

Fig. 7. Model for transcriptional regulation of macrophage colony-stimulating factor receptor (*Csf1r*) and *Hox* genes by mixed lineage leukemia (MLL) fusion proteins. MLL fusions induce the rapid onset of acute myeloid leukemia by activating two classes of pathways, the CSF-1R and *Hox* pathways. Left, MLL fusions stimulate constitutive CSF-1R expression by binding to PU.1 to induce leukemia. Right, MLL fusion proteins form a complex with menin and LEDGF to regulate the expression of *Hox* genes.

survival, proliferation, and/or differentiation of macrophages, osteoclasts, and Paneth cells.^(34–36) A tyrosine kinase inhibitor specific for CSF-1R slowed the progress of MLL-AF10-induced leukemia. CSF-1R upregulation has been detected in LSCs in MOZ-TIF2-induced AML⁽²⁵⁾ and human AML patients.⁽²⁶⁾

CSF-1R expression is critical for AML initiation but not for immortalization *in vitro*. MLL leukemias are invariably associated with the expression of *Hox* genes. Upregulation of *Hox* genes in MLL leukemias is critical for LSC maintenance; however, *Hox* upregulation alone does not recapitulate all the biological and clinical features of MLL leukemias and is unlikely to support malignancy and the high LSC frequency observed in MLL leukemias. Forced expression of the *HOXA9* gene can immortalize myeloid progenitors *in vitro*, but is not sufficient to initiate AML *in vivo*. By contrast, expression of MLL fusions such as MLL-AF10, MLL-AF9, and MLL-ENL is sufficient for both immortalization *in vitro* and initiation of AML *in vivo*. Our results indicate that cells expressing high levels of Csf-1r show strong AML initiation *in vivo* (Fig. 1b,c) whereas cells expressing high and low levels of Csf-1r showed equivalent colony formation *in vitro* (Fig. 1e). These findings suggest that CSF-1R expression is not important for immortalization *in vitro* but is critical for initiation of AML *in vivo*.

The LSC activity of MLL leukemia is known to be reduced after culture *in vitro*,⁽³⁷⁾ suggesting that a certain *in*

in vivo microenvironment is required for LSC maintenance, as is also the case for hematopoietic stem cells. Our results show that the expression of Csf-1r is greatly reduced after culture *in vitro* (data not shown), suggesting that expression of CSF-1R is regulated by microenvironment-dependent epigenetics.

Differential regulation of CSF-1R and *Hox*. Both MLL and MLL fusion proteins form a complex with menin and LEDGF to regulate the expression of *Hox* genes.^(30,38) Our results show that PU.1 mediates the regulation of *Csf1r* transcription by MLL/MLL fusion proteins. The menin and LEDGF-interacting domains of MLL are not required for interaction with PU.1 or transactivation at the *Csf1r* promoter, suggesting that menin and LEDGF are unlikely to be involved in the regulation of CSF-1R expression. While expression of Csf-1r rapidly decreased after deletion of the *Pu.1* gene (Fig. 5d,e), *HoxA9* mRNA levels were stable at least 4 days after *Pu.1* deletion (Fig. 5e). Moreover, *HoxA9* mRNA levels were equivalent in AML cells expressing high and low levels of CSF-1R (Fig. 1h). These results suggest that expression of the *Csf1r* and *Hox* genes is independently regulated by MLL fusion proteins.

The MLL-AF10-induced AML was shown to be cured by deletion of *Pu.1* (Fig. 5c). However, deletion of Csf-1r prolonged survival time but did not cure the AML completely. These facts suggest that CSF-1R is not the sole critical PU.1-dependent mediator of leukemogenesis, and that there are other PU.1-target genes critical for maintenance of MLL fusion-induced AML. Such genes may be involved in the leukemogenesis in collaboration with CSF-1R and *Hox* genes.

It has been suggested that second mutations, such as activating point mutations, in receptor tyrosine kinases (e.g., FLT3 and c-KIT) are required for fusion genes such as AML1-ETO, PML-RAR α , or CBF β -MYH11 to induce acute leukemia.⁽³⁹⁾ In contrast, MLL fusions alone can induce the rapid onset of AML.⁽⁴⁰⁾ Our data suggest that MLL fusions induce the upregulation of the receptor tyrosine kinase *Csf-1r* and *Hox* genes, thereby inducing the rapid onset of AML by activating two classes of pathways (Fig. 7). These pathways provide potential molecular targets for new approaches in the treatment of these forms of leukemia.

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Disclosure Statement

The authors have no conflicts of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. (A) Bone marrow (BM) cells from normal mice were analyzed by flow cytometry for expression of GFP and macrophage colony-stimulating factor receptor (Csf-1r). (B) Csf-1r^{high} and Csf-1r^{low/-} cells from normal mixed lineage leukemia (MLL)-AF10-induced AML mice were sorted by flow cytometry. Levels of *Csf1r* (H) mRNAs were measured in Csf-1r^{high} and Csf-1r^{low/-} cells prepared from normal and acute myeloid leukemia mouse BM. (C) *Mll*^{+/+} and *Mll*^{-/-} Fetal liver cells were analyzed by flow cytometer for expression of Csf-1r.

Specific monoclonal antibodies against IDH1/2 mutations as diagnostic tools for gliomas

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Abstract Mutations of isocitrate dehydrogenase 1/2 (IDH1/2) have been reported in gliomas and other types of tumors, such as acute myeloid leukemias, cartilaginous tumors, intrahepatic cholangiocarcinomas, osteosarcomas, and giant cell tumors of bone. In gliomas, IDH mutations uniformly occur in the functionally critical arginine 132 residue (R132) of IDH1 and arginine 172 residue (R172) of IDH2. IDH1 and IDH2 catalyze the oxidative carboxylation of isocitrate to α -ketoglutarate (α -KG) in the cytosol and mitochondria, respectively. In contrast, mutated IDH1/2 proteins possess a neomorphic enzymatic function that changes α -KG into the oncometabolite, *R*(-)-2-hydroxyglutarate, resulting in genomic hypermethylation, histone methylation, genetic instability, and malignant transformation. To date, several monoclonal antibodies (mAbs) specific for IDH1/2 mutations such as anti-IDH1-R132H mAbs (clone H09, clone IMab-1, and clone HMAb-1) or an anti-IDH1-R132S mAb (clone SMab-1) have been established. Furthermore, one of multi-specific mAbs, MsMab-1, recognizes IDH1 mutants (R132H, R132S, R132G) and IDH2 mutants (R172S, R172G), which are observed in gliomas. Another mAb, MsMab-2, recognizes IDH1-R132L and IDH2-R172M. These IDH1/2 mutation-specific mAbs are useful for the immunohistochemical determination of IDH1/2 mutation-bearing gliomas.

Keywords Isocitrate dehydrogenase 1 · Isocitrate dehydrogenase 2 · Monoclonal antibody · Mutations · Multi-specific mAb

Abbreviations

IDH1/2 Isocitrate dehydrogenase 1/2
mAb Monoclonal antibody

Isocitrate dehydrogenase (IDH) mutations in gliomas and other tumors

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 create a novel capability of the enzyme, specifically, to change α -KG into the oncometabolite *R*(-)-2-hydroxyglutarate (2-HG). 2-HG accumulates in the inherited metabolic disorder, 2-hydroxyglutaric aciduria, as a result of 2-HG dehydrogenase deficiency because 2-HG dehydrogenase converts 2-HG to α -KG [2]. Patients with 2-HG dehydrogenase deficiencies are known to accumulate 2-HG in the brain, to develop leukoencephalopathy and to have an increased risk of developing brain tumors. Furthermore, elevated 2-HG levels in the brain result in increased reactive oxygen species (ROS) concentrations, potentially contributing to an increased risk of malignant tumors [3]. IDH1/2 mutations occur in some gliomas [4–19], acute myeloid leukemias [20–22], intrahepatic cholangiocarcinomas [23–25], cartilaginous tumors [26–29], osteosarcomas [30], and giant cell tumors of bone [31].

In astrocytomas, oligodendrogliomas, oligoastrocytomas, and secondary glioblastomas, IDH1/2 mutations have been

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identified as early and frequent genetic alterations (50–90 %) [32]. They might also be the initiating event in these glioma subtypes. 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 [33]. 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 [34–36]. 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 %) [33]. Although all IDH1/2 mutations possess the same neomorphic enzymatic function that enables the conversion of α -KG into 2-HG, the potential for IDH mutations to produce 2-HG was reported to depend on allele specificity and subcellular compartmentalization [37]. The production of 2-HG from a cytosolic IDH1 mutation is dependent on the activity of a retained wild-type IDH1 allele. This result is consistent with another report [38]. In contrast, the expression of mitochondrial IDH2 mutations leads to robust 2-HG production in a manner that is independent of wild-type mitochondrial IDH function [37]. Furthermore, among the IDH2 mutations at R172 and R140, IDH2-R172 mutations consistently lead to greater 2-HG accumulation than the IDH2-R140 mutations. The expression of an IDH1 construct engineered to localize to the mitochondria using the mitochondrial-targeting sequence caused greater 2-HG accumulation. These data demonstrate that the non-equivalent 2-HG accumulation resulting from several IDH mutations might underlie their differential prognosis and prevalence in various tumors. Although the value of IDH1/2 mutations as an independent prognostic marker remains controversial, glioma patients with IDH1/2 mutations have shown good prognosis in almost all studies [4, 6, 7, 13, 39–45]. In contrast, AML patients with IDH1/2 mutations have shown both good and poor prognosis [34, 46]. The prognosis of AML patients with IDH1/2 mutations remains controversial, most likely because IDH2-R172K, which is a high 2-HG producer, and IDH2-R140Q, which is a low 2-HG producer, are present at different rates. Further studies are necessary to conclude the relationship between IDH mutation types and patient prognosis in various tumors.

Molecular targeting therapy and immunotherapy against IDH1/2 mutations

Two novel drugs against mutated IDH1 and mutated IDH2 were recently developed. One inhibitor of mutated IDH1,

AGI-5198, blocks the ability of IDH1-R132H to produce 2-HG in a dose-dependent manner [47]. Under conditions of 2-HG inhibition, AGI-5198 induces the demethylation of histone H3K9me3 and results in the expression of genes associated with gliogenic differentiation. Blockade of IDH1-R132H impaired the growth of IDH1-R132H-possessing glioma cells but not that of IDH1 wild-type glioma cells, without appreciable changes in genome-wide DNA methylation. Another inhibitor of mutated IDH2, AGI-6780, inhibits the tumor-associated mutant IDH2-R140Q potently and selectively [48]. Treatment with AGI-6780 induced the differentiation of TF-1 erythroleukemia and primary human AML cells *in vitro*. Of interest, a crystal structure of AGI-6780 complexed with IDH2-R140Q showed that the inhibitor binds in an allosteric manner at the dimer interface. Schumacher et al. [49] recently reported that IDH1-R132H is a potential target for immunotherapy because IDH1-R132H is a cancer-specific potential antigen in gliomas. The IDH1-R132H peptides are presented on major histocompatibility complexes (MHC) class II and induce mutation-specific CD4⁺ T helper type-1 (Th1) responses. Interestingly, antibodies and Th1 cells, which occur spontaneously in IDH1-R132H-positive glioma patients, recognize IDH1-R132H specifically. Because IDH1-R132H is present in slow-growing gliomas, an anti-IDH1-R132H vaccine could be a novel therapeutic strategy for IDH1-R132H-mutated gliomas. Because these molecular targeting therapies and immunotherapies are specific against each IDH1/2 mutation, not only the direct DNA sequencing but also the development of mutation-specific mAbs are important to determine the IDH1/2 mutation types.

Production of monoclonal antibodies against IDH1/2 mutations

In 2009, we successfully developed an anti-IDH1-R132H mAb (clone IMab-1) [50]. Capper et al. [51] also reported another anti-IDH1-R132H mAb (clone H09) in the same year, which was the first commercially available anti-mutated IDH1/2 mAb. Both IMab-1 and H09 can be used in enzyme-linked immunosorbent assay (ELISA), Western-blot analysis, and immunohistochemical analysis using glioma sections. Preusser et al. [52] compared IMab-1 and H09 in immunohistochemical analysis using formalin-fixed and paraffin-embedded biopsy samples of 95 diffuse gliomas. Concordant immunostaining results were shown using both antibodies in 94 (98.9 %) of the 95 gliomas. However, H09 generally showed a higher signal-to-background ratio than IMab-1. In a single tiny biopsy, both antibodies showed immunoreactivity. In contrast, genetic testing was inconclusive, indicating that anti-IDH1-R132H immunostaining

is a reliable method for evaluation of IDH1 mutation status. We further compared the immunohistochemistry results obtained using IMab-1 to direct DNA sequencing in 49 glioma samples [11], and showed that that IMab-1 immunohistochemistry is more sensitive than direct DNA sequencing for detecting IDH1-R132H. The IDH1-R132H mutation was confirmed after subcloning in 3 cases that were determined to be wild-type by routine direct DNA sequencing. In contrast, immunohistochemical detection with IMab-1 was positive for these 3 cases. The reasons for this discrepancy might include the following: (1) the IDH1-R132H mutation was not detected by direct DNA sequencing of highly contaminated tumor samples; (2) the fraction of tumor cells in the three samples was very small. Although anti-IDH1-R132H mAbs are very useful for detecting IDH1-R132H, the most frequent IDH1 mutation in gliomas, there are other IDH-mutations described above that are not detected by anti-IDH1-R132H mAbs. Therefore, we started to develop other anti-mutated IDH1/2 mAbs.

Although IDH1-R132C or IDH2-R172K have been reported to be the second most frequent IDH1/2 mutations in several studies [33], IDH1-R132S was observed as the second most frequent IDH1/2 mutation in our independent two studies [12, 15]. To establish IDH1-R132S-specific mAbs, mice were immunized with an IDH1-R132S peptide [53]. One mAb, SMab-1, reacted with the IDH1-R132S peptide but not with other IDH1 mutants in ELISA. Western-blot analysis showed that SMab-1 reacted with only the IDH1-R132S protein and not with IDH1-WT protein or IDH1 mutants, indicating that SMab-1 is IDH1-R132S-specific. Recent studies have revealed that SMab-1 can react with not only IDH1-R132S but also IDH1-R132A and IDH1-R132T peptides in ELISA [30], but those mutations have not reported in gliomas. SMab-1 specifically stained the IDH1-R132S-expressing glioblastoma cells in immunocytochemistry and immunohistochemistry but did not react with IDH1-WT or IDH1-R132H-containing glioblastoma cells, indicating that SMab-1 can be used in the diagnosis of IDH1-R132S-bearing gliomas. Next, the importance of immunohistochemistry was investigated using the combination of anti-IDH1-R132H and anti-IDH1-R132S mAbs [12]. For this study, another anti-IDH1-R132H mAb (clone: HMab-1) was produced, and 164 cases of glioma were evaluated immunohistochemically for IDH1 mutations (R132H and R132S) using the anti-IDH1 mAbs, HMab-1 and SMab-1. IDH1 mutations were 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. In grade III gliomas, progression-free survival (PFS) was significantly longer in cases that were IDH1 mutation-positive (80 months) than those that were IDH1 mutation-negative (23 months) ($p = 0.001$). Additionally, overall survival

(OS) was significantly longer in cases that were IDH1 mutation-positive (119 months) than those that were IDH1 mutation-negative (33 months). In grade IV gliomas, the PFS was significantly longer in cases that were IDH1 mutation-positive (20 months) than those that were IDH1 mutation-negative (9 months). Additionally, OS was significantly longer in cases that were IDH1 mutation-positive (26 months) than those that were IDH1 mutation-negative (14 months). These results show that mutated IDH1 screened by the combination of HMab-1/SMab-1 is much more advantageous compared with immunohistochemistry using only anti-IDH1-R132H mAbs (H09, IMab-1, and HMab-1). Having successfully demonstrated the importance of anti-IDH1/2 mAbs, additional anti-IDH1/2 mAbs were produced. At present, GMab-r1 against IDH1-R132G [54], LMab-1 against IDH1-R132L [54], KMab-1 against IDH2-R172K [55], MMab-1 against IDH2-R172M [55], and WMab-1 against IDH2-R172W [56] were developed. These mAbs are summarized in Table 1.

Next, we sought to produce multi-specific mAbs against mutated IDH1/2 [57]. For this study, mice were immunized with synthetic peptides of IDH1-R132G, and the wells of hybridomas that produced IDH1-R132G-reactive/IDH1-wild type (WT)-nonreactive antibodies were screened by ELISA. After limiting dilution, clone MsMab-1 (mouse IgG_{2a}, kappa) against IDH1-R132G was established. MsMab-1 reacts with all peptides bearing the common IDH1 mutations, IDH1-R132H, IDH1-R132C, IDH1-R132S, IDH1-R132G, and IDH1-R132L, in ELISA but not with IDH-WT peptide, but the MsMab-1 reaction against IDH1-R132C was very weak. Western-blot analysis showed that MsMab-1 strongly recognizes the MBP-IDH1-R132H, MBP-IDH1-R132S, and MBP-IDH1-R132G proteins but only weakly reacted with MBP-IDH1-R132L. MsMab-1 also reacts with MBP-IDH2-R172M but not with MBP-IDH2-WT; however, 19 amino acids of the IDH1-R132G peptides, which were used for immunization, showed only 73.7 % homology with the corresponding amino acids of the IDH2-R172M peptides. These results indicate that MsMab-1 is applicable for detecting IDH1-R132H/R132S/R132G and IDH2-R172M proteins. MsMab-1 also stained almost all tumor cells of IDH1-R132H-positive gliomas, IDH1-R132S-positive gliomas, and IDH1-R132G-positive gliomas via immunohistochemistry, but no staining was observed in IDH1/2-WT gliomas (Fig. 1). Importantly, MsMab-1 immunohistochemistry is more sensitive than direct DNA sequencing for detecting IDH1/2 mutations, because IDH1 mutations were detected after subcloning method in direct DNA sequencing [57]. These results demonstrate that MsMab-1 is useful in immunohistochemical analyses for the detection of IDH1-R132H/R132S/R132G and IDH2-R172M.

Table 1 Commercially available monoclonal antibodies against mutated IDH1/2

Gene	Mutation	mAb (clone/cat. #)	Original/cross reactivities	Host	Immunogen	References	Companies	
IDH1	R132H	MsMab-1	Cross	Mouse	Peptide (R132G)	[57]	M, MM, W	
		HMab-1	Original	Mouse	Peptide (R132H)	[12]	I, M, MM, S, W	
		H09	Original	Mouse	Peptide (R132H)	[51]	D	
		#26081	Original	Mouse	Recombinant (R132H)	–	N	
	R132S	MsMab-1	Cross	Mouse	Peptide (R132G)	[57]	M, MM, W	
		SMab-1	Original	Mouse	Peptide (R132S)	[53]	I, M, MM, S, W	
		#26160	Original	Mouse	Peptide (R132S)	–	N	
	R132G	MsMab-1	Cross	Mouse	Peptide (R132G)	[57]	M, MM, W	
		GMAb-r1	Original	Rat	Peptide (R132G)	[54]	M	
	R132L	MsMab-2	Original	Rat	Peptide (R132L)	[58]	M	
		H09	Cross	Mouse	Peptide (R132H)	[84]	D	
		Wild type	RMab-3	–	Mouse	Peptide (R132C)	[12]	M, MM, W
	IDH2	R172K	KMAb-1	Original	Rat	Peptide (R172K)	[55]	M
			#26163	Original	Mouse	Peptide (R172K)	[24]	N
		R172M	MsMab-2	Cross	Rat	Peptide (R132L)	[58]	M
			MMab-1	Original	Rat	Peptide (R172M)	[55]	M
		R172W	WMab-1	Original	Rat	Peptide (R172W)	[56]	M
#26164			Original	Mouse	Peptide (R172W)	–	N	
R172S		MsMab-1	Cross	Mouse	Peptide (R132G)	[57]	M, MM, W	
R172G		MsMab-1	Cross	Mouse	Peptide (R132G)	[57]	M, MM, W	
		#26321	Original	Mouse	Peptide (R172G)	–	N	
R140Q ^a		#26165	Original	Mouse	–	[24]	N	
Wild type	RMab-22	–	Mouse	Peptide (R172K)	[55]	M, MM, W		
	KrMab-3	–	Mouse	Peptide (R172K)	[55]	M		

D Dianova GmbH, I Immuno-Biological Laboratories Co. Ltd., M Medical Biological Laboratories Co. Ltd., MM Merck Millipore Corp., N NewEast Biosciences, S Sigma-Aldrich Corp., W Wako Pure Chemical Industries Ltd.

^a R140Q has not been observed in gliomas

Because MsMab-1 does not react with all IDH1/2 mutations, we immunized rats with IDH1-R132L peptide, and screened IDH1-R132L-reactive/IDH1-wild type non-

reactive mAbs in ELISA [58]. The developed MsMab-2 mAb recognized not only IDH1-R132L but also IDH2-R172M in Western blot, neither of which was detected by

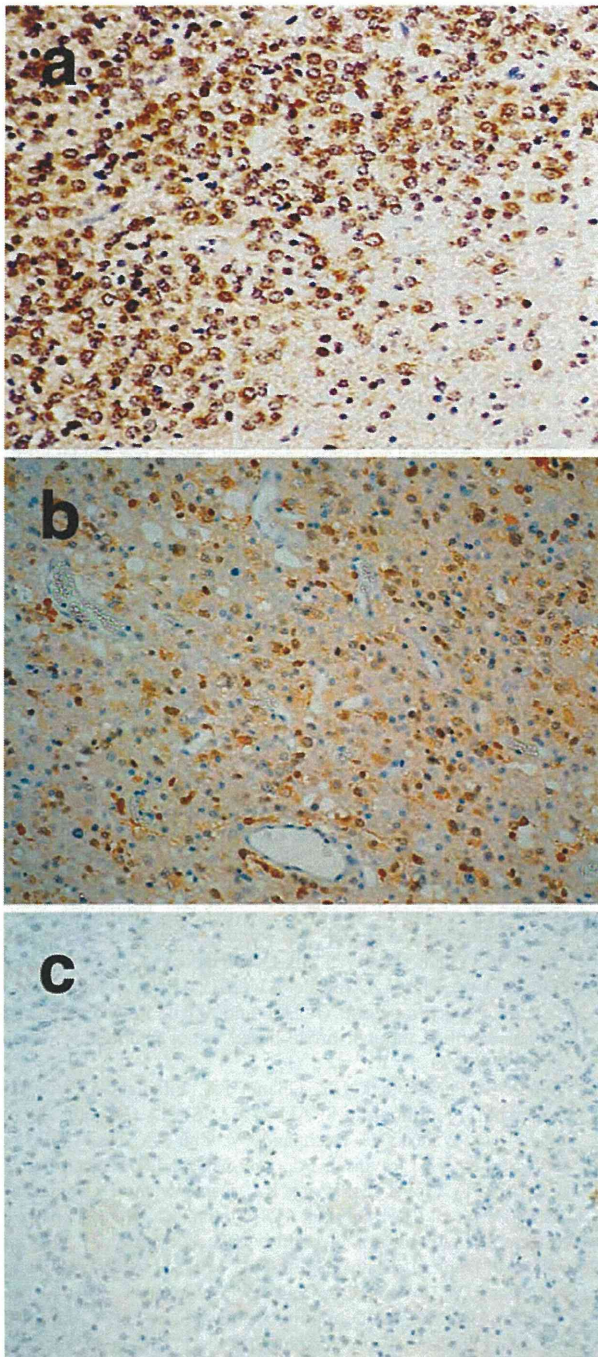


Fig. 1 Immunohistochemical analyses by MsMab-1 against glioma tissues. Glioma tissues possessing IDH1-R132H (a, anaplastic oligodendroglioma), IDH1-R132S (b, glioblastoma), and IDH1/2-WT (c, glioblastoma) were stained with MsMab-1. Magnification $\times 200$

MsMab-1. Taken together, the combination of MsMab-1 and MsMab-2 could be used in the diagnosis of mutated IDH1/2-bearing gliomas.

Conclusions

Until now, many anti-mutated IDH1/2 mAbs have been developed. Although only anti-IDH1-R132H mAbs (H09, IMab-1, HMab-1) have been used in the pathological diagnosis of gliomas [59–85], the combination of anti-mutated IDH1/2 mAbs [12] or the application of multi-specific, anti-mutated IDH1/2 mAbs [57, 58] in immunohistochemistry could lead to high and sensitive detection of IDH1/2 mutations. The platform to produce cancer-specific monoclonal antibodies (CasMabs), which targets membranous glycoproteins, was recently developed, and several CasMabs against gliomas have been already produced [86]. The combination of anti-mutated IDH mAbs and CasMabs could be more effective for diagnosis of gliomas.

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Conflict of interest The authors have no conflict of interest to declare.

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