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A high-sensitive HMab-2 specifically detects IDH1-R132H, the most common IDH mutation in gliomas

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ABSTRACT

Isocitrate dehydrogenase 1 (IDH1) mutations have been detected in gliomas and other tumors. Although IDH1 catalyzes the oxidative carboxylation of isocitrate to α-ketoglutarate (α-KG) in cytosol, mutated IDH1 proteins possess the ability to change α-KG into the oncometabolite D-2-hydroxyglutarate (D-2HG). Several monoclonal antibodies (mAbs) specific for IDH1 mutations have been established, such as H09, IMab-1, and HMab-1 against IDH1-R132H, which is the most frequent IDH1 mutation in gliomas. In this study, we established a novel high-sensitive mAb HMab-2, which reacts with IDH1-R132H but not with wild type IDH1 in ELISA. HMab-2 reacted only with IDH1-R132H, not with wild type IDH1/2 and other IDH1/2 mutants in Western-blot analysis. Furthermore, HMab-2 recognized IDH1-R132H more sensitively compared with our previously established HMab-1. HMab-2 detected endogenous IDH1-R132H protein expressed in glioblastoma in immunohistochemical analysis. HMab-2 is expected to be useful for the diagnosis of IDH1-R132H-bearing tumors.

1. Introduction

Isocitrate dehydrogenase (IDH) 1 and IDH2 catalyze the oxidative carboxylation of isocitrate to α-ketoglutarate (α-KG) in the cytosol and mitochondria, respectively [1]. IDH1/2 mutations have been found to change α-KG into the oncometabolite D-2-hydroxyglutarate (D-2HG). IDH1/2 mutations occur in some gliomas [2–7], acute myeloid leukemias [8], intrahepatic cholangiocarcinomas [9], and cartilaginous tumors [10]. In astrocytomas, oligodendrogliomas, oligoastrocytomas, and secondary glioblastomas, IDH1/2 mutations have been identified as early and frequent genetic alterations (50–90%) [11]. In contrast, primary glioblastomas rarely contain IDH1/2 mutations (3–16%). The vast majority of IDH1/2 mutations are heterozygous, and IDH1 mutations and IDH2 mutations are mutually exclusive [12]. IDH1 mutations are remarkably specific to a single codon in the conserved and functionally important arginine 132 residue (R132). IDH2 mutations are specific to a single codon in arginine 172 residue (R172) in gliomas; however, in AML, IDH2 mutations in arginine 140 residue (R140) have been discovered and were found more frequently than R172 [13–15]. In gliomas, previously reported IDH1/2 mutations include IDH1-R132H (89.7%), IDH1-R132C (2.8%), IDH2-R172K (2.8%), IDH1-R132S (1.4%), IDH1-R132G (1.1%), IDH2-R172M (0.8%), IDH1-R132L (0.6%), IDH2-R172W (0.6%), and IDH2-R172S (0.2%) [12]. In the present study, we report a novel high-sensitive mAb HMab-2, which reacts with IDH1-R132H but not with wild type IDHs and other mutated IDHs.

2. Materials and methods

2.1. Cell lines and specimens

P3X63Ag8U.1 (P3U1) mouse myeloma cell line and LN229 glioblastoma cell line were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. P3U1 was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque Inc., Kyoto, Japan), and LN229 was cultured in Dulbecco's modified Eagle medium (DMEM; Nacalai Tesque Inc.). Both media include 2 mM L-glutamine (Nacalai Tesque Inc.), 100

Abbreviations: IDH, isocitrate dehydrogenase; mAb, monoclonal antibody.

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units/ml of penicillin, 100 μg/ml of streptomycin, and 25 μg/ml of amphotericin B (Nacalai Tesque, Inc.) and 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific Inc., Waltham, MA). Two patients who underwent primary surgery at Tsukuba University Hospital are included in this study. Informed consent was obtained from each patient or the patient's caretaker for obtaining samples and subsequent data analysis.

2.2. Hybridoma production

BALB/c mice were immunized by intraperitoneal (i.p.) injections of 100 μg of synthetic peptides of CKPIIIGHHAYGD (IDH1-R132H), conjugated with keyhole limpet hemocyanin (KLH) together with Imject Alum (Thermo Fisher Scientific Inc.). One week later, secondary i.p. immunization of 100 μg of synthetic peptides was performed. After several additional immunizations of 100 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 RPMI medium with hypoxanthine, aminopterin, and thymidine (HAT) selection medium supplement (Thermo Fisher Scientific Inc.). The culture supernatants were screened using enzyme-linked immunosorbent assay (ELISA).

2.3. Enzyme-linked immunosorbent assay (ELISA)

Recombinant proteins were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific Inc.) at 1 μg/ml with a volume of 50 μl at 37 °C for 30 min. After blocking with 1% BSA in phosphate buffered saline (PBS) with a volume of 200 μl at 4 °C over night, the plates were incubated with culture supernatant with a volume of 50 μl at 37 °C for 30 min with subsequent 1:3000 diluted peroxidase-conjugated anti-mouse IgG (Dako, Glostrup, Denmark) in 0.1% BSA/PBS with a volume of 50 μl at 37 °C for 30 min. The enzymatic reaction was conducted with 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific Inc.) with a volume of 50 μl at room temperature for 10 min. The optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories Inc., Philadelphia, PA).

2.4. Protein expression using bacteria cells

Competent *Escherichia coli* TOP-10 cells (Thermo Fisher Scientific Inc.) were transformed with appropriate amounts of plasmids, pMAL-IDH1-WT or pMAL-IDH1-R132H [16]. Then, they were cultured overnight at 37 °C in LB medium (Thermo Fisher Scientific Inc.) containing 100 μg/ml ampicillin (Sigma–Aldrich Corp.). Cell pellets were resuspended in 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., Ipswich, MA). 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

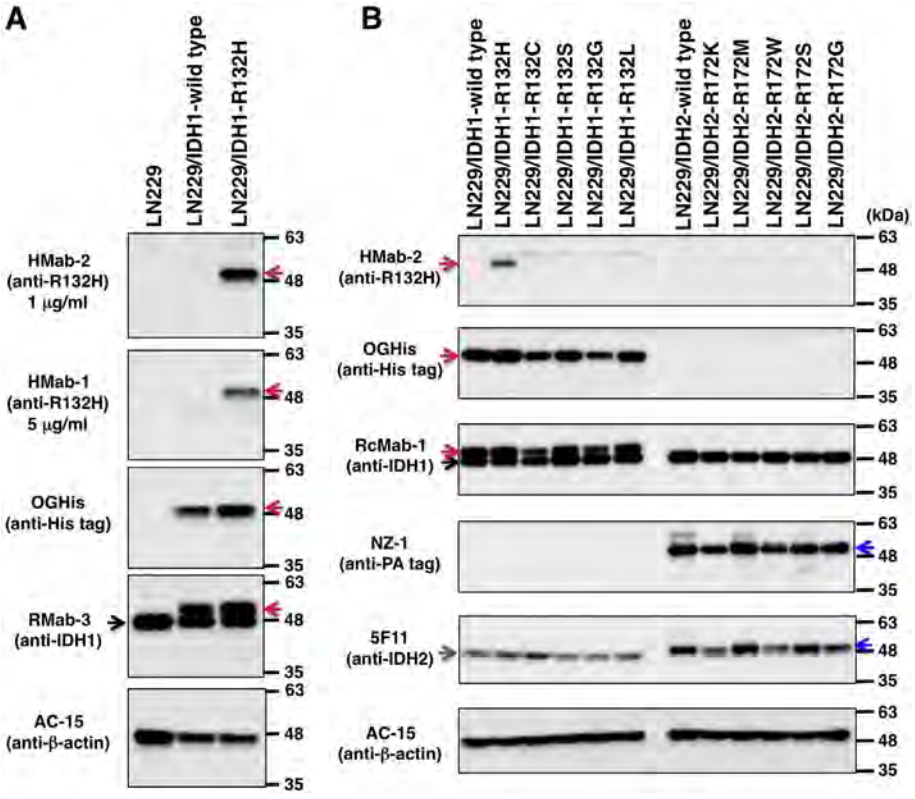

Fig. 1. (A) Western-blot analyses by anti-IDH1/2 mAbs against IDH1-wild type or IDH1-R132H-expressing LN229 cells. Cell lysates were prepared for Western blot analyses by boiling in SDS sample buffer. They were electrophoresed on 5–20% polyacrylamide gels. The separated proteins were transferred to a PVDF membrane. After blocking, the membrane was incubated with HMab-2 (1 μg/ml), HMab-1 (5 μg/ml), OGHis (anti-His tag; 1 μg/ml), RMab-3 (1 μg/ml), and AC-15 (anti-β-actin; 1 μg/ml). Then the membrane was incubated with peroxidase-conjugated anti-mouse antibody, and developed with ImmunoStar LD Chemiluminescence Reagent using Sayaca-Imager. Red arrow, exogenous IDH1; black arrow, endogenous IDH1. (B) Western-blot analyses by anti-IDH1/2 mAbs against mutated IDH1/2-expressing LN229 cells. The membrane was incubated with HMab-2 (1 μg/ml), OGHis (anti-His tag; 1 μg/ml), RcMab-1 (1 μg/ml), NZ-1 (anti-PA tag; 1 μg/ml), 5F11 (1 μg/ml), and AC-15 (anti-β-actin; 1 μg/ml). Red arrow, exogenous IDH1; black arrow, endogenous IDH1; blue arrow, exogenous IDH2; green arrow, endogenous IDH2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

buffer with 10 mM maltose.

2.5. Protein expression using mammalian cells

LN229 cells were transfected with appropriate amounts of plasmids, pcDNA3.1V5tag-Histag (VH)/IDH1-WT, pcDNA3.1VH/IDH1-R132H, pcDNA3.1VH/IDH1-R132C, pcDNA3.1VH/IDH1-R132S, pcDNA3.1VH/IDH1-R132G, pcDNA3.1VH/IDH1-R132L, pcDNA3-PAtag (PA)C-terminal(c)high-binding(H)/IDH2-WT, pcDNA3-PAcH/IDH2-R172K, pcDNA3-PAcH/IDH2-R172M, pcDNA3-PAcH/IDH2-R172W, pcDNA3-PAcH/IDH2-R172S, pcDNA3-PAcH/IDH2-R172G using Lipofectamine LTX (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions [17]. The expression level of IDH1/2 was confirmed using Western blot analyses.

2.6. 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 by boiling in SDS sample buffer (Nacalai Tesque, Inc.). They were electrophoresed on 5–20%

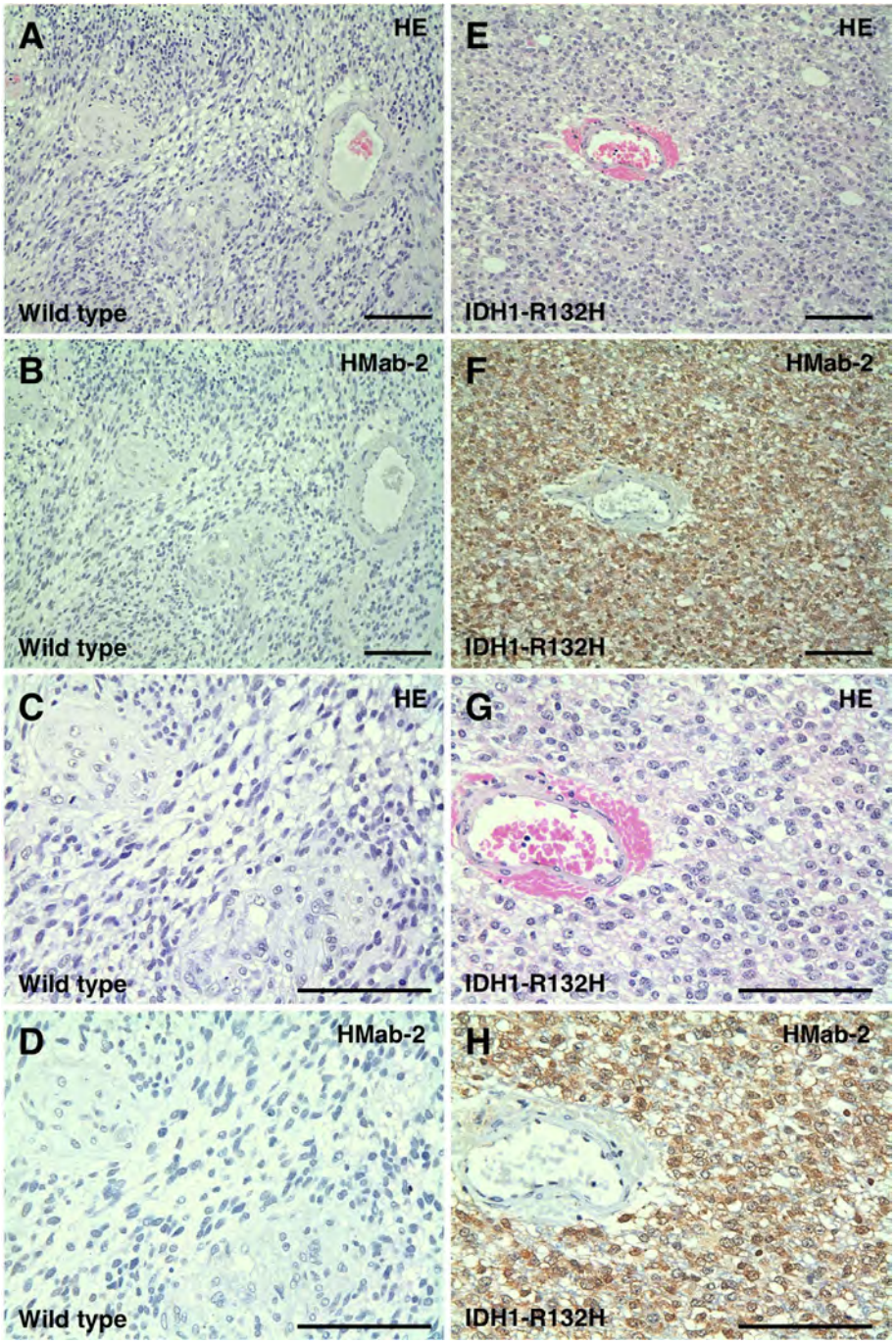

Fig. 2. Immunohistochemical analysis by HMab-2. Sections of IDH1-wild type glioblastoma (A–D) and IDH1-R132H-posssesing glioblastoma (E–H) were incubated with 2 μg/ml of HMab-2 (B, D, F, H), followed by Envision + kit, and color was developed using DAB and counterstained with hematoxylin. Serial sections were also stained by hematoxylin-eosin (A, C, E, G). Scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

polyacrylamide gels (Wako Pure Chemical Industries Ltd., Osaka, Japan). 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 HMab-2, HMab-1 [18], RMab-3 [18], RcMab-1 [19], 5F11 (anti-IDH2; Sigma–Aldrich Corp.), OGHis (anti-His tag; MBL Co. Ltd., Nagoya, Japan), NZ-1 (anti-PA tag) [20,21], 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.) using Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

2.7. Immunohistochemical analysis

IDH1-R132H protein expression was determined immunohistochemically in paraffin-embedded tumor specimens, as described previously [22,23]. Briefly, 5-μm-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 2 μg/ml of HMab-2, 5 μg/ml of RMab-3, 5 μg/ml of RcMab-1, 2 μg/ml of isotype control (clone PMab-2; mouse IgG_1, kappa), or PBS overnight at 4 °C. Blocking peptides were used at a concentration of 20 μg/ml. The Envision + kit (Dako) or biotinylated anti-rat IgG (Dako) + Vectastain Elite ABC kit (Vector laboratories Inc., Burlingame, CA) was used for secondary mAbs and amplification. Color was developed using 3, 3-diaminobenzidine tetrahydrochloride (DAB) for 10 min, and counterstained with hematoxylin.

3. Results

3.1. Production of a mutated IDH1-R132H-specific mAb

We immunized mice with synthetic peptides of IDH1-R132H, and screened IDH1-R132H-reactive/IDH1-wild type (WT)-non-reactive mAbs using ELISA. After limiting dilution, a clone HMab-2 (mouse IgG_1, kappa) against IDH1-R132H was established. HMab-2 also reacted with MBP-fused IDH1-R132H protein, not with MBP-fused IDH1-WT protein using Western-blot analysis (data not shown). We next investigated the reactivity of HMab-2 against IDH1-R132H, which is expressed in mammalian cells in Western-blot analysis. HMab-2 detected IDH1-R132H, which is transfected in LN229 cells, whereas it did not react with IDH1-WT, which is transfected in LN229 cells or endogenous IDH1 (Fig. 1A). Previous established HMab-1, which is very useful in immunohistochemistry [18,24], recognized IDH1-R132H at a concentration of 5 μg/ml. In contrast, HMab-2 detected IDH1-R132H at a concentration of 1 μg/ml. Furthermore, the signal intensity of HMab-2 at a concentration of 1 μg/ml was stronger than that of HMab-1 at a concentration of 5 μg/ml. RcMab-1 (anti-IDH) detected both exogenous and endogenous IDH1 proteins. OGHis (anti-His tag) reacted with exogenous IDH1 proteins.

3.2. Specificity of HMab-2 against IDH proteins in Western blot analyses

To determine the specificity of HMab-2, we performed Western-blot analyses against IDH1/2 mutations, which are expressed in LN229 glioblastoma 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 LN229, and Western-blotted with anti-IDH mAbs. As shown in Fig. 1B, RcMab-1 detected both exogenous and endogenous IDH1 proteins. 5F11 reacted with both exogenous and endogenous IDH2 proteins. Anti-tag mAbs, OGHis (anti-His tag) and NZ-1 (anti-PA tag) reacted with exogenous IDH1 and IDH2 proteins, respectively. Interestingly, HMab-2 specifically detected IDH1-R132H, but did not react with IDH1/2-WTs and other mutations.

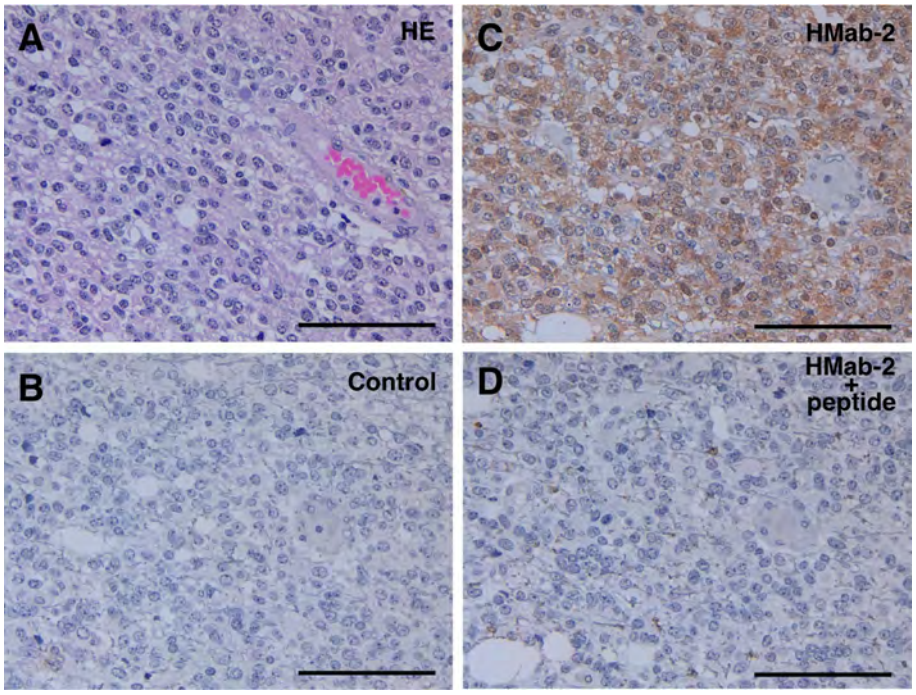

Fig. 3. HMab-2 reaction was blocked by immunized peptide in immunohistochemical analysis. The section of IDH1-R132H-posssesing glioblastoma was stained by hematoxylin-eosin (A). Serial sections were incubated with 2 μg/ml of isotype control (B), 2 μg/ml of HMab-2 (C), or 2 μg/ml of HMab-2 + 20 μg/ml of immunized peptide (D) followed by Envision + kit, and color was developed using DAB and counterstained with hematoxylin. Scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. HMab-2 reacts with endogenous IDH1-R132H expressed in glioblastoma tissues

We selected two glioblastoma tissues: one possesses only IDH1-WT (Fig. 2A–D); the other possesses IDH1-R132H, which is previously investigated using DNA direct sequencing (Fig. 2E–H) [25]. As shown in Fig. 2F and H, HMab-2 clearly stained IDH1-R132H-possessing glioma cells, whereas it did not react with vascular endothelial cells. In contrast, HMab-2 did not stain glioma cells of IDH-WT tissue (Fig. 2 B and D). HMab-2 reaction was completely inhibited by the immunized peptide, demonstrating that HMab-2 reaction is specific against IDH1-R132H protein of glioma cells (Fig. 3). These data indicate that HMab-2 is specific against IDH1-R132H of gliomas in immunohistochemical analyses. Anti-IDH1 mAbs such as RMab-3 and RcMab-1 strongly reacted with glioma cells of both IDH-WT tissue and IDH1-R132H tissue, indicating that IDH1 expression was not suppressed in IDH-WT tissue (Fig. 4).

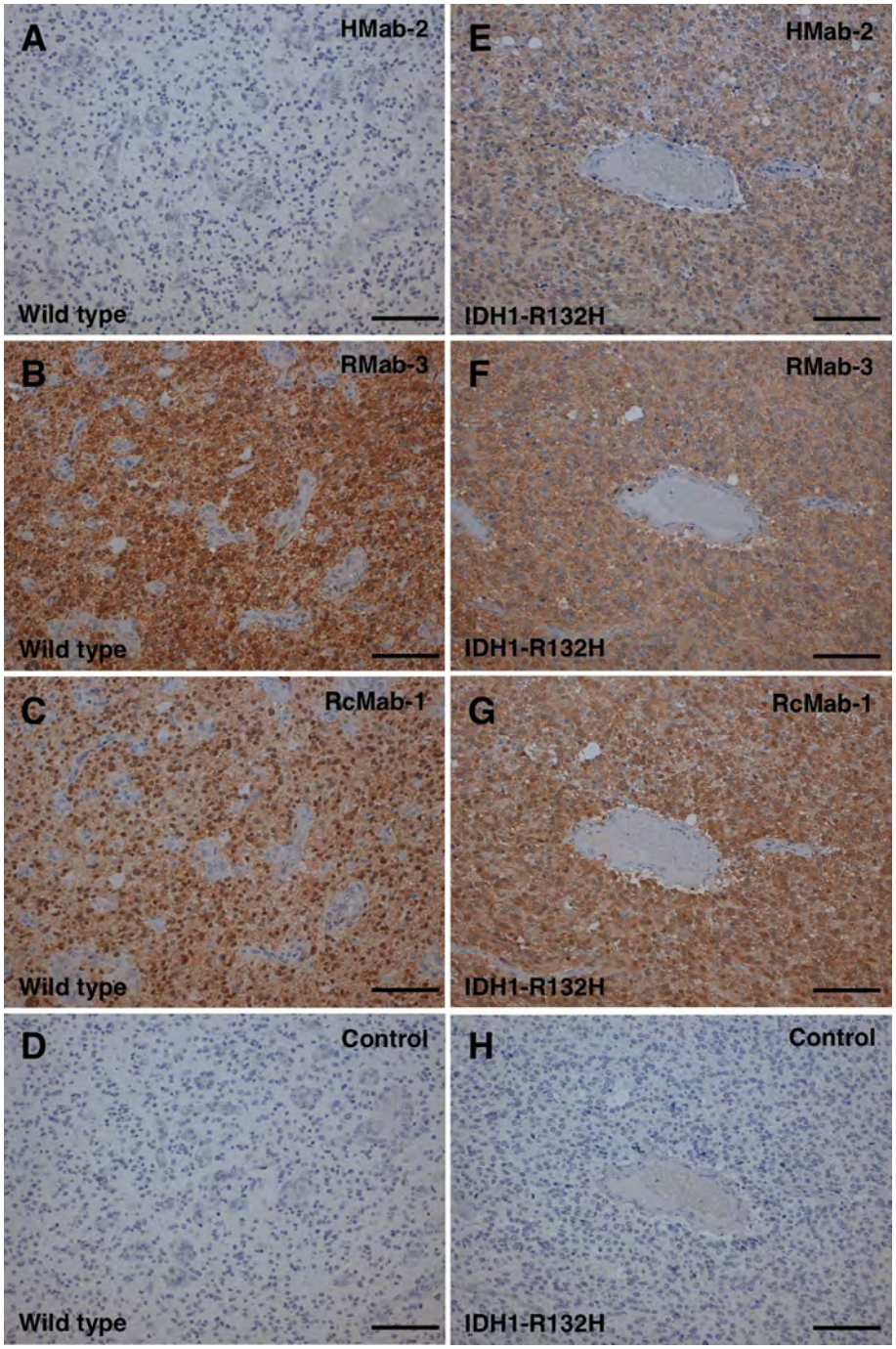

Fig. 4. Anti-IDH1 mAbs strongly reacted with glioma cells of both IDH-WT tissue and IDH1-R132H tissue. The section of IDH1-wild type glioblastoma (A–D) and IDH1-R132H-posssesing glioblastoma (E–H) were incubated with 2 μg/ml of HMab-2 (A, E), 5 μg/ml of RMab-3 (B, F), 5 μg/ml of RcMab-1 (C, G), or PBS (D, H) followed by Envision + kit (A, B, D, E, F, H) or biotinylated anti-Rat IgG and Vectastain Elite ABC kit (C, G), and color was developed using DAB and counterstained with hematoxylin. Scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

There are several methods for testing IDH mutation status, which targets DNA sequence, protein, and D-2-hydroxyglutarate (D-2HG) [26]. DNA direct sequencing (Sanger method) and immunohistochemistry are two conventional methods, which are applied for daily diagnostic practice. Recently, several special methods such as melting curve analysis [27], pyrosequencing [28], and magnetic resonance spectroscopy (MRS) [29] were developed; however, special equipment are required for those methods [26]. Although Sanger method detects all types of mutations, at least 20% of the mutant allele is required for detection of IDH mutations [28]. In contrast, immunohistochemistry detects only one mutation-bearing tumor cell; however, many types of monoclonal antibodies (mAbs) against IDH mutations should be developed. We have established two multi-specific IDH mAbs [24]: MsMab-1 [16] and MsMab-2 [19]. We also established several mono-specific anti-mutated IDH mAbs [24]: IDH1-R132H-specific mAbs (IMab-1 [30] and HMab-1 [18]), IDH1-R132S-specific mAb SMab-1 [31], IDH2-R172K-specific mAb KMab-1 [32], IDH2-R172M-specific mAb MMab-1 [32], and IDH2-R172W-specific mAb WMab-1 [33]. In contrast, clone H09 against IDH1-R132H, which was established by another group, has been used in many studies [34]. However, H09 is cross-reactive with IDH-R132L, and IMab-1 and HMab-1 are low-sensitive [24].

In the present study, we established a novel high-sensitive and high-specific mAb HMab-2, which reacts with IDH1-R132H but not with IDH1-WT and other IDH mutants. Compared with our previous trials of producing anti-IDH1-R132H mAbs, we used three different methods for the first screening: i) the short peptide of 12 amino acids (+N-terminal cysteine) was used for immunization; ii) the recombinant proteins of IDH1, which were fused with maltose-binding protein (MBP), were used for the first ELISA screening because recombinant protein-binding mAbs in ELISA have been reported to be more advantageous in immunohistochemistry [24]; iii) our previous studies used Chinese hamster ovary (CHO)-transfectants for the second screening, whereas LN229 glioblastoma-transfectants were used in this study in anticipation of the direct application of immunohistochemistry against gliomas. Previous established HMab-1, which is very useful in immunohistochemistry [18,24], is unable to detect IDH1-R132H at a concentration of 1 μg/ml in Western-blot, although HMab-2 can detect it at a concentration of less than 1 μg/ml. Furthermore, the signal intensity of HMab-2 at a concentration of 1 μg/ml is stronger than that of HMab-1 at a concentration of 5 μg/ml in immunohistochemistry, demonstrating that HMab-2 is high-sensitive against IDH1-R132H. Taken together, HMab-2 is expected to be useful for the diagnosis of IDH1-R132H-bearing tumors, especially gliomas. HMab-2 is not only high-sensitive compared with HMab-1 mAb but also high-specific compared with H09 mAb. The combination of HMab-2 with previously established anti-mutated IDH1/2 mAbs might lead to high-sensitive detection of IDH1/2 mutation in clinical diagnosis.

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Author disclosure statement

The authors have no financial interest to disclose.

Conflict of interest

None.

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