



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



Xing Liu ^{a, b}, Satoshi Ogasawara ^a, Mika K. Kaneko ^a, Hiroharu Oki ^{a, b}, Yasukazu Hozumi ^c, Kaoru Goto ^c, Michiaki Takagi ^b, Yukinari Kato ^{a, *}

^a Department of Regional Innovation, Tohoku University Graduate School of Medicine, 2-1 Seiryomachi, Aoba-ku, Sendai, Miyagi 980-8575, Japan

^b Department of Orthopaedic Surgery, Yamagata University Faculty of Medicine, 2-2-2 Iida-nishi, Yamagata 990-9585, Japan

^c Department of Anatomy and Cell Biology, Yamagata University Faculty of Medicine, 2-2-2 Iida-nishi, Yamagata 990-9585, Japan

ARTICLE INFO

Article history:

Received 25 February 2015

Available online 6 March 2015

Keywords:

IDH2

IDH2 mutation

R172S

Monoclonal antibody

SMab-2

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

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

* Corresponding author. Fax: +81 22 717 8207.

E-mail addresses: yukinari-k@bea.hi-ho.ne.jp, yukinarikato@med.tohoku.ac.jp (Y. Kato).

<http://dx.doi.org/10.1016/j.bbrc.2015.02.162>

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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-R172K-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

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% CO₂ 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 CGGVKPTIGSHAHGDDQYKA (IDH2-R172S) or CMWKSPTNGTIQNILGGTVFR (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 (Roche Diagnostics, Indianapolis, IN). The hybridomas were grown in RPMI1640 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., St. Louis, MO). 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-PAC/IDH2-WT, pcDNA3-PAC/IDH2-R172K, pcDNA3-PAC/IDH2-R172M, pcDNA3-PAC/IDH2-R172W, pcDNA3-PAC/IDH2-R172S, pcDNA3-PAC/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'-CAAGCT-GAAGAAGATGTGGAA-3') and human IDH2 antisense (5'-CAGAGA-CAAGAGGATGGCTA-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

generator, where the droplets were then manually transferred with a multichannel pipet to a 96-well PCR plate. The plate was heat-sealed with a foil seal and then placed on a conventional thermal cycler and amplified to the end-point (39 cycles). After PCR, the 96-well PCR plate was loaded on the droplet reader (Bio-Rad Laboratories Inc.). Analysis of the ddPCR data was performed with QuantaSoft analysis software (Bio-Rad Laboratories Inc.) that accompanied the droplet reader.

2.8. Immunocytochemical analyses

Cultured cells were fixed with 4% paraformaldehyde (Wako Pure Chemical Industries Ltd.) 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 30 µg/ml of SMab-2 or control (PBS) containing 0.1% Triton X-100 overnight at 4 °C in a moist chamber. They were incubated with goat anti-mouse IgG-Alexa 488 (dilution 1:200; Life Technologies Corp.) in PBS containing 0.1% Triton X-100 for 1 h at room temperature. Cells were also treated with TO-PRO-3

(Life Technologies Inc.) to stain the cell nuclei. They were examined using confocal laser-scanning microscopy (LSM700; Carl Zeiss Inc., Jena, Germany). Then fluorescent images were processed using image-processing software (Adobe Photoshop; Adobe Systems Inc.).

3. Results

3.1. Production of a mutated IDH2-R172S-specific monoclonal antibody

We immunized mice with synthetic peptides of IDH2-R172S, and screened IDH2-R172S-reactive/IDH2-wild type (WT)-non-reactive mAbs using ELISA. After limiting dilution, clone SMab-2 (mouse IgG₁, kappa) against IDH2-R172S was established. SMab-2 also reacted with MBP-fused IDH2-R172S protein, not with MBP-fused IDH2-WT protein using ELISA. We further immunized mice with synthetic peptides of IDH2-R140Q, and screened mAbs reacting with both IDH1-WT and IDH2-WT using ELISA. After limiting dilution, clone RqMab-3 (mouse IgG₁, kappa) against IDH1/2-WT was established.

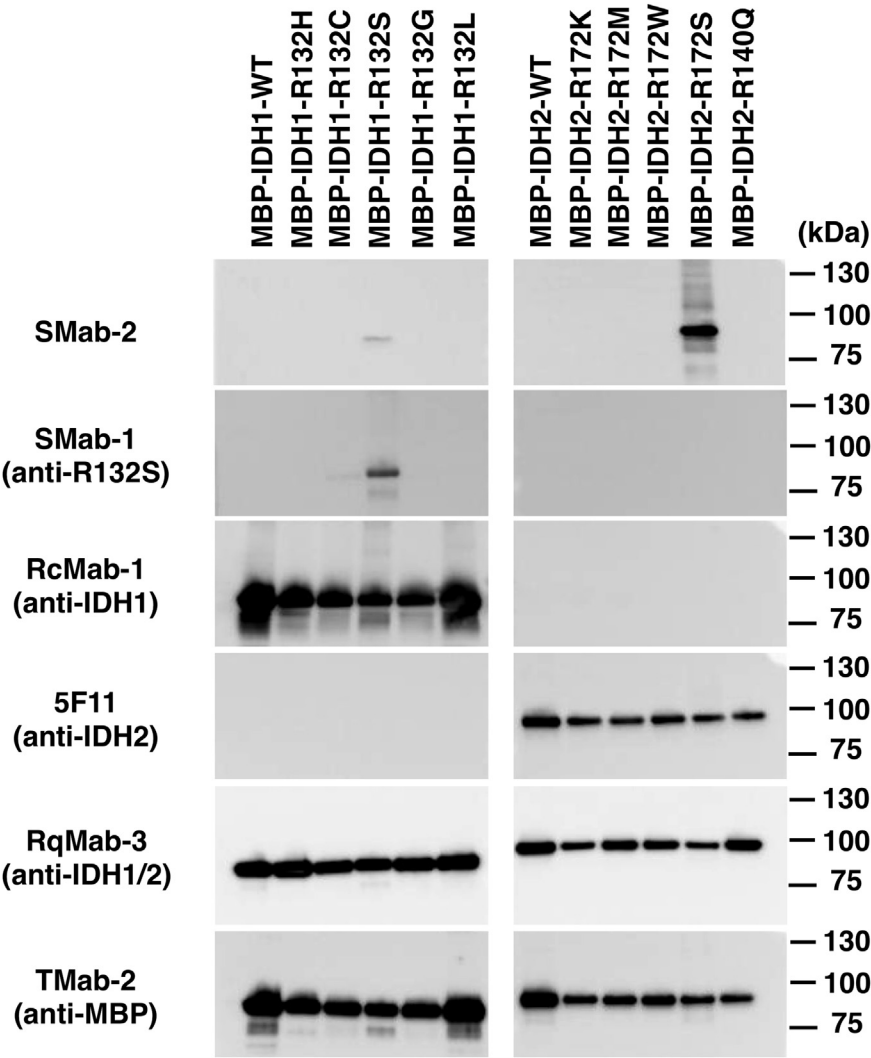


Fig. 1. Western-blot analyses by anti-IDH1/2 mAbs against MBP-IDH1/2 proteins. Purified proteins (50 ng/lane) of *E. coli*-expressing IDH1 wild type (WT; lane 1), IDH1 mutants (lane 2, IDH1-R132H; lane 3, IDH1-R132C; lane 4, IDH1-R132S; lane 5, IDH1-R132G; lane 6, IDH1-R132L), IDH2-WT (lane 7), IDH2 mutants (lane 8, IDH2-R172K; lane 9, IDH2-R172M; lane 10, IDH2-R172W; lane 11, IDH2-R172S; lane 12, IDH2-R140Q) were electrophoresed under reducing condition using 5–20% gels, and were Western-blotted with SMab-2, SMab-1 (anti-IDH1-R132S), RcMab-1 (anti-IDH1), 5F11 (anti-IDH2), RqMab-3 (anti-IDH1/2), and TMab-2 (anti-MBP).

3.2. Specificity of SMab-2 against IDH in western blot analyses

To determine the specificity of SMab-2, IDH1/2-WT and IDH1/2 mutants (MBP-IDH1-R132H, MBP-IDH1-R132C, MBP-IDH1-R132S, MBP-IDH1-R132G, MBP-IDH1-R132L, MBP-IDH2-R172K, MBP-IDH2-R172M, MBP-IDH2-R172W, MBP-IDH2-R172S, MBP-IDH2-R140Q) were Western-blotted using several anti-IDH1/2 mAbs. As shown in Fig. 1, RcMab-1 (anti-IDH1) recognized IDH1-WT and all IDH1 mutations. 5F11 (anti-IDH2) reacted with IDH2-WT and all IDH2 mutations. RqMab-3 (anti-IDH1/2) detected IDH1/2-WT and all IDH1/2 mutations. TMab-2 (anti-MBP tag) detected all IDH1/2 proteins. In contrast, SMab-2 strongly recognized MBP-IDH2-R172S, and weakly reacted with MBP-IDH1-R132S. SMab-1 reacted with only MBP-IDH1-R132S, not with MBP-IDH2-R172S.

We next performed Western-blot analyses against IDH1/2 mutations, which are expressed in mammalian cells. IDH1/2-WTs and IDH1/2 mutants (IDH1-R132H, IDH1-R132C, IDH1-R132S, IDH1-R132G, IDH1-R132L, IDH2-R172K, IDH2-R172M, IDH2-R172W, IDH2-R172S, IDH2-R172G) were transfected in U-2 OS, and Western-blotted with anti-IDH mAbs. As shown in Fig. 2, RcMab-1 detected both exogenous and endogenous IDH1 proteins. 5F11 reacted with both exogenous and endogenous IDH2 proteins. RqMab-3 (anti-IDH1/2) recognized both exogenous and endogenous IDH1/2 proteins. Anti-tag mAbs, 1H6 (anti-V5 tag) and NZ-1 (anti-PA tag) reacted with exogenous IDH1 and IDH2 proteins, respectively. SMab-2 strongly recognized IDH2-R172S, but not reacted with IDH1/2-WTs. In contrast, SMab-1 recognized IDH1-R132S, not with other mutations. Interestingly, SMab-2 detected

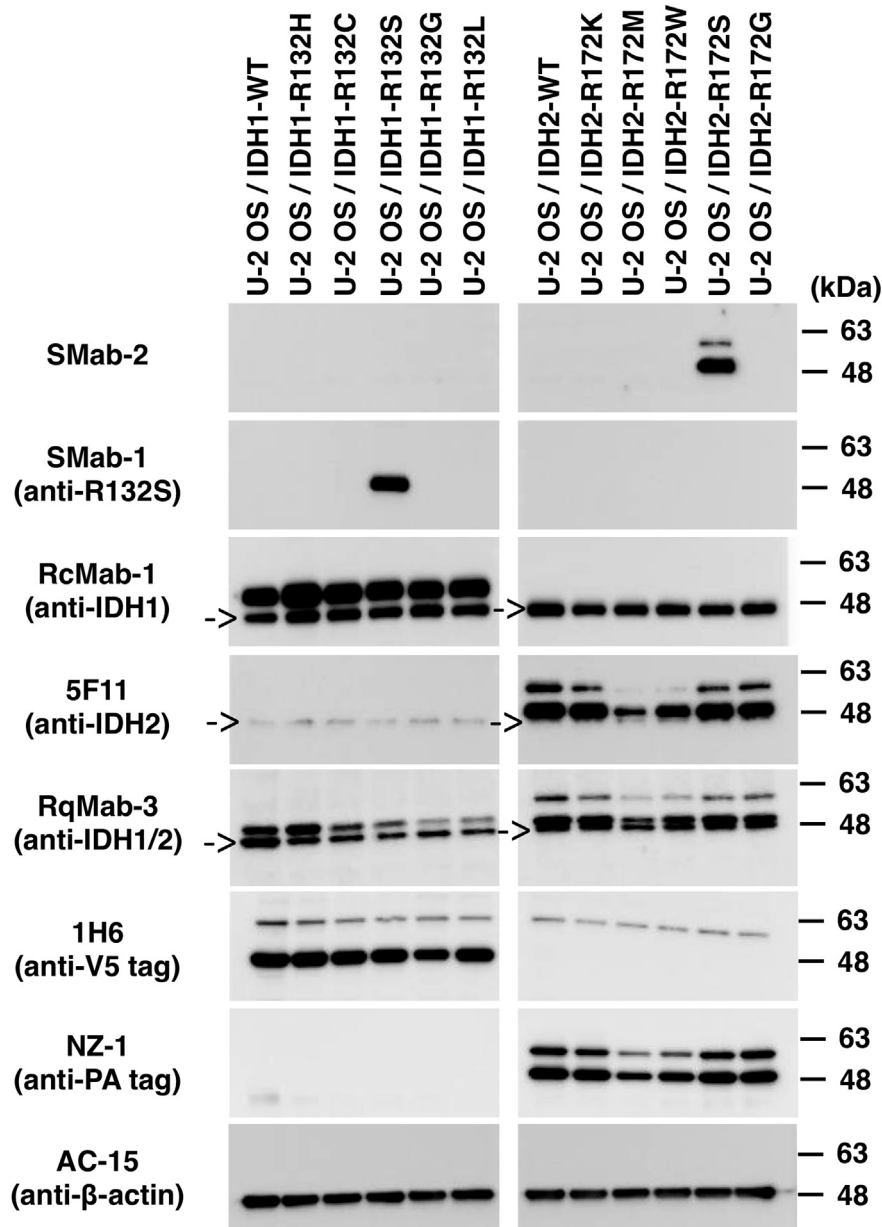


Fig. 2. Western-blot analyses by anti-IDH1/2 mAbs against mutated IDH1/2-expressing U-2 OS. Total cell lysate (10 µg/lane) from U-2 OS cells expressing IDH1 wild type (WT, lane 1) and IDH1 mutants (lane 2, IDH1-R132H; lane 3, IDH1-R132C; lane 4, IDH1-R132S; lane 5, IDH1-R132G; lane 6, IDH1-R132L), IDH2-WT (lane 7), IDH2 mutants (lane 8, IDH2-R172K; lane 9, IDH2-R172M; lane 10, IDH2-R172W; lane 11, IDH2-R172S; lane 12, IDH2-R172G) were electrophoresed under reducing condition using 5–20% gels, and were Western-blotted with SMab-2, SMab-1 (anti-IDH1-R132S), RcMab-1 (anti-IDH1), 5F11 (anti-IDH2), RqMab-3 (anti-IDH1/2), 1H6 (anti-V5 tag), NZ-1 (anti-PA tag), and AC-15 (anti-β-actin). Arrows: endogenous IDH1/2.

with only IDH2-R172S in mammalian cells in the different manner with *E. coli* proteins, indicating that SMab-2 is mono-specific against IDH2-R172S in cancers.

3.3. SMab-2 reacts with endogenous IDH2-R172S expressed in a chondrosarcoma cell line

A chondrosarcoma cell line SW1353 is harboring endogenous IDH2-R172S (Fig. 3A). The IDH2-R172S mutation of SW1353 was confirmed using droplet digital PCR analysis (Fig. 3B). In Western-blot analysis, SMab-2 detected both exogenous IDH2-R172S in U-2 OS and endogenous IDH2-R172S in SW1353 (Fig. 3C). An anti-IDH2 mAb, 5F11 reacted both wild type and mutated IDH2 proteins. An anti-PA tag mAb, NZ-1 detected only exogenous IDH2 proteins. These results demonstrate that SMab-2 recognizes not only exogenous IDH2-R172S but also endogenous one, indicating that SMab-2 is useful for detecting IDH2-R172S in tumors.

We next performed immunocytochemical analyses using SMab-2 against SW1353 chondrosarcoma cell line. As shown in Fig. 4B, SMab-2 clearly stained SW1353 cells. In contrast, SMab-2 did not stain HeLa cells, which do not possess IDH2-R172S mutation (Fig. 4B), indicating that SMab-2 is also specific against IDH2-R172S in immunocytochemical analyses.

4. Discussion

We previously established several mono-specific anti-mutated IDH2 mAbs: IDH2-R172K-specific KMab-1, IDH2-R172M-specific MAb-1, and IDH2-R172W-specific WMAb-1 [15]. We also established two multi-specific IDH1/2 mAb: MsMab-1 and MsMab-2 [15]. Those mAbs have been used as a useful diagnostic tool for detecting IDH2 mutations in several tumors. However, anti-IDH2-R172S-specific mAbs have not been developed. In this study, we developed clone SMab-2 (mouse IgG1, kappa) against IDH2-R172S. IDH2-R172S has been discovered in several tumors such as gliomas, osteosarcomas, and chondrosarcomas; therefore, SMab-2 is advantageous for detecting IDH2-R172S in IDH2-R172S-bearing tumors.

Although SMab-2 strongly recognized MBP-IDH2-R172S, and weakly reacted with MBP-IDH1-R132S, it detected with only IDH2-R172S in mammalian cells. Indeed, 19 amino acids of IDH2-R172S peptide (GGTKPITIGSHAHGDQYKA) show 78.9% homology with the equivalent portion of IDH1-R132S (GGVKPIIGSHAYGDQYRA); therefore, the weak reaction of SMab-2 to MBP-IDH1-R132S is thought to be the cross-reaction. The same cross-reaction of SMab-1 against IDH1-R132S was observed in ELISA (data not shown). A clone RqMab-3, which was produced by immunizing synthetic peptide (NGTIQNILGG), detected both IDH1 and IDH2 (Figs. 1 and

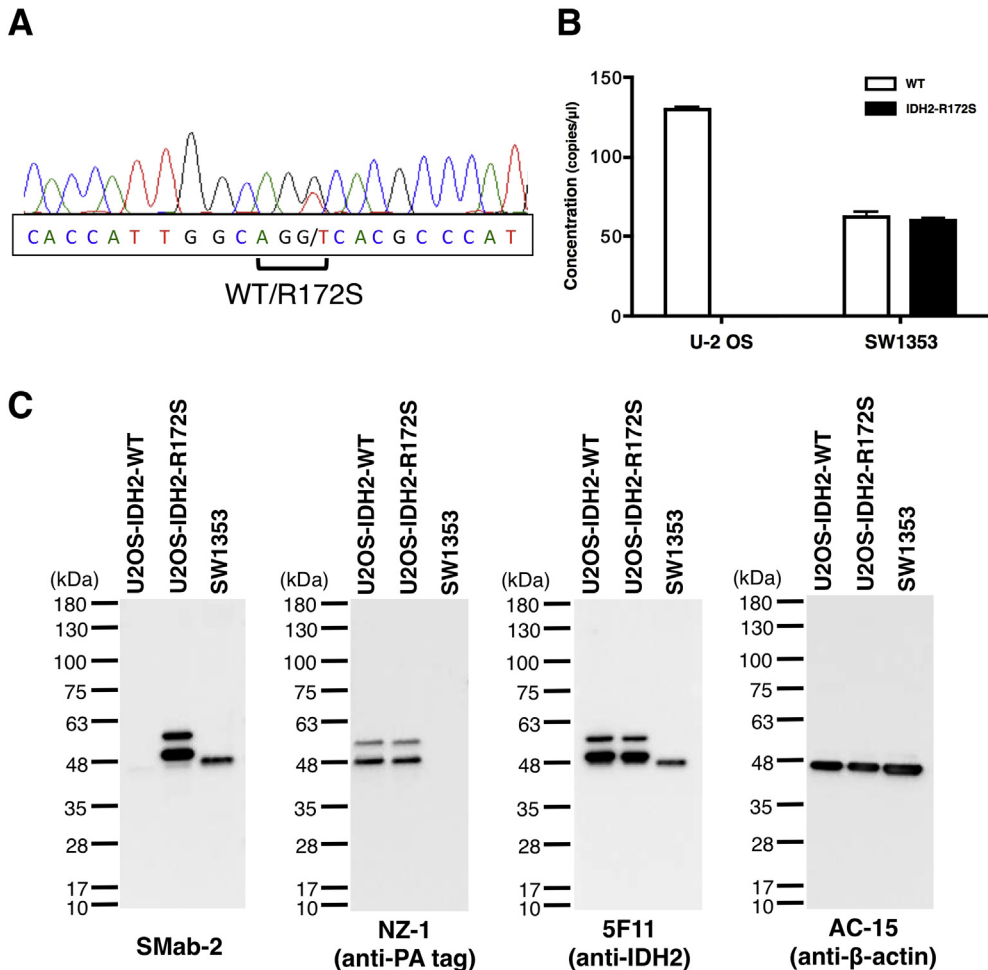


Fig. 3. Endogenous IDH2-R172S mutation in SW1353 cells was detected using three methods. (A) Direct sequencing. Both AGG (WT) and AGT (R172S) were detected. (B) Digital PCR analysis. Both WT and R172S were detected in SW1353. (C) Western blot analysis. Total cell lysate from U-2 OS cells expressing IDH2-WT (lane 1) and IDH2-R172S (lane 2) or SW1353 (lane 3) were electrophoresed under reducing condition using 5–20% gels, and were Western-blotted with SMab-2, NZ-1 (anti-PA tag), 5F11 (anti-IDH2), and AC-15 (anti-β-actin).

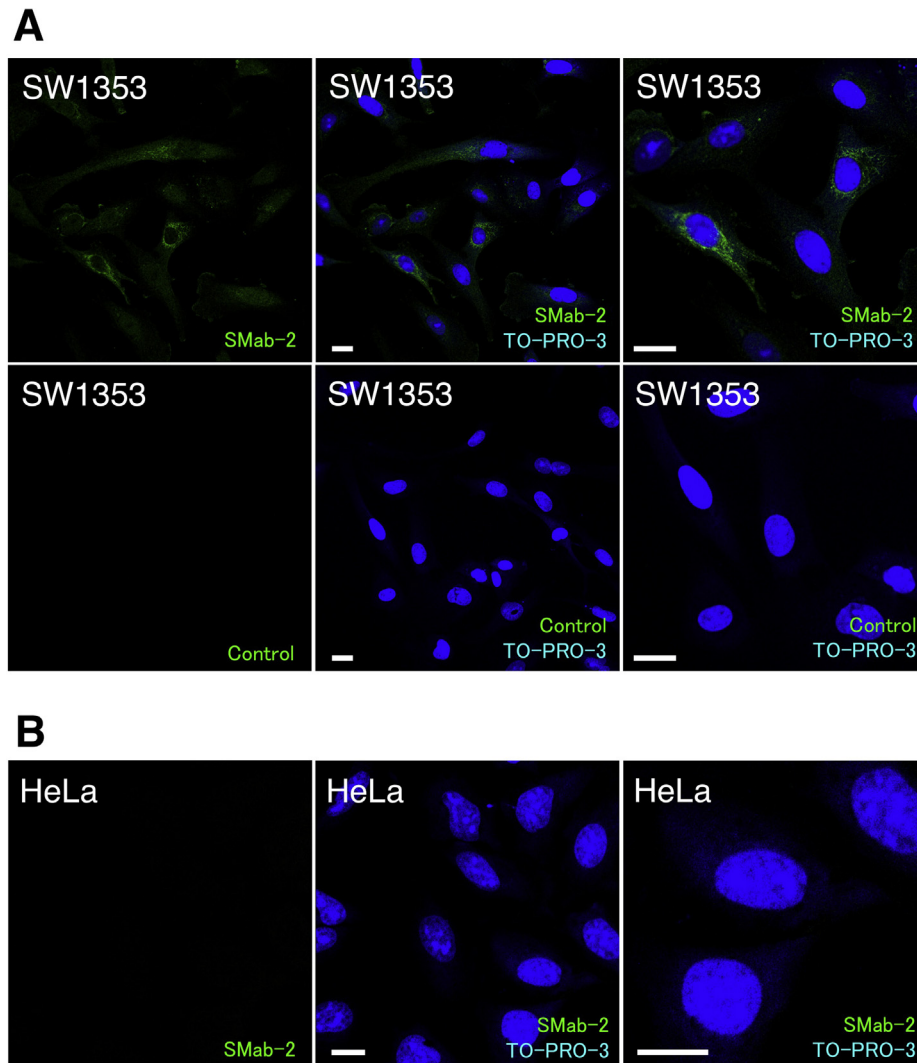


Fig. 4. Immunocytochemical analyses using SMab-2. SW1353 cells (A) and HeLa cells (B) were stained by 30 $\mu\text{g}/\text{ml}$ of SMab-2 or control (PBS), followed by anti-mouse IgG-Alexa 488 antibody (green). Cells were also treated with TO-PRO-3 to stain the cell nuclei (blue). Scale bar: 20 μm . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2), and a previously established multi-specific mAb, clone MsMab-1 also detected both IDH1-R132S and IDH2-R172S [18]. An anti-IDH1-R132H mAb, clone H09 cross-reacts with IDH1-R132L [15], indicating that the cross-reaction has been often observed in anti-IDH1/2 mAbs. A chondrosarcoma cell line SW1353 was reported to possess endogenous IDH2-R172S [6]. Because anti-IDH2-R172S mAbs have not been established, the detection of IDH2-R172S was performed using Sanger sequencing, next generation sequencing, pyrosequencing, or digital PCR. In this study, we revealed that SMab-2 detected exogenous IDH2-R172S in SW1353 in Western-blot (Fig. 3C) and immunocytochemical analyses (Fig. 4A), indicating that SMab-2 is effective for a diagnostic tool of IDH2-R172S-harboring tumors.

Taken together, SMab-2 is expected to be useful for diagnosis of IDH2-R172S-bearing tumors. The combination of SMab-2 with previously established anti-mutated IDH1/2 mAbs might lead to high-sensitive detection of IDH1/2 mutation in clinical diagnosis. The concentration of SMab-2 needs to be more than 30 $\mu\text{g}/\text{ml}$ in immunocytochemical analyses, showing that SMab-2 is not so sensitive for detecting IDH2-R172S in immunocytochemical analyses. Indeed, SMab-2 could not detect IDH2-R172S protein in immunohistochemical analyses using paraffin-embedded tissues.

We should further produce much more sensitive mAbs against endogenous IDH2-R172S protein, which are clinically useful in immunohistochemical analyses.

Author disclosure statement

The authors have no financial interest to disclose.

Conflict of interest

None.

Acknowledgments

We thank Takuro Nakamura, Kanae Yoshida, and Noriko Saidoh for their excellent technical assistance. This work was supported in part by the Platform for Drug Discovery, Informatics, and Structural Life Science (PDIS) from Japan Agency for Medical Research and Development, AMED (Y.K.); by the Basic Science and Platform Technology Program for Innovative Biological Medicine from AMED (Y.K.); by the Regional Innovation Strategy Support Program from the Ministry of Education, Culture, Sports, Science and Technology

(MEXT) of Japan (Y.K.); by Health Labour Sciences Research Grant from AMED (Y.K.); and by a Grant-in-Aid for Scientific Research (C) (M.K.K., Y.K.) from MEXT of Japan.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.bbrc.2015.02.162>.

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