Establishment of a novel monoclonal antibody SMab-1 specific for IDH1-R132S mutation

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A B S T R A C T
Isocitrate dehydrogenase 1 (IDH1) mutations, which are early and frequent genetic alterations in gliomas, are specific to a single codon in the conserved and functionally important Arginine 132 (R132) in IDH1. We earlier established a monoclonal antibody (mAb), IMab-1, which is specific for R132H-containing IDH1 (IDH1-R132H), the most frequent IDH1 mutation in gliomas. To establish IDH1-R132S-specific mAb, we immunized mice with R132S-containing IDH1 (IDH1-R132S) peptide. After cell fusion using Sendai virus envelope, IDH1-R132S-specific mAbs were screened in ELISA. One mAb, SMab-1, reacted with the IDH1-R132S peptide, but not with other IDH1 mutants. Western-blot analysis showed that SMab-1 reacted only with the IDH1-R132S protein, not with IDH1-WT protein or IDH1 mutants, indicating that SMab-1 is IDH1-R132S-specific. Furthermore, SMab-1 specifically stained the IDH1-R132S-expressing glioblastoma cells in immunocytochemistry and immunohistochemistry, but did not react with IDH1-WT or IDH1-R132H-containing glioblastoma cells. We newly established an anti-IDH1-R132S-specific mAb SMab-1 for use in diagnosis of mutation-bearing gliomas.

1. Introduction
Isocitrate dehydrogenase 1 (IDH1) mutations, which have been identified as early and frequent genetic alterations (50–93%) in astrocytomas, oligodendrogliomas, and oligoastrocytomas, as well as in secondary glioblastomas, might be the initiating event in these glioma subtypes [1–4]. In contrast, primary glioblastomas, as well as other systemic cancers, rarely contain IDH1 mutations. The IDH1 mutations are remarkably specific to a single codon in the conserved and functionally important Arginine 132 residue (R132) in IDH1. Mutations in the analogous IDH2-R172 codon also occur at a lower rate (3–5%) in these cancers. Of interest, IDH1/2 mutations were observed subsequently in 22% of acute myeloid leukemias [5]. IDH1 mutations were found to result in the ability of the enzyme to catalyze the reduced NADP-dependent reduction of α-ketoglutarate to R(-)-2-hydroxyglutarate (2-HG) [6]. Reduction of α-ketoglutarate by 2-HG or mutant IDH1 results in a lower level of prolyl hydroxylases and promotes the accumulation of hypoxia-inducible factor (HIF) 1α [7]. HIF-1α levels were greater in human gliomas harboring an IDH1 mutation than in tumors without the mutation. Therefore, IDH1 appears to function as a tumor suppressor that, when mutationally inactivated, contributes to tumorigenesis in part through induction of the HIF-1 pathway.

To date, two monoclonal antibodies against IDH1 mutations have been reported [8,9]. Of those antibodies, we established IMab-1, which is specific for IDH1-R132H, the most frequent IDH1 mutations (>90%) [8]. IMab-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 reacted only with the IDH1-R132H protein, not the IDH1-WT protein or other IDH1 mutants. Recently, we investigated the usefulness of IMab-1 for immunohistochemical analysis of numerous human glioma samples in comparison with the direct DNA sequencing method [10]. IMab-1 detected 12 out of 49 cases. However, only nine cases were deter-
mined to be IDH1-R132H by direct DNA sequencing because of the small population of IDH1-R132H mutation-possessing tumor cells, indicating that IMab-1 immunohistochemistry is useful for detecting IDH1-R132H in immunohistochemistry.

Although several IDH1 mutations exist, including R132H, R132C, R132S, and R132L, IMab-1 and another mAb, H09, can detect only IDH1-R132H mutation [11]. Therefore, novel antibodies that recognize the other IDH1 mutants should be developed to cover all IDH1 mutations in immunohistochemistry. We newly report an anti-IDH1-R132S-specific monoclonal antibody SMab-1, which is expected to be extremely useful for diagnosis of mutation-bearing gliomas and acute myeloid leukemia.

2. Materials and methods

2.1. Female BALB/c mice were obtained from CLEA Japan Inc. (Tokyo, Japan). P3U1 and LN299 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). P3U1 or LN299 cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air in RPMI 1640 or DMEM medium (Wako Pure Chemical Industries Ltd., Osaka, Japan), respectively, including 2 mM L-glutamine (Wako Pure Chemical Industries Ltd.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen Corp., Carlsbad, CA). Three glioblastoma patients who underwent primary surgery between 2009 and April 2010 at Tsukuba University Hospital were included in this study [10]. Informed consent was obtained from each patient or the patient’s guardian for obtaining samples and for subsequent data analysis.

2.2. Hybridoma production

BALB/c mice were immunized by i.p. injections of 100 μg of synthetic peptide CKPIIIGSHAYGD (IDH1-R132S peptide; Operon Biotechnologies, K.K., Tokyo, Japan), corresponding to amino acids 126–137 of human IDH1-R132S plus N-terminus cysteine conjugated with KLH together with Imject Alum (Thermo Scientific, Rockford, IL). One week later, secondary i.p. immunization of 30 μg of IDH1-R132S peptide was performed. After several additional immunization of 30 μg of IDH1-R132S 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 using Sendai virus (Hemagglutinating Virus of Japan: HV) envelope: GenomONE-CF (Ishihara Sangyo Kaisha Ltd., Osaka, Japan) according to the manufacturer’s instructions [8,12]. The hybridomas were grown in RPMI medium with hypoxanthine, aminopterin, and thymidine selection medium supplement (Invitrogen Corp.). The culture supernatants were screened using ELISA for binding to the IDH1-R132S peptide and the IDH1-wild type (IDH1-WT), as described in Section 2.3.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Synthetic peptides (Operon Biotechnologies, K.K.) corresponding to amino acids 126–137 of the human IDH1 plus N-terminus cysteine: IDH1-WT: (CKPHIGHAYGD), IDH1-R132H (CKPHIGHAYGD), IDH1-R132C (CKPHIGHAYGD), IDH1-R132S (CKPHIGHAYGD), and IDH1-R132L (CKPHIGHAYGD) were immobilized, respectively, on Maxisorp 96-well immunoplates (Thermo Fisher Scientific Inc., Waltham, MA) at 1 μg/ml for 30 min, respectively. After blocking with 1% BSA 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 H₂SO₄, 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 IDH1 cDNA (GenBank Accession No. AF113917 or BC012846) encoding a full-length open reading frame (ORF) was obtained by PCR using a human lung cDNA library (Cosmo Bio Co. Ltd., Tokyo, Japan) as a template. The primer set for IDH1 was as follows: EcoRI-IDH1-F1, 5’-cagcaattcATGTCTCCAATAAAAA TCAGTGG-3’ and Sall-IDH1-R1, 5’-gtggtgagCTTTAAGGTGGCCCTG AGCTA-3’. The amplified cDNA was subcloned into a pcDNA3.1/V5-His-TOPO vector (Invitrogen Corp.). Substitution of the Arginine 132 (R132) to appropriate amino acid codons (Histidine, Cysteine, or Serine) in IDH1 cDNAs was accomplished using the QuikChange Lightning site-directed mutagenesis kit (Stratagene, La Jolla, CA). The full-length IDH1-WT, R132H, R132C, and R132S were subcloned into an expression vector, pMAL-c2 (New England Biolabs Inc., Beverly, MA), via EcoRI and Sall restriction sites.

2.5. Protein expression using bacteria cells and mammalian cells

Competent Escherichia coli TOP-10 cells (Invitrogen Corp.) were transformed with the plasmid, pMAL-IDH1-WT, -R132H, -R132C, and -R132S. Then they were cultured overnight at 37°C in LB medium containing 100 μg/ml ampicillin. Cell pellets were resuspended in phosphate buffered saline (PBS) with 1% Triton X-100 with 50 μg/ml aprotinin. After sonication, the crude extracts were collected by centrifugation (9000g for 30 min, 4°C). The supernatants were loaded onto Amylose resin. The loaded resin was 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. Mammalian cells were transfected with appropriate amounts of plasmids, pcDNA3.1/IDH1-R132H or -R132S using Metafectene (Biontex Laboratories GmbH, Martinsried, Germany) according to the manufacturers’ instructions. Stable transfectants were selected by cultivating the transfectants in medium containing 0.5 mg/ml of Geneticin (Invitrogen Corp.). The expression level of IDH1 was confirmed using Western-blot analysis.

2.6. Western-blot analyses

Cultured cell pellets were lysed with PBS with 1% Triton X-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 5 μg of total protein were prepared for Western-blot analysis by boiling in SDS sample buffer (50 mM Tris, 2% SDS, 5% glycerol, 10% 2-mercaptoethanol, pH 6.8). They were electrophoresed on 5–20% polyacrylamide gels (Wako Pure Chemical Industries Ltd., Osaka, Japan). The separated proteins were transferred to a PVDF membrane (Bio-Rad Laboratories Inc.). After blocking with 4% skim milk in PBS, the membrane was incubated with anti-IDH1 antibody (1/1000 diluted; Santa Cruz Biotechnology Inc., Santa Cruz, California), IMab-1 culture supernatant (kindly donated by Dr. Darell D. Bigner, Duke University Medical Center), or SMab-1 culture supernatant; then with peroxidase-conjugated secondary antibodies (1/1000 diluted) and developed with ECL reagents (GE Healthcare, Buckinghamshire, UK) using EZ-capture II (Atto Corp., Tokyo, Japan).
2.7. Immunocytochemical analysis

Cultured cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 min at room temperature and then 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 SMab-1 or IMab-1 culture supernatant containing 0.1% Triton X-100 overnight at 4 °C in a moist chamber. Then they were incubated with goat anti-mouse 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.).

2.8. Immunohistochemical analysis

IDH1-R132S protein expression was determined immunohistochemically in paraffin-embedded tumor specimens, as described previously [10]. Briefly, 5-μm-thick histologic sections were deparaaffinized in xylene and rehydrated. Then they were heated at 100 °C in citrate buffer (pH 6.0) for 5 min. Sections were incubated with culture supernatants of SMab-1 or IMab-1 overnight at 4 °C. The LSAB Kit (Dako, Glostrup, Denmark) was used for the post-primary antibody blocker and secondary antibody. Color was developed using 3, 3-diaminobenzidine tetrahydrochloride (DAB) for 10 min, and counterstained with hematoxylin.

3. Results and discussion

3.1. Production of an IDH1-R132S-specific antibody

The IDH1 gene at 2q33 encodes isocitrate dehydrogenase 1 (IDH1), which catalyzes the oxidative carboxylation of isocitrate to α-ketoglutarate [7]. IDH1 mutations occur frequently in some malignant gliomas [2] and less frequently in acute myeloid leukemias [5]. To date, almost all IDH1 mutations have been identified at the critical isocitrate binding site R132 codon. The vast majority of changes are heterozygous [1–4]. In gliomas, mutations in the IDH1 homolog IDH2 have been identified at the aligned R172 codon [2]. These discoveries mark unique findings in the field of cancer genetics.

We previously established IMab-1, which is specific for IDH1-R132H, the most frequent of IDH1 mutations (>90%) [8]. However, antibodies against other IDH1 mutations have not been reported. For this study, we immunized mice with synthetic peptides of IDH1-R132S mutant. After cell fusion using the Sendai virus envelope, the wells of hybridomas, which produced IDH1-R132S-specific antibody were selected in ELISA. In total, 46 wells, which reacted with IDH1-R132S, not with IDH1-WT, were selected. However, 45/46 wells did not react with IDH1-R132S protein in ELISA. After reacted with IDH1-R132S, not IDH1-WT, were selected. However, specific antibodies, were selected in ELISA. In total, 46 wells, which loped, the wells of hybridomas, which produced IDH1-R132S-specific antibody blocker and secondary antibody. Color was developed using 3, 3-diaminobenzidine tetrahydrochloride (DAB) for 10 min, and counterstained with hematoxylin.

3.2. Specificity of SMab-1 against IDH1 mutants

To determine the specificity of SMab-1 monoclonal antibody, the reactivities against IDH1-wild type (IDH1-WT) and the IDH1 mutants (R132H, R132C, R132S, R132G, R132L) peptides were investigated using ELISA. Results show that SMab-1 reacted with IDH1-R132S peptide, not with IDH1-WT or other IDH1 mutants (R132H, R132C, R132S, R132G, R132L) peptides, whereas IMab-1 reacted with IDH1-R132H peptide, not with IDH1-WT or other IDH1 mutants (R132C, R132S, R132G, R132L) peptides (data not shown), indicating that SMab-1 is a specific antibody against IDH1-R132S peptide. To confirm the reactivity of SMab-1 antibody against not only IDH1 peptides but also against IDH1 proteins, IDH1-WT and three IDH1 mutants (R132H, R132C, R132S) were expressed in E. coli for Western-blot analyses. As portrayed in Fig. 1A, SMab-1 recognized only IDH1-R132S protein, not the other proteins (WT, R132H, R132C), indicating that SMab-1 is a specific antibody against IDH1-R132S protein. IMab-1 recognized only IDH1-R132H protein (Fig. 1B); an anti-IDH1 antibody reacted with all types (Fig. 1C). These results indicate that SMab-1 is also useful in detecting not only IDH1-R132S peptide, but also the IDH1-R132S protein.

3.3. Immunocytochemical and immunohistochemical analyses by SMab-1 against glioma tissues with IDH1-R132S

SMab-1 was confirmed to be an anti-IDH1-R132S-specific antibody in ELISA and Western-blot analysis. Therefore, we first performed immunocytochemical analyses using IDH1-R132S or IDH1-R132H-transfected LN229 glioblastoma cells. Results show that SMab-1 reacted with IDH1-R132S-transfected cells (Fig. 2A), not with IDH1-R132H-transfected cells (Fig. 2C), whereas IMab-1 reacted with IDH1-R132H-transfected cells (Fig. 2C), but not with IDH1-R132S-transfected cells (Fig. 2E), indicating that both SMab-1 and IMab-1 are mutation-specific antibodies in immunocytochemistry. We next performed immunohistochemistry of SMab-1 against IDH1-R132S-positive or IDH1-R132H-positive gliomas. Typical results are shown in Fig. 3. SMab-1 stained almost all tumor cells of IDH1-R132S-positive gliomas (Fig. 3A), although no staining was observed in IDH1-R132H-positive gliomas or IDH1-WT gliomas (Fig. 3C and E). In fact, SMab-1 did not stain endothelial cells in IDH1-R132S-positive gliomas (Fig. 3A). Furthermore, IMab-1, also used as mouse IgG1 isotype control, did not stain any tumor cells in IDH1-R132S-positive gliomas (Fig. 3B), whereas IMab-1 stained IDH1-R132H-positive gliomas (Fig. 3D). These results indicate that SMab-1 is useful in immunohistochemical analyses for detection of IDH1-R132S mutation. Our previous reports described that one R132S-positive glioblastoma among 13 IDH1-mutation-bearing gliomas was undetectable by IMab-1 antibody [10]. We were able to raise the detection ratio of IDH1 mutation

![Fig. 1. Western-blot analysis by SMab-1. Cell lysates from E. coli-expressing IDH1-wild type (lane 1) and IDH1 mutants (lane 2, R132H; lane 3, R132C; lane 4, R132S) were electrophoresed under reducing conditions using 5–20% gels, and were Western-blotted with SMab-1 (A), IMab-1 (B), and anti-IDH1 (C).](image-url)
Fig. 2. Immunocytochemical analysis by Smab-1 against IDH1-R132S-transfected glioblastoma cells. LN229 cells were transfected by IDH1-R132S (A, B, E, and F) or IDH1-R132H (C, D, G, and H). Each cell was stained by Smab-1 (A–D) or by IMab-1 (E–H) followed by anti-mouse IgG-Alexa 488 antibody (green). Cells were also treated with TO-PRO-3 to stain the cell nuclei (blue) (B, D, F, and H). Bars 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
in immunohistochemistry by using both IMab-1 and SMab-1 for those glioma samples. IDH1 mutation were reported as very early events, before TP53 mutations or loss of 1p/19q occurs [13], which might engender diffused staining by SMab-1 or IMab-1 in almost every cell of IDH1-R132S or IDH1-R132H-positive glioma, respectively (Fig. 3A and D).

Recently, IDH1/2 mutations have been found in 129 of 805 acute myeloid leukemia (AML)—IDH1 in 61 patients (7.6%) and IDH2 in 70 patients (8.7%) [14]. It is particularly interesting that five of 61 IDH1-mutant-bearing patients (8.2%) were R132S, although only 1.3% of IDH1 mutations were R132S in gliomas [11], indicating that SMab-1 might be more useful for diagnosis of IDH1-R132S-positive AMLs. The R132C mutations were observed at high frequency in AML (20/61 IDH1 mutations; 33%), although they were observed in only 3.7% of glioma specimens [11,14], suggesting that an anti-IDH1-R132C specific antibody might be useful for diagnosis of AML as well as glioma subtypes.

In conclusion, the established anti-IDH1-R132S-specific monoclonal antibody SMab-1 is expected to be extremely useful for diagnosis and biological evaluation of mutation-bearing gliomas in combination with anti-IDH1-R132H-specific monoclonal antibody IMab-1. Additional IDH1/2 mutation-specific antibodies must be developed for pathological diagnosis of gliomas and AML.

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**References**


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