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Short Communications

# Novel Monoclonal Antibodies GMab-r1 and LMab-1 Specifically Recognize IDH1-R132G and IDH1-R132L Mutations

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Isocitrate dehydrogenase 1 (IDH1) catalyzes the oxidative carboxylation of isocitrate to α-ketoglutarate in cytosol. IDH1 mutations, which are specific to a single codon in the conserved and functionally important Arginine 132 (R132), 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). IDH1 mutations, which are early and frequent genetic alterations that occur in gliomas, cartilaginous tumors, and leukemias. We previously established two monoclonal antibodies (MAbs) that are specific for IDH1 mutations: clone HMab-1 against IDH1-R132H and clone SMab-1 against IDH1-R132S. However, specific MAbs against IDH1-R132G or IDH1-R132L have not been reported. To establish IDH1-R132G-specific or IDH1-R132L-specific MAbs, we immunized rats with each mutation-containing IDH1 peptides, and IDH1-R132G-specific or IDH1-R132L-specific MAbs were screened in ELISA. Established MAb GMab-r1 reacted with the IDH1-R132G peptide, but not with IDH1-wild type (WT) in ELISA. In contrast, LMab-1 reacted with the IDH1-R132L peptide, but not with IDH1-WT. Western blot analysis also showed that GMab-r1 and LMab-1 reacted with the IDH1-R132G and IDH1-R132L recombinant proteins, respectively, but not with IDH1-WT or other IDH1 mutants, indicating that GMab-r1 and LMab-1 are IDH1-mutation-specific. Furthermore, GMab-r1 and LMab-1 specifically stained the IDH1-R132G- and IDH1-R132L-expressing cells in immunocytochemistry, respectively. This is the first report to establish anti-IDH1-R132G-specific or IDH1-R132L-specific MAbs, which could be useful in the diagnosis of mutation-bearing tumors.

## Introduction

The *IDH1* gene at 2q33 encodes isocitrate dehydrogenase 1 (IDH1), which catalyzes the oxidative carboxylation of isocitrate to α-ketoglutarate. IDH1 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).[1] 2-HG is reported to accumulate in the inherited metabolic disorder 2-hydroxyglutaric aciduria, which is caused by deficiency of 2-hydroxyglutarate dehydrogenase, because 2-hydroxyglutarate dehydrogenase converts 2-HG to α-ketoglutarate.[2] Patients with 2-hydroxyglutarate dehydrogenase deficiencies are known to accumulate 2-HG in the brain, develop leukoencephalopathy, and possess an increased risk of brain tumors. Moreover, elevated 2-HG levels in brain result in increased ROS levels, potentially contributing to an increased risk of cancer.[3]

IDH1 mutations occur in some malignant gliomas,[4] cartilaginous tumors,[5,6] and acute myeloid leukemias.[7] 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.[4,8–10] 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. Hartmann and colleagues 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%).[11] Another R132V mutation was reported in 1212 IDH1 mutations (1/1212: 0.1%) by von Deimling and colleagues.[12] To date, several monoclonal antibodies (MAbs) against IDH1 mutations have been reported.[13–17] Of those antibodies, we established IDH1-R132H-specific MAb HMab-1[16] and IDH1-R132S-specific MAb SMab-1.[15] HMab-1 reacted with the IDH1-R132H peptide, but not with the

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wild-type IDH1 (IDH-WT) peptide in ELISA. In Western blot analysis, HMab-1 reacted only with the IDH1-R132H protein, not with the IDH1-WT protein or other IDH1 mutants. SMab-1 reacted with IDH1-R132S specifically in the same way.[15] 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.[13] 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, other IDH1 mutations have been missed unfortunately in immunohistochemistry. Therefore, novel antibodies that recognize other IDH1 mutations should be developed to cover all of IDH1 mutations in immunohistochemistry. Here, we newly report anti-IDH1-mutation-specific monoclonal antibodies (GMab-r1 and LMab-1) that are expected to be extremely useful for diagnosis of mutation-bearing gliomas.

## Materials and Methods

### *Cell lines*

Chinese hamster ovary (CHO) cells and Sp2/0 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, Osaka, Japan), respectively, including 2 mM L-glutamine (Wako Pure Chemical Industries) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies Corp., Carlsbad, CA).

### *Hybridoma production*

Three rat monoclonal antibodies (GMab-r1, LMab-1, and RcMab-1) were produced using rat medial iliac lymph node methods.[18–21] Briefly, WKY/Izm rats were immunized by injecting 170 μg of synthetic peptides of CGGVKPIIIGG HAYGDQYRA (IDH1-R132G) for GMab-r1 or CGGVKPIIIG LHAYGDQYRA (IDH1-R132L) for LMab-1, conjugated with KLH together with Freund's complete adjuvant (FCA; Sigma-Aldrich, St. Louis, MO) or recombinant IDH1 protein for RcMab-1[20] 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 mutations (IDH1-R132G or IDH1-R132L) and IDH1-WT peptides conjugated with BSA for GMab-r1 and LMab-1 or recombinant IDH1 protein for RcMab-1.

### *Enzyme-linked immunosorbent assay*

Synthetic peptides were immobilized, respectively, on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, 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-rat IgG (Dako, Glostrup, Denmark). The enzymatic reaction was conducted with a substrate solution containing TMB (Thermo Fisher Scientific). The optical density was measured at 655 nm with an iMark microplate reader (Bio-Rad Laboratories, Philadelphia, PA). These reactions were performed with a volume of 50 μL at 37°C.

### *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., Tokyo, Japan) as a template. The primer set for IDH1 was as follows: EcoRI-IDH1-F1, 5′-cacgaattc ATGTCCAAAAAAATCAGTGG-3′; and SalI-IDH1-R1, 5′-gtggtcgacTTAAAGTTTGGCCTGAGCTA-3′. The amplified cDNA was subcloned into a pcDNA3.1/V5-His-TOPO vector (Life Technologies 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 (Stratagene, La Jolla, CA). The full-length IDH1-WT, IDH1-R132G, and IDH1-R132L were subcloned into an expression vector, pMAL-c2 (New England Biolabs, Beverly, MA) via EcoRI and SalI restriction sites.

### *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. They were cultured overnight at 37°C in LB medium (Life Technologies) 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. 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, pcDNA3.1/IDH1-WT, pcDNA3.1/IDH1-R132G, pcDNA3.1/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). The expression level of IDH1 was confirmed using Western blot analysis.

### *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 10 μg of total protein were prepared for Western blot analysis by boiling in SDS sample buffer (Nakalai Tesque, Kyoto, Japan). They were electrophoresed on 5–20% polyacrylamide gels (Wako Pure Chemical Industries). The separated proteins were transferred to a PVDF membrane (EMD Millipore Corp., Billerica, MA). After blocking with 4% skim milk in PBS for 15 min, the membrane was incubated with GMab-r1 (1 μg/mL), LMab-1 (1 μg/mL), RcMab-1 (1 μg/mL), anti-V5 tag (1:1000 dilution; MBL, Nagoya, Japan), anti-

MBP (TMab-2; 1 μg/mL),[19] or anti-GST tag (1:1000 dilution; Wako Pure Chemical Industries) for 15 min, then with peroxidase-conjugated secondary antibodies (1:1000 dilution; Dako) for 15 min, and developed with Pierce Western Blotting Substrate Plus (Thermo Fisher Scientific, Inc.) using SAYACA-IMAGER (DRC Co., Tokyo, Japan).

### *Immunocytochemical 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 non-specific binding sites, and were incubated with 5 μg/mL of GMab-r1, LMab-1, or RcMab-1 containing 0.1% Triton X-100 overnight at 4°C in a moist chamber. They were incubated with goat rat IgG-Alexa 488 (1:400 dilution; Molecular Probes, 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, Jena, Germany). Then fluorescent images were processed using image-processing software (Adobe Photoshop, Adobe Systems, San Jose, CA).

## Results and Discussion

### *Production of IDH1-R132G-specific and IDH1-R132L-specific antibodies*

We previously established two anti-mutated IDH1 MAbs: HMab-1[13] against IDH1-R132H and SMab-1[15] against IDH1-R132S. However, specific antibodies against R132G or R132L have not been reported. In this study, we immunized rats with synthetic peptides of IDH1-R132G or IDH1-R132L. After cell fusion, the wells of hybridomas, which produced IDH1-R132G-reactive/IDH1-wild type (WT)-nonreactive or IDH1-R132L-reactive/IDH1-WT-nonreactive antibodies, were screened in ELISA. After limiting dilution, clone GMab-r1 (rat $IgG_{2a}$ subclass) against IDH1-R132G and clone LMab-1 (rat $IgG_{2a}$ subclass) against IDH1-R132L were established. One anti-IDH1-WT MAb, clone RcMab-1 (rat $IgG_{2a}$), was also established. GMab-r1 and LMab-1 were shown to be IDH1-mutation-specific MAbs, not reacting with IDH1-WT, using ELISA against both synthetic peptides and recombinant proteins (data not shown). In contrast, RcMab-1 was shown to be reactive with both IDH1 mutants and IDH1-WT using ELISA against both recombinant proteins (data not shown).

### *Specificity of anti-mutated IDH1 MAbs against IDH1 mutants*

To determine the specificity of GMab-r1, LMab-1, and RcMab-1 MAbs, the reactivity against IDH1-WT and IDH1 mutants (MBP-IDH1-R132G and MBP-IDH1-R132L) were investigated using Western blot analyses. Figure 1 shows that GMab-r1 recognized only IDH1-R132G protein, not the other proteins (IDH1-WT and IDH1-R132L), indicating that GMab-r1 is specific antibodies against IDH1-R132G protein. In contrast, LMab-1 reacted with only IDH1-R132L protein, not the other proteins (IDH1-WT and IDH1-R132G), indicating that LMab-1 is a specific antibody against IDH1-R132L protein.

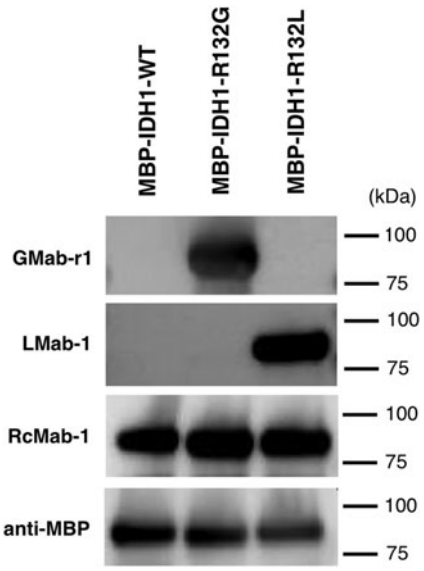


**FIG. 1.** Western blot analyses by anti-IDH1 MAbs against MBP-fusion proteins. Purified proteins (0.05 μg/lane) of *E. coli*-expressing IDH1 wild type (WT; lane 1) and IDH1 mutants (lane 2, IDH1-R132G; lane 3, IDH1-R132L) were electrophoresed under reducing conditions using 5–20% gels, and were Western blotted with GMab-r1, LMab-1, RcMab-1, and anti-MBP tag (TMab-2).

RcMab-1 recognized IDH1-WT, IDH1-R132G, and IDH1-R132L proteins. These results indicate that GMab-r1 and LMab-1 are useful in detecting IDH1-R132G or IDH1-R132L, respectively. To exclude the possibility that the specificities of these MAbs were caused by MBP-tag, which was used for the expression of IDH1 mutants in *E. coli*; another GST-tag was also used in the expression of IDH1 mutants GST-IDH1-WT, GST-IDH1-R132G, and GST-IDH1-R132L. Results show that GST-tagged IDH1-WT and IDH1 mutants were also detected 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.

We next performed Western blot analyses against mutated IDH1-expressing CHO cells. As shown in Figure 2A, GMab-r1 recognized only IDH1-R132G protein expressed in CHO cells, but not the other proteins (IDH1-WT and IDH1-R132L). LMab-1 recognized only IDH1-R132L protein expressed in CHO cells, not the other proteins (IDH1-WT and IDH1-R132G). These results indicate that GMab-r1 and LMab-1 are useful in detecting IDH1-R132G or IDH1-R132L expressed in mammalian cells, respectively. As shown in Figure 2A, RcMab-1 strongly recognized IDH1-WT, IDH1-R132G, and IDH1-R132L. RcMab-1 also detects endogenous hamster IDH1.

### *Immunocytochemical analyses by anti-mutated IDH1 MAbs against mutated IDH1-expressing cell lines*

Newly established anti-IDH1 MAbs GMab-r1 and LMab-1 were confirmed to be anti-mutated IDH1-specific antibodies in Western blot analyses; therefore, we performed immunocytochemical (ICC) analyses using IDH1-R132G- or IDH1-R132L-transfected CHO cells. Results show that GMab-r1 and LMab-1 reacted with IDH1-R132G- or IDH1-R132L-transfected cells, respectively. RcMab-1 reacted with IDH1-

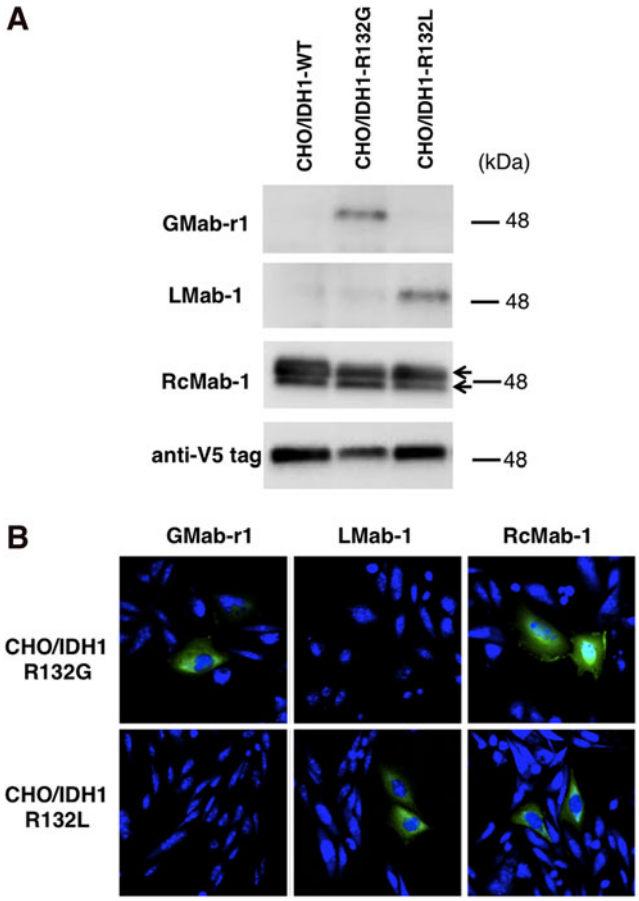


**FIG. 2.** (**A**) Western blot analyses by anti-IDH1 MAbs against mutated IDH1-expressing CHO cells. Total cell lysate from CHO cells expressing IDH1 wild type (WT; lane 1) and IDH1 mutants (lane 2, IDH1-R132G; lane 3, IDH1-R132L) were electrophoresed under reducing conditions using 5–20% gels, and were Western blotted with GMab-r1, LMab-1, RcMab-1, and anti-V5 tag. RcMab-1 detects two bands, including recombinant human IDH1 (upper band) and endogenous hamster IDH1 (lower band). (**B**) Immunocytochemical analyses by anti-IDH1 MAbs. CHO cells were transfected by IDH1-R132G or IDH1-R132L. Each cell was stained by GMab-r1, LMab-1, or RcMab-1, followed by rat IgG-Alexa 488 antibody (green). Cells were also treated with TO-PRO-3 to stain the cell nuclei (blue).

WT-expressing CHO cells, suggesting that RcMab-1 is also useful in ICC. Next, we investigated the specificity of GMab-r1 and LMab-1 against mutated IDH1-expressing CHO cells. As depicted in Figure 2B, GMab-r1 reacted with only CHO/IDH1-R132G, but not with CHO/IDH1-R132L. Immunostaining of GMab-r1 for CHO/IDH1-R132G demonstrated cytosolic patterns. In contrast, LMab-1 reacted with only CHO/IDH1-R132L, not with CHO/IDH1-R132G. Immunostaining of LMab-1 for CHO/IDH1-R132L also demonstrated cytosolic patterns. These results indicate that GMab-r1 and LMab-1 are useful in detecting IDH1-R132G or IDH1-R132L, respectively, suggesting that GMab-r1 and LMab-1 could be also useful in immunohistochemical analyses for detection of IDH1-R132G or IDH1-R132L. In the same way with Western blot analyses, RcMab-1 reacted with both IDH1-R132G and IDH1-R132L (Fig. 2B).

In conclusion, the newly established anti-mutated IDH1-specific MAbs GMab-r1 and LMab-1 are expected to be extremely useful for diagnosis and biological evaluation of mutation-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 AML.

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## Author Disclosure Statement

The authors have no financial interests to disclose.

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