(Table 2). Plx-inv CD204%^{high} was independently associated with peritoneal dissemination (HR, 2.886; $P < 0.001$) and locoregional recurrence (HR, 2.483; $P < 0.001$) (Table 2 and Fig. 2c and d).

3.4. Prognostic analyses stratified by presence of adjuvant chemotherapy

Adjuvant chemotherapy represented an independent prognostic factor for OS and DFS as a definitive therapeutic modality (Table 1, Fig. 3a and b). Multivariate analyses to test prognostic factors with adjuvant chemotherapy were re-examined and revealed that only plx-inv CD204%^{high} was associated with both shorter OS (HR, 2.624; $P = 0.011$) and shorter DFS (HR, 2.257; $P = 0.038$) in patients with plx-inv who underwent postoperative adjuvant chemotherapy (Table 3).

4. Discussion

The present study demonstrated that the accumulation of CD204-positive cells, representing M2 macrophages, at plx-inv of pancreatic IDC was an independent predictor of shorter OS and DFS in patients who underwent curative pancreaticoduodenectomy for pancreatic IDC. The prognostic impact of plx-inv CD204%^{high} was maintained in patients who received adjuvant chemotherapy. Infiltration of M2 macrophages at plx-inv of pancreatic IDC was revealed as a key factor to explain the aggressiveness of pancreatic IDC for the first time in this study.

Peritoneal dissemination has long been considered a poor prognostic factor for patients with pancreatic IDC [27–29]. Patients with plx-inv CD204%^{high} showed early relapse to the peritoneal cavity in this study. The interaction between M2 macrophages and tumour cells at plx-inv was suggested to play a crucial role in peritoneal recurrence, which led to poor survival. From the perspective of surgical anatomy, nerve fibres of the plexus pancreaticus capitalis might provide a convenient pathway for infiltrating tumour cells. As recent experimental study showed that macrophages around nerves were recruited in response to cytokine secreted by invading tumour cells and increased migration of tumour cells [15], M2 macrophages might promote the invasiveness of tumour cells at plx-inv, leading tumour cells to disperse into the peritoneal space and resulting in peritoneal dissemination. This speculation warrants further studies to observe the distribution of M2 macrophages in metastatic sites of pancreatic IDC and to test the role of M2 macrophages in metastatic tumour models.

Immunophysiologically, neural injury leads to the accumulation of macrophages in the peripheral nerve system, although few macrophages exist in intact nerves [30]. Ceyhan et al. reported that neuritis was caused by the invasion of malignant tumour cells into the pancreas

Table 2
Multivariate analysis for early relapse according to the sites of recurrence in patients with invasive ductal carcinoma of the pancreas ($n = 170$).

Parameter	Liver metastasis ($n = 71$)				Peritoneal dissemination ($n = 57$)				Locoregional recurrence ($n = 76$)				Distant lymph node metastasis (n = 46)			
	n	%	HR	95% CI	*P*	*n*	%	HR	95% CI	*P*	*n*	%	HR	95% CI	*P*	*n*	%	HR	95% CI	*P*
Absence of adjuvant chemotherapy	50	70.4	1.924	1.065–3.476	0.030*	34	59.6	1.107	0.602–2.036	0.743	52	68.4	1.734	0.995–3.022	0.052	32	69.6	1.660	0.794–3.471	0.178
CEA ⩾ 3.4 ng/ml	40	56.3	1.347	0.819–2.215	0.241	23	40.4	0.934	0.531–1.640	0.811	36	47.4	1.238	0.773–1.985	0.374	26	56.5	1.516	0.810–2.839	0.193
Tumour size ⩾ 3.0 cm	38	53.5	1.492	0.925–2.405	0.101	22	38.6	0.968	0.558–1.681	0.909	34	44.7	1.114	0.698–1.780	0.650	23	50.0	1.327	0.732–2.407	0.351
Ly, moderate to severe	28	39.4	2.634	1.574–4.408	<0.001*	14	24.6	0.839	0.436–1.617	0.601	21	27.6	0.878	0.501–1.539	0.649	17	37.0	1.909	0.993–3.671	0.053
V, moderate to severe	46	64.8	1.146	0.660–1.988	0.629	30	52.6	1.126	0.618–2.052	0.698*	47	61.8	1.323	0.774–2.261	0.306	29	63.0	1.133	0.560–2.292	0.729
Peripheral CD204%^{high}	37	52.1	1.517	0.931–2.472	0.095	30	52.6	1.815	1.055–3.124	0.031*	36	47.4	1.501	0.933–2.417	0.094	21	45.7	1.305	0.706–2.412	0.396
Plx-inv CD204%^{high}	18	25.4	0.916	0.518–1.619	0.763	24	42.1	2.886	1.615–5.159	<0.001*	28	36.8	2.483	1.485–4.151	<0.001*	15	32.6	1.564	0.795–3.073	0.195

* $P < 0.05$. Multivariate analysis was carried out using Cox regression hazard model. HR, hazard ratio; 95% CI, 95% confidence interval; CEA, carcinoembryonic antigen; Ly, lymphatic invasion; V, vessel invasion; Peripheral CD204%^{high}, percentage of CD204-positive cells area at the periphery ⩾ 3.34; Plx-inv CD204%^{high}, percentage of CD204-positive cells area at extrapancreatic nerve plexus invasion ⩾ 0.57.

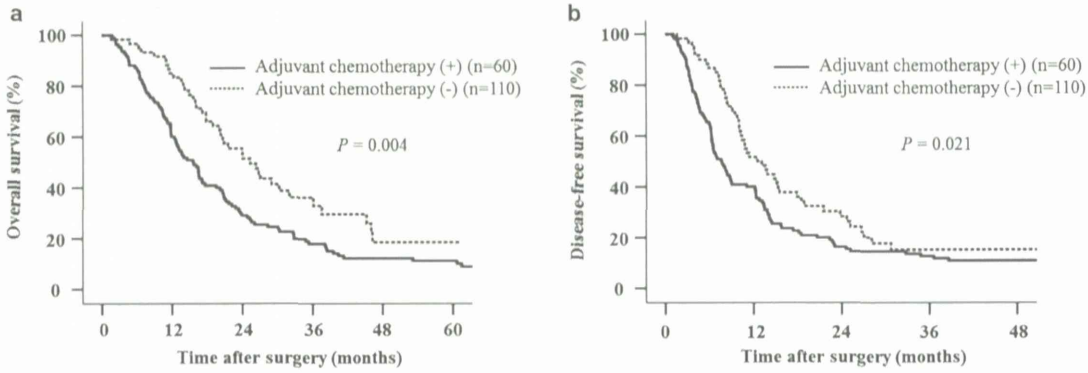

Fig. 3. (a) Kaplan–Meier curve for overall survival stratified by the presence of adjuvant chemotherapy. (b) Kaplan–Meier curve for disease-free survival stratified by the presence of adjuvant chemotherapy.

Table 3
Multivariate analysis for overall survival and disease-free survival in patients who received adjuvant chemotherapy ($n = 60$).

Parameter	n	%	Overall survival			Disease-free survival		
			HR	95% CI	P	HR	95% CI	P
CEA $\geqslant$ 3.4 ng/ml	23	38.3	1.514	0.704–3.257	0.289	1.883	0.969–3.658	0.062
Tumour size $\geqslant$ 3.0 cm	26	43.3	1.283	0.663–2.484	0.460	1.179	0.641–2.170	0.596
Ly, moderate to severe	21	35.0	1.775	0.833–3.782	0.137	1.475	0.667–3.263	0.337
V, moderate to severe	19	31.7	2.178	1.059–4.479	0.034*	1.476	0.769–2.833	0.242
Peripheral CD204%^{high}	32	53.3	1.206	0.601–2.420	0.598	0.890	0.472–1.679	0.719
Plx-inv CD204%^{high}	20	33.3	2.624	1.242–5.544	0.011*	2.257	1.045–4.879	0.038*

* $P < 0.05$. Multivariate analysis was carried out using Cox regression model. HR, hazard ratio; 95% CI, 95% confidence interval; CEA, carcinoembryonic antigen; Ly, lymphatic invasion; V, vessel invasion; Peripheral CD204%^{high}, percentage of CD204-positive cells area at the periphery $\geqslant$ 3.34; Plx-inv CD204%^{high}, percentage of CD204-positive cells area at extrapancreatic nerve plexus invasion $\geqslant$ 0.57.

[31]. In our previous experimental study [12], neural invasion over a long distance could lead to severe neural damage. Additionally, the present study showed strong positive correlations among ne, plx-inv, long plx-inv distance and plx-inv CD204%^{high}. Taken together with the paracrine regulation between macrophages and tumour cells at plx-inv [14,15], severe neural invasion of tumour cells appears to recruit M2 macrophages due to neural damage. Moreover, the neural system was suggested as an expedient structure for interaction between tumour cells and M2 macrophages that promotes pancreatic cancer cell proliferation.

Adjuvant chemotherapy after complete resection of pancreatic IDC has been established as the definitive standard of care within the last decade [24,32,33]. In the present study, plx-inv CD204%^{high} was the only independent prognostic factor for poor OS and DFS in the group of patients with adjuvant chemotherapy. According to recent reports, immunoregulatory cytokines such as interleukin-6 and prostaglandin E2, which are present in the tumour microenvironment, are associated with chemoresistance and tumour-induced differentiation of tumour-promoting M2 macrophages [34,35]. Additional therapy to suppress M2 macrophages might thus prove effective, particularly against cases with plx-inv and high accumulation of M2 macrophages. Depletion of macrophages by zoledronic acid has been reported to enhance the effects of sorafenib in an *in vivo* model of metastatic liver cancer [36]. A phase II randomised controlled study of tasquinimod (oral quinolone-3-carboxamide) for metastatic castrate-resistant prostate cancer patients prolonged progression-free survival and confirmed the pharmacological efficacy of this agent for inhibiting S100A9 [37], which is a protein expressed in inflammatory cells that induces the maturation of macrophages [38]. Therefore, anti-M2 macrophage therapy may have potential as an innovative treatment for pancreatic IDC.

Limitations of this study include the retrospective manner of the investigation. Adjuvant chemotherapy was performed in 60 patients and was an independent factor predictive of OS and DFS, but the indication was influenced by time trends, and some degree of selection bias might have been present. Although OS and DFS for our patient cohort were comparable with the other previous studies [24,32,33], further investigation in patients with standardised adjuvant chemotherapy is needed. Moreover, since only resectable pancreatic cancer was studied, it is unknown whether the results can be extrapolated to the much higher numbers of unresectable cases.

In conclusion, pancreatic cancer patients with high accumulation of CD204-positive cells at plx-inv who underwent curative resection showed a high incidence

of recurrence in the form of peritoneal dissemination and locoregional recurrence and shorter OS and DFS. The impact of CD204-positive cells at plx-inv on OS and DFS was maintained in the setting of adjuvant chemotherapy. Increased infiltration of M2 macrophages at plx-inv may represent an important finding for detecting patients with aggressive IDC of the pancreas.

Conflict of interest statement

None declared.

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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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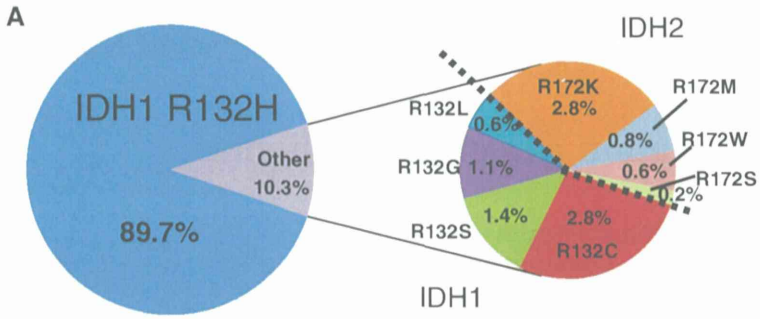

B

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

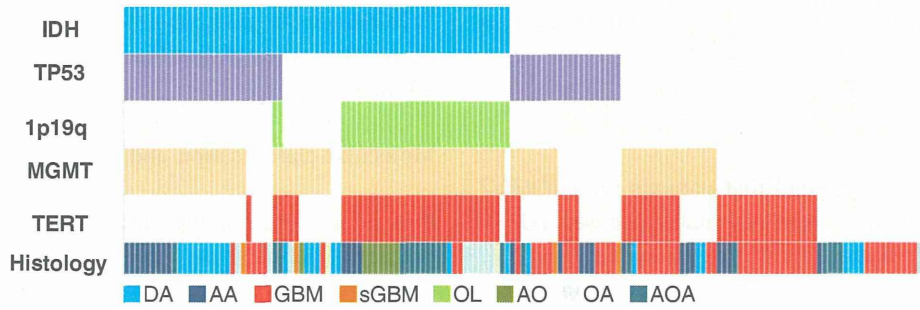

Fig. 2 Relationships among *IDH1/2* mutations, other genetic alterations, and histological subtypes. *Each column* represents individual tumors. *IDH1/2* mutations are strongly associated with *TP53* mutation or total 1p19q loss. Most *IDH1/2* mutated tumors show *MGMT* methylation. The data for the 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

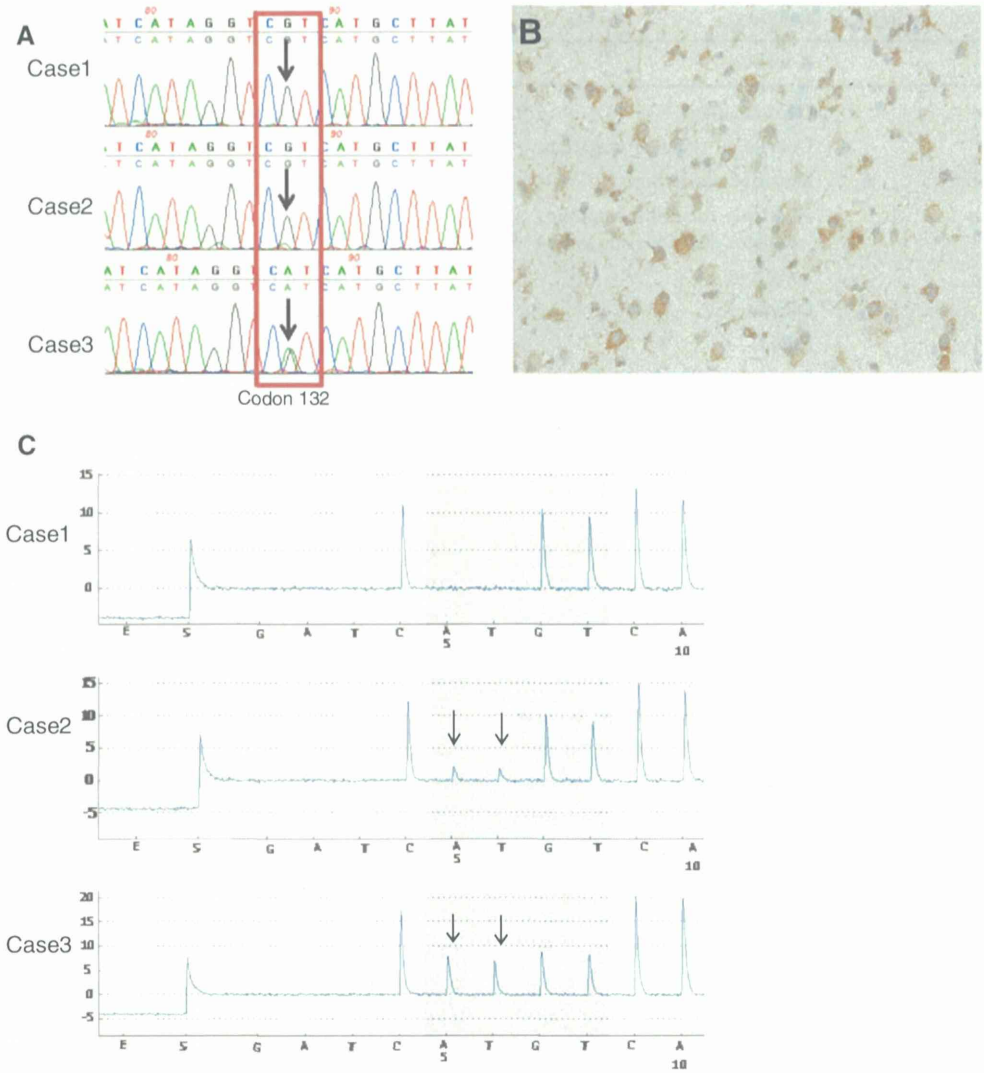

Fig. 3 **a** Sanger sequencing. Chromatograms of Sanger sequencing for *IDH1* in representative cases. The three cases are all anaplastic astrocytoma cases. *Case 1* presents a wild-type *IDH1*, while *cases 2* and *3* present the R132H mutant of *IDH1*. In case 3, a peak of adenine (*green*) indicates 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