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ORIGINAL ARTICLE

Diagnostic advantage of double immunohistochemistry using two mutation-specific anti-IDH antibodies (HMab-1 and MsMab-1) in gliomas

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Abstract Isocitrate dehydrogenase (IDH) mutation is a valuable prognostic marker and a tool for decision-making for glioma treatment. An algorithm for IDH mutation screening was recently proposed—it consists of a two-step process of an initial search for the most common IDH1-R132H mutation using immunohistochemistry (IHC)-based assay, followed by DNA-based analysis of IHC-negative or -equivocal cases. Here, we report that immunohistochemistry using two mutation-specific anti-IDH monoclonal antibodies (mAbs)—an IDH1-R132H-specific mAb (clone HMab-1) and a multi-specific mAb (clone MsMab-1)—is easy and reliable for IDH mutation screening. We investigated the IDH status of 54 grade III gliomas. For the first screening, we used HMab-1 IHC and for the second, (of HMab-1-negative cases) we used MsMab-1 IHC. The double IHC screening results were confirmed using sequence analysis (100 % specificity and 100 % sensitivity). Thirty of 54 cases (55.6 %) had IDH mutations and the remaining 24 were of the IDH wild type; moreover, the screening results predicted grade III glioma prognosis. IDH sequencing procedures are popular but inconsistent across laboratories. By contrast, double IHC screening using HMab-1 and MsMab-1 is very reliable for detecting IDH1/2 mutations and can predict survival in grade III glioma patients.

Keywords Glioma · IDH · Immunohistochemistry · Monoclonal antibody · Mutation

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Introduction

Isocitrate dehydrogenase (IDH) mutation is a valuable prognostic marker in glioma. Currently available data suggest that IDH status may become part of the standard diagnostic assessment of gliomas [1]. In addition, the major prognostic impact of IDH mutations on outcome implies that mutation needs to be considered as a stratification factor in trials on glioma [2]. Very recently, updated data from the RTOG 9402 trial showed that IDH mutations predict the benefit of adjuvant chemotherapy in grade III glioma, even in the absence of 1p19q co-deletion [3]. Therefore, reliable and robust assays are needed for the detection of these mutations, especially in grade III glioma.

An algorithm for IDH mutation screening has recently been proposed [4]. It is a two-step process, with an initial search for the most common IDH1-R132H mutation using an immunohistochemistry (IHC)-based assay, followed by DNA-based analysis of IHC-negative or -equivocal cases. Among DNA-based analyses, Sanger sequencing still represents the gold standard for the identification of somatic mutations. However, sequencing sensitivity is low (at around 15–20 % mutation load), and this may lead to false negative results in analyses of tumor specimens with

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insufficient neoplastic cells in a background of normal cells [5, 6]. Immunohistochemical detection of the IDH1-R132H mutation is well recognized [7, 8]. However, there are several other types of IDH mutations that are not detected by anti-IDH1-R132H antibodies [9]. We have, therefore, established MsMab-1 antibody, which recognizes several IDH mutations, namely IDH1-R132S, IDH1-R132G, IDH2-R172S, IDH2-R172G, and IDH2-R172M, as well as IDH1-R132H [10, 11]. Previously, we reported that the anti-IDH1-R132H-specific monoclonal antibody HMab-1 was very useful for detecting IDH1-R132H in IHC and for predicting time to progression in grade III gliomas [8] and overall survival in grade II, III, and IV gliomas [12]. In this study, we investigated the usefulness of IDH IHC with a combination of the two mAbs, HMab-1 and MsMab-1, in grade III gliomas. We propose the importance of this IDH screening with double IHC and without sequence analysis.

Materials and methods

Patients

Fifty-four patients who underwent primary surgery between 1994 and 2011 at Tsukuba University Hospital were included as subjects. Mean patient age at the time of primary surgery was 48.7 ± 14.5 years (range 20–82 years). All tumors were diagnosed as grade III gliomas (26 anaplastic astrocytomas, 9 anaplastic oligodendrogliomas, 19 anaplastic oligoastrocytomas) according to the WHO classification. Postoperative therapies were uniform. All patients were treated maximum surgical resection followed by 54–60 Gy of radiation and ACNU-based chemotherapy. Median follow-up period was 54.5 months (6–179 months). Informed consent was obtained from each patient or the patient's caretaker to obtain samples and subject the associated data to analysis.

Sample preparation

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

Algorithm of IDH status analysis

First, glioma paraffin sections from the 54 patients were stained with HMab-1 to screen for the IDH1-R132H mutation. Second, glioma paraffin sections from HMab-1-negative patients were stained with MsMab-1 to screen for other types of IDH mutation. Third, DNA was extracted from glioma paraffin sections prepared from 54 patients and identical to those used for IHC; IDH status was then analyzed by direct sequencing to determine the accuracy of IHC. Finally, cases in which the results of IHC and direct sequencing disagreed were analyzed by subcloning and sequencing to confirm IDH status. This algorithm is demonstrated in Fig. 1.

IHC with HMab-1 and MsMab-1

IDH1-R132H protein expression was determined immunohistochemically in paraffin-embedded tumor specimens using HMab-1 at a concentration of 5 μg/ml and an LSAB2 kit (Dako, Glostrup, Denmark), as described previously [12]. Expression of the other mutant proteins in paraffin sections was determined using MsMab-1 [13] at a concentration of 5 μg/ml and an Envision+kit (Dako). Because preliminary staining using MsMab-1 with an LSAB2 kit had resulted in diffuse background staining (Supplementary Figure 1), we chose the Envision+kit for MsMab-1 staining. Although both HMab-1 and MsMab-1 detect IDH1-R132H, preliminary staining (Supplementary Figure 2) resulted in higher sensitivity for IDH1-R132H with HMab-1. Therefore, we chose HMab-1 staining for the first screening. Expression of IDH mutations was determined by semi-quantitatively assessing the proportions of positively stained tumor cells. We defined cases in which $\geq$10 % cells were stained as positive. All sections were immunostained with MIB-1 antibody (Immunotech Laboratories Inc., Monrovia, CA, USA), p53 antibody (clone DO7, Dako), and von-Willebrand factor (vWF) antibody (Dako), as described previously [12]. Positivity of nuclei for MIB-1 and p53 was determined by counting at least 1000 tumor cells in a homogeneously stained area. For both MIB-1 and p53, cases with $\geq$10 % stained cells were rated as positive and cases with $<$10 % stained cells were rated as negative. Vascular density was defined by averaging the number of vWF-stained vessels (1.0 mm^2) in the three most vascularized areas.

Direct DNA sequencing of IDH1 and IDH2 mutations and subcloning

Genomic DNA was extracted from formalin-fixed, paraffin-embedded tissue sections using MightyAmp for FFPE (Takara Bio Inc., Shiga, Japan) in accordance with the manufacturer's instructions. The PCR primers for the genomic region corresponding to IDH1 exon 4 (which encodes codon R132) and the flanking intronic sequences were human IDH1

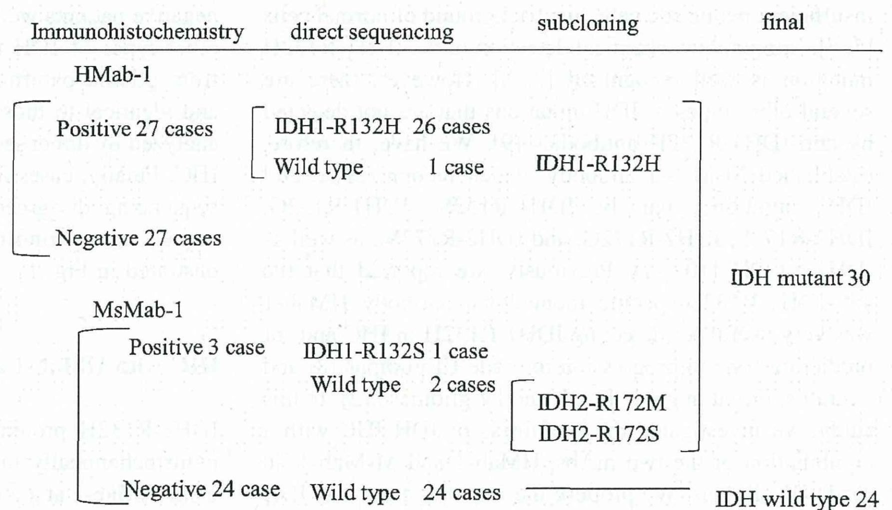

Fig. 1 Algorithm of IDH status analysis for 54 grade III gliomas (26 anaplastic astrocytomas, 9 anaplastic oligodendrogliomas, 19 anaplastic oligoastrocytomas). First screening is HMab-1 immunostaining, and second screening is MsMab-1 staining for HMab-1 negative cases. All cases were confirmed with sequence analysis. Finally, IDH mutation is found in 30 cases and wild type in 24 cases

sense (5′-CGGTCTTCAGAGAAGCCATT-3′) and human IDH1 antisense (5′-GCAAAATCACATTATTGCCAAC-3′). The PCR primers for the genomic region corresponding to IDH2 exon 4 (which encodes codon R172) and the flanking intronic sequences were human IDH2 sense (5′-CA-AGCTGAAGAAGATGTGGAA-3′) and human IDH2 anti-sense (5′-CAGAGACAAGAGGATGGCTA-3′). The PCR conditions were 98 °C for 2 min (1 cycle), followed by 40 cycles of 98 °C for 10 s, 60 °C for 15 s, 68 °C for 30 s, and extension at 68 °C for 10 min with MightyAmp DNA polymerase (Takara Bio Inc.). Cycle sequencing was conducted using sequencing primers for IDH1 (5′-CCATTATCTGCAA AAATATC-3′) and IDH2 (5′-AGCCCATCATCTGCAAAA AC-3′). Subsequently, the PCR products were subcloned into pCR4-TOPO vectors (Life Technologies Inc., Carlsbad, CA), after which 20 clones were sequenced to confirm the IDH2-R172 mutations.

Statistical analysis

Overall survival was calculated from the time of surgery until death or the last follow-up examination; we used the Kaplan–Meier method and the log-rank test for comparison between groups. The Cox proportional hazards model was used to test prognostic factors in the univariate and multivariate analyses. Results are expressed as relative risk and its 95 % confidence interval (CI).

Results

IDH double IHC analyses

Figure 1 summarizes the results of IHC detection of IDH. Among 54 grade III gliomas, 27 (50 %) were positive for HMab-1. Direct sequencing of these positive 27 cases demonstrated that 26 were IDH1-R132H and 1 was of the IDH wild type. Subcloning of the sequence of this single IDH wild-type case demonstrated that it was IDH1-R132H. Among the 27 HMab-1-negative grade III gliomas, 3 were positive for MsMab-1 and the remaining 24 were negative. Direct sequencing of the 3 MsMab-1-positive specimens revealed IDH1-R132S in 1 of them and the IDH wild type in 2. Subcloning sequencing of these 2 wild-type specimens revealed that 1 was IDH2-R172 M and the other IDH2-R172S. Direct sequencing of the 24 MsMab-1-negative specimens revealed that they were all of the IDH wild type. Taken together, these findings show that, in this group of 54 grade III gliomas, IDH mutation was detected in 30 (55.6 %) using double IHC with HMab-1 and MsMab-1. IDH status was confirmed by sequence analysis in all cases. Representative cases demonstrating the usefulness of double IHC and the association of the results with those of sequencing are shown in Fig. 2. HMab-1 detected only IDH1-R132H (Fig. 2a), whereas MsMab-1 detected IDH1-R132S (Fig. 2e), IDH2-R172S (Fig. 2h), IDH2-R172 M (Fig. 2k), and the commonly found IDH1-R132H (Fig. 2b). HMab-1 did not detect IDH1-R132S (Fig. 2D), IDH2-R172S (Fig. 2g), and IDH2-R172 M (Fig. 2j). Neither HMab-1 nor MsMab-1 detected IDH1-wild type (Fig. 2m, n). HMab-1 was more sensitive than MsMab-1 without background staining for detecting IDH1-R132H. HMab-1 screening as a first step was essential.

Clinical significance of double IHC

In patients with 54 grade III gliomas, clinical parameters such as age, pathology, degree of tumor removal, and other pathological parameters such as MIB-1 positivity, number of vessels stained with vWF, and p53 expression were

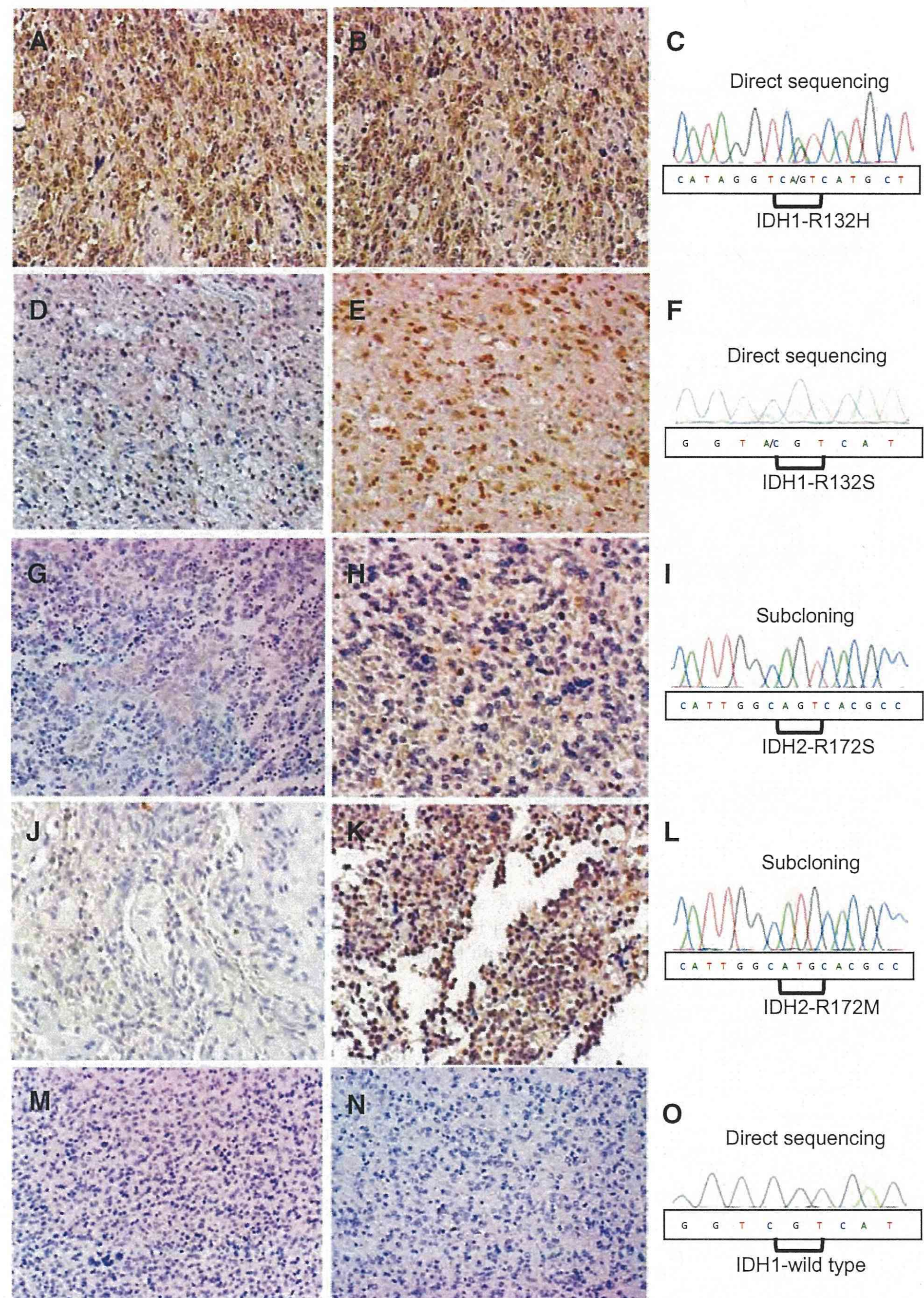

Fig. 2 Immunohistochemistry and sequencing: IDH1-R132H sample (**a**, **b**, **c**), IDH1-R132S sample (**d**, **e**, **f**), IDH2-R172S sample (**g**, **h**, **i**), IDH2-R172M sample (**j**, **k**, **l**) and IDH1 wild sample (**m**, **n**, **o**). HMab-1 staining (**a**, **d**, **g**, **j**, **m**), MsMab-1 staining (**b**, **e**, **h**, **k**, **n**), and sequence analyses (**c**, **f**, **i**, **l**, **o**). HMab-1 is positive for IDH1-R132H alone (**a**). MsMab-1 is positive for IDH1-R132H (**b**), IDH1-R132S (**e**), IDH2-R172S (**h**), and IDH2-R172M (**k**). Original magnification ×400

evaluated in addition to IDH mutation status to determine overall survival. Overall survival was first determined using the results of HMab-1 alone and then using the results of HMab-1–MsMab-1 double IHC. When we used the HMab-1 results alone, the Kaplan–Meier curve showed better survival in HMab-1-positive cases ($n = 27$: median overall survival 111.1 months) than in negative cases ($n = 27$: median overall survival 36.5 months): (log-rank test $P = 0.02103$, hazard ratio 0.464, 95 % CI 0.217–0.883) (Fig. 3). When we used the results of HMab-1–MsMab-1 double IHC (Fig. 4), the Kaplan–Meier curve showed better survival in IDH-positive cases ($n = 30$: median overall survival 111.1 months) than in negative cases ($n = 24$: median overall survival 33.9 months). Statistical significance was apparent with double IHC detection (log-rank test $P = 0.00997$, hazard ratio 0.428, 95 % CI 0.190–0.798). Overall survival was also evaluated using various clinical parameters (Table 1). IDH status

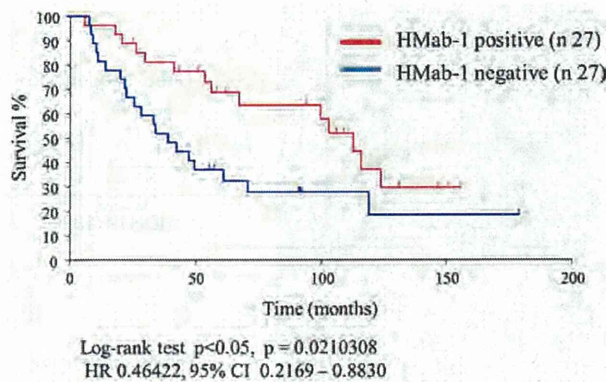

Fig. 3 Kaplan–Meier curve for overall survival with Grade III gliomas tested by HMab-1 immunohistochemistry

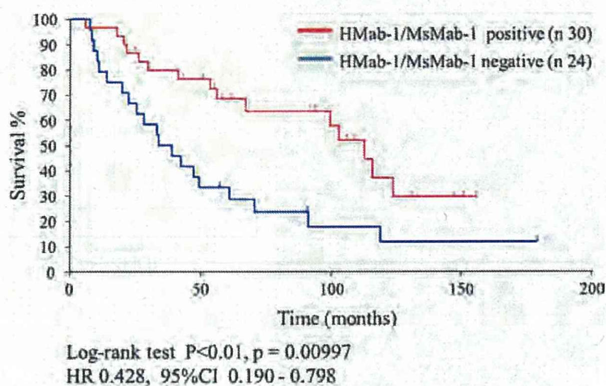

Fig. 4 Kaplan–Meier curve for overall survival with Grade III gliomas tested by double IHC HMab-1/MsMab-1 immunohistochemistry; Significant prognostic value is stronger with double immunohistochemistry compared to single HMab-1 (Fig. 3)

(wild type vs. mutant type) upon HMab-1–MsMab-1 double IHC, MIB1 positivity (≥10 % vs. <10 %), and degree of removal (total vs. other) were significant prognostic factors in the univariate and multivariate analyses.

Table 1 Prognostic value of grade III gliomas: cox hazard model

	p value	Hazard ratio	95% CI
Univariate analysis			
Age			
>50: 1	0.6094	1.186	0.616–2.284
<50: 0			
Pathology			
AA: 1	0.1968	1.539	0.800–2.961
AOA, AO:0			
IDH			
WT: 1	0.0359	2.001	1.047–3.858
mut: 0			
MIB1			
>10 %: 1	0.0232	2.197	1.113–4.334
<10 %: 0			
Density			
>30: 1	0.1512	1.630	0.836–3.176
<30: 0			
p53			
>10 %: 1	0.4176	1.313	0.680–2.536
<10 %: 0			
Removal			
Subtotal: 1	0.0151	4.348	1.329–14.221
Total: 0			
Multivariate analysis			
IDH			
WT: 1	0.0484	1.998	1.005–3.973
mut: 0			
MIB1			
>10 %: 1	0.0077	2.589	1.287–5.211
<10 %: 0			
Removal			
Subtotal: 1	0.0257	3.943	1.181–13.164
Total: 0			

Discussion

We demonstrated the usefulness of HMab-1–MsMab-1 double IHC in terms of two important features—prediction of grade III glioma prognosis and the specificity of IHC compared with direct sequencing. Double IHC resulted in stronger survival prediction than that obtained with HMab-1 detection alone. MsMab-1 IHC detected IDH1-R132S, IDH2-R172 M, and IDH2-R172S as well as IDH1-R132H in glioma samples. Testing for the presence of IDH1/2 mutations is now considered by international guidelines to be part of glioma management [14]. Current IDH mutation screening is performed with an IHC assay specific for IDH1-R132H, the most common mutation. Sequencing is recommended as a second-step test for IHC-negative or -equivocal cases [15]. Recently, a new IDH1/2 PCR assay has been reported as a useful part of the IDH mutation detection algorithm. The assay has a high technical success rate and is more sensitive than Sanger sequencing. Positive concordance rates are 98 % with IHC for IDH1-R132H detection and 100 % with sequencing. The PCR assay can be performed reliably on formalin-fixed paraffin-embedded samples [16, 17]. However, the need for strict quality control of DNA-based biomarker analyses of formalin-fixed, paraffin-embedded tumor samples has been pointed out—IDH sequencing procedures yielded inconsistent results in 2 out of 6 laboratories [18]. Quality assurance is,

therefore, pivotal before IDH testing is made part of the clinical management of patients [1619]. By contrast, IHC is an easy and quick method for detecting IDH1-R132H mutations without false negativity [20]. Furthermore, IHC for the IDH1-R132H mutation is very reliable and consistent across laboratories—it was very consistent across six laboratories, even though different staining procedures were used [21]. The differences in IHC included the use of automated procedures in some laboratories and manual IHC in others, as well as differences in antibody dilution and antibody incubation time and differences in the visualization systems used. Despite that, 100 % concordance was noted. Although almost all laboratories use the monoclonal antibody H09, we used HMab-1, which has shown IHC results concordant with those of H09 antibody in 94 (98.9 %) of 95 cases [4].

Using HMab-1 IHC, we found IDH1-R132H in 27 patients presenting with the R132H mutation (sensitivity 27/27, 100 % for this mutation). Using MsMab-1, we found other IDH mutations in 3 patients who presented with IDH1-R132S, IDH2-R172 M, or IDH2-R172S (specificity 30/30, 100 % for IDH mutation). None of the tumors presenting with the wild-type IDH gene were stained positively (specificity 24/24, 100 %). In a study by Capper et al. [7], direct sequencing was negative in eight cases (8/186) that were found positive by IHC. After resequencing of the tumors, these cases were found to have IDH1 mutations. Although IHC is an easy, quick, and reliable method of detecting IDH-R132H mutations, the most important problem in current IHC screening of IDH mutations is the limitations on detecting antibodies other than IDH1-R132H. Our previous data showed that IDH1-R132S in glioma samples was immunostained with IDH1-R132S-specific antibody (clone SMab-1) [12, 22]. Furthermore, we have developed MsMab-1, and IDH2-R172S was detected in osteosarcomas [11]. Therefore, our screening with a combination of HMab-1 and MsMab-1 is very useful. Double IHC screening was a more precise predictor of prognosis in grade III patients than was HMab-1 single screening. We propose that double IHC IDH screening, without the need for sequencing, can be used routinely in laboratories to manage decision-making on further treatment strategies. IDH testing has not yet been fully introduced into glioma treatment strategies. Van den Bent et al. [19] conducted a prospective randomized study in WHO grade III anaplastic oligodendrogliomas. They demonstrated that IDH1 mutations were of major prognostic significance in grade III oligodendrogliomas but were not predictive of treatment (PCV chemotherapy) response. Cairncross et al. [3] reported the importance of both IDH testing and 1p/19q LOH screening as an individualized approach to grade III gliomas. Reliable and routinely available IDH screening is needed for the treatment of grade III gliomas. Double IHC IDH mutation screening still has limitations, because MsMab-1 does not react with rare mutations such as IDH1-R132C and IDH2-R172K.

In conclusion, IDH screening is important in the clinical management of grade III glioma patients. IDH sequencing procedures are popular but not consistent across laboratories. By contrast, double IHC with HMab-1 and MsMab-1 for IDH screening has diagnostic advantage in detecting IDH1/2 mutations and is able to predict the survival of grade III glioma patients.

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Conflict of interest The authors declare no conflicts of interest.

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A novel monoclonal antibody SMab-2 recognizes endogenous IDH2-R172S of chondrosarcoma

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ABSTRACT

Isocitrate dehydrogenase 2 (IDH2) mutations have been reported in gliomas, osteosarcomas, cartilaginous tumors, giant cell tumors of bone, and acute myeloid leukemias. Although IDH2 catalyzes the oxidative carboxylation of isocitrate to α-ketoglutarate (α-KG) in mitochondria, mutated IDH2 proteins possess the ability to change α-KG into the oncometabolite R(-)-2-hydroxyglutarate (2-HG). To date, several monoclonal antibodies (mAbs) specific for IDH2 mutations have been established, such as KMab-1 against IDH2-R172K, MMab-1 against IDH2-R172M, and WMab-1 against IDH2-R172W. Although a multi-specific mAb MsMab-1 reacted with IDH2-R172G and IDH2-R172S, a mono-specific mAb against IDH2-R172S has not been established. In this study, we established a novel mAb SMab-2, which recognizes IDH2-R172S but not with wild type IDH2 in ELISA. Although SMab-2 reacted with both IDH1-R132S and IDH2-R172S expressed in *Escherichia coli*, it reacted with only IDH2-R172S expressed in U-2 OS osteosarcoma cells. Furthermore, SMab-2 recognized endogenous IDH2-R172S protein expressed in SW1353 chondrosarcoma cells in Western blot and immunocytochemical analyses. SMab-2 is expected to be useful for diagnosis of IDH2-R172S-bearing tumors.

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1. Introduction

Somatic mutations of isocitrate dehydrogenase 2 (IDH2) were first found in gliomas [1]. IDH2 convert α-ketoglutarate (α-KG) to oncometabolite R(-)-2-hydroxyglutarate (2-HG), although IDH2 catalyzes the oxidative carboxylation of isocitrate to α-ketoglutarate in mitochondria [2]. IDH2 mutations, which are specific to a single codon in arginine 172 residue (R172), were also reported in osteosarcomas [3], giant cell tumors of bone [4], and in cartilaginous tumors [5–7]. In contrast, IDH2 mutations of acute myeloid leukemias [8,9] were discovered in not only R172 but also arginine 140 residue (R140), which is found more frequently than R172 [10]. Equivocal microscopic diagnosis often occurs from several factors such as small sample size, sampling site, or sample quality. IDH status provides clinically important information in those cases especially in gliomas, because IDH status is correlated with glioma patient prognosis [1]. There are several methods for testing IDH status, which targets DNA sequence, protein, and 2-HG [11]. DNA direct sequencing (Sanger method) and immunohistochemistry are two conventional methods, which are applied for daily diagnostic practice. Recently, several special methods such as pyrosequencing [12], melting curve analysis [13], and magnetic resonance spectroscopy (MRS) [14] were developed; however, those methods need special equipment [11]. Although Sanger method detects all types of mutations, at least 20% of the mutant allele is required for detection of IDH mutations [12]. In contrast, immunohistochemistry detects only one mutation-bearing tumor cell; however, many types of monoclonal antibodies (mAbs) against IDH mutations should be developed. To date, we established several mono-specific anti-mutated IDH2 mAbs [15]: IDH2-R172-specific KMab-1 [16], IDH2-R172M-specific MMab-1 [16], and IDH2-R172W-specific WMab-1 [17]. We also established two multi-specific IDH mAbs: MsMab-1 [18] and MsMab-2 [19]. In this study, we report a novel

Abbreviations: IDH1/2, isocitrate dehydrogenase 1/2; mAb, monoclonal antibody.

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mAb SMab-2, which recognizes IDH2-R172S but not with wild type IDH.

2. Materials and methods

2.1. Cell lines

U-2 OS osteosarcoma cell line, SW1353 chondrosarcoma cell line, HeLa cervical adenocarcinoma cell lines, and P3X63Ag8U.1 (P3U1) mouse myeloma cell line were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. U-2 OS, SW1353, and HeLa were cultured in Dulbecco's modified Eagle medium (DMEM; Nacalai Tesque Inc., Kyoto, Japan) and P3U1 was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque Inc.), including 2 mM L-glutamine (Nacalai Tesque Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies Inc., Carlsbad, CA).

2.2. Hybridoma production

BALB/c mice were immunized by intraperitoneal (i.p.) injections of 125 μg of synthetic peptides of CGGVKPITIGSHAHGDQYKA (IDH2-R172S) or CNGTIQNILGG (IDH2-R140Q), conjugated with keyhole limpet hemocyanin (KLH) together with Imject Alum (Thermo Fisher Scientific Inc., Waltham, MA). One week later, secondary i.p. immunization of 62.5 μg of synthetic peptides was performed. After several additional immunizations of 62.5 μg of synthetic peptides, a booster injection was given i.p. 2 days before spleen cells were harvested. The spleen cells were fused with P3U1 cells using PEG1500 (Sigma-Aldrich Corp., St. Louis, MO). The hybridomas were grown in RPMI medium with hypoxanthine, aminopterin, and thymidine (HAT) selection medium supplement (Life Technologies Corp.). The culture supernatants were screened using enzyme-linked immunosorbent assay (ELISA).

2.3. Enzyme-linked immunosorbent assay (ELISA)

Synthetic peptides or recombinant proteins were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific Inc.) at 1 μg/ml for 30 min. Synthetic peptides are as follows: GGTKPITIGRHAHGDQYKA (IDH2-WT), GGTKPITIGSHAHGDQYKA (IDH2-R172S). After blocking with SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific Inc.), the plates were incubated with culture supernatant or purified mAbs (1 μg/ml) with subsequent 1:1000 diluted peroxidase-conjugated anti-mouse IgG (Dako, Glostrup, Denmark). The enzymatic reaction was conducted with 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific Inc.). The optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories Inc., Philadelphia, PA). These reactions were performed with a volume of 50 μl at 37 °C.

2.4. Protein expression using bacteria cells and mammalian cells

Competent *Escherichia coli* TOP-10 cells (Life Technologies Inc.) were transformed with appropriate amounts of plasmids, pMAL-IDH1-WT, pMAL-IDH1-R132H, pMAL-IDH1-R132C, pMAL-IDH1-R132S, pMAL-IDH1-R132G, pMAL-IDH1-R132L, pMAL-IDH2-WT, pMAL-IDH2-R172K, pMAL-IDH2-R172M, pMAL-IDH2-R172W, pMAL-IDH2-R172S, and pMAL-IDH2-R140Q [18]. Then, they were cultured overnight at 37 °C in LB medium (Life Technologies Inc.) containing 100 μg/ml ampicillin (Sigma–Aldrich Corp.). Cell pellets were resuspended in phosphate buffered saline (PBS) with 1% Triton X-100 with 50 μg/ml aprotinin (Sigma–Aldrich Corp.). After sonication using Branson Advanced Sonifier (Branson Ultrasonics Corp., Danbury, CT), the crude extracts were collected by centrifugation (9000 × g, 30 min, 4 °C). The supernatants were loaded onto amylose resin (New England Biolabs Inc.). The loaded resins were washed extensively with column buffer (20 mM Tris–HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA). The fusion proteins were eluted by column buffer with 10 mM maltose. Then U-2 OS cells were transfected with appropriate amounts of plasmids, pcDNA3.1VH/IDH1-WT, pcDNA3.1VH/IDH1-R132H, pcDNA3.1VH/IDH1-R132C, pcDNA3.1VH/IDH1-R132S, pcDNA3.1VH/IDH1-R132G, pcDNA3.1VH/IDH1-R132L, pcDNA3-PAcH/IDH2-WT, pcDNA3-PAcH/IDH2-R172K, pcDNA3-PAcH/IDH2-R172M, pcDNA3-PAcH/IDH2-R172W, pcDNA3-PAcH/IDH2-R172S, pcDNA3-PAcH/IDH2-R172G using Lipofectamine LTX (Life Technologies Inc.) according to the manufacturer's instructions [3]. The expression level of IDH1/2 was confirmed using Western blot analyses.

2.5. Western blot analyses

Cultured cell pellets were lysed with 1% RIPA buffer (Thermo Fisher Scientific Inc.) for 15 min on ice. The lysate supernatants were centrifuged for 15 min at 15,000 rpm to remove cellular debris. Cell lysates were prepared for Western blot analyses by boiling in SDS sample buffer (Nacalai Tesque, Inc.). They were electrophoresed on 5–20% polyacrylamide gels (Wako Pure Chemical Industries Ltd.). The separated proteins were transferred to a PVDF membrane (EMD Millipore Corp., Billerica, MA). After blocking with 4% skim milk in PBS with 0.05% Tween 20 for 15 min, the membrane was incubated with 10 μg/ml of SMab-2, 5 μg/ml of RqMab-3, or 1 μg/ml of SMab-1 [20], RcMab-1 [19], 5F11 (anti-IDH2; Sigma–Aldrich Corp.), 1H6 (anti-V5 tag; Medical & Biological Laboratories Co. Ltd., Nagoya, Japan), NZ-1 (anti-PA tag) [21,22], and AC-15 (anti-β-actin; Sigma–Aldrich Corp.) for 60 min. Then the membrane was incubated with peroxidase-conjugated secondary antibodies (1:1000 diluted; Dako) for 30 min, and developed with ImmunoStar LD Chemiluminescence Reagent (Wako Pure Chemical Industries, Ltd., Osaka, Japan) using Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

2.6. Direct DNA sequencing of IDH2 mutation

Genomic DNA was extracted from SW1353 chondrosarcoma cell line according to the manufacturer's instructions (Takara Bio Inc., Shiga, Japan). PCR primers for the genomic region corresponding to IDH2 exon 4, which encodes codon R172, and the flanking intronic sequences were the following: human IDH2 sense (5′-CAAGCTGAAGAAGATGTGGAA-3′) and human IDH2 antisense (5′-CAGAGACAAGAGGATGGCTA-3′). The PCR conditions were 98 °C for 2 min (1 cycle), followed by 40 cycles of 98 °C for 10 s, 60 °C for 15 s, 68 °C for 30 s, and extension at 68 °C for 10 min with MightyAmp DNA polymerase (Takara Bio Inc.). Cycle sequencing was conducted using the sequencing primer for IDH2 (5′-AGCCCATCATCTGCAAAAAC-3′).

2.7. Droplet digital PCR analysis

The droplet digital PCR analysis was performed as described previously [23]. Briefly, the TaqMan PCR reaction mixture was assembled from a 2 × ddPCR Supermix (Bio-Rad Laboratories Inc.), 20 × primer and probe (wild type), 20 × primer and probe (IDH2-R172S), and template in a final volume of 20 μl. Each assembled ddPCR reaction mixture was then loaded into the sample well of an eight-channel disposable droplet generator cartridge (Bio-Rad Laboratories Inc.). A volume of 70 μl of droplet generation oil (Bio-Rad Laboratories Inc.) was loaded into the oil well for each channel. The cartridge was placed into the droplet generator (Bio-Rad Laboratories Inc.). The cartridge was removed from the droplet

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