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Establishment of novel monoclonal antibodies KMab-1 and MMab-1 specific for IDH2 mutations

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ABSTRACT

Isocitrate dehydrogenase 1/2 (IDH1/2) mutations have been detected in gliomas, cartilaginous tumors, and leukemias. IDH1/2 mutations are early and frequent genetic alterations, are specific to a single codon in the conserved and functionally important Arginine 132 (R132) in IDH1 and Arginine 172 (R172) in IDH2. We previously established several monoclonal antibodies (mAbs), which are specific for IDH1 mutations: clones IMab-1 or HMab-1 against IDH1-R132H or clone SMab-1 against IDH1-R132S. However, specific mAbs against IDH2 mutations have not been reported. To establish IDH2-mutation-specific mAbs, we immunized mice or rats with each mutation-containing IDH2 peptides including IDH2-R172K and IDH2-R172M. After cell fusion, IDH2 mutation-specific mAbs were screened in Enzyme-Linked Immunosorbent Assay (ELISA). Established mAbs KMab-1 and MMab-1 reacted with the IDH2-R172K and IDH2-R172M peptides, respectively, but not with IDH2-wild type (WT) in ELISA. Western-blot analysis also showed that KMab-1 and MMab-1 reacted with the IDH2-R172K and IDH2-R172M recombinant proteins, respectively, not with IDH2-WT or other IDH2 mutants, indicating that KMab-1 and MMab-1 are IDH2-mutation-specific. Furthermore, MMab-1 specifically stained the IDH2-R172M-expressing cells in immunocytochemistry, but did not stain IDH2-WT and other IDH2-mutation-containing cells. In immunohistochemical analysis, MMab-1 specifically stained IDH2-R172M-expressing glioma. This is the first report to establish anti-IDH2-mutation-specific mAbs, which could be useful in diagnosis of mutation-bearing tumors.

1. Introduction

Isocitrate dehydrogenase 1/2 (IDH1/2) mutations have been detected in some gliomas [1–4], cartilaginous tumors [5–7], and acute myeloid leukemias (AML) [8,9]. In astrocytomas, oligodendrogliomas, oligoastrocytomas, and secondary glioblastomas, IDH1/2 mutations have been identified as early and frequent genetic alterations (IDH1: 50–93%; IDH2: 3–5%), and might be the initiating event in these glioma subtypes [1–4]. In contrast, primary glioblastomas rarely contain IDH1/2 mutations. The IDH mutations are remarkably specific to a single codon in the conserved and functionally important Arginine 132 residue (R132) in IDH1 and Arginine 172 residue (R172) in IDH2 in gliomas. Of interest, IDH1/2 mutations were observed subsequently in 19–22% of AML, because the IDH mutations were also discovered in Arginine 140 residue (R140) of IDH2, which are more frequent than R172 [8,9]. IDH1/2 mutations were found to result in the ability of the enzyme to catalyze the reduced NADP-dependent reduction of α-ketoglutarate to onco-metabolite R(−)-2-hydroxyglutarate (2-HG) [10]. Excess accumulation of 2-HG leads to an elevated risk of malignant brain tumors in patients with inborn errors of 2-HG metabolism.

Abbreviations: IDH2, isocitrate dehydrogenase 2; mAb, monoclonal antibody.

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To date, four monoclonal antibodies (mAbs) against IDH1 mutations have been reported [11–15]. Of those antibodies, we established IMab-1 and HMab-1, which are specific for IDH1-R132H, the most frequent IDH1 mutations (>90%) [14] and SMab-1: anti-IDH1-R132S-specific mAb [13]. IMab-1 and HMab-1 reacted with the IDH1-R132H peptide, but not with the wild type IDH1 (IDH-WT) peptide in ELISA. In Western-blot analysis, IMab-1 and HMab-1 reacted only with the IDH1-R132H protein, not the IDH1-WT protein or other IDH1 mutants. SMab-1 reacted with IDH1-R132S specifically in the same way [13]. In our recent study, 164 cases of glioma were evaluated immunohistochemically for IDH1 mutations (R132H and R132S) using anti-IDH1 mAbs: HMab-1 and SMab-1 [11]. 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. IDH1 mutation as evaluated using immunohistochemistry might be of greater prognostic significance than histological grading alone in grade III and grade IV gliomas.

Although HMab-1 and SMab-1 are able to find more than 90% of IDH1 mutations of gliomas in immunohistochemistry, IDH2 mutations, which are detected in 3–5% of gliomas, have been missed unfortunately in immunohistochemistry. Therefore, novel antibodies that recognize IDH2-mutations should be developed to cover all of IDH1/2 mutations in immunohistochemistry. Here, we newly report anti-IDH2-mutation-specific monoclonal antibodies (KMab-1 and MMab-1), which are expected to be extremely useful for diagnosis of mutation-bearing gliomas.

2. Materials and methods

2.1. Cell lines and tissues

P3X63Ag8U.1 (P3U1), Sp2/0, and Chinese hamster ovary (CHO)-K1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). These cell lines were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air in RPMI 1640 medium (Wako Pure Chemical Industries Ltd., Osaka, Japan), including 2 mM L-glutamine (Wako Pure Chemical Industries Ltd.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies Corp., Carlsbad, CA). One glioma patient (anaplastic astrocytoma, WHO grade III), who underwent primary surgery at Tohoku University Hospital and one glioma patient (glioblastoma, WHO grade IV), who underwent primary surgery at Tsukuba University Hospital, were included in this study. Informed consent was obtained from each patient or the patient's guardian for obtaining samples and for subsequent data analysis.

2.2. Hybridoma production

One mouse monoclonal antibody, RMab-22 against IDH2-wild type and one rat monoclonal antibody against Maltose-binding protein (MBP) were produced using traditional spleen method [13]. Briefly, BALB/c mice or SD rats (CLEA Japan Inc., Tokyo, Japan) were immunized by i.p. injections of 30 μg of synthetic peptide CKPITIGKHAHGD (IDH2-R172K peptide), corresponding to amino acids 166–177 of human IDH2-R172K plus N-terminus cysteine conjugated with KLH or 100 μg of recombinant MBP protein, respectively, together with Imject Alum (Thermo Scientific Inc., Rockford, IL). One week later, secondary i.p. immunization of 30 μg of IDH2-R172K peptide or 100 μg of recombinant MBP protein was performed. After several additional immunization of 30 μg of IDH2-R172K peptide or 100 μg of recombinant MBP protein, a booster injection was given i.p. 2 days before spleen cells were harvested. The spleen cells were fused with mouse myeloma P3U1 cells using Sendai virus (Hemagglutinating Virus of Japan: HVJ) envelope: GenomONE-CF (Ishihara Sangyo Kaisha Ltd., Osaka, Japan) according to the manufacturer's instructions [11,13,14]. The hybridomas were grown in RPMI medium with hypoxanthine, aminopterin, and thymidine selection medium supplement (Life Technologies Corp.). The culture supernatants were screened using ELISA for binding to the both IDH2-R172K and IDH2-wild type (IDH2-WT) peptides, as described previously [13]. Two rat monoclonal antibodies (KMab-1 and MMab-1) against IDH2-mutations and one mouse monoclonal antibody (KrMab-3) against IDH2-WT were produced using rat or mouse medial iliac lymph node methods [16]. Briefly, WKY/Izm rats and BDF1 mice (Japan SLC Inc., Shizuoka, Japan) were immunized by injecting 170 μg or 80 μg of synthetic peptides of CGGTKPITIGKHAHGDQYKA (IDH2-R172K) and CGGTKPITIGMHAHGDQYKA (IDH2-R172M), conjugated with KLH together with Freund's complete adjuvant (FCA; Sigma–Aldrich, St. Louis, MO) into footpad or tailbase, respectively. The lymphocytes were fused with mouse myeloma Sp2/0 cells using polyethylene glycol (PEG) methods. The culture supernatants were screened using ELISA for binding to the IDH2-mutations (IDH2-R172K and IDH2-R172M) and IDH2-WT peptides, conjugated with BSA.

2.3. Enzyme-Linked Immunosorbent Assay (ELISA)

Synthetic peptides were immobilized, respectively, on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific Inc., Waltham, MA) at 1 μg/ml for 30 min, respectively. After blocking with 1% BSA (Sigma–Aldrich) in PBS, the plates were incubated with culture supernatant, followed by 1:1000 diluted peroxidase-conjugated anti-mouse IgG (Dako, Glostrup, Denmark). The enzymatic reaction was conducted with a substrate solution containing TMB (Thermo Fisher Scientific Inc.). After the reaction was stopped with 2 M H_2SO_4, the optical density was measured at 450 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.

2.4. Plasmid preparation

Human IDH2 cDNA (Accession No. NM_002168) encoding a full-length open reading frame (ORF) was obtained by PCR using a cDNA derived from the U373 glioblastoma cell line [17] as a template. The primer set for IDH2 was as follows: EcoRI-IDH2.F1, 5′-ccGAATTCgggATGGCCGGCTACCTGCGGG-3′ and SalI-IDH2wterR1359, 5′-gccgtcgacCTACTGCCTGCCCAGGGCTCT-3′. The amplified cDNA was subcloned into a pcDNA3.1/V5-His-TOPO vector (Life Technologies Corp.). Substitution of the Arginine 172 (R172) to appropriate amino acid codons (Lysine, Methionine, or Tryptophan) in IDH2 cDNAs was accomplished using the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). The full-length IDH2-WT, IDH2-R172K, IDH2-R172M, and IDH2-R172W were subcloned into an expression vector, pMAL-c2 (New England Biolabs Inc., Beverly, MA), via EcoRI and SalI restriction sites. The full-length IDH2 and each mutated ORF were amplified using the primer set: EcoRI-IDH2.F1 and IDH2wotерR1356-XhoI, 5′- taaCTCGAGcgCTGCCTGCCCAGGGCTCTG-3′. These PCR products were digested with EcoRI and XhoI restriction enzymes, and subcloned into pcDNA3 vector (Life Technologies Corp.) together with the nucleotide sequence (ctcgagTGGCGTTGCCATGCCA GGTGCCGAAGATGATGTGGTGTAAtctaga), which encodes 12 amino acids, GVAMPGAEDDVV (PA tag).

2.5. Protein expression using bacteria cells and mammalian cells

Competent *Escherichia coli* TOP-10 cells (Life Technologies Corp.) were transformed with the plasmid, pMAL-IDH2-WT,

pMAL-IDH2-R172K, pMAL-IDH2-R172M, and pMAL-IDH2-R172W. Then, they were cultured overnight at 37 °C in LB medium (Life Technologies Corp.) containing 100 μg/ml ampicillin (Sigma–Aldrich). Cell pellets were resuspended in phosphate buffered saline (PBS) with 1% Triton X-100 with 50 μg/ml aprotinin (Sigma–Aldrich). After sonication, 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), and the fusion proteins were eluted by column buffer with 10 mM maltose. CHO cells were transfected with appropriate amounts of plasmids, pcDNA3/IDH2-WT, pcDNA3/IDH2-R172K, pcDNA3/IDH2-R172M, or pcDNA3/IDH2-R172W using Lipofectamine LTX (Life Technologies Corp.) according to the manufacturer's instructions. Stable transfectants were selected by cultivating the transfectants in medium containing 1 mg/ml of G418 (Wako Pure Chemical Industries Ltd.). The expression level of IDH2 was confirmed using Western-blot analysis.

2.6. Western-blot analyses

Cultured cell pellets were lysed with PBS with 1% TritonX-100 for 30 min on ice. The lysate supernatants were centrifuged for 15 min at 15,000 rpm to remove cellular debris. Cell lysates containing 10 μg of total protein were prepared for Western-blot analysis by boiling in SDS sample buffer (Nakalai Tesque, Inc., Kyoto, Japan). They were electrophoresed on 5–20% polyacrylamide gels (Wako Pure Chemical Industries Ltd.). The separated proteins were transferred to a PVDF membrane (Bio-Rad Laboratories Inc.). After blocking with 4% skim milk in PBS for 15 min, the membrane was incubated with KMab-1 (5 μg/ml), MMab-1 (1 μg/ml), RMab-22 (5 μg/ml), KrMab-3 (5 μg/ml), TMab-2 (anti-MBP tag; 1 μg/ml), or NZ-1 (anti-PA tag; 1 μg/ml) for 15 min; then with peroxidase-conjugated secondary antibodies (Dako, 1/1000 diluted) for 15 min, and developed with ECL-plus reagents (GE Healthcare, Buckinghamshire, UK) using SAYAKA-IMAGER (DRC Co. Ltd., Tokyo, Japan).

2.7. Immunocytochemical (ICC) analyses

Cultured cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 min at room temperature, and permeabilized with 0.1% Triton X-100 in PBS for 15 min. Cells were then treated with 10% normal goat serum in PBS (NGS/PBS) to block nonspecific binding sites, and were incubated with 5 μg/ml of MMab-1, RMab-22, KrMab-3, or anti-PA tag (NZ-1) containing 0.1% Triton X-100 overnight at 4 °C in a moist chamber. They were incubated with goat anti-mouse or rat IgG-Alexa 488 (dilution 1:400; Molecular Probes Inc., Eugene, OR) in PBS containing 0.1% Triton X-100 for 1 h at room temperature. Cells were also treated with TO-PRO-3 (Molecular Probes) to stain the cell nuclei. They were examined using confocal laser-scanning microscopy (LSM5 PASCAL; Carl Zeiss Inc., Jena, Germany). Then fluorescent images were processed using image-processing software (Adobe Photoshop; Adobe Systems Inc.).

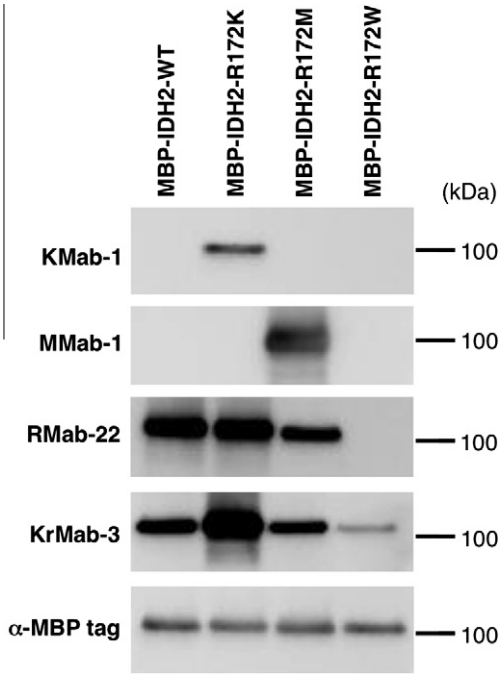

Fig. 1. Western-blot analyses by anti-IDH2 mAbs against MBP-fusion proteins. Purified proteins (0.05 μg/lane) of *E. coli*-expressing IDH2 wild type (WT; lane 1) and IDH2 mutants (lane 2, IDH2-R172K; lane 3, IDH2-R172M; lane 4, IDH2-R172W) were electrophoresed under reducing conditions using 5–20% gels, and were Western-blotted with KMab-1, MMab-1, RMab-22, KrMab-3, and anti-MBP tag (TMab-2).

2.8. Immunohistochemical (IHC) analysis

IDH2-R172M protein expression was determined immunohistochemically in paraffin-embedded tumor specimens. Briefly, 4-μm-thick histologic sections were deparaffinized in xylene and rehydrated. Then, they were autoclaved in citrate buffer (pH 6.0) for 20 min. Sections were incubated with 10 μg/ml of MMab-1 for 1 h at room temperature. Biotin-conjugated secondary anti-rat IgG (Dako) was incubated for 30 min at room temperature followed by the peroxidase-conjugated biotin-streptavidin complex (Vectastain ABC Kit; Vector Laboratories Inc., Burlingame, CA) for 30 min at room temperature. Color was developed using 3, 3-diaminobenzidine tetrahydrochloride (DAB) for 10 min, and counterstained with hematoxylin.

3. Results and discussion

3.1. Production of mutated IDH2-specific antibodies

The *IDH2* gene at 15q26.1 encodes isocitrate dehydrogenase 2 (IDH2), which catalyzes the oxidative carboxylation of isocitrate to a-ketoglutarate in mitochondria [18]. IDH2 mutations occur in some malignant gliomas [2], cartilaginous tumors [5,6], and in acute myeloid leukemias [8]. To date, almost all IDH2 mutations have been identified at the critical isocitrate binding site R172 co-

Table 1
Reactivities of anti-IDH2 monoclonal antibodies against antigens.

Antigen	Clone	Speices	Subclass	ELISA	WB (*E. coli*)	WB (CHO)	ICC
IDH2-R172K	KMab-1	Rat	IgG2b, kappa	+++	+++	+	–
IDH2-R172M	MMab-1	Rat	IgG2b, kappa	+++	+++	+++	+++
IDH2-wild type	RMab-22	Mouse	IgG2b, kappa	+++	+++	++	++
IDH2-wild type	KrMab-3	Mouse	IgG2b, kappa	+++	+++	++	++

+++: high reactivity; ++: medium reactivity; +: low reactivity; –: negative.

don and R140 codon. The vast majority of changes of IDH2 are heterozygous in the same way of IDH1 mutation [1–4]. These discoveries mark unique findings in the field of cancer genetics.

We have established three anti-mutated IDH1 mAbs: IMab-1 [14] or HMab-1 [11] against IDH1-R132H and SMab-1 [13] against IDH1-R132S. Hartmann et al. reported that IDH1 mutations include R132H (664/716: 92.7%), R132C (29/716: 4.2%), R132S (11/716: 1.5%), R132G (10/716: 1.4%), and R132L (2/716: 0.2%); therefore, combination of HMab-1 and SMab-1 cover 94.2% of IDH1 mutations. However, specific antibodies against IDH2 mutations have not been reported. Hartmann et al. also reported that IDH2 mutations include R172K (20/31: 64.5%), R172M (6/31: 19.3%), and R172W (5/31: 16.2%) in gliomas [19]. For this study, we immunized mice or rats with synthetic peptides of several IDH2 mutants including R172K and R172M. After cell fusion, the wells of hybridomas, which produced IDH2-R172K-reactive/IDH2-wild type (WT)-nonreactive or IDH2-R172M-reactive/IDH2-WT-nonreactive were screened in ELISA. After limiting dilution, clone KMab-1 (rat IgG_{2b} subclass) against IDH2-R172K and clone MMab-1 (rat IgG_{2b} subclass) against IDH2-R172M were established (Table 1). Two anti-IDH2-WT mAbs, clone RMab-22 (mouse IgG_{2b}) and clone KrMab-3 (mouse IgG_{2b}), were also established from R172K-immunized mice (Table 1). KMab-1 and MMab-1 were shown to be each IDH2 mutation-specific mAbs, not reacting with IDH2-WT, using ELISA against both synthetic peptides and recombinant proteins (data not shown). In contrast, RMab-22 and KrMab-3 were shown to be reactive with both IDH2-R172K and IDH2-WT using ELISA against both synthetic peptides and recombinant proteins (data not shown).

3.2. Specificity of anti-mutated IDH2 mAbs against IDH2 mutants

To determine the specificity of KMab-1, MMab-1, RMab-22, and KrMab-3 mAbs, the reactivity against IDH2-WT and IDH2 mutants (MBP-IDH2-R172K, MBP-IDH2-R172M, MBP-IDH2-R172W) were investigated using Western-blot analyses. Fig. 1 shows that KMab-1 recognized only IDH2-R172K protein, not the other proteins (IDH2-WT, IDH2-R172M, IDH2-R172W), indicating that KMab-1 is a specific antibody against IDH2-R172K protein. In contrast, MMab-1 reacted with only IDH2-R172M protein, not the other proteins (IDH2-WT, IDH2-R172K, IDH2-R172W), indicating that MMab-1 is a specific antibody against IDH2-R172M protein. RMab-22 recognized IDH2-WT, IDH2-R172K, and IDH2-R172M proteins, not IDH2-R172W protein. In contrast, KrMab-3 recognized all IDH2 mutants, although the reactivity against IDH2-R172W is comparatively weak. Both RMab-22 and KrMab-3 were produced from IDH2-R172K-immunized mice; therefore, both mAbs might be able to react strongly with IDH2-R172K compared with IDH2-R172M or IDH2-R172W. These results indicate that KMab-1 and MMab-1 are useful in detecting IDH2-R172K or IDH2-R172M, respectively.

We next performed Western-blot analyses against mutated IDH2-expressing CHO cells. As shown in Fig. 2A, KMab-1 recognized only IDH2-R172K protein expressed in CHO cells, not the other proteins (IDH2-WT, IDH2-R172M, IDH2-R172W). MMab-1 recognized only IDH2-R172M protein expressed in CHO cells, not the other proteins (IDH2-WT, IDH2-R172K, IDH2-R172W). These results indicate that KMab-1 and MMab-1 are useful in detecting IDH2-R172K or IDH2-R172M expressed in mammalian cells,

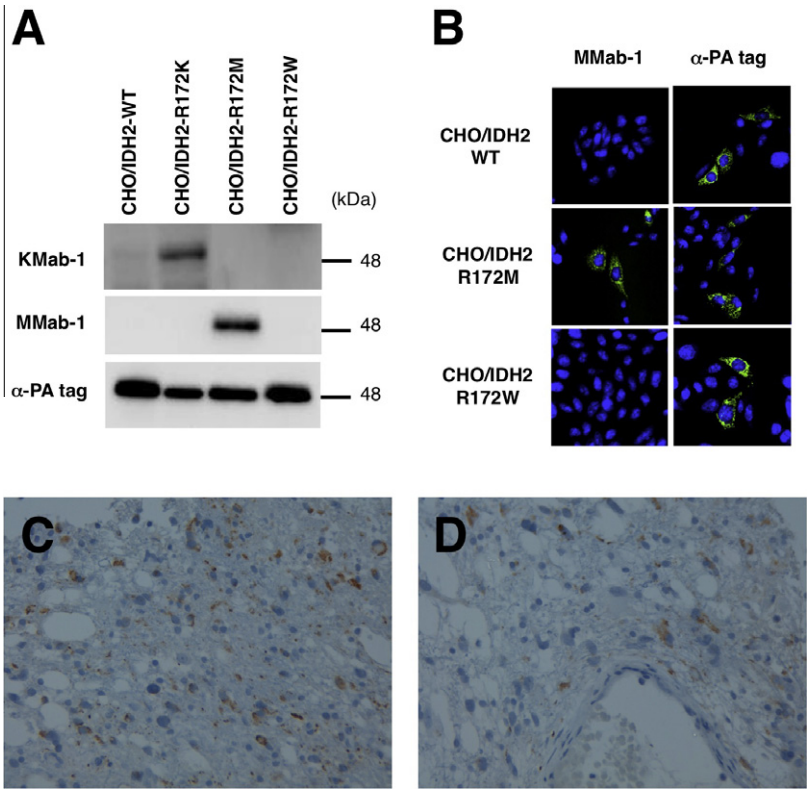

Fig. 2. The specificity of anti-mutated IDH2 mAbs. (A) Western-blot analyses by anti-IDH2 mAbs against mutated IDH2-expressing CHO cells. Total cell lysate from CHO cells expressing IDH2 wild type (WT; lane 1) and IDH2 mutants (lane 2, IDH2-R172K; lane 3, IDH2-R172M; lane 4, IDH2-R172W) were electrophoresed under reducing conditions using 5–20% gels, and were Western-blotted with KMab-1, MMab-1, and anti-PA tag (NZ-1). (B) Immunocytochemical analysis by anti-mutated IDH2 mAbs against mutated IDH2-transfected CHO cells. CHO cells were transfected by mutated IDH2. Each cell was stained by MMab-1 or anti-PA tag (NZ-1) followed by anti-rat IgG-Alexa 488 antibody (green). Cells were also treated with TO-PRO-3 to stain the cell nuclei (blue). (C and D) Immunohistochemical analysis by MMab-1 against one glioma tissue. Glioma tissue having IDH2-R172M was stained with MMab-1. Magnification: ×400. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

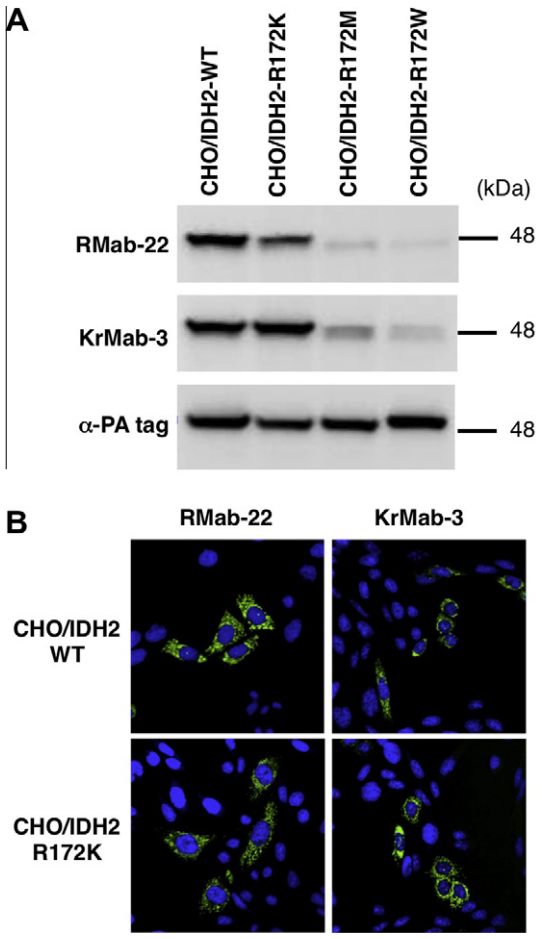

Fig. 3. The specificity of anti-IDH2-wild type (WT) mAbs. (A) Western-blot analyses by anti-IDH2 mAbs against mutated IDH2-expressing CHO cells. Total cell lysate from CHO cells expressing IDH2-WT (lane 1) and IDH2 mutants (lane 2, IDH2-R172K; lane 3, IDH2-R172M; lane 4, IDH2-R172W) were electrophoresed under reducing conditions using 5–20% gels, and were Western-blotted with RMab-22, KrMab-3, and anti-PA tag (NZ-1). (B) Immunocytochemical analysis by anti-IDH2 mAbs against mutated IDH2-transfected CHO cells. CHO cells were transfected by IDH2-WT and IDH2-R172K. Each cell was stained by RMab-22 and KrMab-3, followed by anti-mouse IgG-Alexa 488 antibody (green). Cells were also treated with TO-PRO-3 to stain the cell nuclei (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

respectively. As shown in Fig. 3A, both RMab-22 and KrMab-3 strongly recognized only IDH2-WT and IDH2-R172K. Although KrMab-3 detects both endogenous hamster IDH2 and exogenous human IDH2 in cell lysate from CHO/IDH2-R172M and CHO/IDH2-R172W, RMab-22 slightly reacts with only endogenous hamster IDH2 in cell lysate from CHO/IDH2-R172M and CHO/IDH2-R172W. Because an anti-PA tag antibody (NZ-1) equally reacts with all mutated IDH2 proteins from each cell lysate, RMab-22 and KrMab-3 are able to recognize IDH2-R172K more strongly than IDH2-R172M and IDH2-R172W.

3.3. Immunocytochemical (ICC) analyses by anti-mutated IDH2 mAbs against mutated IDH2-expressing cell lines

Newly established anti-IDH2 mAbs, KMab-1 and MMab-1 were confirmed to be anti-mutated IDH2 specific antibodies in Western-blot analyses; therefore, we performed immunocytochemical (ICC) analyses using IDH2-R172K- or IDH2-R172M-transfected CHO cells. Results show that MMab-1 reacted with IDH2-R172M-transfected cells; however, the reaction of KMab-1 against IDH2-R172K was not observed (Table 1). To investigate the reason why KMab-1 did not react with IDH2-R172K in ICC, dissociation constant (K_D) was determined using ELISA method [20]. As a result, K_D of KMab-1 was determined to be 1.5×10^{-7} M against recombinant protein of IDH2-R172K, whereas that of KMab-1 was 2.8×10^{-9} M against synthetic peptide IDH2-R172K, indicating that the binding-affinity of KMab-1 is extremely low against IDH2-R172K protein. Affinity maturation of KMab-1 should be necessary to increase the binding affinity of KMab-1 against IDH2-R172K protein. RMab-22 and KrMab-3 reacted with IDH2-WT-expressing CHO cells, suggesting that both RMab-22 and KrMab-3 are also useful in ICC (Table 1). Next, we investigated the specificity of MMab-1 against mutated IDH2-expressing CHO cells. As depicted in Fig. 2B, MMab-1 reacted with only CHO/IDH2-R172M, not with CHO/IDH2-WT and CHO/IDH2-R172W. Immunostaining of MMab-1 for CHO/IDH2-R172M demonstrated cytosolic patterns. These results indicate that MMab-1 is useful in detecting IDH2-R172M, suggesting that MMab-1 could be also useful in immunohistochemical analyses for detection of IDH2-R172M. In the same way with Western-blot analyses, both RMab-22 and KrMab-3 reacted with IDH2-WT and IDH2-R172K (Fig. 3B).

3.4. Immunohistochemical (IHC) analysis by MMab-1 against IDH2-R172M-bearing gliomas

We next performed immunohistochemistry of MMab-1 against IDH2-R172M-positive gliomas. Typical results are shown in Fig. 2C and D. MMab-1 stained almost all tumor cells of IDH2-R172M-positive gliomas (Fig. 2C), although no staining was observed in IDH2-WT gliomas (data not shown). Further, MMab-1 did not stain endothelial cells in gliomas (Fig. 2D). These results indicate that MMab-1 is useful in immunohistochemical analyses for detection of IDH2-R172M mutation. In near future, combination of MMab-1 with previously established anti-mutated IDH1 mAbs (IMab-1, HMab-1, and SMab-1) should increase the diagnostic rate using immunohistochemistry against glioma patients.

In conclusion, the established anti-mutated IDH2-specific mAbs are expected to be extremely useful for diagnosis and biological evaluation of mutation-bearing gliomas in combination with anti-mutated IDH1-specific monoclonal antibodies. Additional IDH1/2 mutation-specific antibodies such as anti-IDH1-R132G, anti-IDH1-R132L, or anti-IDH1-R132C must be developed for pathological diagnosis of gliomas, cartilaginous tumors, and AML.

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