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ORIGINAL ARTICLE

Detection of IDH1 mutation in human gliomas: comparison of immunohistochemistry and sequencing

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Abstract Isocitrate dehydrogenase 1 (IDH1) mutations have recently been identified as early and frequent genetic alterations in astrocytomas, oligodendrogliomas, and oligoastrocytomas, as well as secondary glioblastomas, whereas primary glioblastomas very rarely contain IDH1 mutations. Furthermore, a specific monoclonal antibody, IMab-1, which recognizes IDH1-R132H—the most frequent IDH1 mutation—has been generated. IMab-1 has been reported to react with the IDH1-R132H protein, but not the wild-type IDH1 or the other IDH1 mutant proteins in Western-blot analysis. However, the importance of immunohistochemistry using IMab-1 has not yet been elucidated. In this study, we compared the findings from IMab-1 immunohistochemistry and direct DNA sequencing using 49 glioma samples. IMab-1 detected 12 out of 49 cases; however, only nine cases were found to be IDH1-R132H by direct DNA sequencing because of a small population of IDH1-R132H mutation-possessing tumor cells, indicating that IMab-1 immunohistochemistry is useful for detecting IDH1-R132H. We conducted immunohistochemical detection in 52 cases of grade III astrocytomas. The median time to progression (TTP) was significantly longer in the cases with the IDH1 mutation (86.7 months) compared to the cases without the IDH1 mutation (wild type, 10.4 months) ($p < 0.01$). In conclusion, the anti-IDH1-R132H-specific monoclonal antibody IMab-1 is very useful for detecting IDH1-R132H in immunohistochemistry, and predicting the time to progression in grade III anaplastic astrocytomas. Therefore, IMab-1 is likely to be useful for the diagnosis of mutation-bearing gliomas and for determining the treatment strategy of grade III gliomas.

Keywords IDH1 · Mutation · Immunohistochemistry · Monoclonal antibody · Glioma

Introduction

IDH1 (isocitrate dehydrogenase 1) mutations have been identified as early and frequent genetic alterations in astrocytomas, oligodendrogliomas, and oligoastrocytomas, as well as secondary glioblastomas [1–3]. In contrast, primary glioblastomas, as well as other systemic cancers, very rarely contain IDH1 mutations. The IDH1 mutations are remarkably specific to a single codon in the conserved and functionally important Arg132 residue in IDH1 [4].

A monoclonal antibody, IMab-1, which is specific for the IDH1-R132H has recently been developed [5]. IMab-1 reacted with the IDH1-R132H peptide, but not with the wild-type IDH1 (IDH-wt) peptide in ELISA. In Western-blot analysis, IMab-1 reacted only with the IDH1-R132H protein, not the IDH1-wt protein or the other IDH1

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mutants. IMab-1 could be useful for the diagnosis and biological evaluation of mutation-bearing gliomas.

In this study, the usefulness of IMab-1 in the immunohistochemical analysis of a large number of human glioma samples was investigated in comparison with the direct DNA sequencing method. Furthermore, we demonstrated the clinical significance of IMab-1 as a prognostic factor in grade III glioma.

Materials and methods

Patients

Eighty-four consecutive patients who underwent primary surgery between 1994 and April 2010 at Tsukuba University Hospital were included in this study. The mean patient age at the time of the primary surgery was 47.8 ± 16.7 years (range 2–83). Pathological grading was performed according to the WHO classification. The tumors comprised 26 grade IV (22 glioblastomas, 3 glioblastomas with oligodendroglioma components, and 1 primitive neuroectodermal tumor), 52 grade III (24 anaplastic astrocytomas, and 9 anaplastic oligodendrogliomas and 19 anaplastic oligoastrocytomas), 4 grade II (4 diffuse astrocytomas) and 2 grade I (2 pilocytic astrocytomas). The samples from glioblastomas include 25 primary tumors and one secondary tumor. Primary glioblastomas develop very rapidly after a short clinical history, without clinical or histological evidence of a pre-existing, less malignant precursor lesion. Secondary glioblastoma was categorized as WHO grade IV on the basis of histologic criteria but had developed slowly through progression from low-grade diffuse astrocytoma (WHO grade II) or anaplastic astrocytoma (WHO grade III) [6].

All cases underwent operation and achieved maximal resection. Postoperative therapies were uniform depending upon the histological findings. For grade IV and grade III tumors, the patients received 54–60 Gy radiation therapy followed by ACNU and temozolomide based chemotherapy. For grade II, the patients underwent no further treatment after surgery except for re-resection and radiation therapy after recurrence.

Informed consent was obtained from each patient or the patient's caretaker to obtain samples and perform the subsequent data analysis.

Sample preparation

The sample was removed during the surgery and the most viable part of the tumor that was devoid of macroscopically evident necrosis was taken as the specimen. The specimen was divided into two. One was fixed in 10% formalin and the other was frozen for subsequent analysis.

Immunohistochemical analysis

IDH1-R132H protein expression was determined immunohistochemically in paraffin-embedded tumor specimens, as described previously. Briefly, histologic sections, 5 μm in thickness, were deparaffinized in xylene, rehydrated, and heated at 100°C in citrate buffer (pH 6.0) for 5 min. Sections were incubated with the monoclonal IMab-1 antibody (kindly donated by Dr. Darell D. Bigner, Duke University Medical Center) that specifically recognizes IDH1-R132H, the most common glioma-derived mutation [5], overnight at 4°C at a concentration of 5 μg/ml. The DAKO LSAB kit was used for the post-primary antibody blocker and secondary antibody. Color was developed using 3,3-diaminobenzidine tetrahydrochloride (DAB) for 10 min, and counterstained with hematoxylin.

The expression of IDH1-R132H was determined by semiquantitatively assessing the proportion of positively-stained tumor cells. The percentage of positive cells was rated as follows: cases with ≥10% cells were rated as positive, and cases with <10% cells were rated as negative, although there is no previous reference for this definition. In our study, IMab-1 stained diffusely without heterogeneity in grade III and IV tumors, as shown in Figs. 1 and 2. In a positive case, almost 90% of the tumor cells were positive, whereas they are almost completely negative in IMab-1-negative case, and there were no cases with positivities of a few percent. However, in some grade II tumors, IMab-1-positive percentages could be underestimated due to low tumor cell density (Fig. 3a, b). Capper et al. [7] stated that immunoreactivity was scored positive when tumor cells showed strong cytoplasmic staining, even if only one positive cell was detected. However, we defined cases with >10% cells as positive for accuracy.

Direct DNA sequencing of IDH1 mutations and subcloning

Genomic DNA was extracted from formalin-fixed, paraffin-embedded tissue sections using MyghtyAmp for FFPE (Takara) according to the manufacturer's instructions. PCR primers for the genomic region corresponding to IDH1 exon 4, which encodes codon R132, and the flanking intronic sequences were as follows: human IDH1 sense (5′-AATGAGCTCTATATGCCATCACTG-3′) and human IDH1 antisense (5′-TTCATACCTTGCTTAATGGGTG T-3′). The PCR conditions were 95°C for 15 min (1 cycle), followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 30 s, and extension at 72°C for 10 min with HotStarTaq polymerase (Qiagen). Cycle sequencing was

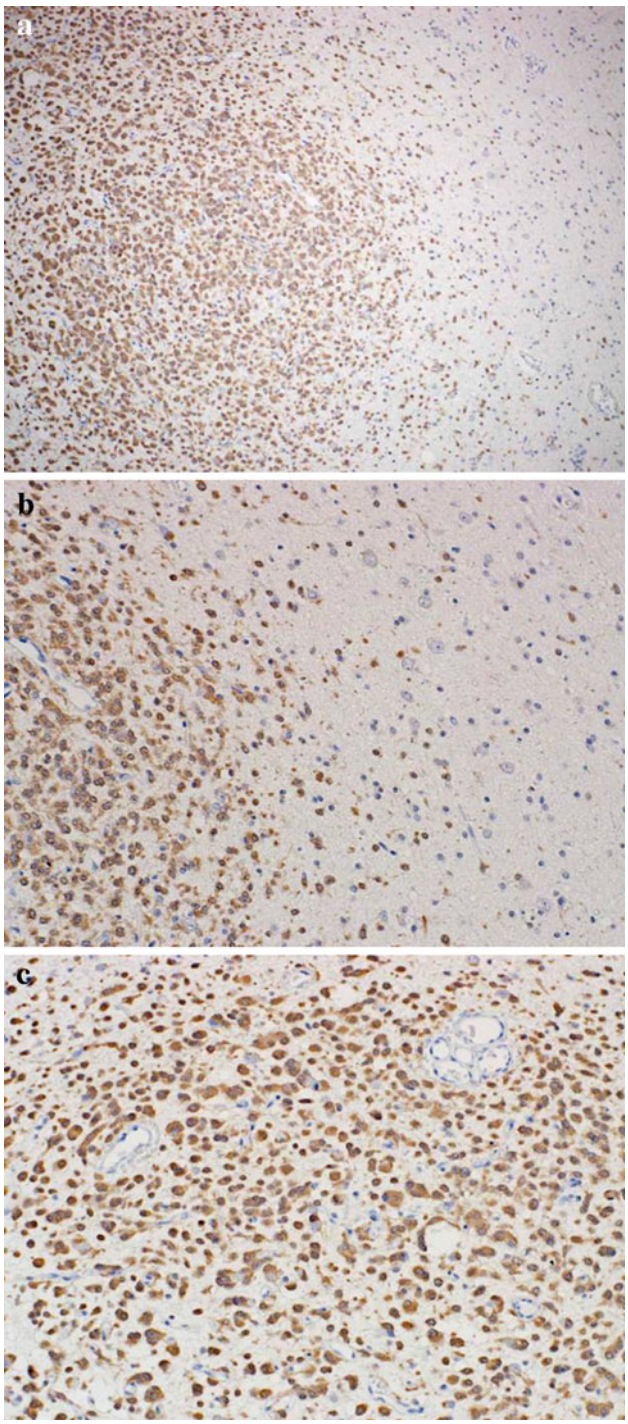

Fig. 1 IDH1 immunohistochemistry. Anaplastic astrocytoma. **a** Tumor periphery at low magnification (original magnification ×50), **b** tumor periphery at high magnification (original magnification ×100), **c** tumor center (original magnification ×100). Please note that IDH1 is strongly positive for tumor cell cytoplasm but negative for adjacent normal glial cells

carried out using the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with the sequencing primer (5′-CCATTATCTG CAAAAATATC-3′). Subsequently, the PCR products were subcloned into pcDNA3.1/V5-His TOPO vectors (Invitrogen), and 48 clones were sequenced to confirm the R132 mutation.

MGMT immunohistochemistry

Immunohistochemistry was carried out according to the streptavidin–biotin peroxidase (LSAB) method (DAKO LSAB2 system, DAKO, Carpinteria, CA, USA). The mouse monoclonal antibody (MGMT Ab-1; clone MT 3.1, Neomarker, Westinghouse, Fremont, CA, USA) was diluted 1:20. Sections were counterstained with hematoxylin. MGMT scoring was accompanied by the determination of the percentage of positive nuclei from regions of maximal nuclear staining after counting 1,000 tumor cells at 400× magnification. Cells were counted as MGMT positive if diffuse nuclear staining was present. Endothelial cells and perivascular lymphocytes were excluded from the positive cell count. The cutoff value for MGMT positive cells was 20% [8–10].

Statistics

The effects of IDH1 mutation on clinical outcome were assessed by plotting survival curves using the Kaplan–Meier method and the log-rank test for comparison between groups. The Cox proportional hazards model was used to test prognostic factors in univariate and multivariate analysis. Results are expressed with relative risk and its 95% confidence interval (CI). Time to progression was defined from the day of surgery until disease progression. Overall survival was defined from the day of surgery until the death of the patient. Data regarding patients who survived until the end of the observation period were censored at their last follow-up visit. Differences were considered statistically significant when the p values were ≤ 0.05.

Results

Immunolocalization of IDH-1-R132H detected by IMab-1 in human gliomas

Representative cases of anaplastic astrocytoma are shown in Fig. 1. The cytoplasm of almost all tumors was strongly stained with IMab-1 antibody, whereas adjacent brain tissue was not (Fig. 1). IMab-1 antibody clearly stained for every grade of glioma, grade II diffuse astrocytoma (Fig. 2a), grade III anaplastic astrocytoma (Fig. 2b), grade III anaplastic oligoastrocytoma (Fig. 2c), and grade IV secondary glioblastoma (Fig. 2d). By contrast, IMab-1 antibody did not stain any grade IV primary glioblastomas (Fig. 2e). A

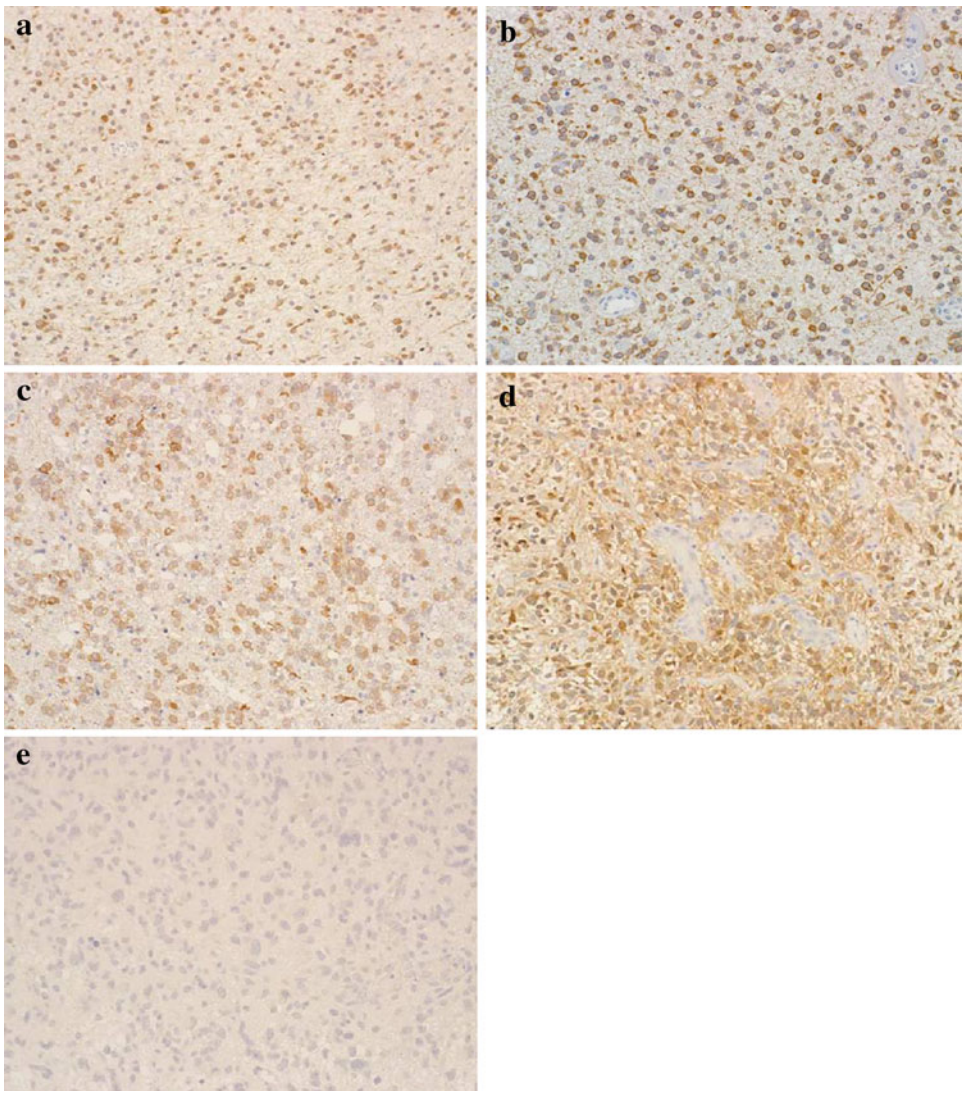

Fig. 2 IDH1 immunohistochemistry. **a** Diffuse astrocytoma, **b** anaplastic astrocytoma, **c** anaplastic oligoastrocytoma, **d** secondary glioblastoma, **e** primary glioblastoma. Original magnification ×100

summary of the immunohistochemical detection of IDH1 mutation (R132H) in gliomas is shown in Table 1.

Comparison between immunohistochemistry and direct DNA sequencing

In 49 cases, the IDH1 mutation status was determined by both IMab-1 immunohistochemistry and direct DNA sequencing. As shown in Table 2, three cases (two grade II astrocytomas and one grade III anaplastic astrocytoma) that were determined to be wild type by direct DNA sequencing were immunohistochemically positive for the IDH1-R132H mutation using the IMab-1 antibody (Fig. 3). For those three cases, the first PCR products were subsequently subcloned, and the IDH1 sequence was checked against 44–64 subclones per case. Consequently, all three cases included the IDH1-R132H mutant sequence (9.4–16%), indicating that not all IDH1 mutations were detected by routine direct DNA sequencing. These results show that the IMab-1 antibody is more sensitive than direct DNA sequencing, and was able to identify the IDH1-R132H mutation. Among the 49 cases, all wild-type cases and only one IDH1 mutation (R132S) were not detectable using the IMab-1 antibody, indicating that IMab-1 is specific for the IDH1-R132H mutation. A previous report has also shown that IMab-1 is specific against the IDH1-R132H mutation, and does not react with the other IDH1 mutations, such as R132C, R132L, R132S, and R132G, in Western-blot analysis [5]. The IMab-1 antibody appears to be superior to routine direct DNA sequencing in the detection of IDH1-R132H mutation.

Clinical significance of IMab-1 immunohistochemistry for grade III gliomas

Based on the superior detection ability of the IMab-1 antibody, we performed immunostaining with the IMab-1 antibody in a large number of grade III gliomas. Fifty-two cases of grade III gliomas (24 anaplastic astrocytomas and 28

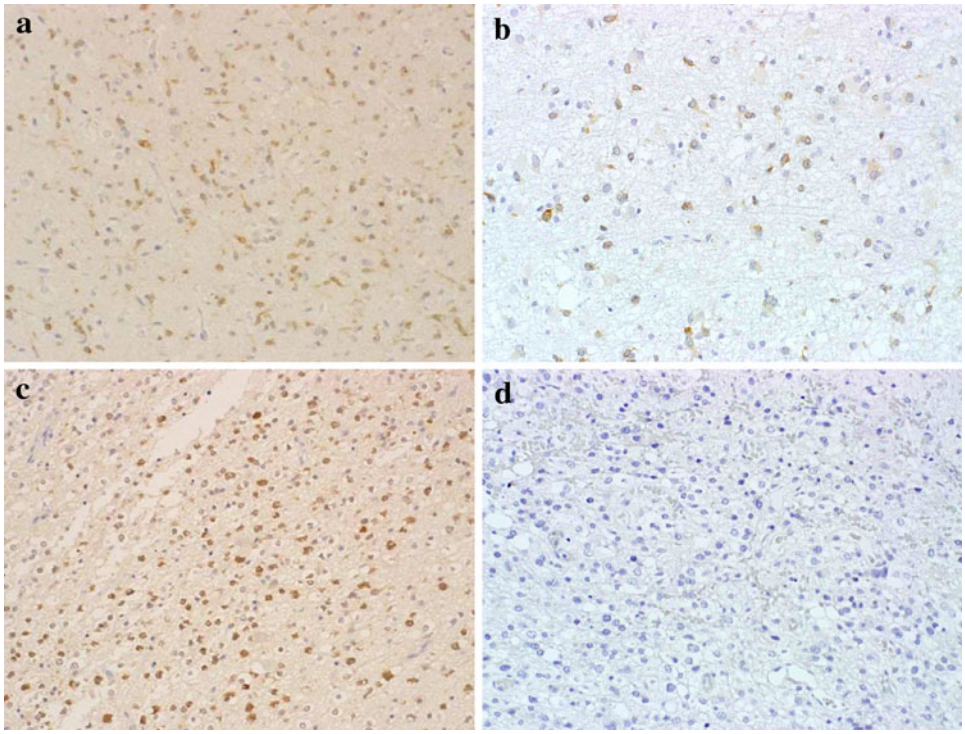

Fig. 3 Four cases of wild-type IDH1 with routine sequencing for R132. **a** Oligodendroglioma, **b** diffuse astrocytoma, **c** anaplastic astrocytoma that was IDH1 positive with immunohistochemistry, **d** anaplastic oligoastrocytoma that was immunohistochemically negative for IDH1 with R132S mutation. Original magnification ×100

Table 1 Summary of immunohistochemical detection of IDH1 mutation (R132H) in gliomas

WHO grade	Pathology	Immunohistochemistry		Percent (%)
Grade IV	GBM	Positive	2	7.7
n 26		Negative	24	
Grade III	AA	Positive	26	50.0
n 52	AOA	Negative	26	
	AO			
Grade II	DA	Positive	4	100
n 4		Negative	0	
Grade I	Pilocytic	Positive	0	0
n 2		Negative	2	

GBM glioblastoma, *AA* anaplastic astrocytoma, *AOA* anaplastic oligoastrocytoma, *AO* anaplastic oligodendroglioma, *DA* diffuse astrocytoma, *Pilocytic* pilocytic astrocytoma

anaplastic oligoastrocytomas/anaplastic oligodendrogliomas) were immunostained and evaluated for clinical parameters, time to progression (TTP) and overall survival. Univariate analysis showed that the significant prognostic factors were preoperative Karnofsky Performance Scale (KPS) score, frontal lobe involvement and IDH1 mutation for TTP, and preoperative KPS for OS (Table 3). Kaplan–Meier curves with and without IDH1 mutation in grade III astrocytomas are shown in Fig. 4. In grade III astrocytomas, the median TTP was significantly longer in the cases with the IDH1 mutation (86.7 months) compared to those without the IDH1 mutation (wild type, 10.4 months) ($p < 0.01$). TTP and OS were not significant under other settings (anaplastic oligoastrocytoma/anaplastic oligodendroglioma: Fig. 5; and all grade III gliomas: Fig. 6) between the IDH1 mutant and wild types. A Cox proportional hazards model in multivariate analysis revealed that the IDH1 mutation, age <50 years and preoperative KPS >80 were independent factors for longer TTP and preoperative KPS for OS (Table 4).

Because the presence of IDH1 mutations was correlated with MGMT promoter methylation in anaplastic oligodendroglial tumors [11], and MGMT low immunoreactivity has been associated with better progression-free survival in patients with glioblastoma [10], we also evaluated MGMT immunoreactivity in this study. Fifty-one of the 52 cases of grade III gliomas were available for this analysis. Sixteen cases were MGMT positive and 35 cases MGMT negative. Among the 26 IDH1 mutant cases, MGMT was positive in 6 cases and negative in 20 cases. Among 25 IDH1 wild-type cases, MGMT was positive in 10 cases and negative in 15 cases. There was no significant correlation between IDH1 mutation and MGMT immunoreactivity in all grade III gliomas. Also, MGMT immunoreactivity was not associated with progression and overall survival in patients with anaplastic astrocytoma (Tables 3, 4) and grade III gliomas (data not shown).

Discussion

We demonstrated the usefulness of the immunohistochemical detection of IDH1-R132H compared to routine direct

Table 2 Summary of IDH status with immunohistochemistry and direct sequencing in gliomas

WHO grade	Pathology	IHC		Sequence		Match (%)	Subcloning		Match (%)
Grade IV	GBM	Positive	2	R132H	2	100			100
n 26		Negative	24	Wild type	24	100			100
Grade III	AA	Positive	6	R132H	5	83.3			100
n 17	AO			Wild type	1		Mutant	1	
	AOA	Negative	11	R132S	1	90.9			100
				Wild type	10				
Grade II	DA	Positive	4	R132H	2	50			100
n 4				Wild type	2		Mutant	2	
		Negative	0						
Grade I	Pilocytic	Positive	0						
n 2		Negative	2	R132H	0	100			100
				Wild type	2				

Table 3 Predictors of time to progression and overall survival in the patients with anaplastic astrocytoma

Variables	*n*	Time to progression		Overall survival	
		Median (mo)	*p* value	Median (mo)	*p* value
Age (years)					
<50	13	38.3		71.9	
>50	11	46.9	0.674	77.7	0.527
Sex					
Male	11	24.3		73.5	
Female	13	48.8	0.552	75.0	0.533
Preoperative KPS					
>80	8	38.6		56.2	
<80	16	34.6	0.046	62.0	0.019
Total resection					
Yes	5	60.0		85.3	
No	19	38.7	0.308	75.1	0.914
Frontal involvement					
Yes	15	52.0		83.4	
No	9	22.2	0.020	39.6	0.806
IDH1 mutation					
Yes	15	52.1		86.7	
No	9	10.4	0.009	23.2	0.449
MGMT expression					
<20%	18	42.1		77.7	
>20%	6	16.0	0.854	25.5	0.718

KPS Karnofsky performance status

DNA sequencing in glioma specimens. We confirmed the IDH1-R132H mutation after subcloning in three cases with the wild type using routine direct DNA sequencing. By contrast, immunohistochemical detection with the

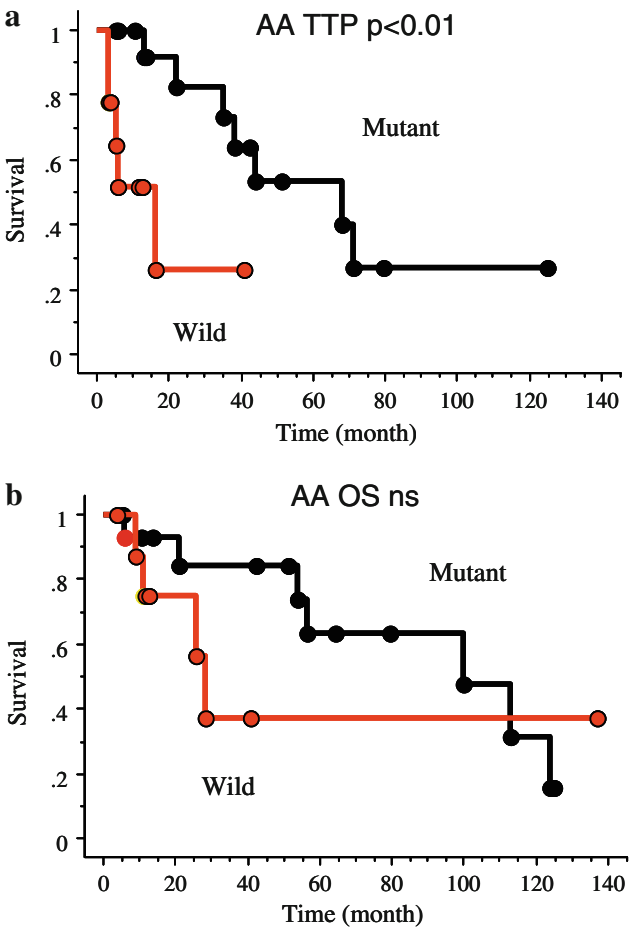

Fig. 4 Kaplan–Meier curve with and without IDH1 mutation in anaplastic astrocytomas. **a** Time to progression, **b** overall survival. TTP is significantly ($p < 0.01$) longer in anaplastic astrocytomas with IDH1 mutation than in those without IDH1 mutation

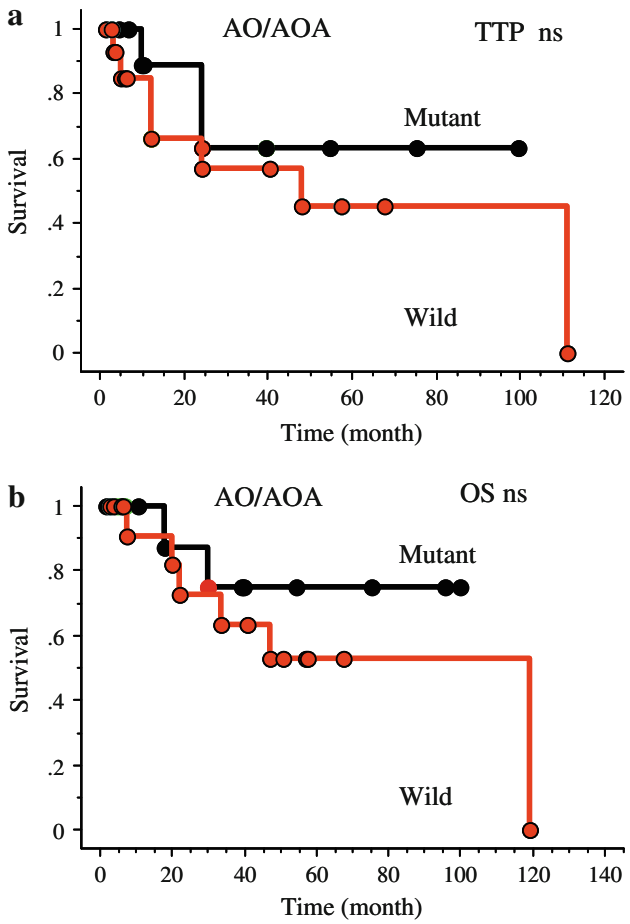

Fig. 5 Kaplan–Meier curve with and without IDH1 mutation in anaplastic oligodendroglioma and anaplastic oligoastrocytomas. **a** Time to progression, **b** overall survival

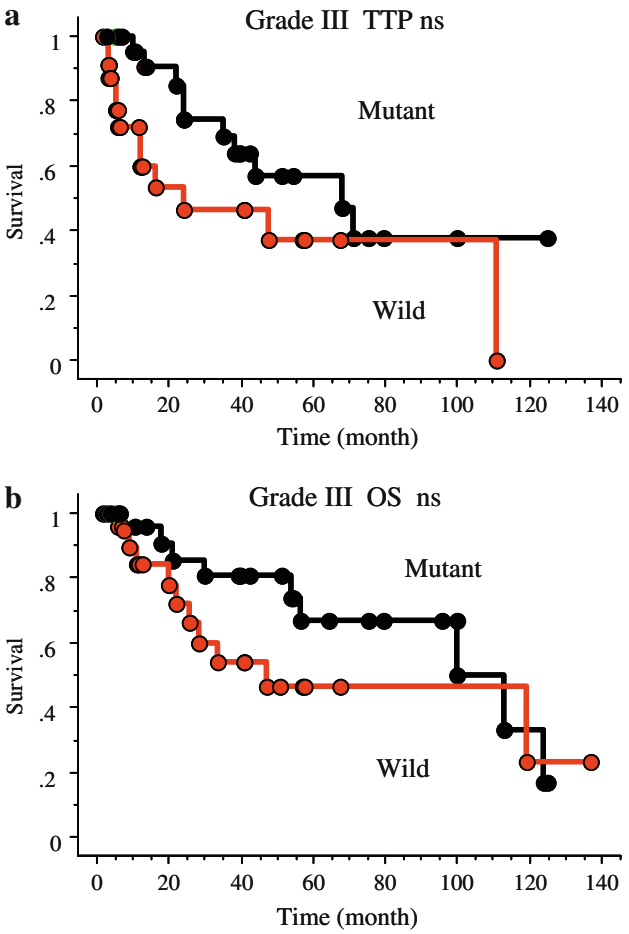

Fig. 6 Kaplan–Meier curve with and without IDH1 mutation in all grade III gliomas. **a** Time to progression, **b** overall survival

IDH1-R132H-specific monoclonal antibody, IMab-1, was positive for these three cases. The reason for this discrepancy might be that direct DNA sequencing of highly contaminated tumor samples could not detect the IDH1-R132H mutation, or that the tumor cell contents in three samples were very small. To solve these problems, we should try several approaches: (1) tumor cell-rich areas should be histopathologically identified and used for DNA sequencing by microdissection; (2) double COLD PCR combined with high-resolution melting could be applied [12], resulting in ~100-fold more sensitivity than Sanger sequencing; (3) frozen specimens could be used for mutation analysis. Furthermore, IMab-1 can only detect IDH1-R132H mutation, whereas there are other IDH mutations, including IDH1-R132C, IDH1-R132L, IDH1-R132S, IDH1-R132G, IDH2-R172K, IDH2-R172M, and IDH2-R172G [3]. Therefore, novel antibodies that recognize the other IDH1 mutants or IDH2 mutants should be developed to cover all IDH mutations in immunohistochemistry.

Three distinct forms of isocitrate dehydrogenase (IDH1, IDH2, and IDH3) have been identified. In contrast to other IDHs in the mitochondria, IDH1 is present in the cytosol, and its biochemical role is not yet fully understood [2]. IDH1 mutations were found to result in the ability of the enzyme to catalyze the reduced NADP-dependent reduction of α-ketoglutarate to *R*(−)-2-hydroxyglutarate (2-HG) [13]. Reduction of α-ketoglutarate by 2-HG or mutant IDH1 results in a lower level of prolyl hydroxylases and promotes the accumulation of hypoxia-inducible factor (HIF) 1α. HIF-1α levels were greater in human gliomas harboring an IDH1 mutation than in tumors without the mutation. Thus, IDH1 appears to function as a tumor suppressor that, when mutationally inactivated, contributes to tumorigenesis in part through induction of the HIF-1 pathway. HIF-1α expression is also a prognostic factor in glioma patients [14]. The link between IDH1 and HIF-1α highlights an emerging theme in which mutationally altered metabolic enzymes are thought to contribute to tumor growth by stimulating the HIF-1α pathway and tumor angiogenesis [15].

Table 4 Multivariate analysis of factors associated with survival

Variable	Time to progression		Overall survival	
	Hazard ratio (95% CI)	*p* value	Hazard ratio (95% CI)	*p* value
IDH1 (mutation vs. wild type)	0.005 (0.000–0.141)	0.002	0.163 (0.022–1.234)	0.079
MGMT (<20 vs. ≥20%)	22.158 (0.760–646.126)	0.072	3.535 (0.209–59.924)	0.382
Age (<50 vs. ≥50 years)	15.068 (1.580–143.695)	0.018	1.804 (0.390–8.347)	0.45
Sex (female vs. male)	0.210 (0.029–1.518)	0.122	1.398 (0.296–6.606)	0.672
Frontal involvement (yes vs. no)	0.129 (0.014–1.234)	0.076	0.878 (0.164–4.706)	0.88
KPS (80–100 vs. 10–70)	0.042 (0.004–0.496)	0.012	0.067 (0.006–0.721)	0.026
Total resection (yes vs. no)	0.644 (0.036–11.667)	0.766	1.273 (0.167–9.682)	0.816

Furthermore, IMab-1 immunohistochemistry showed clinical significance as a prognostic factor in grade III anaplastic astrocytomas. The patients with IMab-1 immunoreactive anaplastic astrocytomas had significantly longer progression-free survival than those who were IMab-1 negative. Clinically, the IDH1 mutation strongly correlated with good prognosis in patients with gliomas. Median overall survival in GBM patients with IDH mutation was significantly longer than that in GBM patients with wild-type IDH1 and IDH2 [3]. Mutations of IDH1 have also been associated with improved prognosis in patients with anaplastic astrocytomas [1, 3]. Multivariate analysis has confirmed that IDH1 mutations are independent favorable prognostic markers in GBMs and anaplastic gliomas after adjustment for other genomic profiles and treatment modalities [4]. A more recent report with anaplastic oligodendroglial tumors showed the presence of IDH1 mutations correlated with 1p/19q codeletion and MGMT promoter methylation. IDH1 mutations and 1p/19q codeletion but not MGMT promoter methylation were independent prognostic factors [11]. These reports were based on the finding of IDH1 mutation by direct DNA sequencing analysis; therefore, our study is noteworthy since it highlights the utility of focusing on the immunohistochemical detection of IDH1 mutation as a prognostic factor in grade III gliomas.

In our study of anaplastic oligodendroglioma (*n* 9) and anaplastic oligoastrocytoma (*n* 19), there was no statistical difference in TTP and OS between mutant-type and wild-type IDH1 tumors. Median TTP was 47.7 months for patients with wild-type IDH1 tumors, and this was not reached by patients with mutated IDH1 tumors. Also, the median OS was 119 months for patients with wild-type IDH1 tumors, and this was not reached by patients with mutated IDH1 tumors. There seems to be a tendency for better survival benefits in terms of both TTP and OS with mutated IDH1 tumors. There could be three reasons why a statistical difference was not obvious in our study. First, the follow-up period for patients was relatively short in the AO and AOA group (average: 36 months) compared to the anaplastic astrocytoma group (average: 48 months). Second, patient survival data in our study, even for wild-type IDH1 tumors, was much better than in a previous report [11] (see the table in the Electronic supplementary material). In AOA and AO with wild-type IDH1, the median TTP was 7.8 and 47.7 months in the previous report and our study, respectively. Also, in AOA and AO with wild-type IDH1, the median OS was 14.2 and 119 months in the previous report and our study, respectively. Third, in our study, the number of cases (*n* 28) is still small compared to the previous report (*n* 76). Longer follow-up periods and more cases are needed to draw further conclusions.

Conclusions

An anti-IDH1-R132H-specific monoclonal antibody, IMab-1, is useful for detecting IDH1-R132H in immunohistochemistry, and predicted time to progression in grade III anaplastic astrocytomas. Therefore, IMab-1 should be useful for the diagnosis of mutation-bearing gliomas and in determining the treatment strategy for grade III gliomas.

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