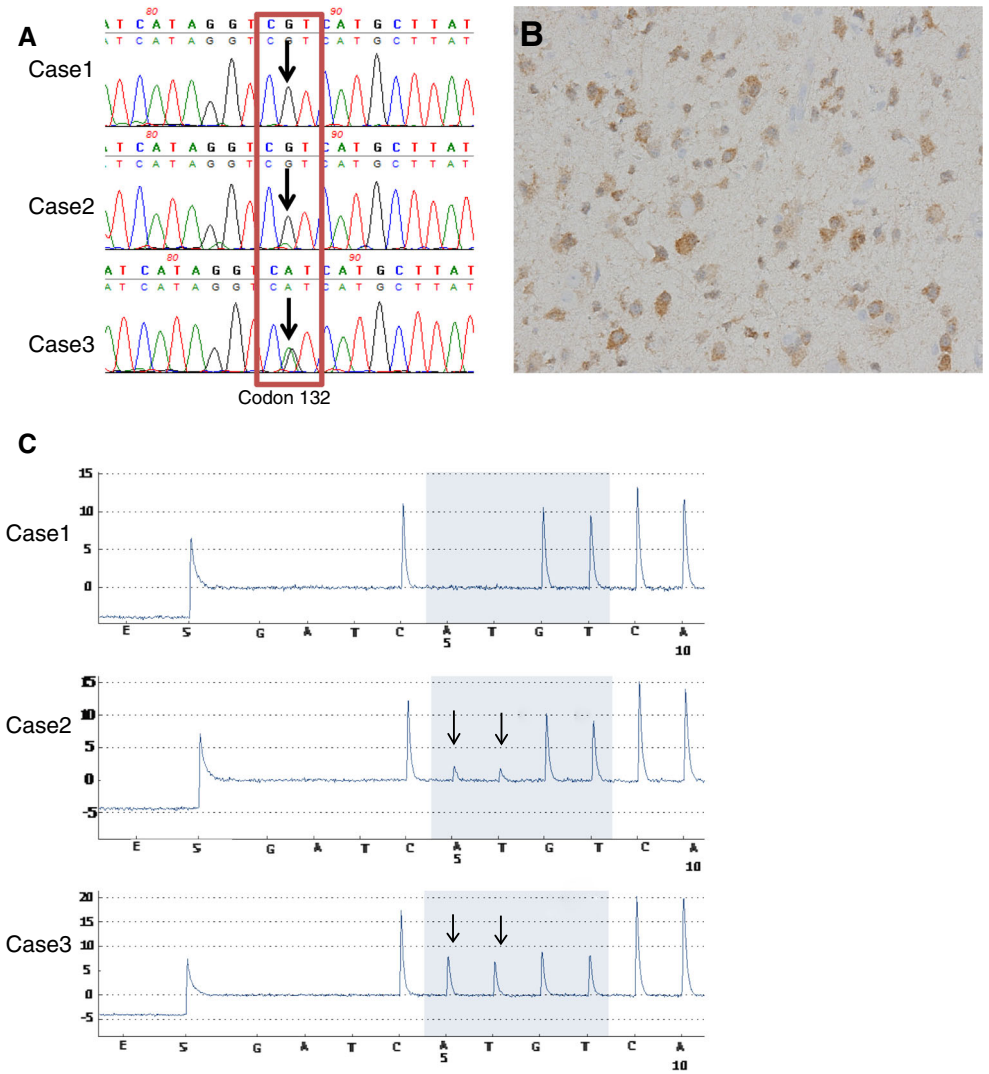

Fig. 3 **a** Sanger sequencing. Chromatograms of Sanger sequencing for *IDH1* in representative cases. The three cases are all anaplastic astrocytoma cases. *Case 1* presents a wild-type *IDH1,* while *cases 2* and *3* present the R132H mutant of *IDH1*. In case 3, a peak of adenine (*green*) indicates the c.395G>A transition. In case 2, a peak indicating the mutation is too low to be distinguished from other nonspecific peaks. **b** Immunohistochemistry. Immunohistochemical staining using anti-*IDH1* R132H mutant antibody. The cytoplasm of tumor cells with IDH R132H is strongly stained, while endothelial cells are not stained. (Original magnification ×200). **c** Pyosequencing. Pyrograms for *IDH1* in representative cases. The *arrows* indicate the specific peaks for the R132H mutants. The cases and analyzed samples are identical to those used for Sanger sequencing. The quantitative analysis of pyrosequencing reported that the frequency of R132H mutant alleles in each case was 0, 16, and 45 %, respectively. In case 2, the R132H mutation is apparent in pyrograms, although the result of Sanger sequencing is inconclusive

and that it enables differentiation of tumor cells from reactive glia [50]. The results of IHC using IDH1 R132H-specific antibodies are consistent across laboratories, even though different staining procedures were used [45].

The obvious limitation is that these antibodies cannot detect non-R132H mutations in IDH1/2 that correspond to approximately 10 % of all the IDH1/2 mutations [50]. Antibodies specific for other IDH1/2 mutations than R132H have also been developed and some of them are commercially available for IHC [52, 53]. A multispecific anti-mutated IDH1/2 antibody recognizing a subset of IDH1 and IDH2 mutations has also been reported [54]. The reactivity of this antibody varies depending on the methodology used, IHC, enzyme-linked immunosorbent assay (ELISA), and/or Western blotting. These approaches, especially the use of the multispecific anti-mutated IDH1/2 antibody, need to be further validated for clinical application.

Pyrosequencing

The principle of this method is a sequencing-by-synthesis analysis based on the real-time detection of nucleotide incorporation by DNA polymerase [55]. In practice, a DNA template amplified by PCR is hybridized to a primer for

pyrosequencing after purification, followed by pyrosequencing reactions using a pyrosequencer. Pyrosequencing reactions consist of four reactions: a DNA polymerase reaction, a sulfyrase reaction, a luciferase reaction, and nucleotide degradation by apyrase. Pyrosequencing reagents include the enzymes and the substrates for these reactions as well as adenosine 5′ phosphosulfate (APS) and luciferin (the four enzyme system) [56]. After adding the substrates and enzymes to the templates, each deoxynucleotide triphosphate (dNTP) is added to the samples stepwise, as programmed by the operator (dispensation order). If the injected dNTP is complementary to the template, the dNTP is incorporated by the DNA polymerase and the pyrophosphate is released. The concentration of ATP is then increased through the conversion of pyrophosphate and APS to ATP by the ATP sulfyrase, followed by the luciferase reaction. The light emitted by the luciferase reaction is quantitatively detected by a charge-coupled device (CCD) camera and represented as a peak at each nucleotide dispensation in the pyrogram. The unincorporated dNTP is rapidly degraded by the apyrase. The signal strength of the pyrosequencing reaction is proportional to the amount of pyrophosphate released and dNTP incorporated; hence the allele dosage. Therefore, the signal strength can be decreased when different sequences are mixed (i.e., heterozygous mutations) and also proportionally increased in a sequence containing homopolymers (a continuous stretch of the same nucleotide such as TT or CCC). The allele frequencies contained in the sample are automatically calculated from the signal strength by using a software developed for pyrosequencing analysis [56].

Several studies have applied pyrosequencing for *IDH1* testing [46, 57–59], some of which have validated the advantages of this technique over Sanger sequencing.

This method is highly sensitive. The minimum detectable frequency of the mutant allele is 5–7 % for pyrosequencing [57, 58], while Sanger sequencing sensitivity is at least 20 % for reliable detection (Fig. 3a, c) [15, 46]. Fragmented DNA template from the formalin-fixed paraffin-embedded (FFPE) specimen can be utilized for analysis [57, 59], as pyrosequencing allows the use of DNA templates under 100 bp [46].

Pyrosequencing requires expensive equipment and is available only in limited centers and laboratories. The robustness of pyrosequencing depends on the assay design, including the primers and the dispensation order [46].

Melting curve analysis

Melting curve analysis is a technique that allows the generation of a melting temperature profile of the double-stranded DNA. The melting temperature is unique to each nucleotide sequence, and even a single nucleotide substitution could alter the melting temperature. Thus, a missense mutation can be accurately detected by measuring the melting curve. Two different variants of this method were reported as an application for *IDH1/2* testing: fluorescent melting curve analysis (FMCA) and high-resolution DNA melting (HRM) [60–62].

In studies using FMCA, the PCR product of the target sequence is hybridized with a pair of fluorescent probes designed to complement the sequence, including mutation hotspot or its adjacent sequence [61, 62]. A real-time PCR system detects the fluorescent change derived by the denaturation of the probes during the gradual heating. In a mutant sequence, lower temperature is required for denaturation, unlike that in a wild-type sequence, because the probe imperfectly binds the mutant sequence. These differences appear in the patterns of the melting curve. A sample with only wild-type alleles shows a single peak in the melting curve, while a sample containing a mutant allele shows an additional peak at lower temperature [62]. This technique is also reported to be highly sensitive and rapid in detecting *IDH1/2* status even in FFPE tissue samples; the entire duration of this assay is about 80 min, and the minimum amount of mutated allele for the detection is 10 % [62].

A fluorescent dye intercalating double-stranded DNA is used in HRM. The amplified template with a saturating fluorescent dye is first denatured by heating, and then annealing is performed at the lower temperature. The fluorescence of the double-stranded DNA reduces by the gradual heating in a melting curve analysis. The samples containing mutant sequences show different melting curves because of the formation of heteroduplexed DNA after the denaturation phase; a lower melting temperature is observed [63]. The detection limit of this assay after conventional PCR amplification is similar to that of Sanger sequencing (25 %) [60].

These methods detect all types of mutations by detecting the melting temperature specific to each mutation [60, 62]. The disadvantage is an unsatisfactory sensitivity, but the sensitivity can be improved by combining co-amplification at lower denaturation temperature PCR (COLD-PCR) (reviewed below). COLD-PCR HRM and FMCA assays allowed the detection of 2 or 1 % mutant allele, respectively [60, 61]. Another disadvantage is that these methods require expensive equipment, which is usually used for another purpose in clinical settings [62].

Co-amplification at lower denaturation temperature PCR (COLD-PCR)

COLD-PCR is a method used to amplify a specific allele with mutation selectively. COLD-PCR itself is not an independent technique for DNA analysis, and this

technique is combined with another method. This method increases the sensitivity to detect a mutant allele contained in a sample [64].

The novelty of COLD-PCR is the use of critical denaturation temperature (Tc), which is lower than the standard denaturation temperature. At the Tc, the mismatched DNA formed by mutant and wild-type sequences is denatured, while the homo-duplex DNA of the mutant or wild-type sequences remains double stranded. Primer annealing and DNA extension by DNA polymerase follow the denaturation at the Tc in this procedure. Since the mismatched DNA formed by the mutant and wild-type sequences is selectively denatured, mutant sequences at low concentration are selectively amplified. This method improves the sensitivity of Sanger sequencing, pyrosequencing, or melting curve analysis [64]. Some studies have reported highly sensitive assays for *IDH1* mutations by combining COLD-PCR with melting curve analysis [60, 61].

Molecular imaging

Another approach to detect *IDH1/2* mutations is molecular imaging. The accumulated (R)-2HG in *IDH1/2* mutated tumors has been considered as a good target for this approach, because this aberrant metabolite is only present at low levels in the normal tissue [65]. Recent studies have shown successful in vivo detection of (R)-2HG using magnetic resonance spectroscopy (MRS) [65–67]. The challenging point of this approach in vivo is that the (R)-2HG spectrum overlaps with that of other metabolites, including glutamate, glutamine, *N*-acetyl-L-aspartate (NAA), or gamma-aminobutyric acid (GABA) [65–67]. These studies have claimed that they overcame this by different MRS sequence optimized in each study (reviewed elsewhere [65]). The signal of (R)-2HG is present in background noises and further improvement will be needed for the robust detection in clinical use. These preliminary reports necessitate further MRS validation and standardization in clinical cases. Nonetheless, this approach potentially poses several advantages over the other *IDH1/2* testing methods. This approach enables noninvasive and quantitative analysis of (R)-2HG, and hence *IDH1/2* status. The concentration of (R)-2HG possibly reflects the tumor status, including cellularity [66]. Identification of (R)-2HG hotspots might also provide information that may help the planning of targeted biopsy [65]. Moreover, the most significant value of this method is the availability for repetitive and temporal measurement during the clinical course. Temporal testing using MRS might enable the dynamic monitoring of the effects of therapeutic agents, including inhibitors of mutant IDH1/2 in the future [68].

Which method should we use for *IDH1/2* testing?

Several studies have compared the sensitivity and robustness of each method. In general, IHC using mutation-specific antibodies, pyrosequencing, or melting curve analysis has a higher sensitivity than Sanger sequencing [46, 49, 57, 62]. The high sensitivity, as reported in each method, is however not always required for all situations, if sufficient tissue specimen is adequately obtained. The optimal method for *IDH1/2* testing varies depending on various factors, including the purpose, sample types (FFPE or frozen tissue), sample number (high throughput analysis is required or not), or laboratory equipment [46].

IHC is sensitive, robust, and accessible. Therefore, this technique is recommended for initial screening [15]. If *IDH1/2* status is critical for a clinical decision, another method should be used in cases showing negative results in IHC, to avoid missing non-R132H mutations. The high throughput techniques, including pyrosequencing and melting curve analysis, are suitable for large-scale studies in which DNA samples are already prepared [46]. Sanger sequencing provides accurate information, if specimens with sufficient tumor cell content are obtained.

Conclusion

As reviewed in this report, *IDH1/2* mutations have distinct relationships with clinical features, pathology, or other genetic/epigenetic alterations. *IDH1/2* status is currently among the most important molecular markers of gliomas. The significance of *IDH1/2* status argues for further improvement and standardization of *IDH1/2* testing methods for practical use. The development of an accurate and robust method for molecular markers is a prerequisite for the establishment of molecular classification of gliomas in the future.

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