



## A monoclonal antibody IMAb-1 specifically recognizes IDH1<sup>R132H</sup>, the most common glioma-derived mutation

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### ABSTRACT

IDH1 (isocitrate dehydrogenase 1) mutations have been identified as early and frequent genetic alterations in astrocytomas, oligodendrogliomas, and oligoastrocytomas as well as secondary glioblastomas. In contrast, primary glioblastomas very rarely contain IDH1 mutations, although primary and secondary glioblastomas are histologically indistinguishable. The IDH1 mutations are remarkably specific to a single codon in the conserved and functionally important Arg132 in IDH1. In gliomas, the most frequent IDH1 mutations (>90%) were G395A (R132H). In this study, we immunized mice with R132H-containing IDH1 (IDH1<sup>R132H</sup>) peptide. After cell fusion using Sendai virus envelope, the monoclonal antibodies (mAbs), which specifically reacted with IDH1<sup>R132H</sup>, were screened in ELISA. One of the mAbs, IMAb-1 reacted with the IDH1<sup>R132H</sup> peptide, but not with wild type IDH1 (IDH1<sup>WT</sup>) peptide in ELISA. In Western-blot analysis, IMAb-1 reacted with only the IDH1<sup>R132H</sup> protein, not IDH1<sup>WT</sup> protein or the other IDH1 mutants, indicating that IMAb-1 is IDH1<sup>R132H</sup>-specific. Furthermore, IMAb-1 specifically stained the IDH1<sup>R132H</sup>-expressing cells in astrocytomas in immunohistochemistry, whereas it did not react with IDH1<sup>R132H</sup>-negative primary glioblastoma sections. In conclusion, we established an anti-IDH1<sup>R132H</sup>-specific monoclonal antibody IMAb-1, which should be significantly useful for diagnosis and biological evaluation of mutation-bearing gliomas.

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### Introduction

Gliomas are the most common primary brain tumors, and are grouped into four grades (grade I–IV) according to the World Health Organization (WHO) criteria. Among them, glioblastomas (GBMs) are the most frequent and malignant type of gliomas [1]. Despite advances in surgical techniques, radiation therapy and adjuvant chemotherapy, their prognoses remain poor: the median survival time for patients with GBMs is only one year [2]. GBMs may occur de novo (primary GBM) or may result from progression of low-grade astrocytomas (secondary GBM) [3,4].

An unbiased, genome-wide analysis of the somatic mutations occurring in GBMs revealed recurrent mutations in arginine (amino acid position 132: R132), which is the active site of IDH1, in 12% of tumors analyzed [5]. In the subsequent genetic studies, IDH1 has been found to be mutated in 50–88% of secondary GBMs, whereas only 3–12% of primary GBMs possess these

alterations [6–13]. IDH1 seems to function as a tumor suppressor; therefore, mutated IDH1 contributes to tumorigenesis in part through induction of the HIF-1 pathway [14]. Analyses of progressive glioma samples, including malignant diffuse astrocytomas, anaplastic astrocytomas, well-differentiated oligodendrogliomas, anaplastic oligodendrogliomas, and the mixed oligoastrocytomas, and anaplastic oligoastrocytomas revealed that mutations in IDH1-R132 (IDH1<sup>R132H</sup>) are in fact common (>70%) in these tumors, and also occur less frequently in primary glioblastomas and other cancers [6–13]. Furthermore, the IDH1 mutation was only rarely detected in any WHO grade I pilocytic astrocytomas [6,7,9,15], a tumor type with infrequent malignant progression, indicating that these tumors arise through a different mechanism. A monoclonal antibody, which can specifically recognize the IDH1<sup>R132</sup> is urgently needed for both research and clinical purposes. Furthermore, an immunohistochemical assay would be advantageous in the evaluation of the diagnosis and prognosis of a glioma biopsy as well as for biological studies such as evaluating the extent of invasion in mutation-bearing gliomas. In this study, we report a monoclonal antibody, IMAb-1, which specifically detects IDH1<sup>R132H</sup> in ELISA, Western-blot analysis, and immunohistochemistry.

Abbreviations: IDH1, isocitrate dehydrogenase 1; WHO, World Health Organization; CHO, Chinese hamster ovary.

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## Materials and methods

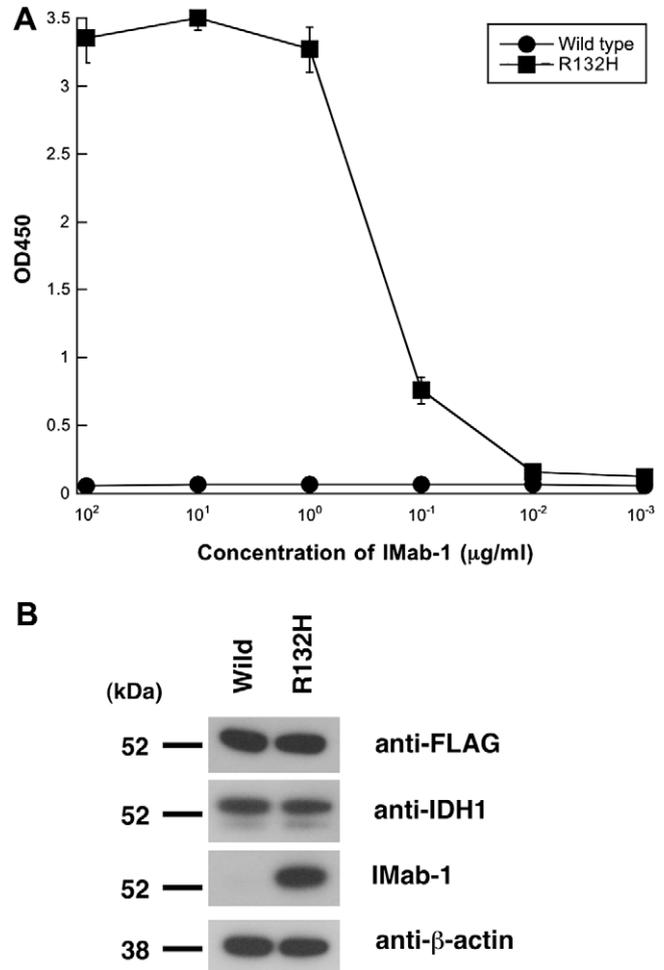
**Animals, cell lines, and tissues.** Female BALB/c mice were obtained from The Jackson laboratory (Bar Harbor, Maine). Chinese hamster ovary (CHO) and P3U1 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). CHO and P3U1 were cultured at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air in RPMI 1640 medium including 2 mM L-glutamine (Invitrogen Corp., Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma, St. Louis, MO) and 1% of penicillin–streptomycin solution (Invitrogen Corp.). HOG cells were cultured as described previously [7]. Human tissues slides were obtained from the Tissue Bank at the Preston Robert Tisch Brain Tumor Center at Duke University as described previously [7]. Four anaplastic astrocytoma (WHO Grade III) tissues slides and one diffuse astrocytoma (WHO Grade II) tissue slides, which possess IDH1-R132H mutation (IDH1<sup>R132H</sup>), and two glioblastoma (WHO grade IV) tissues, which do not possess IDH1<sup>R132H</sup>, were previously analyzed [7].

**Hybridoma production.** BALB/c mice were immunized by neck s.c. injections of 100 µg of synthetic peptide KPIIIG<sup>H</sup>HAYGDQYRC (IDH1<sup>R132H</sup> peptide), corresponding to amino acids 126–140 of the human IDH1<sup>R132H</sup>, plus C-terminus cystein conjugated with KLH with Imject Freund's Complete Adjuvant (Thermo Scientific, Inc., Rockford, IL). One week later, secondary i.p. immunization of 100 µg of IDH1<sup>R132H</sup> peptide was performed. After additional immunization of 100 µg of IDH1<sup>R132H</sup> peptide, the 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: HVJ) envelope: GenomONE-CF<sub>EX</sub> (Cosmo Bio USA, Inc., Carlsbad, CA) according to the manufacturers' instructions. The hybridomas were grown in RPMI medium including hypoxanthine, aminopterin and thymidine selection medium supplement (Sigma), 2 mM L-glutamine (Invitrogen Corp.), 10% heat-inactivated FBS (Sigma), 5% BriClone (QED Bioscience Inc., San Diego, CA), and 1% of penicillin–streptomycin, solution (Invitrogen Corp.). The culture supernatants were screened by ELISA for the binding to the IDH1<sup>R132H</sup> peptide and the IDH1-wild type (IDH1<sup>WT</sup>) as shown in ELISA section. Single cell cloning was performed using ClonaCell-HY Hybridoma Selection Medium (Medium D; StemCell Technologies Inc., Vancouver, Canada).

**Enzyme-linked immunosorbent assay (ELISA).** Synthetic peptides corresponding to amino acids 126–140 of the human IDH1<sup>WT</sup>: (KPIIIG<sup>R</sup>HAYGDQYRC) and IDH1<sup>R132H</sup> (KPIIIG<sup>H</sup>HAYGDQYRC) plus C-terminus cystein conjugated with BSA were immobilized on 96-well plates at 1 µg/ml for 30 min, respectively. After blocking with 1% BSA in PBS, the plates were incubated with I Mab-1 at several concentrations, followed by 1:1000 diluted peroxidase-conjugated anti-mouse IgG (GE Healthcare UK Ltd., Buckinghamshire, England). The enzymatic reaction was conducted with a substrate solution containing TMB (Thermo Scientific, Inc.). After the reaction was stopped with 2 M H<sub>2</sub>SO<sub>4</sub>, the optical density was measured at 450 nm with an auto-plate reader (Bio-Rad Laboratories, Inc., Philadelphia, PA). These reactions were performed with a volume of 50 µl at room temperature.

**Establishment of stable transfectants of IDH1<sup>WT</sup> and IDH1<sup>R132H</sup>.** CHO cells were transfected with vectors (pCMV6, Invitrogen Corp.) containing the coding sequences of IDH1<sup>WT</sup> and IDH1<sup>R132H</sup> [7] using LipofectAMINE 2000 reagent (Invitrogen Corp.) according to the manufacturers' instructions. Stable CHO transfectants were selected by cultivating the transfectants in medium containing 1 mg/ml of geneticin (Invitrogen Corp.), and the expression levels of IDH1<sup>WT</sup> and IDH1<sup>R132H</sup> were confirmed by Western-blot analysis.

**Western-blot analysis.** Stable CHO transfectants of IDH1<sup>WT</sup> and IDH1<sup>R132H</sup> were lysed with lysis buffer (1% Triton X-100 in PBS con-



**Fig. 1.** Production of a specific monoclonal antibody against IDH1<sup>R132H</sup>. (A) Synthetic peptides corresponding to amino acids 126–140 of the human IDH1<sup>WT</sup> and IDH1<sup>R132H</sup> plus C-terminus cystein conjugated with BSA were immobilized on 96-well plates. After blocking, the plates were incubated with I Mab-1 at several concentrations, followed by peroxidase-conjugated anti-mouse IgG. The enzymatic reaction was conducted with a substrate solution containing TMB. After the reaction was stopped with 2 M H<sub>2</sub>SO<sub>4</sub>, the optical density was measured at 450 nm. (B) Cell lysates from CHO-stable transfectants expressing IDH1<sup>WT</sup> or IDH1<sup>R132H</sup> were electrophoresed under reducing conditions using 4–10% gels, and Western-blotted with anti-FLAG, anti-IDH1, I Mab-1, and anti-β-actin.

taining 50 mg/ml aprotinin). HOG cells were transfected with pCMV6 vectors containing the coding sequences of IDH1<sup>WT</sup> and five IDH1 mutants, and lysed as described previously [7]. Samples of the supernatant fraction were collected after centrifuging at 15,000g for 5 min. Ten micrograms of the proteins were electrophoresed under reducing conditions on 4–10% NuPAGE gel (Invitrogen). The separated proteins were transferred to a PVDF membrane (Bio-Rad Laboratories, Inc.). After blocking with 5% skim milk in PBS with 0.05% Tween 20, the membrane was incubated with mouse anti-FLAG (M2; 1 µg/ml; Sigma), goat anti-IDH1 (1/200 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), I Mab-1 (1 µg/ml), mouse anti-Myc (1/2000; Origene Technologies, Inc., Rockville, MD), mouse anti-β-actin antibody (1/5000 dilution; Sigma), or rabbit anti-GAPDH (1/4000 dilution; Santa Cruz Biotechnology), and subsequently with peroxidase-conjugated anti-goat (1/40,000 dilution; Sigma), anti-rabbit (1/5000 dilution; GE Healthcare UK Ltd.) or anti-mouse antibodies (1/25,000 dilution; GE Healthcare UK Ltd.). It was then developed for 5 s to 1 min with ECL (GE Healthcare UK Ltd.), ECL-plus

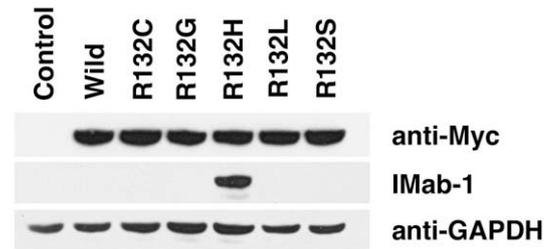
reagents (GE Healthcare UK Ltd.), or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) using Amersham Hyperfilm ECL (GE Healthcare UK Ltd.).

**Immunohistochemical analysis.** All procedures were performed using Bond Polymer Refine Detection of Bond™ Automated Immunohistochemistry system (Leica Microsystems, Inc., Bannockburn, IL). Briefly, formalin fixed, paraffin embedded 5  $\mu$ m thick sections were deparaffinized, rehydrated, and heated at 100 °C using citrate buffer (pH 6.0) for 5 min, and incubated first with IMab-1 (5  $\mu$ g/ml) for 15 min, then with post-primary blocker for 8 min, and finally with polymer for 8 min. Color was developed using 3,3-diaminobenzidine tetrahydrochloride (DAB) for 10 min, and counterstained by hematoxylin.

## Results

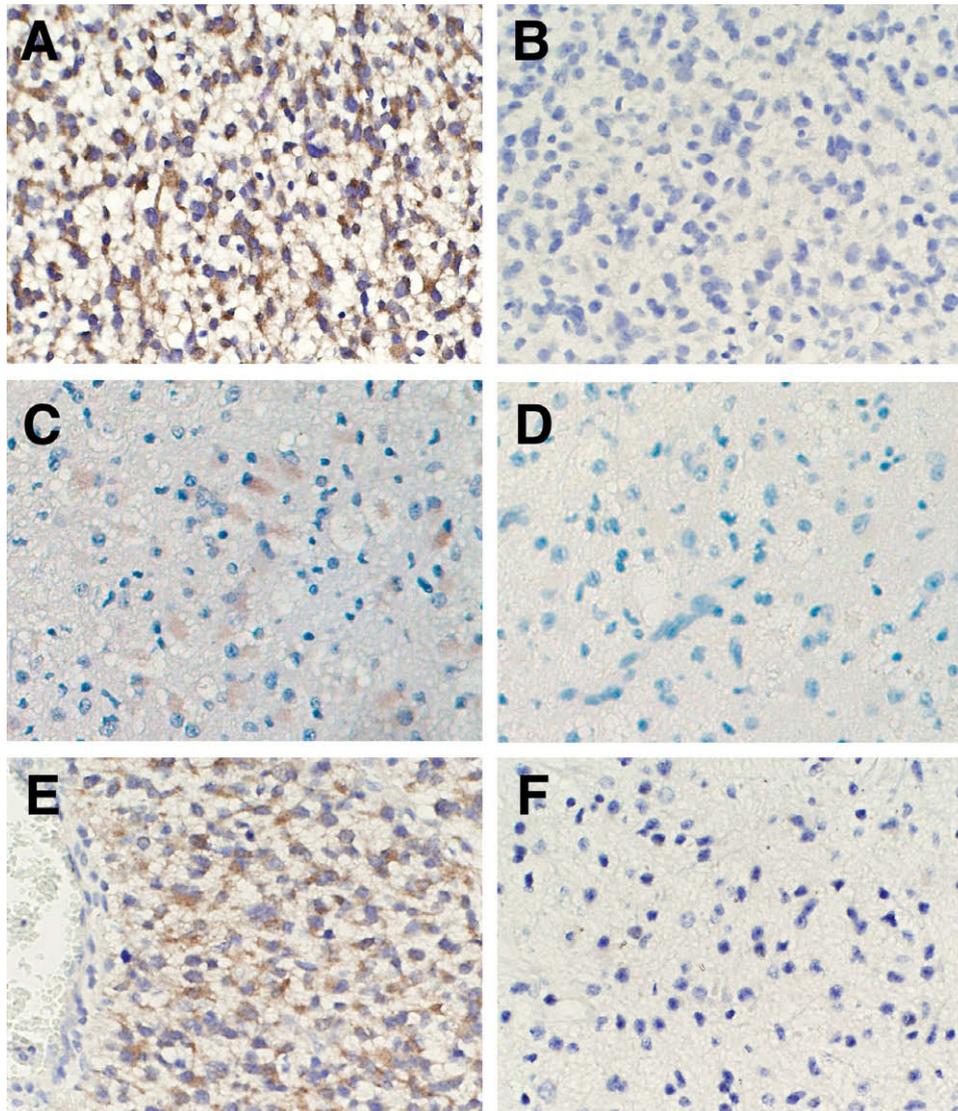
### Production of an anti-IDH1<sup>R132H</sup>-specific antibody

We immunized mice with synthetic peptides of IDH1-R132H mutant (IDH1<sup>R132H</sup>). After cell fusion using Sendai virus envelope, the wells of hybridomas, which were producing anti-IDH1<sup>R132H</sup>-



**Fig. 2.** IMab-1 reacts with only IDH1<sup>R132H</sup>. Cell lysates from HOG transient transfectants expressing IDH1<sup>wt</sup> and five IDH1 mutants were electrophoresed under reducing conditions using 4–10% gels, and Western-blotted with anti-Myc, IMab-1, and anti-GAPDH.

specific antibodies, were selected in ELISA. After limiting dilution, one of the clones, IMab-1 (IgG<sub>1</sub> subclass) was established. As shown in Fig. 1A, IMab-1 reacted with IDH1<sup>R132H</sup> peptide in a dose-dependent manner, but not with IDH1-wild type (IDH1<sup>wt</sup>) in ELISA, indicating that IMab-1 specifically recognized IDH1<sup>R132H</sup>.



**Fig. 3.** Immunohistochemical analysis by IMab-1 against glioma tissues with IDH1<sup>R132H</sup>. Anaplastic astrocytoma tissues which have IDH1<sup>R132H</sup> (A, B, and E), diffuse astrocytoma tissues which have IDH1<sup>R132H</sup> (C, D), and primary glioblastoma tissues which have no IDH1<sup>R132H</sup> (F) were stained by IMab-1 (A, C, E, and F) or isotype control (B, D). Magnification: 200 $\times$ .

Furthermore, IDH1<sup>wt</sup> and IDH1<sup>R132H</sup> proteins were stably expressed in Chinese hamster ovary (CHO) cells, and confirmed by Western-blot analysis (Fig. 1B). Both anti-IDH1<sup>wt</sup> and anti-FLAG antibodies detected IDH1<sup>wt</sup> and IDH1<sup>R132H</sup> proteins at the same expression level. Endogenous IDH1<sup>wt</sup> was also detected slightly by anti-IDH1<sup>wt</sup> antibody in both stable transfectants. Importantly, IMAb-1 recognized only IDH1<sup>R132H</sup>, not IDH1<sup>wt</sup>. These results indicate that IMAb-1 is also useful in detecting not only IDH1<sup>R132H</sup> peptide, but also the IDH1<sup>R132H</sup> protein.

#### Specificity of IMAb-1 against IDH1 mutants

All IDH1 mutants reported until now were transiently transfected into HOG cells (Fig. 2). IMAb-1 recognized only IDH1<sup>R132H</sup>, not the other mutants: IDH1<sup>R132C</sup>, IDH1<sup>R132G</sup>, IDH1<sup>R132L</sup>, and IDH1<sup>R132S</sup>, indicating that IMAb-1 is a specific antibody against IDH1<sup>R132H</sup>.

#### Immunohistochemical analysis by IMAb-1 against glioma tissues with IDH1<sup>R132H</sup>

IMAb-1 was confirmed to be an anti-IDH1<sup>R132H</sup>-specific antibody in ELISA and Western-blot analysis; therefore, we first performed immunohistochemistry using CHO-stable transfectants of IDH1<sup>wt</sup> or IDH1<sup>R132H</sup>, which were embedded in paraffin. Using those paraffin sections, we optimized conditions of immunohistochemistry. IMAb-1 reacted with only IDH1<sup>R132H</sup>-transfectant paraffin sections in the conditions as described in Materials and methods (data not shown). We next performed immunohistochemistry of IMAb-1 against several IDH1<sup>R132H</sup>-positive gliomas and IDH1<sup>R132H</sup>-negative gliomas, which were previously characterized [7]. Typical results were shown in Fig. 3. IMAb-1 stained almost all tumor cells of IDH1<sup>R132H</sup>-positive gliomas (Fig. 3A, C, and E), whereas no staining was observed in IDH1<sup>R132H</sup>-negative gliomas (Fig. 3F). IMAb-1 did not stain endothelial cells or red blood cells in IDH1<sup>R132H</sup>-positive gliomas (Fig. 3E). The concentration-matched isotype control of mouse IgG<sub>1</sub> did not stain any tumor cells (Fig. 3B and D), although at higher concentrations, non-specific binding of gemistocytes is encountered with this isotype control antibody (data not shown). These results indicate that IMAb-1 is useful in immunohistochemistry for detection of IDH1<sup>R132H</sup> mutation.

#### Discussion

The IDH1 gene at 2q33 encodes isocitrate dehydrogenase 1 (IDH1), which catalyzes the oxidative carboxylation of isocitrate to  $\alpha$ -ketoglutarate, which leads to the production of NADPH in the citric acid cycle [14]. IDH1 mutations occur frequently in some types of malignant gliomas, and less frequently in other gliomas [7]. To date, all mutations have been identified at the critical isocitrate binding site R132 and the vast majority of changes are heterozygous [6–13]. In gliomas, mutations in the IDH1 homolog IDH2 have been identified at the aligned R172 [7]. These discoveries mark unique findings in the field of cancer genetics. In this study, we produced an anti-IDH1-R132H (IDH1<sup>R132H</sup>)-specific antibody, IMAb-1. IMAb-1 did not react with wild type of IDH1 (IDH1<sup>wt</sup>) or any other IDH1 mutants in ELISA and Western-blot analysis (Figs. 1 and 2). Importantly, using IMAb-1, we have developed an immunohistochemical method which allows us to view IDH1<sup>R132H</sup>-positive cells in the clinical samples. IMAb-1 displays specific cytosolic staining pattern in the tumor cells, whereas shows no staining in normal cells such as endothelial cells or blood cells (Fig. 3). IDH1 mutations were reported to be very early events, before TP53 mutations or loss of 1p/19q occur [10], which may lead to diffused staining by IMAb-1 in almost every single cell of IDH1<sup>R132H</sup>-positive gliomas.

IDH1 is mutated in the majority of secondary GBMs, whereas less than 10% of primary GBMs possess these alterations [7,10]. IMAb-1 can serve as a molecular tool for distinguishing subtypes of GBMs. Furthermore, IMAb-1 could help distinguish pilocytic astrocytomas (WHO grade I), which rarely express IDH1<sup>R132H</sup> from diffuse astrocytomas (WHO grade II), since these lesions can sometimes be difficult to categorize solely on the basis of histopathological criteria [15]. Most recently, IDH1 mutation have been found in 15 of 187 acute myeloid leukemia samples (AML) [16]. The other tumors, in which IDH1 mutations are reported, are one of colorectal cancer [17], two prostate cancers [18], one B-acute lymphoblastic leukemia (B-ALL) [18], and three adult supratentorial primitive neuroectodermal tumors (spNET) [6]. IMAb-1 might be also useful for diagnosis of these kinds of IDH1<sup>R132H</sup>-positive tumors. The R132C mutations (IDH1<sup>R132C</sup>) were observed at high frequency in AML (8/16 IDH1 mutations; 50%), whereas it was observed in only 4% in glioma specimens [16]. In contrast, IDH1<sup>R132H</sup> was observed in only 44% of IDH1 mutations in AML, whereas it was observed in 88% in glioma specimens [7]. These results suggest that an anti-IDH1<sup>R132C</sup>-specific antibody may be useful for diagnosis of AML as well as glioma subtypes.

Therefore, the established anti-IDH1<sup>R132H</sup>-specific monoclonal antibody IMAb-1 should be significantly useful for diagnosis and biological evaluation of mutation-bearing gliomas.

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