



## A novel monoclonal antibody GMAb-m1 specifically recognizes IDH1-R132G mutation

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### ABSTRACT

Isocitrate dehydrogenase 1 (IDH1) mutations occur in gliomas, acute myeloid leukemias, and cartilaginous tumors. While IDH1 catalyzes the oxidative carboxylation of isocitrate to  $\alpha$ -ketoglutarate in cytosol, mutated IDH1 proteins possess the ability to change  $\alpha$ -ketoglutarate into onco-metabolite R(-)-2-hydroxyglutarate (2-HG). To date, two monoclonal antibodies (mAbs), which are specific for IDH1 mutations have been established: clone HMab-1 against IDH1-R132H and clone SMab-1 against IDH1-R132S. However, specific mAbs against IDH1-R132G, which are useful for immunohistochemical analysis, have not been reported. To establish IDH1-R132G-specific mAbs, we immunized mice with IDH1-R132G-containing peptides. Established mAb GMAb-m1 reacted with the IDH1-R132G peptide, but not with IDH1-wild type (WT) in ELISA. Western-blot analysis also showed that GMAb-m1 reacted with the IDH1-R132G recombinant proteins, not with IDH1-WT or other IDH1 mutants, indicating that GMAb-m1 is IDH1-R132G-specific. Furthermore, GMAb-m1 specifically stained the IDH1-R132G-expressing glioma cells in immunohistochemistry. This is the first report to establish anti-IDH1-R132G-specific mAbs, which is useful in immunohistochemistry of IDH1-R132G-bearing tumors.

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### 1. Introduction

Isocitrate dehydrogenase 1 (IDH1) mutations were found to result in the ability of the enzyme to change  $\alpha$ -ketoglutarate to onco-metabolite R(-)-2-hydroxyglutarate (2-HG), although IDH1 usually catalyzes the oxidative carboxylation of isocitrate to  $\alpha$ -ketoglutarate in cytosol [1]. Excess accumulation of 2-HG leads to an elevated risk of malignant brain tumors in patients. IDH1 mutations occur in some gliomas [2], cartilaginous tumors [3,4], and in acute myeloid leukemias [5]. In astrocytomas, oligodendrogliomas, oligoastrocytomas, and secondary glioblastomas, IDH1 mutations have been identified as early and frequent genetic alterations (50–93%), and might be the initiating event in these glioma subtypes [2,6–8]. In contrast, primary glioblastomas rarely contain IDH1 mutations.

The IDH1 mutations are remarkably specific to a single codon in the conserved and functionally important Arginine 132 residue (R132). The vast majority of changes are heterozygous. IDH1 muta-

Abbreviations: IDH1, isocitrate dehydrogenase 1; mAb, monoclonal antibody.  
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tions were reported as IDH1-R132H (664/716: 92.7%), IDH1-R132C (29/716: 4.2%), IDH1-R132S (11/716: 1.5%), IDH1-R132G (10/716: 1.4%), and IDH1-R132L (2/716: 0.2%) [9]. Only one IDH1-R132V mutation was also reported in 1212 IDH1 mutations (1/1212: 0.1%) [10]. Although one monoclonal antibody (mAb) against IDH1-R132H has been reported by another group [11], we established IDH1-R132H-specific mAb HMab-1 [12] and IDH1-R132S-specific mAb SMab-1 [13]. We previously evaluated 164 cases of glioma for IDH1 mutations (R132H and R132S) using anti-IDH1 mAbs: HMab-1 and SMab-1 [14]. IDH1 mutation was detected in 9.7%, 63.6%, 51.7%, and 77.8% of primary grade IV, secondary grade IV, grade III, and grade II gliomas, respectively. Evaluation of IDH1 mutation using immunohistochemistry might be of greater prognostic significance than histological grading alone in grade III and grade IV gliomas. Combination of HMab-1 and SMab-1 is able to find more than 90% of IDH1 mutations of gliomas in immunohistochemistry; however, other IDH1 mutations have been missed in immunohistochemistry. Therefore, novel antibodies that recognize other IDH1-mutations should be developed to cover all of IDH1 mutations in immunohistochemistry. Here, we report a novel anti-IDH1-mutation-specific monoclonal antibody, GMAb-m1, which is expected to be extremely useful for diagnosis of IDH1-R132G-bearing gliomas.

## 2. Materials and methods

### 2.1. Cell lines and tissues

Chinese hamster ovary (CHO)-K1 cells (CCL-61) and Sp2/0-Ag14 myeloma (CRL-1581) and 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<sub>2</sub> and 95% air in RPMI 1640 medium including 2 mM L-glutamine (Nakalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies Corp., Carlsbad, CA). One glioma patient (oligodendroglioma, WHO grade II), who underwent primary surgery at Nagoya 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

GMAb-m1 was produced using mouse medial iliac lymph node methods [15]. Briefly, BDF1 mice (Japan SLC Inc., Shizuoka, Japan) were immunized by injecting 80 µg of synthetic peptides of CCGVKPIIIGGHAYGDQYRA (IDH1-R132G), conjugated with KLH together with Freund's complete adjuvant (FCA; Sigma–Aldrich, St. Louis, MO) into tailbase. Rcmab-1 was produced using rat medial iliac lymph node methods [15]. WKY/Izm rats (Japan SLC Inc., Shizuoka, Japan) were immunized by injecting 170 µg of recombinant IDH1 protein expressed in *Escherichia coli* TOP-10 cells into footpad. 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 IDH1-R132G and IDH1-WT peptides, conjugated with BSA.

### 2.3. Enzyme-linked immunosorbent assay (ELISA)

Synthetic peptides were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific Inc., Waltham, MA) at 1 µg/ml for 30 min, respectively. After blocking with SuperBlock T20 (PBS) Blocking Buffer in PBS (Thermo Fisher Scientific Inc.), 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 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific Inc.). The optical density was measured at 655 nm with 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. Plasmid preparation

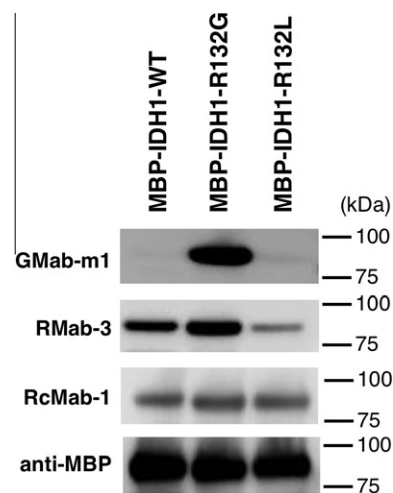
Human IDH1 cDNA (GenBank Accession Nos. AF113917 or BC012846) encoding a full length open reading frame (ORF) was obtained by PCR using a human lung cDNA library (Cosmo Bio Co., Ltd., Tokyo, Japan) as a template. The primer set for IDH1 was as follows: EcoRI-IDH1-F1, 5'-cacgaattcATGTCCAAAAAATCA GTGG-3' and Sall-IDH1-R1, 5'-gtggtcgacTTAAAGTTGGCCTGAGC-TA-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 (Glycine or Leucine) in IDH1 cDNAs was accomplished using the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). The full-length IDH1-WT, IDH1-R132G, and IDH1-R132L 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

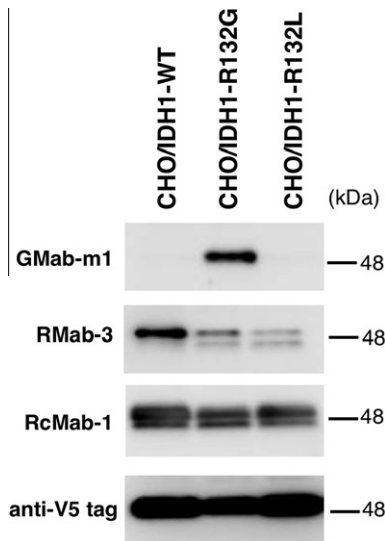
Competent *Escherichia coli* TOP-10 cells (Life Technologies Corp.) were transformed with the plasmid, pMAL-IDH1-WT, pMAL-IDH1-R132G, and pMAL-IDH1-R132L. Then, they were cultured overnight at 37 °C in LB medium (Life Technologies Corp.) containing 100 µg/ml ampicillin (Sigma–Aldrich). Cell pellets were resuspended in phosphate buffered saline (PBS) with 1% Triton X-100 with 50 µg/ml aprotinin (Sigma–Aldrich). After sonication using Branson Advanced Sonifier (Branson Ultrasonics, Danbury, CT), the crude extracts were collected by centrifugation (9000 × g 30 min, 4 °C). The supernatants were loaded onto Amylose resin (GE Healthcare, Buckinghamshire, UK). 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/IDH1-WT, pcDNA3/IDH1-R132G, and pcDNA3/IDH1-R132L 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 IDH1 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 analyses by boiling in SDS sample buffer (Nakalai Tesque, Inc.). 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 GMAb-m1 (1 µg/ml), RMAb-3 [14] (1 µg/ml), Rcmab-1 (1 µg/ml), anti-MBP tag [15,16] (TMab-2; 1 µg/ml), and anti-V5 tag (1:1000 diluted; MBL, Nagoya, Japan) for 30 min; then with peroxidase-conjugated secondary antibodies (Dako, 1/1000 diluted) for 15 min, and developed with ECL-plus reagents (GE Healthcare) using SAYAKA-IMAGER (DRC Co., Ltd., Tokyo, Japan).



**Fig. 1.** Western-blot analyses by anti-IDH1 mAbs against MBP-fusion proteins. Purified proteins (0.05 µg/lane) of *E. coli*-expressing IDH1 wild type (WT; lane 1) and IDH1 mutants (lane 2, IDH1-R132G; lane 3, IDH1-R132L) were electrophoresed under reducing conditions using 5–20% gels, and were Western-blotted with GMAb-m1, RMAb-3, Rcmab-1, and anti-MBP tag (TMab-2).



**Fig. 2.** Western-blot analyses by anti-IDH1 mAbs against mutated IDH1-expressing CHO cells. Total cell lysate from CHO cells expressing IDH1 wild type (WT; lane 1) and IDH1 mutants (lane 2, IDH1-R132G; lane 3, IDH1-R132L) were electrophoresed under reducing conditions using 5–20% gels, and were Western-blotted with GMab-m1, RMaB-3, RCMab-1, and anti-V5 tag.

### 2.7. Immunohistochemical (IHC) analysis

IDH1-R132G protein expression was determined immunohistochemically in paraffin-embedded tumor specimens. Briefly, 4- $\mu$ m-thick histologic sections were deparaffinized in xylene and rehydrated. Then, they were autoclaved in citrate buffer (pH 6.0; Dako) for 20 min. Sections were incubated with 5  $\mu$ g/ml of GMab-m1 over night at 4 °C. Biotin-conjugated secondary anti-mouse 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; Dako) for 10 min, and counterstained with hematoxylin.

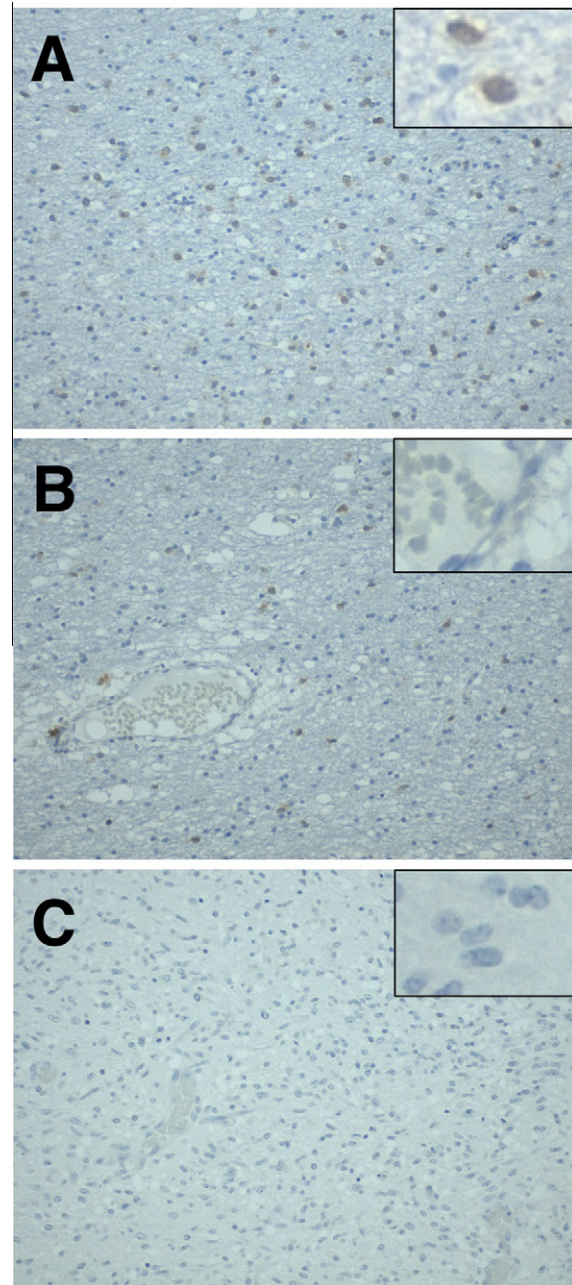
## 3. Results and discussion

### 3.1. Production of IDH1-R132G-specific antibodies

Specific antibodies against R132G have not been reported, although we previously established two anti-mutated IDH1 mAbs: HMab-1 [14] against IDH1-R132H and SMab-1 [13] against IDH1-R132S. In this study, mice were immunized with synthetic peptides of IDH1-R132G, and the wells of hybridomas, which produced IDH1-R132G-reactive/IDH1-wild type (WT)-nonreactive antibodies, were screened in ELISA. After limiting dilution, clone GMab-m1 (mouse IgG<sub>2a</sub>, kappa) against IDH1-R132G was established. GMab-m1 was shown to be an IDH1-R132G-specific mAb, not reacting with IDH1-WT, using ELISA against both synthetic peptides and recombinant proteins (data not shown).

### 3.2. Specificity of GMab-m1 against IDH1 mutants

To determine the specificity of GMab-m1, the reactivity against IDH1-WT and IDH1 mutants (MBP-IDH1-R132G and MBP-IDH1-R132L) were investigated using Western-blot analyses. Fig. 1 shows that GMab-m1 recognized only IDH1-R132G protein, not the other proteins (IDH1-WT and IDH1-R132L), indicating that GMab-m1 is a specific antibody against IDH1-R132G protein. In



**Fig. 3.** Immunohistochemical analyses by GMab-m1 against glioma tissues. Glioma tissues possessing IDH1-R132G, (A and B) or IDH1-WT, (C) were stained with GMab-m1. Magnification:  $\times 200$ .

contrast, RMaB-3 [14] and RCMab-1 recognized IDH1-WT, IDH1-R132G, and IDH1-R132L proteins. These results indicate that GMab-m1 is useful for detecting IDH1-R132G. Another GST-tag was also used in the expression of IDH1 mutants: GST-IDH1-WT, GST-IDH1-R132G and GST-IDH1-R132L. As a result, GST-tagged IDH1-WT and IDH1 mutants were also detected by anti-IDH1 mAbs totally in the same way as MBP-tagged proteins (data not shown), suggesting that the reactivity of those antibodies is observed independently of those tag proteins.

Next, we performed Western-blot analyses against mutated IDH1-expressing CHO cells. As shown in Fig. 2, GMab-m1 recognized only IDH1-R132G protein expressed in CHO cells, not the other proteins (IDH1-WT and IDH1-R132L), indicating that GMab-m1 is useful for detecting IDH1-R132G expressed in mammalian cells. In contrast, RCMab-1 equally recognized



IDH1-WT, IDH1-R132G, and IDH1-R132L, whereas RMab-3 detected IDH1-WT strongly. RcMab-1 and RMab-3 also detects endogenous hamster IDH1.

### 3.3. Immunohistochemical (IHC) analysis by GMab-m1 against IDH1-R132G-bearing gliomas

We next performed immunohistochemistry of GMab-m1 against IDH1-R132G-positive gliomas. Typical results are shown in Fig. 3. GMab-m1 stained almost all tumor cells of IDH1-R132G-positive gliomas (Fig. 3A), although no staining was observed in IDH1-WT gliomas (Fig. 3C). Further, GMab-m1 did not stain endothelial cells in gliomas (Fig. 3B). These results indicate that GMab-m1 is useful in immunohistochemical analyses for detection of IDH1-R132G mutation. In near future, combination of GMab-m1 with previously established anti-mutated IDH1 mAbs (HMab-1 and SMab-1) should increase the diagnostic rate using immunohistochemistry against glioma patients.

In conclusion, the newly established anti-IDH1-R132G-specific mAb, GMab-m1, is expected to be extremely useful for diagnosis and biological evaluation of IDH1-R132G-bearing gliomas in combination with previously established anti-mutated IDH1-specific monoclonal antibodies (HMab-1 and SMab-1). Additional IDH1 mutation-specific antibodies such as anti-IDH1-R132C must be developed for pathological diagnosis of gliomas, cartilaginous tumors, and acute myeloid leukemias.

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