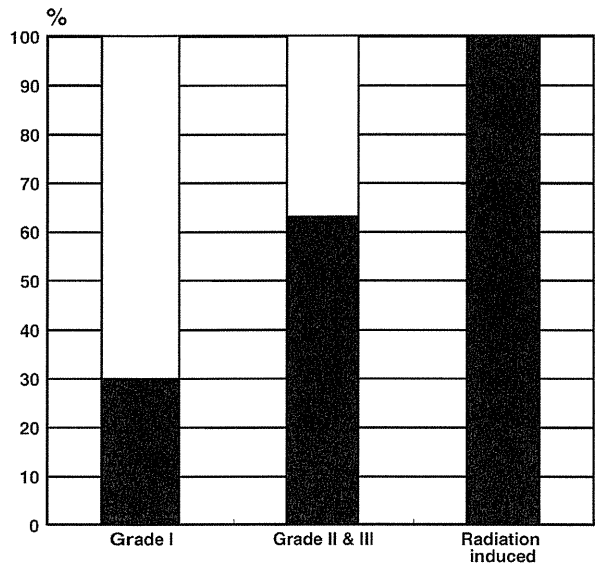


Fig. 2 Comparison of FAS expression among benign (WHO grade I), and aggressive (WHO grades II and III and radiation-induced) meningiomas. FAS positivity (*black bar*) was 29.8 % in grade I, 62.9 % in grade II and III, and 100 % in radiation-induced meningiomas (chi-squared test, $p < 0.001$)

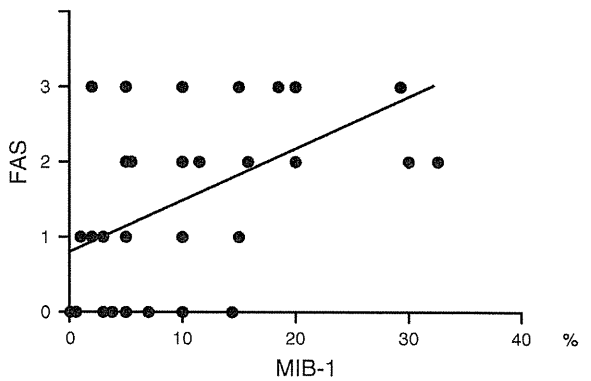
index was a significant prognostic factor for the development of recurrence or regrowth of WHO grade I tumors, as was tumor infiltration into surrounding tissues.

Because it can be used to measure the concentrations of the major metabolites in brain tumors, ^{1}H magnetic resonance spectroscopy (MRS) reveals biochemical tumor characteristics and is, therefore, a noninvasive method for quantitative assessment of tumor metabolism. Pfisterer et al. [32] reported that ^{1}H MRS may facilitate biochemical assessment for early detection of more aggressive tumors and of meningiomas in the process of becoming more aggressive. Herting et al. [33] showed that the level of some enzymes of the energy metabolism, e.g. phosphofructokinase (PFK) and lactate dehydrogenase (LDH), were significantly increased in malignant meningiomas. These observations suggest a correlation between the energy metabolism and the clinical behavior of meningiomas.

Our findings that FAS expression was increased in malignant meningiomas coincide with those of Haase et al. [20]. Panagopoulos et al. [34] showed that expression of brain fatty acid binding protein (BFABP) increased with the tumor grade. We document that FAS expression correlated well with the MIB-1 index. Moreover, Haase et al. reported that treatment with FAS inhibitor reduced meningioma cell survival in vitro and increased cell death in meningioma-bearing xenograft mice [20]. These findings support the hypothesis that FAS expression is involved

Table 2 Correlation between FAS expression and clinico-pathologic factors

Factor	FAS expression		*p* value
	Positive	Negative	
Age			
<60 years	12	24	
≥60 years	15	24	0.644
Sex			
Male	7	12	
Female	20	36	0.930
Site			
Cranial base	15	32	
Convexity	7	9	
Falx/parasagittal	5	7	0.233
Grade			
I	17	40	
II/III	10	8	0.047
Edema			
Yes	19	34	
No	8	14	0.966
Infiltration			
Yes	20	21	
No	7	27	0.011
MIB1 index			
<5 %	13	41	
≥5 %	14	7	0.001
Recurrence			
No	19	40	
Yes	8	8	0.188



Spearman r	**0.4634**
95% confidence interval	**0.1914 to 0.6693**
P value (two-tailed)	**0.0012**

Fig. 3 Correlation between FAS expression and MIB-1 index. Spearman rank order correlation was with GraphPad Prism software

Table 3 Univariate analysis for recurrence or regrowth in 57 patients with grade I tumor

Factor	$\chi 2$	df	p value
Sex	0.016	1	0.898
Age	1.006	1	0.316
Edema	2.191	1	0.139
Infiltration	4.576	1	0.032
MIB-1	4.176	1	0.041
FAS	1.186	1	0.276

df degrees of freedom

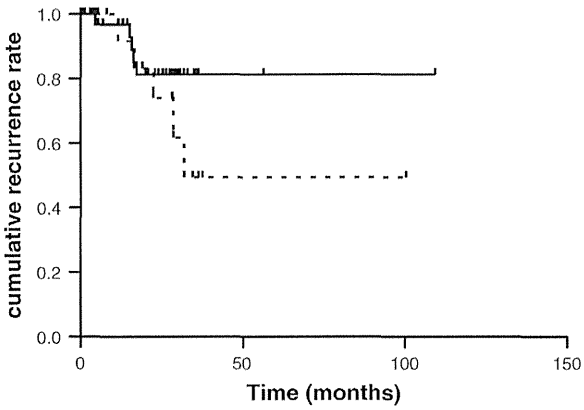


Fig. 4 Kaplan–Meier curve showing disease-free survival for patients with meningiomas that did (n = 17; *dashed line*) or did not (n = 40; *solid line*) express FAS

in the progression of meningiomas and that it may be a useful proliferation marker and a novel therapeutic target.

Although radiation-induced meningiomas tend to be more clinically aggressive than sporadic meningiomas, the meningotheliomatous, transitional, and fibroblastic histological subtypes are more common and their histological features differentiate them from sporadic meningiomas. Soffer et al. [35], who studied 42 patients with radiation-induced meningiomas, found that their tumors had high cellularity, nuclear pleomorphism, an increased mitotic rate, focal necrosis, bone invasion, and tumor cell infiltration of the brain. Rubinstein et al. [8] reported that radiation-induced cerebral meningiomas had high cellularity, pleomorphic nuclei with great variations in their size, shape, and chromatin density, many multinucleated and giant cells, and frequent mitoses. Moreover, among 79 patients with meningiomas induced by high-dose radiation, 23 % had atypical or malignant tumors.

Our series included six radiation-induced meningiomas. Although four of these were grade I tumors (meningothelial), all expressed FAS, as did the other two atypical meningiomas. These data suggest that radiation-induced meningiomas may have biological characteristics that differentiate them from sporadic meningiomas.

In conclusion, FAS expression was significantly correlated with meningioma grade and MIB-1 index, and all radiation-induced meningiomas expressed FAS. As meningiomas with high FAS expression may behave aggressively we recommend they be followed closely. Inhibition of FAS activity may be a new treatment option to address unresectable and malignant meningiomas.

Conflict of interest The authors declare that they have no conflict of interest.

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CLINICAL STUDY

Prediction of high-grade meningioma by preoperative MRI assessment

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Abstract High-grade (World Health Organization grades II and III) meningiomas grow aggressively and recur frequently, resulting in a poor prognosis. Assessment of tumor malignancy before treatment initiation is important. We attempted to determine predictive factors for high-grade meningioma on magnetic resonance (MR) imaging before surgery. We reviewed 65 meningiomas (39 cases, benign; 26 cases, high-grade) and assessed four factors: (1) tumor–brain interface (TBI) on T1-weighted imaging (T1WI), (2) capsular enhancement (CapE), i.e., the layer of the tumor–brain interface on gadolinium-enhanced T1WI (T1Gd), (3) heterogeneity on T1Gd, and (4) tumoral margin on T1Gd. All four factors were useful in distinguishing high-grade from benign meningiomas, according to univariate analysis. On multivariate regression analysis, unclear TBI and heterogeneous enhancement were independent predictive factors for high-grade meningioma. In meningiomas with an unclear TBI and heterogeneous enhancement, the probability of high-grade meningioma was 98%. Our data suggest that this combination of factors obtained from conventional sequences on MR imaging may be useful to predict high-grade meningioma.

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Keywords Meningioma · High-grade · MR imaging · Logistic regression analysis · Predictive factor

Introduction

Meningiomas are common tumors and account for 20–32% of primary intracranial tumors [1–3]. Most meningiomas are benign, corresponding to the World Health Organization (WHO) grade I. However, 22.0–35.5% of meningiomas are classified as grade II and III (high-grade meningiomas) according to the WHO 2000 classification system and show aggressive clinical features [4, 5]. High-grade meningioma has a several-fold increased risk of recurrence, as well as an increased rate of mortality [3]. Early prediction of high-grade meningioma is important, because it aids in preoperative surgical planning and determination of frequency of radiological examination in cases of observation without surgery [6]. The development of magnetic resonance (MR) imaging as a diagnostic tool, and the increased frequency of health screening with no symptoms or unrelated symptoms, have resulted in the detection of unexpected, incidental meningiomas, and ruling out high-grade meningioma is critical to the treatment plan.

A computed tomography (CT) scan is generally chosen as the first-line radiological investigation. Most meningiomas show typical imaging characteristics on CT, which facilitate an unequivocal diagnosis, while intratumoral calcification is a predictive factor of benign histology [2, 7, 8]. With superior image resolution, MR imaging allows us to predict the malignancy of meningioma with greater accuracy. Many studies have reported on the evaluation of meningioma by MR imaging which has revealed

distinguishing characteristics of high-grade meningioma [9–16]. However, there are no reports that clearly show the probability of high-grade meningioma based on assessment of a combination of MR imaging parameters.

To address this issue, we analyzed four factors that were considered to be associated with malignancy of meningioma on MR imaging. Additionally, we performed multivariate logistic regression analysis to predict the probability of high-grade meningioma as a function of the independent variables.

Materials and methods

Patients

We assessed the clinical records of patients with meningioma between 1993 and 2010 at the Departments of Neurosurgery, Kanazawa University and Kumamoto University. Thirty-nine patients (14 males, 25 females; range, 35–84 years old; mean age, 57.8 years old) with benign meningioma and 26 patients (12 males, 14 females; range, 24–81 years old; mean age, 57.5 years old) with high-grade meningioma were included in this study. The data of 39 patients with benign meningioma and 8 patients with high-grade meningioma were collected from Kanazawa University during the period between 2007 and 2010, while those of 18 patients with high-grade meningioma were assembled from Kumamoto University between 1993 and 2010. All patients had received brain MR imaging that allowed adequate image interpretation before surgery. The diagnosis and grade of meningioma were confirmed in all patients by histopathology. The need for informed consent was waived because of the retrospective study design.

MR image analysis

MR imaging was performed at 1.5T or 3T. Axial T1-weighted imaging (T1WI) and gadolinium-enhanced T1WI (T1Gd) of the whole brain were available in all the patients. MR imaging were reviewed independently by 2 neurosurgeons (Y.K. and M.N.) blinded to the histopathological results, and disagreement was resolved by consensus. The parameters evaluated were the tumor–brain interface (TBI) on T1WI, and capsular enhancement (CapE), heterogeneity, and tumoral margin (TM) on T1Gd. These parameters were determined from deductions from previously published literature as well as theoretical considerations [6, 11, 12, 14, 15]. TBI was categorized as "clear" or "unclear". Tumors with a distinct low-intensity border and obvious demarcation from the brain or edema were regarded as "clear" (Fig. 1a), whereas tumors without a distinct border were regarded as "unclear" (Fig. 1b). CapE was defined as the enhanced layer at the tumor–brain interface and was categorized as "positive" (Fig. 1c) or "negative" (Fig. 1d). "Positive" was defined as the

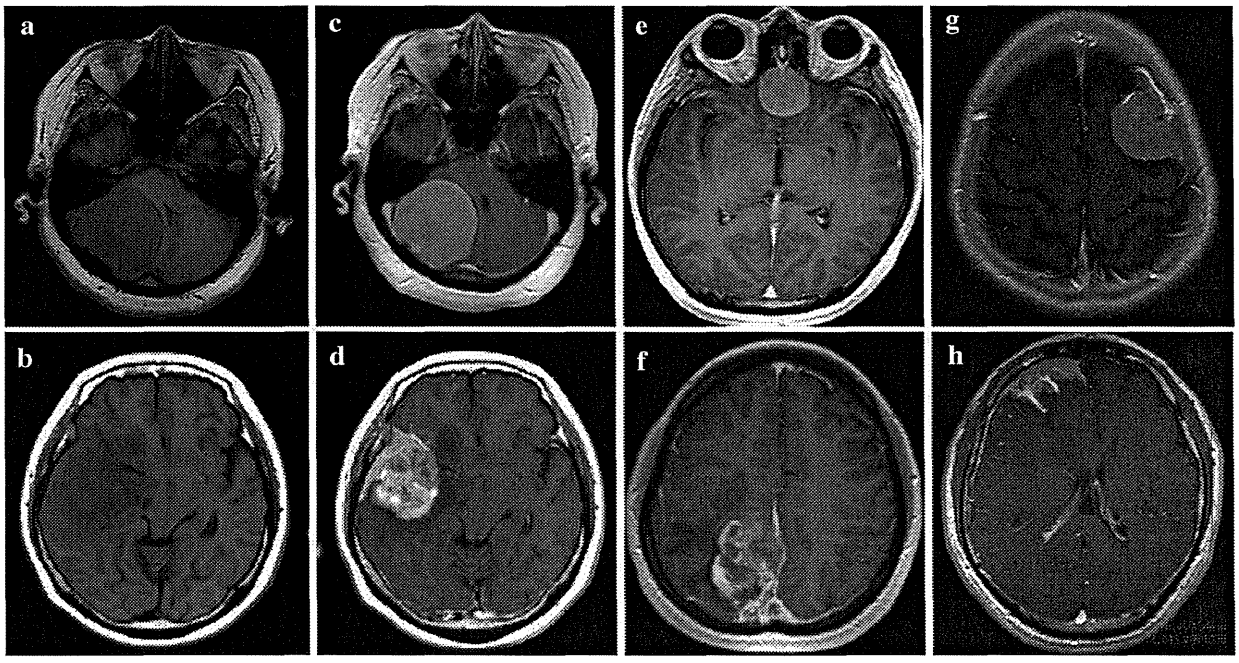

Fig. 1 MR imaging obtained from representative benign meningioma with clear tumor–brain interface (TBI) (**a**), positive capsular enhancement (CapE) (**c**), homogeneous enhancement (**e**) and regular tumoral margin (TM) (**g**). MR imaging obtained from representative high-grade meningioma with unclear TBI (**b**), negative CapE (**d**), heterogeneous enhancement (**f**), and irregular TM (**h**)

presence of the layer on most interfaces between the tumor and the brain. Heterogeneity was determined by the subjective evaluation of intratumoral enhancement and categorized as "homogeneous" (Fig. 1e) or "heterogeneous" (Fig. 1f). TM was categorized as "regular" (Fig. 1g) or "irregular" (Fig. 1h), and tumors with a lobulated appearance, so-called mushrooming tumors, were included in "irregular". The possible predictive factors for high-grade meningioma on MR imaging were unclear TBI, negative CapE, heterogeneous enhancement, and irregular TM.

Statistical analysis

Univariate analysis was used to identify covariates that might affect the combined rate of high-grade meningioma. On univariate analysis, a χ^2 test and Fisher's exact test were performed to compare the predictive factors. A stepwise binary logistic regression analysis was applied to identify significant independent factors for foretelling high-grade meningioma. The significance was set at $P < 0.05$. The predictive formula was based on binary logistic regression analysis [17]. All odds ratios on analyses reflected the odds of high-grade meningioma. The goodness of fit of the regression model was confirmed by demonstrating a nonsignificant P value on the Hosmer–Lemeshow test. All statistical analyses were performed using SPSS v.12.0 J.

Result

MR imaging findings

Each of the four imaging parameters of the 65 patients was assessed. Disagreement between two reviewers was resolved by consensus (4 of 260 items). The sensitivity of high-grade meningioma with unclear TBI, negative CapE, heterogeneous enhancement, and irregular TM was 65.4, 80.8, 65.4, and 69.2%, respectively. In contrast, the specificity with clear TBI, positive CapE, homogeneous enhancement, and regular TM was 97.4, 82.1, 89.7, and 82.1%, respectively (Table 1).

Data analysis

Tables 2 and 3 demonstrate the results of univariate and multivariate logistic regression analyses, respectively. Unclear TBI was the strongest predictive factor of high-grade meningioma. Meningiomas with unclear TBIs were significantly more likely to be high-grade [odds ratio (OR), 71.8; 95% confidence interval (CI), 8.4–612; $P < 0.001$]. This relationship was upheld in a multivariate analysis revealing a 42-fold increased risk of high-grade meningioma with unclear TBI (OR, 42.0; 95% CI, 4.5–390; $P = 0.001$). Heterogeneous enhancement was also strongly associated with increased risk of high-grade meningioma

Table 1 Result of image assessment

	High-grade (%) $n = 26$	Benign (%) $n = 39$
TBI		
Unclear	17 (65.4)	1 (2.6)
Clear	9 (34.6)	38 (97.4)
CapE		
Negative	21 (80.8)	7 (17.9)
Positive	5 (19.2)	32 (82.1)
Heterogeneity		
Heterogeneous	17 (65.4)	4 (10.3)
Homogeneous	9 (34.6)	35 (89.7)
TM		
Irregular	18 (69.2)	7 (17.9)
Regular	8 (30.8)	32 (82.1)

TBI tumor–brain interface, *CapE* capsular enhancement, *TM* tumoral margin

Table 2 Univariate analysis of potential predictive factors for highgrade meningioma

Characteristics, high-grade	OR	95% CI		*P*
		−	+	
Unclear TBI	71.8	8.4	612	<0.001
Negative CapE	19.2	5.4	69	<0.001
Heterogeneous enhancement	16.5	4.4	61	<0.001
Irregular margin	10.3	3.2	33	<0.001

OR odds ratio, *CI* confidence interval, *TBI* tumor–brain interface, *CapE* capsular enhancement

Table 3 Multivariate analysis of potential predictive factors for high-grade meningioma

Characteristics, high-grade	OR	95% CI		*P*
		−	+	
Unclear TBI	42.0	4.5	390	0.001
Heterogeneous enhancement	8.3	1.7	40.4	0.009

OR odds ratio, *CI* confidence interval, *TBI* tumor–brain interface

Table 4 Probability of high-grade meningioma derived from multivariate analysis of potential risk factors

TBI	Heterogeneity	High-grade	Benign	Probability (%)
Clear	Homo	5	34	12.1
	Hetero	4	4	53.3
Unclear	Homo	4	1	85.3
	Hetero	13	0	98.0

Probability $= e^z/(1 + e^z)$, where $z = -1.979 - 3.738A + 2.112B$; A = TBI, where 0 is clear and 1 is unclear, and B = enhancement, where 0 is homongenous and 1 is heterogeneous

TBI tumor–brain interface

on univariate analysis (OR, 16.5; 95% CI, 4.4–61; $P < 0.001$) and was also a strong risk factor on multivariate analysis (OR, 8.3; 95% CI, 1.7–40.4; $P = 0.009$). Patients with negative CapE had significantly increased risk of high-grade meningioma on univariate analysis; however, this relationship was not significant on multivariate regression analysis. Similarly, irregular TM increased the risk of high-grade meningioma on univariate analysis, but was not significant on multivariate regression analysis.

In the logistic regression model, unclear TBI and heterogeneous enhancement contributed to high-grade meningioma. The numbers of each category and the probability of high-grade meningioma are shown in Table 4. The probability was calculated by the logistic equation: $P = e^z/(1 + e^z)$, where $z = -1.979 + 3.738 \times$ TBI $+ 2.112 \times$ heterogeneity, as described previously [17]. Only 12.1% of meningiomas with clear TBI and homogeneous enhancement were high-grade meningiomas. The probability of high-grade meningioma with unclear TBI and homogeneous enhancement was 85.3%. Meningiomas with unclear TBI and heterogeneous enhance0-ment had the highest probability (98%) of high-grade meningioma.

Discussion

This study revealed that certain factors on MR imaging were useful for predicting high-grade meningioma. Univariate analyses indicated that TBI, CapE, TM, and tumor heterogeneity on MR imaging were significant factors for discriminating between benign and high-grade meningiomas, whereas multivariate analysis demonstrated that TBI and heterogeneity were independently linked to high-grade meningioma.

First, we assessed the TBI, i.e., the low-intensity border at the TBI on T1WI. Our results demonstrated that there was a high frequency of unclear TBI in high-grade meningioma. Previous reports have demonstrated that TBI represents cerebrospinal fluid space and/or blood vessels [12–14, 18]. A clear TBI signifies the presence of these physiological barriers between the tumor and adjacent brain parenchyma. In contrast, an unclear TBI indicates their absence, suggesting tight adhesion between the tumor and brain or tumor invasion of the brain [14], which are pathological features of high-grade meningioma [19]. Thus, it is reasonable for unclear TBI to be a predictive factor for high-grade meningioma. Our result demonstrated that unclear TBI was the most significant indicative factor of malignancy on both univariate and multivariate analyses.

Meningioma usually develops connective tissue around the tumor. Previous literature has revealed that it was composed of tumor stroma, arachnoid mater, and arachnoid trabeculaes [11, 12, 20]. Because connective tissue formation is a chronic reaction occurring on the brain surface, it is likely to take time to develop. Accordingly, it is speculated that rapidly growing meningiomas are less likely to produce it. A previous pathological study reported that invasive meningiomas consistently lacked surrounding connective tissue on microscopic examination [11]. This connective tissue is recognized as an enhanced layer of varying thickness at the tumor–brain interface on T1Gd MR imaging [12], which we call CapE. To the best of our knowledge, this is the first study of the correlation between CapE and meningioma malignancy.

Meningiomas are commonly enhanced homogeneously on MR imaging, which reflects uniform pathological features. Non-uniform pathological features caused by intratumoral necrosis, which is a pathological trait of high-grade meningioma, can explain the heterogeneous enhancement of the malignant tumors [15, 21]. Our results agree with those of previous reports that showed that high-grade meningiomas tended to display heterogeneous enhancement [15, 21, 22]. Durand et al. observed that all meningiomas that were enhanced heterogeneously (16/199 cases) were high-grade meningiomas [22].

In addition to necrosis, high-grade meningiomas exhibit heterogeneous distribution of proliferating cells, resulting in an imbalance of cell density in the tumors [6]. This histological heterogeneity may lead to disproportion of intratumoral pressure, resulting in an irregular margin. Invasion into the brain parenchyma also explains the

irregular margin of malignant meningiomas [21]. Excluding en-plaque meningiomas, the absence of clear margins is a radiological feature reflecting histological malignancy [6, 11, 12, 14, 23]. As expected, our study demonstrated that approximately 70% of high-grade meningiomas showed irregular TMs.

The presence of peritumoral edema could be associated with malignancy of meningioma, because extravasation of plasma water and macromolecules through a damaged blood–brain barrier cause peritumoral edema. Previous reports have shown a relationship between edema and malignancy, whereas some other reports could not prove the relationship [7, 9, 15]. We also additionally examined the existence of the edema in our study; however, the edema was not a predictive factor for high-grade meningioma even in univariate analysis (data not shown).

The binary logistic regression analysis showed that unclear TBI and heterogeneous enhancement signified high-grade meningioma, and the combination of these findings could be valuable to distinguish high-grade meningioma from benign meningioma. To our knowledge, this is the first study of meningioma and combinations of factors on MR imaging.

Some advanced MR imaging techniques, such as MR spectroscopy and diffusion tensor imaging, have shown potential for differentiating between benign and high-grade meningiomas [24–26]. However, TBI, CapE, heterogeneity, and TM can be used clinically for predicting high-grade meningioma, because all factors can be assessed by the T1WI and T1Gd on MR imaging, which are widely available and are considered routine imaging methods for examination of meningiomas.

This study was a retrospective case–control study, and the patients were assembled from two institutions. Selection bias such as sampling bias and membership bias may have affected the outcome. Ascertainment bias may influence the assessment even though two reviewers assessed the factors. We used a logistic regression model for statistics, which tends to systematically overestimate odds ratios when the sample size is small. Further study with a large enough number of patients could lead to more accurate probabilities of high-grade meningioma.

In conclusion, we found that unclear TBI, negative CapE, heterogeneous enhancement, and an irregular margin are all noteworthy predictive factors for high-grade meningioma on univariate analysis. Unclear TBI and heterogeneous enhancement were significant predictive factors on multivariate analysis. The prediction of high-grade meningioma from these two parameters on MR imaging may be useful in clinical situations.

Conflict of interest The authors declare that they have no conflict of interest.

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LABORATORY INVESTIGATION

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

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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 α-ketoglutarate to *R*(-)-2-hydroxyglutarate (2-HG) [4]. Results of the initial study demonstrated that reduction of α-ketoglutarate by 2-HG or mutant IDH results in a lower level of prolyl hydroxylases and promotes accumulation of hypoxia-inducible factor (HIF)-1α [5]. Although HIF-1α is upregulated in a subset of gliomas in vivo, activation of the HIF-1α 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].

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 *O*6-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 ± 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 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 μg 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 μg IDH1-R132H peptide was performed. After several additional immunizations of 30 μg 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 (KPIIIGCH AYGD), 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 μg/ml for 30 min. After blocking with Super-Block T20 (PBS) blocking buffer (Thermo Fisher Scientific Inc.), the plates were incubated with 1 μg/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 μl 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 ≥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 ≥10% cells were rated as positive.

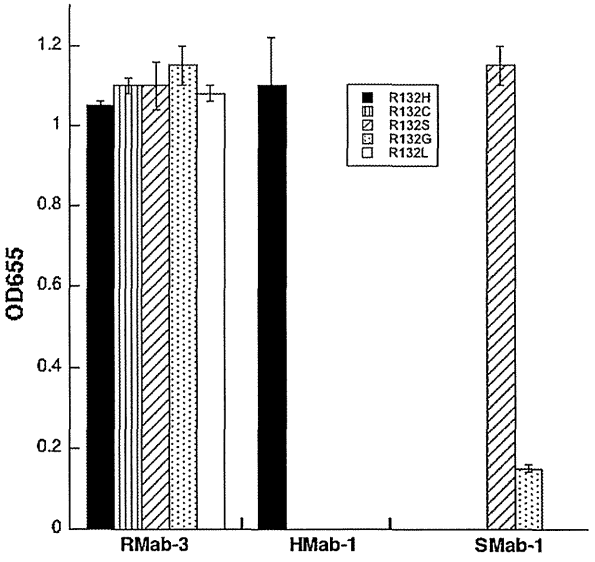

Fig. 1 Production of a specific monoclonal antibody against IDH1-R132H and IDH1 wild type. Synthetic peptides corresponding to amino acids 126–137 of the human IDH wild type and IDH1 mutants were immobilized on 96-well plates. After blocking, the plates were incubated with anti-IDH1 antibodies, followed 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 ≥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× field (1.0 mm^2) 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 (AHBT3; 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-μm 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_1 subclass). We also established RMab-3 (IgG_1 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 ≥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 ≥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 ≥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 ≥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.

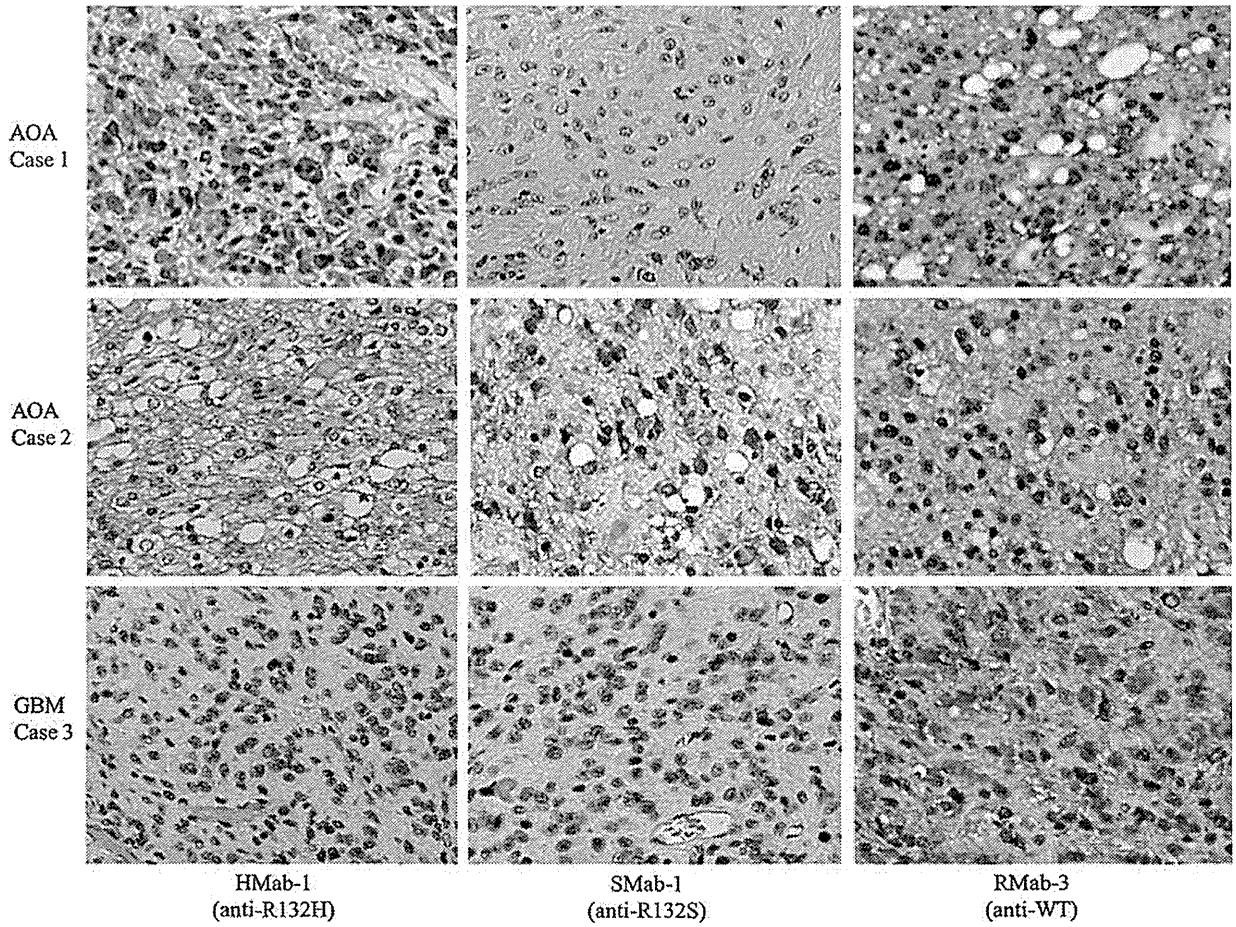

Fig. 2 Immunohistochemical analyses by anti-IDH1 antibodies against glioma tissues. Glioma tissues having IDH1-R132H (AOA: anaplastic oligoastrocytoma, case 1), IDH1-R132S (AOA, case 2), and wild type (GBM: glioblastoma, case 3) were stained with HMab-1 (anti-IDH1-R132H), SMab-1 (anti-IDH1-R132S), and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

Table 1 Summary of immunohistochemical analysis of each glioma grade

			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	*p* 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.

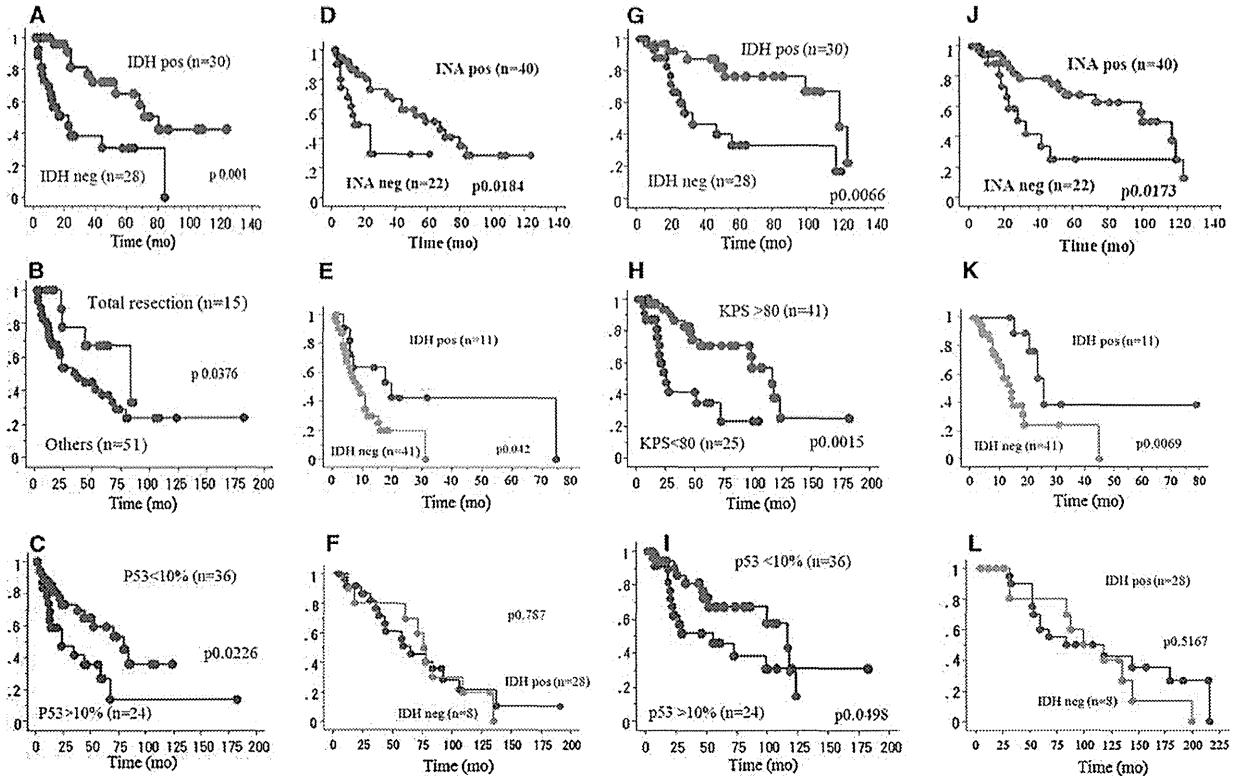

Fig. 3 Kaplan–Meier curves for progression-free (**a–f**) and overall (**g–l**) survival in grade III gliomas (**a–d**, **g–j**), grade IV gliomas (**e**, **k**), and grade II gliomas (**f**, **l**)

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	*p* 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	*p* 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 (≥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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