



Generation of a novel monoclonal antibody WMab-1 specific for IDH2-R172W mutation

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ABSTRACT

Isocitrate dehydrogenase 2 (IDH2) mutations have been detected in gliomas, cartilaginous tumors, and acute myeloid leukemias. IDH2 mutations are specific to a single codon in the conserved and functionally important Arginine 172 (R172) or Arginine 140 (R140). To date, we have established specific monoclonal antibodies (mAbs) against IDH2-R172K and IDH2-R172M. However, specific mAbs against IDH2-R172W have not been reported. To establish IDH2-R172W-specific mAbs, we immunized rats with IDH2-R172W peptides. Western-blot analysis showed that WMab-1 reacted with the IDH2-R172W recombinant protein, not with IDH2-wild type (WT) or other IDH2 mutants, indicating that WMab-1 is IDH2-R172W-specific. Furthermore, WMab-1 specifically stained the IDH2-R172W-expressing cells in immunocytochemistry, but did not stain IDH2-WT and IDH2-R172M-containing cells. WMab-1 also specifically stained the IDH2-R172W-expressing glioma cells in immunohistochemistry. This is the first report to establish an anti-IDH2-R172W-specific mAb, which could be useful in the diagnosis of IDH2-R172W-bearing tumors.

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1. Introduction

Isocitrate dehydrogenase 2 (IDH2) mutations have been detected in some gliomas [1–3], cartilaginous tumors [4–6], and acute myeloid leukemias (AML) [7,8]. IDH2 mutations have been identified as early genetic alterations, and might be the initiating event in these tumors [1–3]. The IDH2 mutations are remarkably specific to a single codon in the conserved and functionally important Arginine 172 residue (R172) in gliomas. Of interest, IDH2 mutations of AML were discovered subsequently in Arginine 140 residue (R140), which are more frequent than R172 [7,8]. IDH2 mutations were found to possess the ability to change α -ketoglutarate to onco-metabolite R(-)-2-hydroxyglutarate (2-HG) [9].

To date, several monoclonal antibodies (mAbs) against IDH1/2 mutations have been reported [10–15]. Of those antibodies, we established HMab-1: an anti-IDH1-R132H-specific mAb [10], SMab-1: an anti-IDH1-R132S-specific mAb [11], GMab-m1: an anti-IDH1-R132G-specific mAb [15], KMab-1: an anti-IDH2-R172K-specific mAb [14], and MAb-1: an anti-IDH2-R172M-specific mAb [14]. In our previous study, IDH1 mutation as evaluated using immunohistochemistry might be of greater prognostic significance than histological grading alone in gliomas [10,16–19].

Here, we report a novel anti-IDH2-R172W-specific monoclonal antibody WMab-1, which is expected to be extremely useful for diagnosis of mutation-bearing gliomas.

2. Materials and methods

2.1. Cell lines and tissues

Sp2/0 and Chinese hamster ovary (CHO) cells 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% CO₂ and 95% air in RPMI 1640 medium (Wako Pure Chemical Industries Ltd., Osaka, Japan), including 2 mM L-glutamine (Wako Pure Chemical Industries Ltd.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies Corp., Carlsbad, CA). One glioma patient (oligodendroglioma, WHO grade II–III), 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

WMab-1 against IDH2-R172W was produced using rat medial iliac lymph node methods [14,15]. Briefly, WKY/lzm rats (Japan

Abbreviations: IDH2, isocitrate dehydrogenase 2; mAb, monoclonal antibody.

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SLC Inc., Shizuoka, Japan) were immunized by injecting 170 µg of synthetic peptides of CGGTKPITIGWHAHGDQYKA, conjugated with KLH together with Freund's complete adjuvant (FCA; Sigma–Aldrich, St. Louis, MO) 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 IDH2-R172W and IDH2-WT peptides, conjugated with BSA.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Synthetic peptides were immobilized, respectively, 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-rat 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 IDH2 cDNA (Accession No. NM_002168) encoding a full-length open reading frame (ORF) was obtained by PCR using a cDNA derived from the U373 glioblastoma cell line [20] as a template. The primer set for IDH2 was as follows: EcoRI-IDH2.F1, 5'-ccGAATTCgggATGGCCGGCTACCTGCGGG-3' and Sall-IDH2wterR1359, 5'-gccgtcgacCTACTGCCTGCCAGGGCTCT-3'. The amplified cDNA was subcloned into a pcDNA3.1/V5-His-TOPO vector (Life Technologies Corp.). Substitution of the Arginine 172 (R172) to appropriate amino acid codons (Lysine, Methionine, or Tryptophan) in IDH2 cDNAs was accomplished using the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). The full-length IDH2-WT, IDH2-R172K, IDH2-R172M, and IDH2-R172W were subcloned into an expression vector, pMAL-c2 (New England Biolabs Inc., Beverly, MA), via EcoRI and Sall restriction sites. The full-length IDH2 and each mutated ORF were amplified using the primer set: EcoRI-IDH2.F1 and IDH2woterR1356-XhoI, 5'-taaCTC-GAGcgCTGCTGCCAGGGCTCTG-3'. These PCR products were digested with EcoRI and XhoI restriction enzymes, and subcloned into pcDNA3 vector (Life Technologies Corp.) together with the nucleotide sequence (ctcgagTGGCGTTGCCATGCCAGGTGCCGAA-GATGATGTGGTGTAActaga), which encodes 12 amino acids, GVAMPGAEDDVV (PA tag) [14].

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-IDH2-wild type (WT), pMAL-IDH2-R172K, pMAL-IDH2-R172M, and pMAL-IDH2-R172W. 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, the crude extracts were collected by centrifugation (9000×g 30 min, 4 °C). The supernatants were loaded onto Amylose resin (New England Biolabs Inc.). 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/IDH2-WT, pcDNA3/IDH2-R172K, pcDNA3/IDH2-R172M, or pcDNA3/IDH2-R172W 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 IDH2 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 analysis by boiling in SDS sample buffer (Nakalai Tesque, Inc., Kyoto, Japan). 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 WMab-1 (1 µg/ml), 5F11 (anti-IDH2; 1 µg/ml; Sigma–Aldrich), TMab-2 (anti-MBP tag [14]; 1 µg/ml), or NZ-1 (anti-PA tag [14]; 1 µg/ml) for 15 min; then with peroxidase-conjugated secondary antibodies (Dako, 1/1000 diluted) for 15 min, and developed with ECL-plus reagents (GE Healthcare, Buckinghamshire, UK) using SAYACA-IMAGER (DRC Co., Ltd., Tokyo, Japan).

2.7. Immunocytochemical (ICC) 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 nonspecific binding sites, and were incubated with 5 µg/ml of WMab-1 containing 0.1% Triton X-100 overnight at 4 °C in a moist chamber. They were incubated with goat anti-rat 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

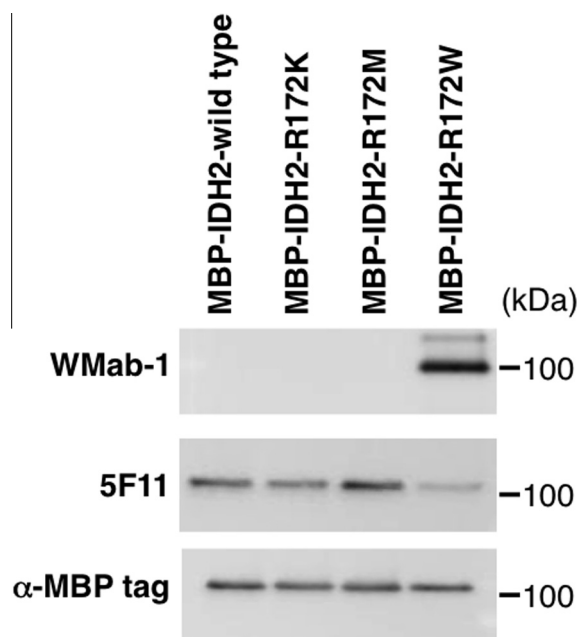


Fig. 1. Western-blot analyses by anti-IDH2 mAbs against MBP-fusion proteins. Purified proteins (0.05 µg/lane) of *E. coli*-expressing IDH2 wild type (lane 1) and IDH2 mutants (lane 2, IDH2-R172K; lane 3, IDH2-R172M; lane 4, IDH2-R172W) were electrophoresed under reducing conditions using 5–20% gels, and were Western-blotted with WMab-1, 5F11, and anti-MBP tag.

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 (IHC) analysis

IDH2-R172W protein expression was determined immunohistochemically in paraffin-embedded tumor specimens [14]. Briefly, 4- μ 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 μ g/ml of WMab-1 overnight at 4 °C. Biotin-conjugated secondary anti-rat 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 an IDH2-R172W-specific antibody

The *IDH2* gene at 15q26.1 encodes isocitrate dehydrogenase 2 (IDH2), which catalyzes the oxidative carboxylation of isocitrate

to α -ketoglutarate in mitochondria [21]. To date, almost all IDH2 mutations have been identified at the critical isocitrate binding site R172 codon or R140 codon in some malignant gliomas [1], cartilaginous tumors [4,5], and in acute myeloid leukemias [7]. The vast majority of changes of IDH2 are heterozygous in the same way of IDH1 mutation [1,2,22,23]. 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%); therefore, combination of our established mAbs: an anti-R132H mAb (HMAb-1) [10], an anti-R132S mAb (SMab-1) [11], and an anti-R132G mAb (GMab-m1) [15] covers 95.6% of IDH1 mutations. It was also reported that IDH2 mutations include R172K (20/31: 64.5%), R172M (6/31: 19.3%), and R172W (5/31: 16.2%) in gliomas [3]. Recently, we have established KMab-1: anti-IDH2-R172K-specific mAb and MMab-1: anti-IDH2-R172M-specific mAb [14]. Combination of our established anti-IDH2 mAbs could cover 83.8% of IDH2 mutations. However, anti-IDH2-R172W mAbs have not been established. For this study, we immunized rats with synthetic peptides of IDH2-R172W. After cell fusion, the wells of hybridomas, which produced IDH2-R172W-reactive/IDH2-wild type (WT)-nonreactive antibodies, were screened in ELISA. After limiting dilution, clone WMab-1 (rat IgG_{2b}, kappa) against IDH2-R172W was established.

3.2. Specificity of an anti-IDH2-R172W mAb against IDH2 Mutants

To determine the specificity of WMab-1, the reactivity against IDH2-WT and IDH2 mutants (MBP-IDH2-R172K, MBP-IDH2-

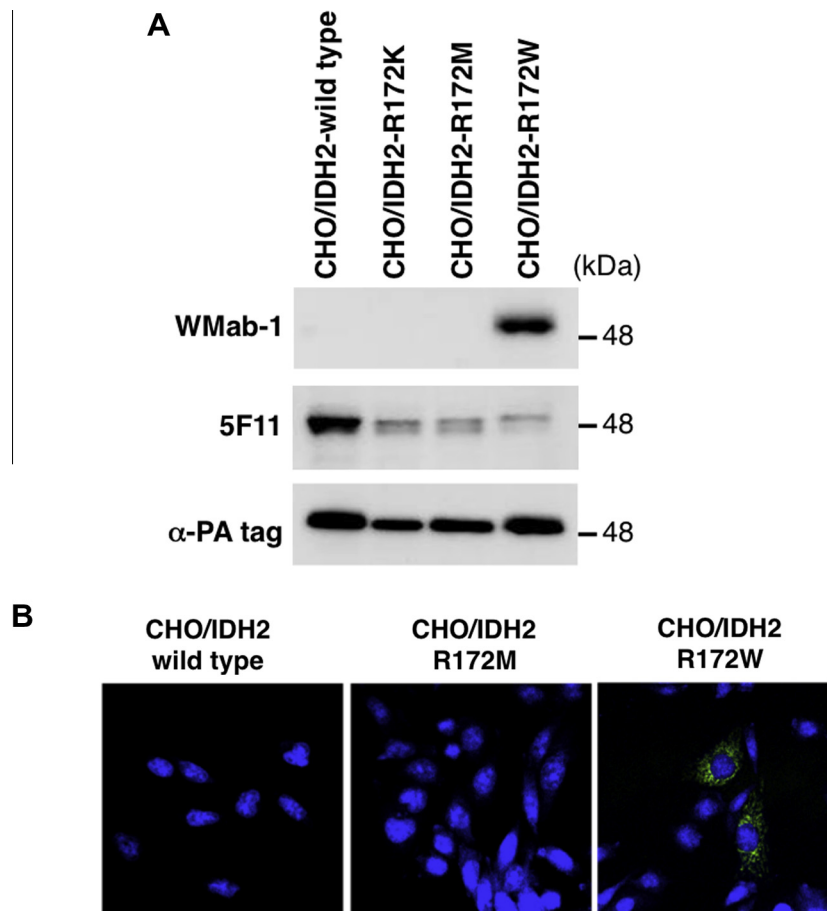


Fig. 2. The specificity of an anti-mutated IDH2 mAb, WMab-1. (A) Western-blot analyses by anti-IDH2 mAbs against mutated IDH2-expressing CHO cells. Total cell lysate from CHO cells expressing IDH2 wild type (lane 1) and IDH2 mutants (lane 2, IDH2-R172K; lane 3, IDH2-R172M; lane 4, IDH2-R172W) were electrophoresed under reducing conditions using 5–20% gels, and were Western-blotted with WMab-1, 5F11, and anti-PA tag. (B) Immunocytochemical analysis by WMab-1 against mutated IDH2-transfected CHO cells. CHO cells were transfected by mutated IDH2. Each cell was stained by WMab-1, followed by anti-rat IgG-Alexa 488 antibody (green). Cells were also treated with TO-PRO-3 to stain the cell nuclei (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

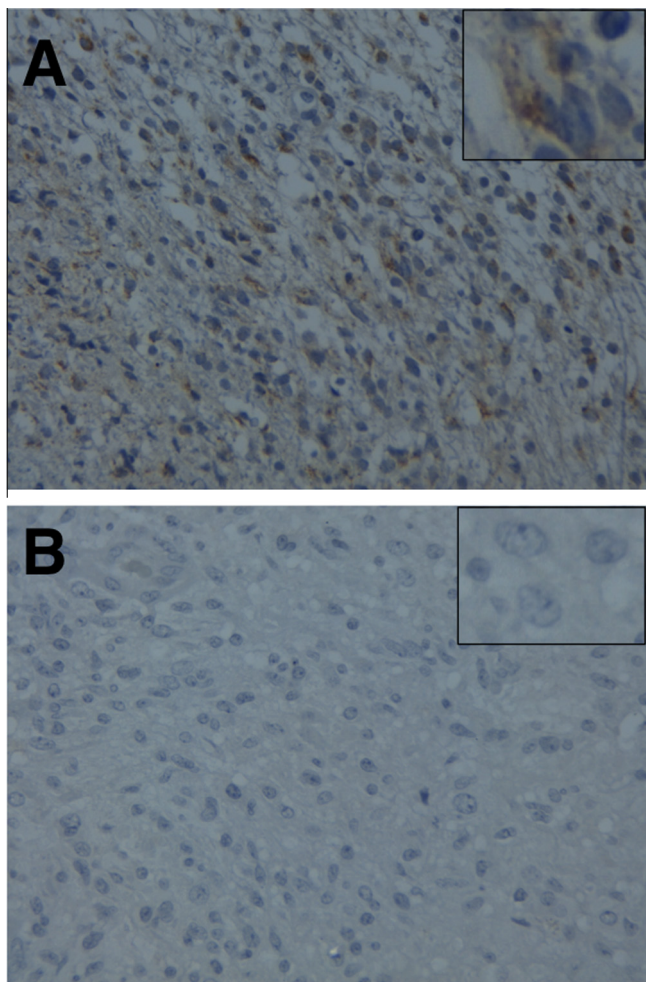


Fig. 3. Immunohistochemical analyses by WMab-1 against glioma tissues. Glioma tissues possessing IDH2-R172W (A) or IDH2-WT (B) were stained with WMab-1. Magnification: $\times 400$.

R172M, MBP-IDH2-R172W) was investigated using Western-blot analyses. As shown in Fig. 1, WMab-1 recognized only MBP-IDH2-R172W protein, not with the other proteins (MBP-IDH2-WT, MBP-IDH2-R172K, MBP-IDH2-R172M), indicating that WMab-1 specifically recognizes MBP-IDH2-R172W protein. In contrast, clone 5F11 recognized MBP-IDH2-WT and all MBP-IDH2-mutant proteins. These results indicate that WMab-1 is useful in detecting IDH2-R172W specifically. To exclude the possibility that the specificity of WMab-1 was affected by MBP-tag, which was used for the expression of IDH2 mutants in *E. coli*, another GST-tag was also used in the expression of IDH2 mutants: GST-IDH2-WT, GST-IDH2-R172K, GST-IDH2-R172M, and GST-IDH2-R172W. Results show that WMab-1 recognized only GST-IDH2-R172W (data not shown), suggesting that the reactivity of WMab-1 is observed independently of those tag proteins.

We next performed Western-blot analyses against mutated IDH2-expressing CHO cells. As shown in Fig. 2A, WMab-1 reacted with only IDH2-R172W protein expressed in CHO cells, not with the other proteins (IDH2-WT, IDH2-R172K, IDH2-R172M). In contrast, anti-IDH2 mAb (clone 5F11) strongly recognized only IDH2-WT; weakly recognized mutated IDH2 proteins. 5F11 also detected endogenous IDH2 expressed in CHO cells. These results indicate that WMab-1 is useful in detecting IDH2-R172W expressed in mammalian cells.

3.3. Immunocytochemical (ICC) analyses by WMab-1 against IDH2-R172W-expressing cell lines

Newly established WMab-1 was confirmed to be an IDH2-R172W-specific antibody in Western-blot analyses; therefore, we performed immunocytochemical (ICC) analyses using IDH2-WT-, IDH2-R172M-, or IDH2-R172W-transfected CHO cells. As depicted in Fig. 2B, WMab-1 reacted with only CHO/IDH2-R172W, not with CHO/IDH2-wild type (WT) and CHO/IDH2-R172M. Immunostaining of WMab-1 for CHO/IDH2-R172W demonstrated cytosolic patterns. These results indicate that WMab-1 is useful for detecting IDH2-R172W in ICC, suggesting that WMab-1 could be also useful in immunohistochemical analyses for detection of IDH2-R172W.

3.4. Immunohistochemical (IHC) analysis by WMab-1 against IDH2-R172W-bearing gliomas

We next performed immunohistochemistry (IHC) of WMab-1 against IDH2-R172W-positive gliomas. Typical results are shown in Fig. 3. WMab-1 stained almost all tumor cells of IDH2-R172W-positive gliomas (Fig. 3A), although no staining was observed in IDH1-WT gliomas (Fig. 3B). These results indicate that WMab-1 is useful in IHC analyses for detection of IDH2-R172W mutation. In near future, combination of WMab-1 with previously established anti-mutated IDH1 mAbs (HMab-1 [10], SMab-1 [11], GMab-m1 [15], GMab-r1 [24], LMab-1 [24]) and anti-mutated IDH2 mAbs (KMab-1 [14], MMab-1 [14]) should increase the diagnostic rate using IHC against glioma patients.

In conclusion, an established anti-IDH2-R172W-specific mAb WMab-1 is expected to be extremely useful for diagnosis and biological evaluation of mutation-bearing gliomas in combination with anti-mutated IDH1/2-specific monoclonal antibodies. Additional IDH1/2 mutation-specific antibodies must be developed for pathological diagnosis of gliomas, cartilaginous tumors, and AML.

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References

- [1] H. Yan, D.W. Parsons, G. Jin, R. McLendon, B.A. Rasheed, W. Yuan, I. Kos, I. Batinic-Haberle, S. Jones, G.J. Riggins, H. Friedman, A. Friedman, D. Reardon, J. Herndon, K.W. Kinzler, V.E. Velculescu, B. Vogelstein, D.D. Bigner, IDH1 and IDH2 mutations in gliomas, *N. Engl. J. Med.* 360 (2009) 765–773.
- [2] Y. Sonoda, T. Kumabe, T. Nakamura, R. Saito, M. Kanamori, Y. Yamashita, H. Suzuki, T. Tominaga, Analysis of IDH1 and IDH2 mutations in Japanese glioma patients, *Cancer Sci.* 100 (2009) 1996–1998.
- [3] C. Hartmann, J. Meyer, J. Bals, D. Capper, W. Mueller, A. Christians, J. Felsberg, M. Wolter, C. Mawrin, W. Wick, M. Weller, C. Herold-Mende, A. Unterberg, J.W. Jeuken, P. Wesseling, G. Reifenberger, A. von Deimling, Type and frequency of IDH1 and IDH2 mutations are related to astrocytic and oligodendroglial differentiation and age: a study of 1,010 diffuse gliomas, *Acta Neuropathol.* 118 (2009) 469–474.
- [4] T.C. Pansuriya, R. van Eijk, P. d'Adamo, M.A. van Ruler, M.L. Kuijper, J. Oosting, A.M. Cleton-Jansen, J.G. van Oosterwijk, S.L. Verbeke, D. Meijer, T. van Wezel, K.H. Nord, L. Sangiorgi, B. Toker, B. Liegl-Atzwanger, M. San-Julian, R. Sciot, N.

- Limaye, L.G. Kindblom, S. Daugaard, C. Godfraind, L.M. Boon, M. Vikkula, K.C. Kurek, K. Szuhai, P.J. French, J.V. Bovee, Somatic mosaic IDH1 and IDH2 mutations are associated with enchondroma and spindle cell hemangioma in Ollier disease and Maffucci syndrome, *Nat. Genet.* 43 (2011) 1256–1261.
- [5] M.F. Amary, S. Damato, D. Halai, M. Eskandarpour, F. Berisha, F. Bonar, S. McCarthy, V.R. Fantin, K.S. Straley, S. Lobo, W. Aston, C.L. Green, R.E. Gale, R. Tirabosco, A. Futreal, P. Campbell, N. Presneau, A.M. Flanagan, Ollier disease and Maffucci syndrome are caused by somatic mosaic mutations of IDH1 and IDH2, *Nat. Genet.* 43 (2011) 1262–1265.
- [6] M.F. Amary, K. Bacsí, F. Maggiani, S. Damato, D. Halai, F. Berisha, R. Pollock, P. O'Donnell, A. Grigoriadis, T. Diss, M. Eskandarpour, N. Presneau, P.C. Hogendoorn, A. Futreal, R. Tirabosco, A.M. Flanagan, IDH1 and IDH2 mutations are frequent events in central chondrosarcoma and central and periosteal chondromas but not in other mesenchymal tumours, *J. Pathol.* 224 (2011) 334–343.
- [7] P.S. Ward, J. Patel, D.R. Wise, O. Abdel-Wahab, B.D. Bennett, H.A. Collier, J.R. Cross, V.R. Fantin, C.V. Hedvat, A.E. Perl, J.D. Rabinowitz, M. Carroll, S.M. Su, K.A. Sharp, R.L. Levine, C.B. Thompson, The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate, *Cancer Cell* 17 (2010) 225–234.
- [8] S. Chotirat, W. Thongnoppakhun, O. Promsuwicha, C. Boonthimat, C.U. Auewarakul, Molecular alterations of isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2) metabolic genes and additional genetic mutations in newly diagnosed acute myeloid leukemia patients, *J. Hematol. Oncol.* 5 (2012) 5.
- [9] S. Gross, R.A. Cairns, M.D. Minden, E.M. Driggers, M.A. Bittinger, H.G. Jang, M. Sasaki, S. Jin, D.P. Schenkein, S.M. Su, L. Dang, V.R. Fantin, T.W. Mak, Cancer-associated metabolite 2-hydroxyglutarate accumulates in acute myelogenous leukemia with isocitrate dehydrogenase 1 and 2 mutations, *J. Exp. Med.* 207 (2010) 339–344.
- [10] S. Takano, Y. Kato, T. Yamamoto, M.K. Kaneko, E. Ishikawa, Y. Tsujimoto, M. Matsuda, K. Nakai, R. Yanagiya, S. Morita, K. Tsuboi, A. Matsumura, Immunohistochemical detection of IDH1 mutation, p53, and internexin as prognostic factors of glial tumors, *J. Neurooncol.* 108 (2012) 361–373.
- [11] M.K. Kaneko, W. Tian, S. Takano, H. Suzuki, Y. Sawa, Y. Hozumi, K. Goto, K. Yamazaki, C. Kitanaka, Y. Kato, Establishment of a novel monoclonal antibody SMab-1 specific for IDH1-R132S mutation, *Biochem. Biophys. Res. Commun.* 406 (2011) 608–613.
- [12] Y. Kato, G. Jin, C.T. Kuan, R.E. McLendon, H. Yan, D.D. Bigner, A monoclonal antibody IMab-1 specifically recognizes IDH1R132H, the most common glioma-derived mutation, *Biochem. Biophys. Res. Commun.* 390 (2009) 547–551.
- [13] D. Capper, H. Zentgraf, J. Balss, C. Hartmann, A. von Deimling, Monoclonal antibody specific for IDH1 R132H mutation, *Acta Neuropathol.* 118 (2009) 599–601.
- [14] M.K. Kaneko, S. Morita, Y. Tsujimoto, R. Yanagiya, K. Nasu, H. Sasaki, Y. Hozumi, K. Goto, A. Natsume, M. Watanabe, T. Kumabe, S. Takano, Y. Kato, Establishment of novel monoclonal antibodies KMab-1 and MMab-1 specific for IDH2 mutations, *Biochem. Biophys. Res. Commun.* 432 (2013) 40–45.
- [15] Y. Kato, A. Natsume, M.K. Kaneko, A novel monoclonal antibody GMab-m1 specifically recognizes IDH1-R132G mutation, *Biochem. Biophys. Res. Commun.* 432 (2013) 564–567.
- [16] S. Takano, W. Tian, M. Matsuda, T. Yamamoto, E. Ishikawa, M.K. Kaneko, K. Yamazaki, Y. Kato, A. Matsumura, Detection of IDH1 mutation in human gliomas: comparison of immunohistochemistry and sequencing, *Brain Tumor Pathol.* 28 (2011) 115–123.
- [17] K. Motomura, A. Natsume, R. Watanabe, I. Ito, Y. Kato, H. Momota, R. Nishikawa, K. Mishima, Y. Nakasu, T. Abe, H. Namba, Y. Nakazato, H. Tashiro, I. Takeuchi, T. Mori, T. Wakabayashi, Immunohistochemical analysis-based proteomic subclassification of newly diagnosed glioblastomas, *Cancer Sci.* 103 (2012) 1871–1879.
- [18] M.J. Kwon, S.T. Kim, D.S. Kong, D. Lee, S. Park, S.Y. Kang, J.Y. Song, D.H. Nam, Y. Kato, Y.L. Choi, Y.L. Suh, Mutated IDH1 is a favorable prognostic factor for type 2 gliomatosis cerebri, *Brain Pathol.* 22 (2012) 307–317.
- [19] A. Yoshikawa, M. Nakada, T. Watanabe, Y. Hayashi, H. Sabit, Y. Kato, S. Suzuki, A. Ooi, H. Sato, J.I. Hamada, Progressive adult primary glioblastoma in the medulla oblongata with an unmethylated MGMT promoter and without an IDH mutation, *Brain Tumor Pathol.* (2012), <http://dx.doi.org/10.1007/s10014-012-0118-9>.
- [20] Y. Kato, M.K. Kaneko, A. Kuno, N. Uchiyama, K. Amano, Y. Chiba, Y. Hasegawa, J. Hirabayashi, H. Narimatsu, K. Mishima, M. Osawa, Inhibition of tumor cell-induced platelet aggregation using a novel anti-podoplanin antibody reacting with its platelet-aggregation-stimulating domain, *Biochem. Biophys. Res. Commun.* 349 (2006) 1301–1307.
- [21] S. Zhao, Y. Lin, W. Xu, W. Jiang, Z. Zha, P. Wang, W. Yu, Z. Li, L. Gong, Y. Peng, J. Ding, Q. Lei, K.L. Guan, Y. Xiong, Glioma-derived mutations in IDH1 dominantly inhibit IDH1 catalytic activity and induce HIF-1 α , *Science* 324 (2009) 261–265.
- [22] D.W. Parsons, S. Jones, X. Zhang, J.C. Lin, R.J. Leary, P. Angenendt, P. Mankoo, H. Carter, I.M. Siu, G.L. Gallia, A. Olivi, R. McLendon, B.A. Rasheed, S. Keir, T. Nikolskaya, Y. Nikolsky, D.A. Busam, H. Tekleab, L.A. Diaz Jr., J. Hartigan, D.R. Smith, R.L. Strausberg, S.K. Marie, S.M. Shinjo, H. Yan, G.J. Riggins, D.D. Bigner, R. Karchin, N. Papadopoulos, G. Parmigiani, B. Vogelstein, V.E. Velculescu, K.W. Kinzler, An integrated genomic analysis of human glioblastoma multiforme, *Science* 321 (2008) 1807–1812.
- [23] J. Balss, J. Meyer, W. Mueller, A. Korshunov, C. Hartmann, A. von Deimling, Analysis of the IDH1 codon 132 mutation in brain tumors, *Acta Neuropathol.* 116 (2008) 597–602.
- [24] M.K. Kaneko, Y. Tsujimoto, Y. Hozumi, K. Goto, Y. Kato, Novel monoclonal antibodies GMab-r1 and LMab-1 specifically recognize IDH1-R132G and IDH1-R132L mutations, *Monoclon. Antib. Immunodiagn. Immunother.*, in press.