

# Immunohistochemical detection of IDH1 mutation, p53, and internexin as prognostic factors of glial tumors

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**Abstract** Isocitrate dehydrogenase 1 (IDH1) mutations, which are early and frequent genetic alterations in astrocytomas, oligodendrogliomas, oligoastrocytomas, and secondary glioblastomas, are specific to arginine 132 (R132). Recently, we established monoclonal antibodies (mAbs) against IDH1 mutations: anti-IDH1-R132H and anti-IDH1-R132S. However, the importance of immunohistochemistry using the combination of those mAbs has not been elucidated. For this study, 164 cases of glioma were evaluated immunohistochemically for IDH1 mutations (R132H and R132S) using anti-IDH1 mAbs (HMab-1 and SMab-1). IDH1 mutation was detected, respectively, in 9.7%, 63.6%, 51.7%, and 77.8% of primary grade IV, secondary grade IV, grade III, and grade II gliomas. For each grade of glioma, prognostic factors for progression-free survival and overall survival were evaluated using clinical and pathological parameters in addition to IDH1 immunohistochemistry. IDH1 mutation, p53 overexpression, and internexin expression, as evaluated using immunohistochemistry with clinical parameters such as degree

of surgical removal and preoperative Karnofsky Performance Status (KPS), might be of greater prognostic significance than histological grading alone in grade III as well as IDH1 mutation in grade IV gliomas.

**Keywords** IDH1 · p53 · Internexin · Mutation · Immunohistochemistry · Monoclonal antibody · Glioma

## Introduction

Isocitrate dehydrogenase 1 (IDH1) and analogous IDH2 mutations, which were identified as early and frequent genetic alterations (IDH1: 50–93%; IDH2: 3–5%) in astrocytomas, oligodendrogliomas, and oligoastrocytomas, as well as in secondary glioblastomas, might be the initiating events in these glioma subtypes [1–3]. In contrast, primary glioblastomas and other systemic cancers rarely contain IDH1 mutations. The IDH mutations are remarkably specific to a single codon in the conserved and functionally important arginine 132 residue (R132) in IDH1 and R172 in IDH2. The IDH1 mutations were found to give the enzyme the ability to catalyze the reduced nicotinamide adenine dinucleotide phosphate (NADP)-dependent reduction of  $\alpha$ -ketoglutarate to *R*(-)-2-hydroxyglutarate (2-HG) [4]. Results of the initial study demonstrated that reduction of  $\alpha$ -ketoglutarate by 2-HG or mutant IDH results in a lower level of prolyl hydroxylases and promotes accumulation of hypoxia-inducible factor (HIF)-1 $\alpha$  [5]. Although HIF-1 $\alpha$  is upregulated in a subset of gliomas in vivo, activation of the HIF-1 $\alpha$  pathway is not regulated primarily by IDH1 mutation in vitro [6] or in vivo [7]. Progression of IDH1 mutant glioma might be related to alternative mechanisms such as excess accumulation of 2-HG [4].

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To date, three monoclonal antibodies against IDH1 mutations have been reported [8–11]. In this study, we newly established an anti-IDH1-R132H-specific monoclonal antibody, HMab-1, which is expected to be extremely useful in immunohistochemistry. This study was conducted to evaluate the prognostic relevance of this IDH1 mutation assessed using two antibodies (HMab-1 and SMab-1) associated with other immunohistochemically detectable factors such as p53, internexin (INA) [12–15], and O6-methylguanine-DNA methyltransferase (MGMT) expression in a large consecutive series of low-grade and high-grade gliomas.

## Patients and methods

### Patients

One hundred sixty-four consecutive patients who underwent primary surgery at Tsukuba University Hospital (grade II between 1994 and 2004, grade III between 1994 and 2010, and grade IV between 2008 and 2010) were included in this study. Mean patient age at time of primary surgery was  $48.6 \pm 14.3$  years (range 18–83 years). Pathological grading was performed according to the World Health Organization (WHO) classification. The tumors comprised 52 grade IV (41 primary glioblastomas and 11 secondary glioblastomas), 66 grade III (32 anaplastic astrocytomas, 10 anaplastic oligodendrogliomas, and 24 anaplastic oligoastrocytomas), and 46 grade II (42 diffuse astrocytomas and 4 oligodendrogliomas). The pathological review was diagnosed in our institution by three pathologists and two neurosurgeons as a routine study. Pathologically difficult cases were sent to Brain Tumor Reference Center (Dr. Yoichi Nakazato; Neuropathological Section of Gunma University) to decide a final diagnosis. Secondary glioblastomas were categorized as WHO grade IV on the basis of histologic criteria, but had been categorized as WHO grade II or III at least 1 year earlier. For patients with secondary glioblastomas, survival was calculated from date of secondary diagnosis [1]. All cases underwent operation and achieved maximal resection without new permanent neurological deficits. Extent of surgery assessment was based on postoperative magnetic resonance imaging (MRI) within 72 h after surgery. The tumoral lesion (T1 gadolinium-enhanced lesion for grade IV and T1 low-intensity lesion for grade II and III) was totally removed on postoperative MRI. 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 carer for obtaining samples and subsequent data analysis.

### Sample preparation

The sample was removed during 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.

### Hybridoma production

BALB/c mice (CLEA Japan Inc., Tokyo, Japan) were immunized by intraperitoneal (i.p.) injection of 100  $\mu$ g synthetic peptide CKPIIIGHHAYGD (IDH1-R132H peptide; Operon Biotechnologies, K.K., Tokyo, Japan), corresponding to amino acids 126–137 of human IDH1-R132H plus N-terminus cysteine conjugated with KLH together with Imject Alum (Thermo Scientific Inc., Rockford, IL). One week later, secondary i.p. immunization of 30  $\mu$ g IDH1-R132H peptide was performed. After several additional immunizations of 30  $\mu$ g IDH1-R132H peptide, a booster injection was given i.p. 2 days before spleen cells were harvested. The spleen cells were fused with mouse myeloma P3U1 cells (American Type Culture Collection, Manassas, VA) using Sendai virus (hemagglutinating virus of Japan, HVJ) envelope: GenomONE-CF (Ishihara Sangyo Kaisha, Ltd., Osaka, Japan) according to the manufacturer's instructions. The hybridomas were grown in Roswell Park Memorial Institute (RPMI) medium with hypoxanthine, aminopterin, and thymidine selection medium supplement (Invitrogen Corp.). The culture supernatants were screened using enzyme-linked immunosorbent assay (ELISA) for binding to the IDH1-R132H peptide and the IDH1 wild type (IDH1-WT).

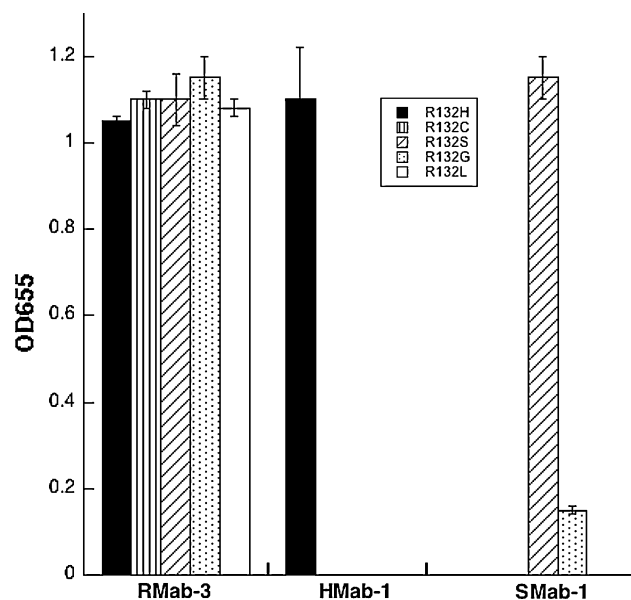
### Enzyme-linked immunosorbent assay (ELISA)

Synthetic peptides corresponding to amino acids 126–137 of the human IDH1: IDH1-WT (KPIIIGRHAYGD), IDH1-R132H (KPIIIGHHAYGD), IDH1-R132C (KPIIIGCHAYGD), IDH1-R132S (KPIIIGSHAYGD), IDH1-R132G (KPIIIGGHAYGD), and IDH1-R132L (KPIIIGLHAYGD) were immobilized, respectively, on Maxisorp 96-well immunoplates (Thermo Fisher Scientific Inc., Waltham, MA) at 1  $\mu$ g/ml for 30 min. After blocking with SuperBlock T20 (PBS) blocking buffer (Thermo Fisher Scientific Inc.), the plates were incubated with 1  $\mu$ g/ml of primary antibodies, followed by 1:1,000 diluted peroxidase-conjugated anti-mouse IgG (Dako, Glostrup, Denmark). The enzymatic reaction was conducted with a substrate solution containing 1-Step Ultra TMB-ELISA (Thermo Fisher

Scientific Inc.). The optical density was measured at 655 nm with a Benchmark microplate reader (Bio-Rad Laboratories Inc., Philadelphia, PA). These reactions were performed with a volume of 50  $\mu$ l at 37°C.

#### Immunohistochemical analysis of IDH1

IDH1-R132H, IDH1-R132S, and IDH1 wild type (WT) protein expression was determined immunohistochemically in paraffin-embedded tumor specimens, as described previously [11, 16]. Anti-IDH1-R132H (HMab-1), anti-IDH1-R132S (SMab-1), and anti-IDH1 (RMab-3), which were established in this study, are now commercially available from Medical & Biological Laboratories Co., Ltd. (MBL; Nagoya, Japan). Expression of IDH1 was determined by semiquantitatively assessing the proportion of positively stained tumor cells. We defined cases with  $\geq 10\%$  cells as positive, and cases with  $< 10\%$  cells were rated as negative, although there is no previous reference for the definition. In our study, anti-IDH1 antibodies stained diffusely without heterogeneity in grade III and IV tumors as shown in Fig. 1. In positive cases almost 90% of tumor cells were positive, whereas cases negative for anti-IDH1 antibodies were almost completely negative, and no cases with a few percent positivity were found. However, in some grade II tumors, positive percentages for anti-IDH1 antibodies could be underestimated due to low tumor cell density. Cases with  $\geq 10\%$  cells were rated as positive.



**Fig. 1** Production of a specific monoclonal antibody against IDH1-R132H and IDH1 wild type. Synthetic peptides corresponding to amino acids 126–137 of the human IDH wild type and IDH1 mutants were immobilized on 96-well plates. After blocking, the plates were incubated with anti-IDH1 antibodies, followed by peroxidase-conjugated anti-mouse IgG. The enzymatic reaction was conducted with a substrate solution containing TMB

Immunohistochemical analyses of MGMT, MIB-1, p53, VEGF, and von Willebrand factor

Immunohistochemistry was carried out according to the streptavidin–biotin–peroxidase method (Dako LSAB2 system). A mouse monoclonal antibody, MGMT Ab-1 (clone MT3.1; Neomarker, Westinghouse, CA) at dilution of 1:20, a monoclonal MIB-1 antibody (Immunotech) at dilution of 1:100, a monoclonal anti-human von Willebrand factor (vWF) antibody (Dako) at dilution of 1:50, a polyclonal anti-vascular endothelial growth factor (VEGF) antibody (A20; Santa Cruz) at dilution of 1:100, and a monoclonal anti-p53 antibody (clone DO7; Dako) at dilution of 1:50 were used as primary antibodies. Nuclei positive for MGMT, MIB-1, and p53 were determined by counting at least 1,000 tumor cells in a homogeneously stained area. The percentage of positive cells was rated as follows: cases with  $\geq 10\%$  cells were rated as positive, and cases with  $< 10\%$  cells were rated as negative for both MGMT [17, 18] and p53 [19, 20]. We decided the threshold of p53 overexpression as  $> 10\%$  [19, 20]. VEGF was defined as positive with  $> 10\%$  of tumor cytoplasmic staining. The number of vessels in a 200 $\times$  field (1.0 mm<sup>2</sup>) was measured in microvessel “hot spots” (i.e., microscopic areas containing the densest collections of microvessels, as initially identified under low-power magnification) under an Olympus microscope (AHTB3; Olympus, Tokyo, Japan) on tissue sections stained for vWF. Vascular density was defined by averaging the number of vessels in the three most vascularized areas.

#### Immunohistochemical analysis of internexin neuronal intermediate filament protein alpha (INA)

INA immunohistochemistry was carried out on 5- $\mu$ m paraffin sections of formalin-fixed tumor samples according to the streptavidin–biotin–peroxidase method (Dako LSAB2 system) using an antibody that targeted INA (clone 2E3MOI; Novus Biologicals, Interchim, Montlucon, France; 1:100 dilution). Positive INA results were observed either as fibrillar, crescent-shaped or more paranuclear, dot-like intracytoplasmic inclusions. Positively stained neural cells were used as internal positive control, especially for negative slides. Labeling was defined as strong ( $> 10\%$  positive cells), weak ( $< 10\%$  positive cells), or negative (no positive tumor cells detected) [12].

#### Statistical analysis

Overall survival (OS) and progression-free survival (PFS) were calculated from time of surgery until death, disease progression, or last follow-up examination according to the Kaplan–Meier method with 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).

## Results

### Production of a novel IDH1-R132H-specific antibody

For this study, we immunized mice with synthetic peptides of IDH1-R132H mutant. After cell fusion using the Sendai virus envelope, the wells of hybridomas, which produced IDH1-R132H-specific antibodies, were selected in ELISA. After limiting dilution, one clone was established: HMab-1 (IgG<sub>1</sub> subclass). We also established RMab-3 (IgG<sub>1</sub> subclass), which reacts with both IDH1-R132H and IDH1 wild type (IDH1-WT). To determine the specificity of HMab-1 monoclonal antibody, the reactivities against IDH1-WT and the IDH1 mutant (R132H, R132C, R132S, R132G, R132L) peptides were investigated using ELISA. Results showed that HMab-1 reacted with IDH1-R132H peptide but not with IDH1-WT or other IDH1 mutant (R132C, R132S, R132G, R132L) peptides, whereas RMab-3 reacted with both IDH1-WT and all IDH1 mutant peptides (Fig. 1), indicating that HMab-1 is a specific antibody against IDH1-R132H peptide and that RMab-3 recognizes the common epitope of IDH1. In addition, SMab-1 showed weak cross-reaction to R132G (Fig. 1).

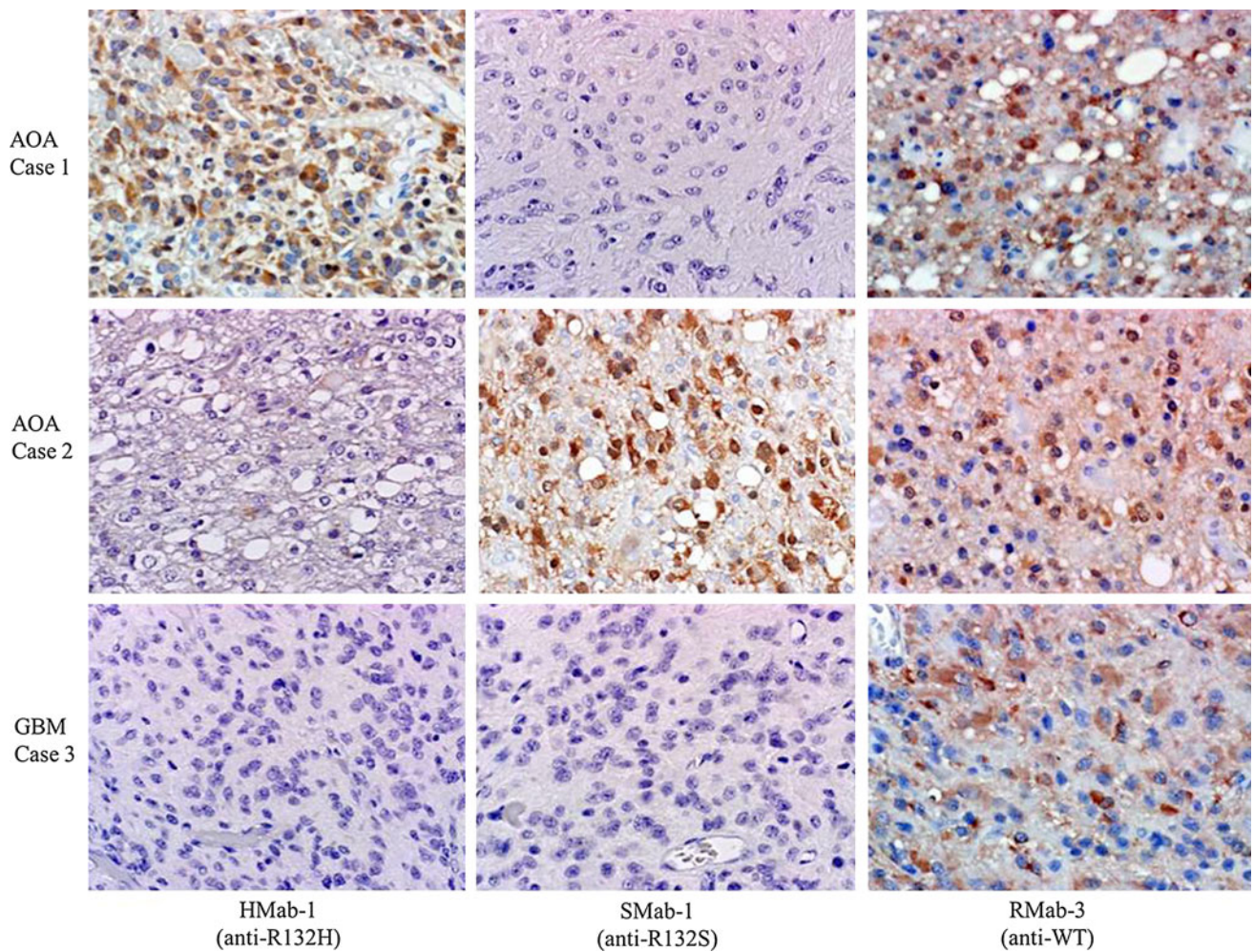
### IDH1 immunohistochemical analysis

HMab-1 and SMab-1 were confirmed as anti-IDH1-R132H-specific and anti-IDH1-R132S-specific antibodies in ELISA, respectively. Therefore, we next performed immunohistochemistry of HMab-1 and SMab-1 against IDH1-R132H-positive or IDH1-R132S-positive gliomas, whose mutations were determined by direct sequencing [11]. Typical results are presented in Fig. 2. HMab-1 stained almost all tumor cells of IDH1-R132H-positive glioma (AOA: anaplastic oligoastrocytoma, case 1), although no staining was observed in IDH1-R132S-positive glioma (AOA, case 2) or IDH1-WT glioma (GBM: glioblastoma, case 3). In fact, HMab-1 stained no endothelial cells (data not shown). Furthermore, SMab-1 stained no tumor cells in IDH1-R132H-positive gliomas (AOA, case 1), although SMab-1 stained IDH1-R132S-positive gliomas (AOA, case 2). RMab-3, which recognizes common epitopes of IDH1, reacted with all glioma types (cases 1, 2, and 3), although the RMab-3 reactivity is apparently heterogeneous. These results indicate that HMab-1 and SMab-1 are useful in respective immunohistochemical analyses for detection of IDH1-R132H and

IDH1-R132S mutations. A summary of the immunohistochemical detection of IDH1 mutations (R132H and R132S) in gliomas is presented in Table 1. IDH1 mutation was detected, respectively, in 9.7%, 63.6%, 57.1%, 46.6%, 75.0%, and 100% of primary grade IV, secondary grade IV, grade III anaplastic astrocytoma, grade III anaplastic oligodendroglioma/anaplastic oligoastrocytoma, grade II diffuse astrocytoma, and grade II oligodendroglioma. The frequency of IDH1-R132H was 42.5% (62/146), and that of IDH1-R132S was 4.8% (7/146).

### Clinical significance of HMab-1 and SMab-1 immunohistochemical analyses for gliomas

A summary of other immunohistochemical detections of p53, MGMT, and INA in the respective grades is presented in Table 1. In patients with 66 grade III gliomas, clinical parameters such as age, sex, preoperative KPS, tumor location, degree of tumor removal, postoperative chemotherapy, and other pathological parameters such as INA expression, MGMT expression, MIB-1 positivity, vWF-stained vessel number, and p53 expression were evaluated to determine progression-free and overall survival in addition to IDH1 mutation. Results of univariate analyses indicated that the significant prognostic factors were IDH1 mutation positivity, MIB-1 <20%, INA positivity, and p53 <10% for PFS, and IDH1 mutation positivity, INA positivity, preoperative KPS  $\geq$ 80, and MIB-1 <20% for OS (Table 2). Results of multivariate analyses showed that the independent significant prognostic factors were IDH1 mutation positivity, total removal, and p53 <10% for PFS, and IDH1 mutation positivity, total removal, MIB-1 <20%, preoperative KPS  $\geq$ 80, and p53 <10% for OS. Kaplan–Meier curves with and without these prognostic factors in grade III gliomas are portrayed in Fig. 3. PFS was significantly longer in cases with IDH1 mutation positivity (80 months) than in those with IDH1 mutation negativity (23 months) ( $p = 0.001$ ) (Fig. 3a), for total removal (84 months) versus without total removal (38 months) (Fig. 3b), for p53 <10% (80 months) versus p53  $\geq$ 10% (24 months) (Fig. 3c), and for INA positivity (68 months) versus INA negativity (14 months) (Fig. 3d). In addition, the figure shows that OS was significantly longer in cases with IDH1 mutation positivity (119 months) than in those with IDH1 mutation negativity (33 months) (Fig. 3g), for preoperative KPS >80 (117 months) versus KPS <80 (26 months) (Fig. 3h), for p53 <10% (117 months) versus p53  $\geq$ 10% (56 months) (Fig. 3i), and for INA positivity (117 months) versus INA negativity (33 months) (Fig. 3j). Taking these facts together, IDH1 mutation positivity and p53 <10% were shown to be the most striking prognostic factors with subsequent INA expression.



**Fig. 2** Immunohistochemical analyses by anti-IDH1 antibodies against glioma tissues. Glioma tissues having IDH1-R132H (AOA: anaplastic oligoastrocytoma, case 1), IDH1-R132S (AOA, case 2), and wild type (GBM: glioblastoma, case 3) were stained with HMab-1 (anti-IDH1-R132H), SMab-1 (anti-IDH1-R132S), and RMab-3 (anti-IDH1-WT). Magnification  $\times 200$

**Table 1** Summary of immunohistochemical analysis of each glioma grade

		n	IDBI			p53	MGMT	INA
			R132H	R132S	Positive	Positive	Positive	Positive
Grade IV	Primary	n = 41	3	1	4/41 (9.7%)	14/35 (40.0%)	7/31 (22.5%)	9/41 (21.9%)
	Secondary	n = 11	6	1	7/11 (63.6%)	5/10 (50.0%)	1/4 (25.0%)	10/11 (90.9%)
Grade III	AA	n = 32	16	0	16/28 (57.1%)	12/32 (37.5%)	10/28 (35.7%)	17/30 (56.7%)
	AO/AOA	n = 34	11	3	14/30 (46.6%)	13/33 (39.4%)	14/31 (45.1%)	23/32 (71.9%)
Grade II	DA	n = 42	22	2	24/32 (75.0%)	11/37 (29.9%)	ND	22/33 (66.7%)
	Oligo	n = 4	4	0	4/4 (100%)	0/1 (0%)	ND	4/4 (100%)

In 52 patients with grade IV gliomas, clinical parameters and other pathological parameters that were used in grade III gliomas were evaluated to determine progression-free and overall survival in addition to IDH1 mutation. Univariate analysis showed that IDH1 mutation positivity had a tendency as a prognostic factor for PFS and was a

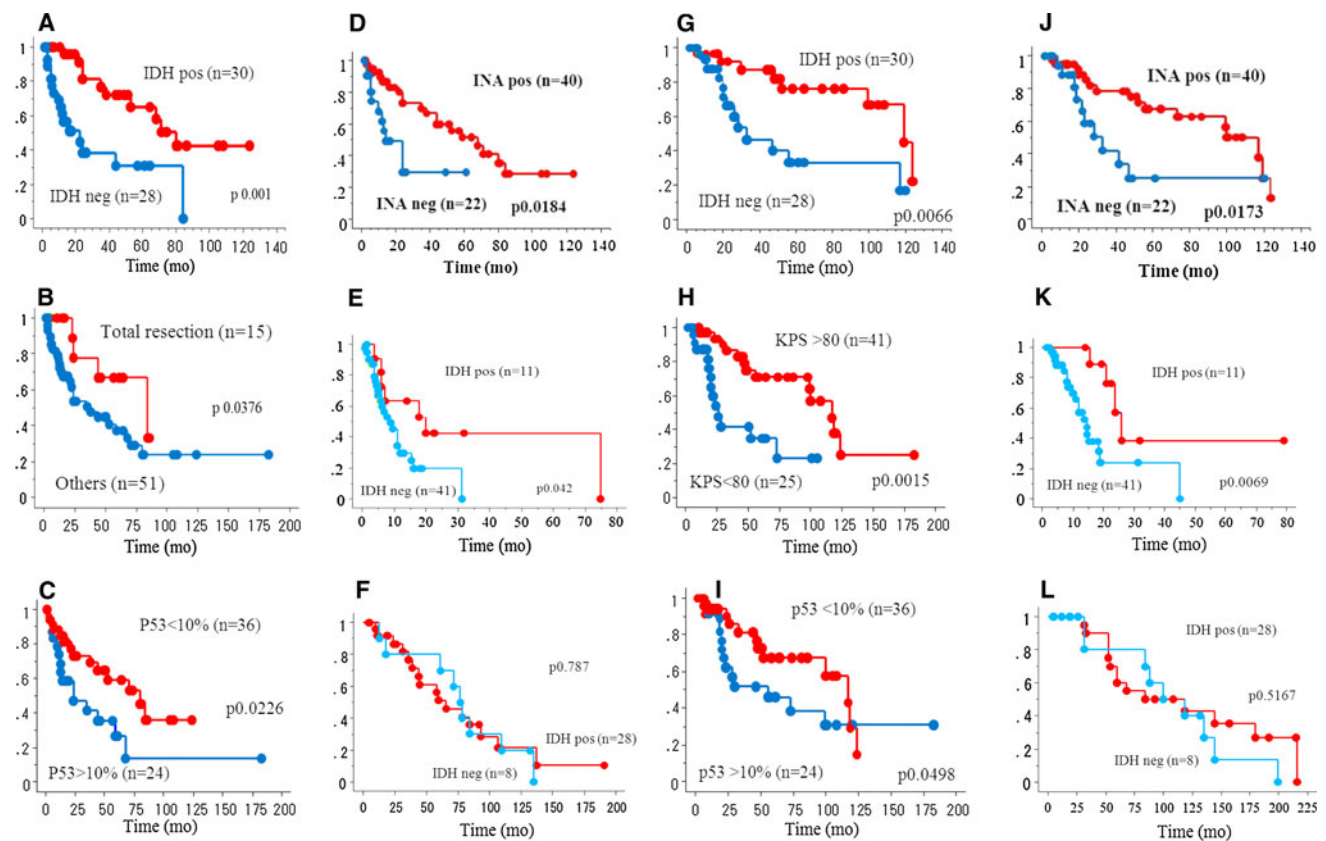
significant prognostic factor for OS. Multivariate analysis showed that independent significant prognostic factors were IDH1 mutation positivity and sex (male predominance) for OS (Table 3A, B). Kaplan–Meier curves showed that PFS was significantly longer in cases with IDH1 mutation positivity (20 months) than in those with

**Table 2** Prognostic factors of (A) grade III glioma: PFS, (B) grade III glioma: OS

		Hazard ratio	95% CI	<i>p</i> value
(A)				
PFS univariate				
Age (years)	<40 vs. >40	0.971	0.466–2.022	0.9375
Sex	M vs. F	0.875	0.432–1.774	0.7114
KPS	>80 vs. <80	0.522	0.254–1.073	0.0771
Location	Frontal vs. others	0.646	0.309–0.350	0.2451
Total removal	Yes vs. no	0.412	0.144–1.175	0.0973
Pathology	AA vs. AO	1.467	0.731–2.944	0.2805
Chemo	ACNU vs. TMZ	0.827	0.187–3.652	0.8017
IDH1	Pos vs. neg	0.272	0.120–0.620	0.0019
INA	Pos vs. neg	0.34	0.151–0.765	0.0091
MGMT	Pos vs. neg	0.864	0.358–2.086	0.7455
MIB-1	>20% vs. <20%	2.593	1.066–6.306	0.0356
Vessel	>50 vs. <50	1.25	0.520–3.007	0.615
p53	Pos vs. neg	2.312	1.097–4.873	0.0276
PFS multivariate				
KPS	>80 vs. <80	0.94	0.342–2.579	0.9038
Total removal	Yes vs. no	0.059	0.012–0.302	0.0007
Pathology	AA vs. AO	1.97	0.714–5.433	0.1901
IDH1	Pos vs. neg	0.088	0.023–0.333	0.008
INA	Pos vs. neg	0.895	0.266–3.014	0.8584
MIB-1	>20% vs. <20%	1.612	0.444–5.849	0.468
p53	Pos vs. neg	7.037	2.504–19.778	0.0002
(B)				
OS univariate				
Age (years)	<40 vs. >40	1.231	0.535–2.835	0.6251
Sex	M vs. F	0.819	0.370–1.811	0.6216
KPS	>80 vs. <	0.28	0.125–0.646	0.0028
Location	Frontal vs. others	1.001	0.434–2.308	0.9984
Total removal	Yes vs. no	0.287	0.067–1.225	0.0917
Pathology	AA vs. AO	1.581	0.724–3.453	0.2505
Chemo	ACNU vs. TMZ	0.501	0.060–4.174	0.5229
IDH1	Pos vs. neg	0.294	0.115–0.747	0.0101
INA	Pos vs. neg	0.38	0.166–0.867	0.0215
MGMT	Pos vs. neg	0.717	0.240–2.147	0.5526
MIB-1	>20% vs. <20%	3.003	1.066–8.454	0.0373
Vessel	>50 vs. <50	1.212	0.434–3.389	0.7134
p53	Pos vs. neg	1.767	0.804–3.880	0.1563
OS multivariate				
KPS	>80 vs. <80	0.502	0.100–0.907	0.0328
Total removal	Yes vs. no	0.12	0.020–0.714	0.0197
Pathology	AA vs. AO	2.429	0.761–7.757	0.134
IDH1	Pos vs. neg	0.256	0.068–0.959	0.0432
INA	Pos vs. neg	2.897	0.683–12.284	0.1488
MIB-1	>20% vs. <20%	7.49	1.389–41.618	0.0203
p53	Pos vs. neg	3.003	1.04–8.673	0.0421

IDH1 mutation negativity (9 months) (Fig. 3e). In addition, OS was significantly longer in cases with IDH1 mutation positivity (26 months) than in those with IDH1

mutation negativity (14 months) (Fig. 3k). IDH1 mutation positivity was shown to be the most striking prognostic factor in grade IV gliomas.



**Fig. 3** Kaplan–Meier curves for progression-free (a–f) and overall (g–l) survival in grade III gliomas (a–d, g–j), grade IV gliomas (e, k), and grade II gliomas (f, l)

In patients with 46 grade II gliomas, postoperative irradiation and tumor size >6 cm were evaluated as additional clinical parameters and VEGF expression as an additional pathological parameter to determine progression-free and overall survival in addition to IDH1 mutation. Univariate analysis showed that the significant prognostic factor was tumor size <6 cm for PFS (Table 3C), and total removal and tumor size <6 cm for OS (Table 3D). Multivariate analysis showed that significant prognostic factors were age <40 years, total removal, and MIB-1 <2.5% for OS. IDH1 mutation positivity was not included in any prognostic factor, and showed no survival benefit on the Kaplan–Meier curve (Fig. 3f, l).

## Discussion

Clinically, the IDH1 mutations correlated strongly with good prognosis in patients with gliomas, suggesting that future clinical trials might require stratification by IDH1 mutation status [1, 2, 21]. Multivariate analyses confirmed that IDH1 mutations are independent favorable prognostic markers in GBMs and anaplastic gliomas after adjustment for other genomic profiles and treatment modalities [22].

IDH1 mutations are also correlated with 1p/19q codeletion and MGMT promoter methylation in anaplastic oligodendroglial tumors [23]. Furthermore, IDH1 mutation status in anaplastic astrocytomas as well as glioblastomas was the most powerful single prognostic factor of overall patient survival, followed by age, tumor type, and MGMT methylation status [24]. Although these reports were based on the finding of IDH1 mutations by direct DNA sequencing analysis, we recently reported that anti-IDH1-R132H mAb immunohistochemistry revealed clinical significance as a prognostic factor in grade III anaplastic astrocytomas [11]. Patients with anti-IDH1-R132H-immunoreactive anaplastic astrocytomas had significantly longer progression-free survival than those who were anti-IDH1-R132H negative. At present, it is recommended that all IDH1-R132H-immunonegative cases be evaluated by direct sequencing [25], because immunohistochemical approach has sensitivity of 94% because of the lack of detection of other types of IDH1 mutations [26]. In this study, HMAb-1/SMab-1-positive patients had significantly longer progression-free as well as overall survival than those who were HMAb-1/SMab-1 negative in grade III and grade IV gliomas. Although HMAb-1 alone does not reveal statistical significance for OS on multivariate analysis (hazard ratio, 0.359;

**Table 3** Prognostic factors of (A) grade IV glioma: PFS, (B) grade IV glioma: OS, (C) grade II glioma: PFS, and (D) grade II glioma: OS

		Hazard ratio	95% CI	<i>p</i> value
(A)				
PFS univariate				
Age (years)	<40 vs. >40	0.415	0.141–1.218	0.1095
Sex	M vs. F	0.693	0.341–1.409	0.3106
KPS	>80 vs. <80	1.048	0.541–2.231	0.7957
Diagnosis	Primary vs. second	1.486	0.714–3.096	0.2895
Location	Frontal vs. others	0.87	0.392–1.933	0.7325
Total removal	Yes vs. no	0.472	0.136–1.645	0.2388
IDH1	Pos vs. neg	0.396	0.155–1.011	0.0527
INA	Pos vs. neg	0.711	0.309–1.635	0.4223
MGMT	Pos vs. neg	0.753	0.249–2.275	0.6155
MIB-1	>20% vs. <20%	1.454	0.688–3.070	0.3265
Vessel	>50 vs. <50	1.048	0.326–3.368	0.9377
p53	Pos vs. neg	1.165	0.544–2.495	0.6935
PFS multivariate				
Age (years)	<40 vs. >40	0.21	0.037–1.199	0.0791
Sex	M vs. F	0.283	0.079–1.014	0.525
KPS	>80 vs. <80	1.413	0.488–4.091	0.5234
Diagnosis	Primary vs. second	1.226	0.410–3.667	0.7158
Total removal	Yes vs. no	0.719	0.048–10.839	0.8115
IDH1	Pos vs. neg	0.173	0.032–0.934	0.0415
INA	Pos vs. neg	3.882	1.155–13.046	0.282
MIB-1	>20% vs. <20%	1.162	0.354–3.813	0.8047
p53	Pos vs. neg	0.472	0.151–1.476	0.1969
(B)				
OS univariate				
Age (years)	<40 vs. >40	0.424	0.123–1.454	0.1721
Sex	M vs. F	0.675	0.208–1.582	0.3659
KPS	>80 vs. <80	0.6	0.257–1.399	0.2368
Diagnosis	Primary vs. second	1.142	0.476–2.737	0.7661
Location	Frontal vs. others	0.958	0.380–2.414	0.9278
Total removal	Yes vs. no	0.425	0.097–1.854	0.255
IDH1	Pos vs. neg	0.239	0.079–0.729	0.0119
INA	Pos vs. neg	0.7	0.274–1.791	0.4565
MGMT	Pos vs. neg	1.845	0.552–6.165	0.3169
MIB-1	>20% vs. <20%	1.992	0.792–4.762	0.1469
Vessel	>50 vs. <50	0.286	0.076–1.077	0.0642
p53	Pos vs. neg	1.269	0.524–3.070	0.5977
OS multivariate				
Age (years)	<40 vs. >40	0.169	0.015–1.897	0.1414
Sex	M vs. F	0.15	0.024–0.924	0.0408
KPS	>80 vs. <80	0.272	0.053–1.404	0.1199
Diagnosis	Primary vs. second	0.506	0.107–2.389	0.3896
Total removal	Yes vs. no	2.731	0.080–93.132	0.5768
IDH1	Pos vs. neg	0.061	0.005–0.795	0.0328
INA	Pos vs. neg	4.37	0.818–23.375	0.0846
MIB-1	>20% vs. <20%	1.563	0.320–7.641	0.5812
p53	Pos vs. neg	0.577	0.158–2.110	0.4059



**Table 3** continued

		Hazard ratio	95% CI	<i>p</i> value
(C)				
PFS univariate				
Age (years)	<40 vs. >40	0.972	0.477–1.982	0.9376
Location	Frontal vs. others	0.596	0.286–1.243	0.1677
Total removal	Yes vs. no	0.453	0.173–1.182	0.1055
Radiation first	Yes vs. no	1.971	0.834–4.627	0.1193
MIB-1	>2.5% vs. <2.5%	1.27	0.585–2.754	0.5454
p53	Pos vs. neg	1.114	0.507–2.444	0.7884
VEGF	Pos vs. neg	1.223	0.510–2.935	0.6516
IDH1	Pos vs. neg	0.981	0.426–2.259	0.9641
INA	Pos vs. neg	0.907	0.383–2.150	0.8249
Tumor size	>6 vs. <6 cm	3.47	1.450–8.303	0.0052
Vessel	>22 vs. <22	1.014	0.428–2.402	0.9744
PFS multivariate				
Age (years)	<40 vs. >40	0.291	0.075–1.133	0.0751
Total removal	Yes vs. no	0.618	0.166–2.237	0.4728
Radiation first	Yes vs. no	3.837	0.805–18.202	0.0913
MIB-1	>2.5% vs. <2.5%	2.186	0.539–8.861	0.2732
p53	Pos vs. neg	0.531	0.142–1.983	0.3459
IDH1	Pos vs. neg	1.688	0.639–4.485	0.2945
INA	Pos vs. neg	2.037	0.432–9.604	0.3633
Tumor size	>6 vs. <6 cm	3.304	0.793–13.768	0.1007
(D)				
OS univariate				
Age (years)	<40 vs. >40	0.903	0.427–1.909	0.7895
Location	Frontal vs. others	0.823	0.380–1.78	0.6219
Total removal	Yes vs. no	0.258	0.078–0.859	0.0272
Radiation first	Yes vs. no	2.197	0.890–5.423	0.0877
MIB-1	>2.5% vs. <2.5%	1.555	0.679–3.559	0.2962
p53	Pos vs. neg	0.917	0.390–2.154	0.8426
VEGF	Pos vs. neg	1.307	0.575–3.314	0.5729
IDH1	Pos vs. neg	0.759	0.322–1.790	0.5286
INA	Pos vs. neg	1.117	0.454–2.747	0.8103
Tumor size	>6 vs. <6 cm	4.354	1.804–10.510	0.0011
Vessel	>22 vs. <22	1.014	0.428–2.402	0.9744
OS multivariate				
Age (years)	<40 vs. >40	0.215	0.050–0.929	0.0395
Total removal	Yes vs. no	0.103	0.016–0.672	0.0175
Radiation first	Yes vs. no	3.551	0.730–17.275	0.1163
MIB-1	>2.5% vs. <2.5%	8.907	1.552–51.1	0.0141
p53	Pos vs. neg	0.389	0.098–1.545	0.1797
IDH1	Pos vs. neg	1.279	0.436–3.757	0.654
INA	Pos vs. neg	2.133	0.350–12.99	0.4112
Tumor size	>6 vs. <6 cm	3.098	0.635–15.106	0.1619

95% CI, 0.124–1.038; *p* value, 0.0587), combined use of HMab-1/SMab-1 revealed statistical significance for OS on multivariate analysis (Table 2B; hazard ratio, 0.256; 95%

CI, 0.068–0.959; *p* value, 0.0432), indicating that combined use of HMab-1/SMab-1 is a powerful tool to make prognoses of patients with grade III glioma.

In our study, IDH1 mutation was not a prognostic factor in grade II gliomas. Some have reported that grade II diffuse glioma patients with mutated IDH1/2 demonstrated better prognosis [7, 22, 27], although another report described that IDH1 mutation was not a prognostic factor in grade II gliomas [28, 29]. Thon et al. reported that IDH1 mutations in grade II astrocytomas are associated with unfavorable progression-free survival and prolonged post-recurrence survival [28]. Significant differences were reported for survival in a group of 77 IDH1-mutated (median OS, 150.9 months) and 23 IDH-nonmutated (median OS, 60.1 months) grade II gliomas ( $p = 0.01$ ) [22]. Positive prognostic impact of IDH1 mutation was reported on OS in a population of 49 low-grade astrocytomas, even if the analysis was performed at time of progression [27]. However, their reports did not demonstrate the role of prognostic factors for PFS in grade II gliomas [22, 27]. A recent report showed that IDH mutations are strongly associated with prolonged survival in low-grade glioma ( $n = 271$ ) on univariate and multivariate analyses, although PFS was not different between IDH1 mutation positivity and mutation negativity [30]. However, these studies do not include important clinical parameters such as tumor size, which was a significant prognostic factor for both PFS and OS on our univariate analysis and others [31]. In addition, for grade II gliomas, various strategies including chemotherapy, radiation therapy, and other modalities after surgery other than IDH1 mutation can cause various genetic changes in the tumor. To study the role of IDH mutation on the spontaneous growth (natural history) of low-grade glioma, PFS was investigated in a series of 171 patients who had no adjuvant treatment after surgery until first progression. Spontaneous PFS did not differ in IDH-mutated and IDH-wild-type patients [30]. Taken together, the IDH1 mutation is a controversial prognostic factor in grade II gliomas. Further studies, particularly addressing special location [32] or uniform treatment group, must be undertaken to determine the exact significance of IDH1 mutation in grade II gliomas.

Increased expression of p53 most likely reflects the presence of loss-of-function mutations in the protein [33]. Mutations of the p53 gene are most commonly found in low-grade gliomas and younger patients [34]. They are believed to be related to invasive and aggressive nature or malignant astrocytomas [19]. In our study, patients with p53 expression ( $\geq 10\%$ ) had significantly shorter progression-free and overall survival in grade III gliomas. IDH mutation positivity and p53  $< 10\%$  have been shown to be the most striking prognostic factors, although cases that were IDH mutation negative with p53  $> 10\%$  (10 of 58 grade III gliomas) had the shortest PFS of 12.0 months. Recently, Birner et al. [35] reported strong correlation between IDH1 mutations and p53 expression. In our study,

no significant correlation was found between IDH1 mutations and p53 expression (data not shown), although p53 expression was an independent prognostic factor for PFS in grade III gliomas. This discrepancy can be explained by the difference of p53-positive criteria. Our criteria are quantitative ( $> 10\%$  nuclear staining), whereas their criteria were semiquantitative, merely reflecting whether it was strong or not.

Although recent studies emphasized the importance of immunostaining as a screening procedure to identify patients with mutant p53 DNA alleles, immunostaining can also reveal an expanded spectrum of diseases because of overexpression of nonmutant or wild-type p53 [36]. The existence of a subset of astrocytic tumors overexpressing p53 protein in the absence of detectable p53 mutations has been demonstrated [20]. Overexpression of p53 protein was not always associated with point mutations in conserved exons of the p53 gene in astrocytic tumors. Evaluation of p53 protein expression as a continuous variable rather than as a binary variable also demonstrated higher levels of statistical significance for PFS, suggesting that overexpression of p53 protein is important in the natural growth of these aggressive tumors [20]. Use of p53 immunostaining as a prognostic indicator, in contrast to mutational DNA analyses, might be a useful adjunct for identifying patients at higher risk of treatment failure. This finding implies a distinct role of wild-type p53 expression in the natural growth of astrocytomas. Taken together, our finding of p53 overexpression as well as IDH1 mutations as having prognostic significance is valuable in terms of the natural growth of astrocytic tumors.

Close functional and genetic relation between IDH1 mutations and 1p/19q codeletion has been reported intensively [37]. In practice, the most widely available techniques to detect 1p/19q codeletion include fluorescent in situ hybridization (FISH), loss of heterozygosity (LOH), multiplex ligation-dependent probe amplification (MLPA), and comparative genomic hybridization (CGH) array. All have their respective limitations: contamination with normal cells might impair genetic analysis; LOH and FISH are inadequate to distinguish whole 1p and whole 19q loss from partial 1p loss frequently associated with 1p and 19q loss. These prognoses are radically different, and the amount of available tissue, in cases of biopsy, might be insufficient for CGH array. Results of the Ducray study suggest that INA expression can serve as a surrogate marker for 1p/19q-codeleted tumors [12]. Their data indicate that absence of INA expression in an oligodendroglial tumor makes 1p/19q deletion very unlikely (1/48: 2%). In contrast, when  $> 10\%$  of cells express INA, there is  $> 80\%$  chance of finding 1p/19q codeletion in the tumor. Recent retrospective analysis revealed that INA expression is related more closely to CGH than to FISH. Furthermore,

INA expression is related closely to MGMT promoter methylation and IDH1/2 mutations. INA expression is also prognostic with IDH1-nonmutated glioma [15]. In our study, INA expression was shown to be a significant prognostic factor for both PFS and OS on univariate analysis, with significant longer survival on the Kaplan–Meier curve in grade III gliomas. However, the exact prognostic and predictive significance of INA status in patients with grade IV and grade II gliomas was not observed. This must be determined based on results of future studies, as results of other studies have suggested [13, 14].

We used an immunohistochemical approach against MGMT expression in this study. A recent report described only weak to moderate correlation between MGMT immunoreactivity and MGMT promoter methylation [38]. However, Christmann et al. [18] found agreement between immunoreactivity and MGMT activity (>30 fmol/mg protein) in 75% of cases. Correlation between promoter methylation and immunoreactivity was only slightly lower (in 72.5% of cases for MSP-P1 and 62.5% of cases for MSP-P2). The combination of several methods might provide more useful prognostic data. Consequently, it was recently demonstrated that the combination of MGMT promoter methylation and negative MGMT expression was significantly associated with increased overall survival, although a correlation between promoter methylation and immunostaining was observed in only 50% of samples [39]. Immunohistochemical approaches for MGMT might be useful in limited circumstances.

IDH mutation status is now regarded as an independent positive predictive factor for glioblastoma [22] and WHO grade II and III astrocytomas [21, 30]. In our study, IDH1 mutation status was confirmed as a strong prognostic factor in grade III and grade IV with longer overall survival as well as longer progression-free survival than their wild-type counterparts, although IDH1 mutation status was not a prognostic factor in grade II gliomas. The most striking finding evaluated by either immunohistochemistry or direct sequencing might be the greater prognostic significance than that of histological grading [25]. Immunohistochemical detection of IDH mutations combined with p53 and INA is expected to be a promising technique for diagnosis and tumor classification in gliomagenesis.

In summary, these striking findings of IDH1 mutations (R132H and R132S) and p53 mutation, evaluated using immunohistochemistry with clinical parameters such as degree of surgical removal and preoperative KPS, might be of greater prognostic use for grade III gliomas than histological grading alone. In addition, IDH1 mutations (R132H and R132S) might be of great prognostic use as factors to evaluate grade IV gliomas. Because of the high mutation frequency, mutated IDH1 screened by the combination

HMAb-1/SMab-1 can potentially form a good target and/or biomarkers for new treatments.

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