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.

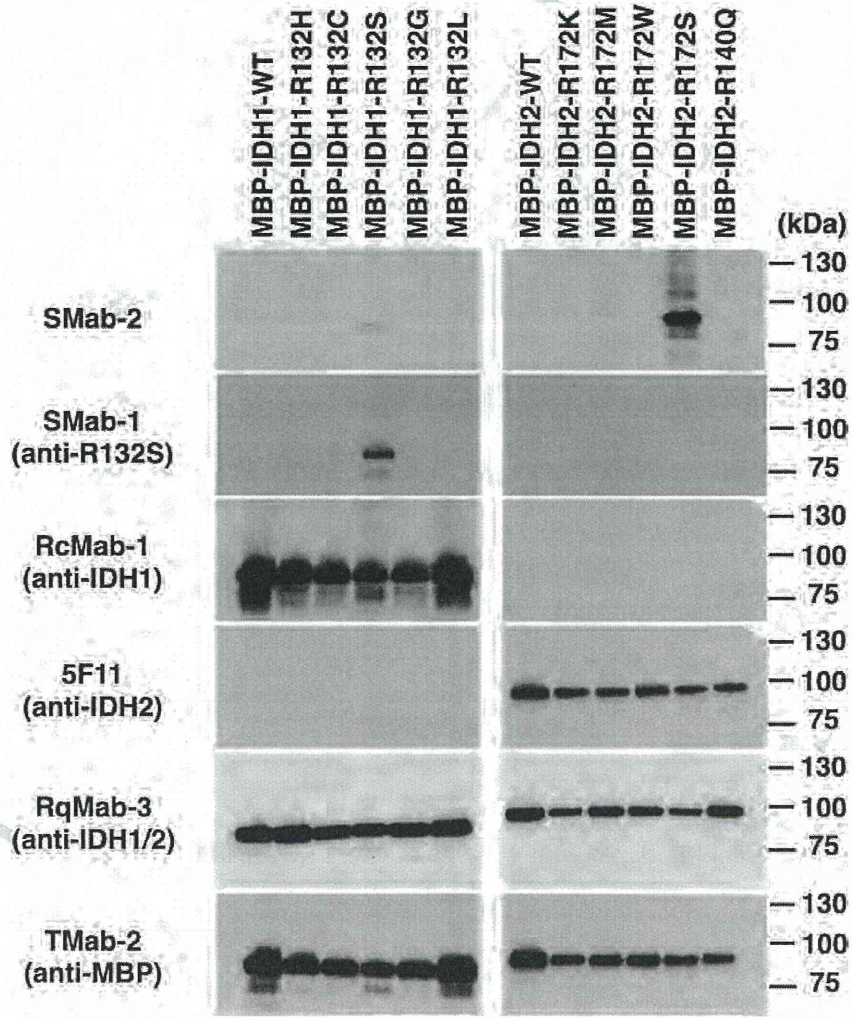

Fig. 1. Western-blot analyses by anti-IDH1/2 mAbs against MBP-IDH1/2 proteins. Purified proteins (50 ng/lane) of *E. coli*-expressing IDH1 wild type (WT; lane 1), IDH1 mutants (lane 2, IDH1-R132H; lane 3, IDH1-R132C; lane 4, IDH1-R132S; lane 5, IDH1-R132G; lane 6, IDH1-R132L), IDH2-WT (lane 7), IDH2 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).

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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

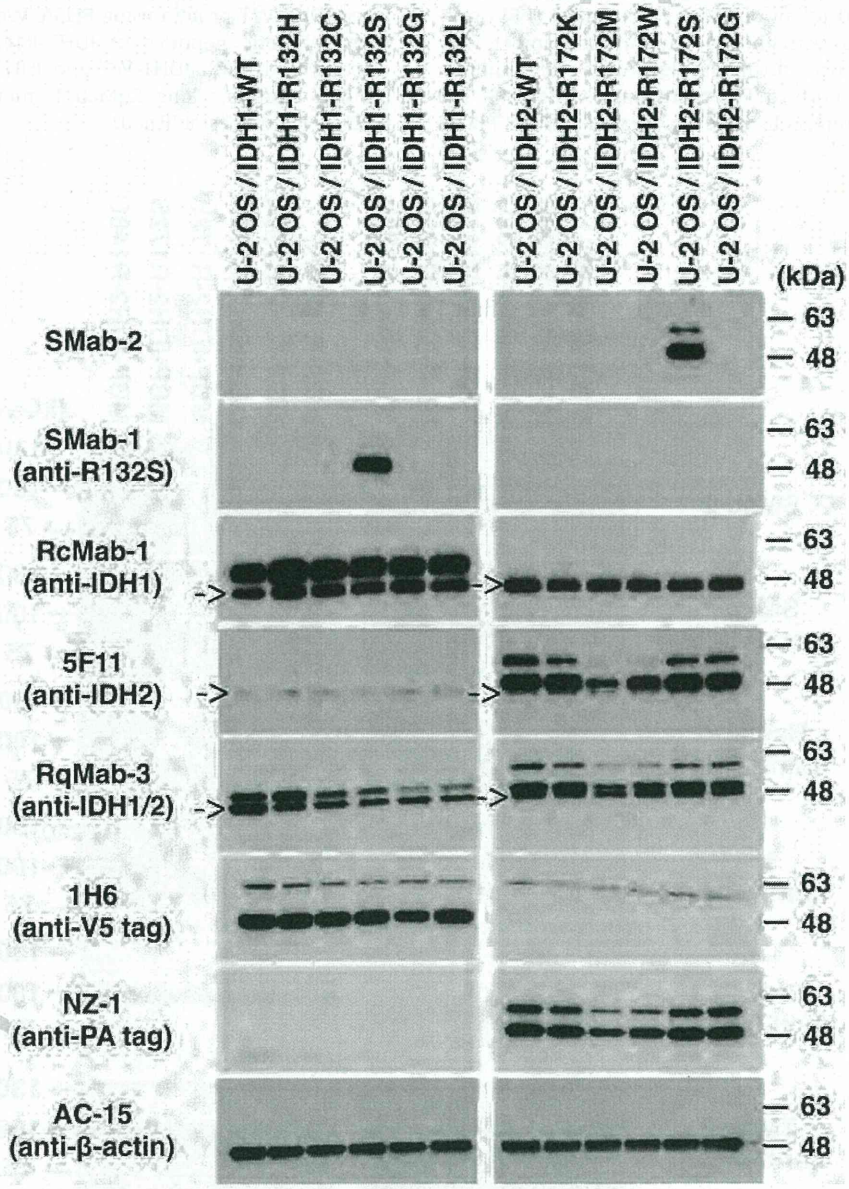

Fig. 2. Western-blot analyses by anti-IDH1/2 mAbs against mutated IDH1/2-expressing U-2 OS. Total cell lysate (10 μg/lane) from U-2 OS cells expressing IDH1 wild type (WT, lane 1) and IDH1 mutants (lane 2, IDH1-R132H; lane 3, IDH1-R132C; lane 4, IDH1-R132S; lane 5, IDH1-R132G; lane 6, IDH1-R132L), IDH2-WT (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), 1H5 (anti-V5 tag), NZ-1 (anti-PA tag), and AC-15 (anti-β-actin). Arrows: endogenous IDH1/2.

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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-R172-specific KMab-1, IDH2-R172M-specific MMab-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 (GGVKPIIIGSHAYGDQYRA); therefore, the weak reaction of SMab-2 to MBP-IDH1-R132S is though 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

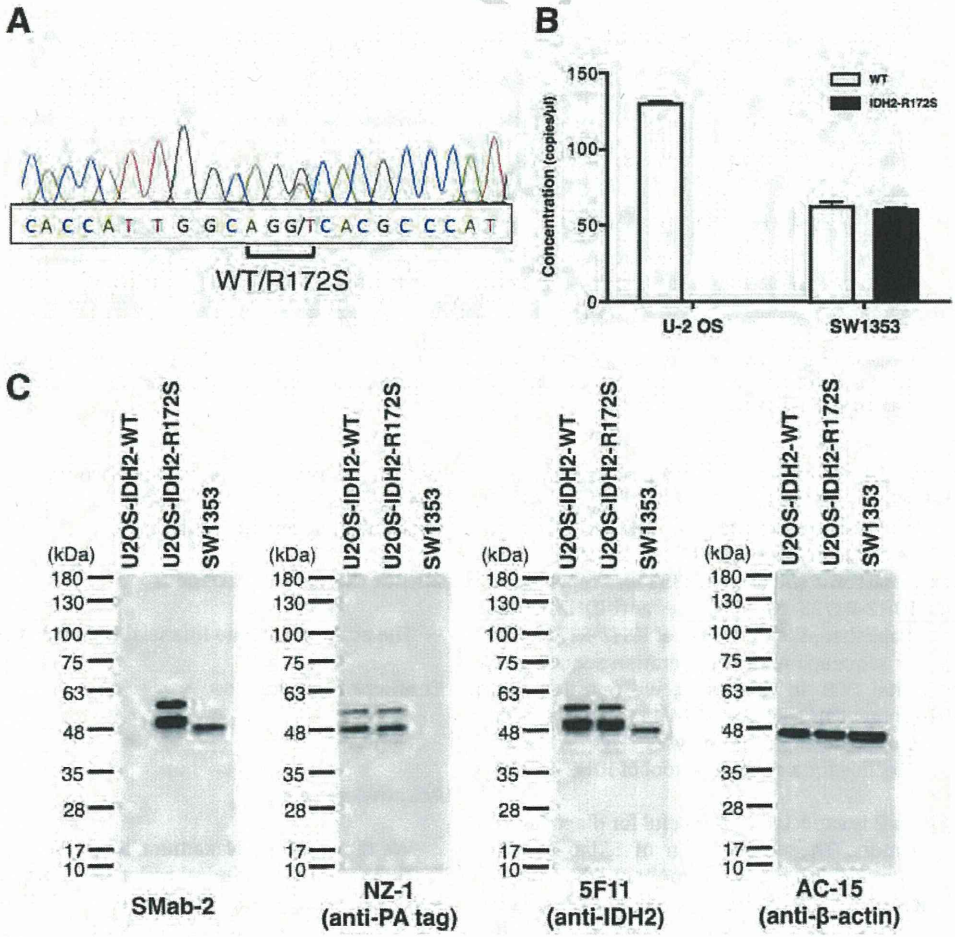

Fig. 3. Endogenous IDH2-R172S mutation in SW1353 cells was detected using three methods. (A) Direct sequencing. Both AGG (WT) and AGT (R172S) were detected. (B) Digital PCR analysis. Both WT and R172S were detected in SW1353. (C) Western blot analysis. Total cell lysate from U-2 OS cells expressing IDH2-WT (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).

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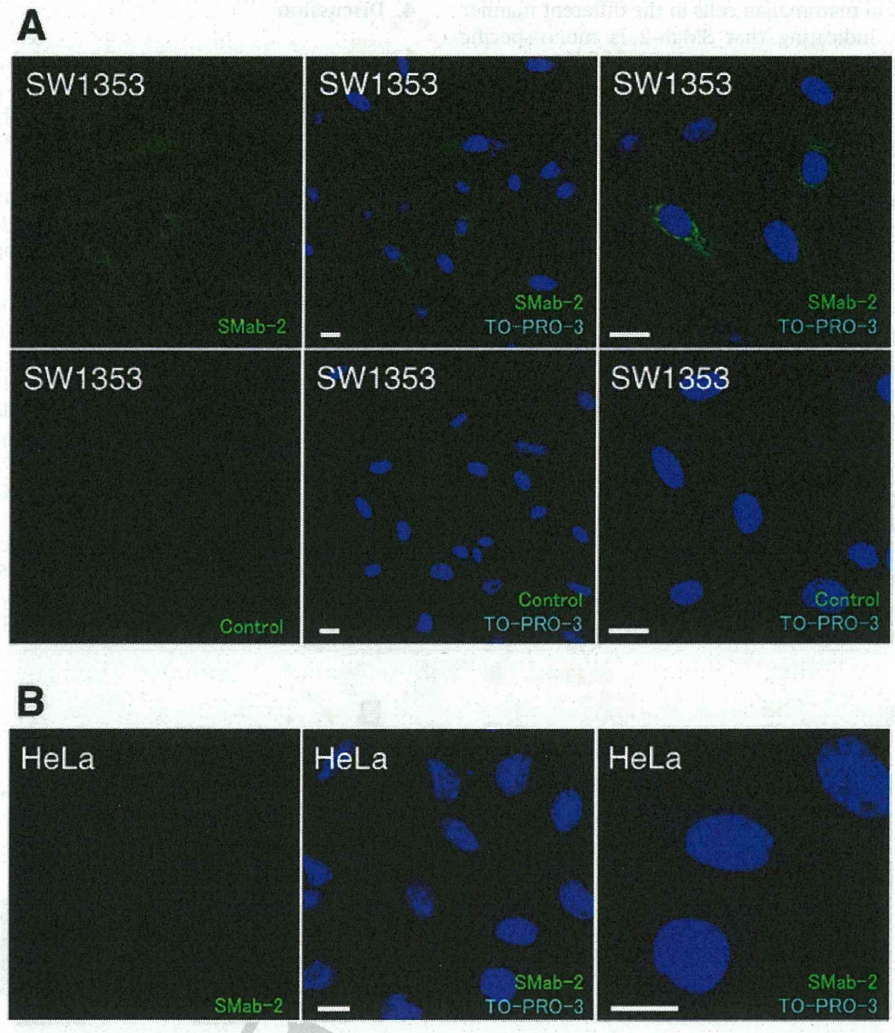

Fig. 4. Immunocytochemical analyses using SMab-2. SW1353 cells (A) and HeLa cells (B) were stained by 30 μg/ml of SMab-2 or control (PBS), followed by anti-mouse IgG-Alexa 488 antibody (green). Cells were also treated with TO-PRO-3 to stain the cell nuclei (blue). Scale bar: 20 μm. (For interpretation 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 μg/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.

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