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RAPID IMMUNOHISTOCHEMISTRY OF IDH-1 FOR THE INTRAOPERATIVE DIAGNOSIS OF GLIOMAS

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Abstract
Immunohistochemistry (IHC) plays a major role in the histopathological diagnosis of central nervous system (CNS) tumors. We developed a rapid immunohistochemistry (R-IHC) method using an alternating current electric field, which enables the completion of IHC in 20 min. We performed intraoperative R-IHC on 16 glioma patients (9 with grade I-III gliomas and 7 with glioblastomas) using the anti-mutant isocitrate dehydrogenase 1 (IDH1) monoclonal antibody (IDH1-R132H ; H09), and the results were validated on permanent formalin-fixed paraffin-embedded samples of the same patients by standard IHC. DNA sequencing of the *IDH1* gene was also performed for the 16 patients. Of 6 gliomas, 5 were shown to be positive by both R-IHC and standard IHC, and *IDH1* mutation was confirmed by DNA sequencing. According to the results of R-IHC compared with both standard IHC results and DNA sequencing, the sensitivity of IDH1 R-IHC was 83% (5/6) ; specificity, 70% (7/10) ; positive predictive value, 63% (5/8) ; negative predictive value, 88% (7/8) ; and accuracy, 75% (12/16). Thus, R-IHC using the anti-mutant IDH1 monoclonal antibody has potential applications in the intraoperative identification of *IDH1* mutations in gliomas.

Key words : IDH-1, rapid immunohistochemistry, glioma, intraoperative diagnosis, DNA sequencing

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Introduction

For the histopathological diagnosis of central nervous system (CNS) tumors, immunohistochemical staining (IHC) is an essential technique owing to the wide variety of morphological appearances even in the same entity of

such tumors[1]. However, the IHC procedure usually takes at least 2–3 h to complete all the steps. Thus, the incorporation of conventional IHC into the intraoperative pathological diagnosis of CNS tumors has not been feasible thus far. In the last 3 decades, several methods for rapid immunohistochemistry (R-IHC) have been developed, such as methods using microwaves[2,3], high-quality reagents[4-8], and ultrasound[9]. However, only a few papers have reported the use of these techniques for CNS tumors[6,7]. Recently, we developed a R-IHC technique in which an alternating current (AC) electric field enables the antigen-antibody reaction[10]. The R-IHC procedure takes only 20 min, enabling its incorporation into the intraoperative diagnosis of CNS tumors[11]. We have reported the application of intraoperative R-IHC for Ki-67/MIB-1 staining to estimate the WHO grading of gliomas and CD20 staining to differentiate between CNS tumors of glial origin and CNS lymphomas[11].

Isocitrate dehydrogenase (*IDH1*) mutations have originally been identified by genome-wide exome sequencing of secondary glioblastomas, and the diagnostic role of *IDH1/2* genes in the treatment of gliomas has been scrutinized because among CNS tumors with favorable prognostic value, their mutations can be found in gliomas[12]. *IDH* mutations uniformly occur in the functionally critical residues arginine 132 (R132) of *IDH1* and arginine 172 (R172) of *IDH2*, and specific antibodies for these mutated proteins have been established[13,14]. Several types of *IDH1* mutations have been reported, including R132H, with the most common types being R132C, R132S, R132L, and R132G[12].

In this study, we performed intraoperative R-IHC using the anti-mutant IDH1 (IDH1-R132H ; H09) monoclonal antibody to determine the mutational status of *IDH1* in 16 glioma cases. The results were validated by conventional IHC of IDH1 on permanent formalin-fixed paraffin embedded (FFPE) samples from the same specimen and confirmed by the DNA sequencing of *IDH1*. R-IHC was also performed with GFAP, Olig2, and Ki-67 antibodies to support the intraoperative diagnosis of gliomas as necessary. The present results show the usefulness and reliability of applying IDH1 R-IHC as an intraoperative test.

Materials and methods

Patients

Sixteen patients surgically treated for astrocytic and oligodendroglial tumors and mixed gliomas at Akita University Hospital, Akita, Japan, from 2011 to 2015 were included in this study. The final diagnoses were as follows : pilocytic astrocytoma, 1 case ; anaplastic astrocytoma, 1 case ; anaplastic oligodendroglioma, 1 case ; anaplastic oligoastrocytoma, 6 cases ; and glioblastoma, 7 cases. The characteristics of the patients are listed in Table 1. The World Health Organization classification of CNS tumors was used for histopathological evaluation[1]. This study was approved by the institutional review board of the Akita University School of Medicine, and written informed consent was obtained from all the patients.

Tissue preparation for intraoperative R-IHC

Specimens for intraoperative diagnosis sized 3–5 mm in diameter were placed into plastic cassettes (Tissue-Tek Cryomold Standard ; Sakura Finetek Japan, Tokyo, Japan), mounted with OCT compound medium (Sakura Finetek Japan), and frozen in Histo-Tek Hyfluid (Sakura Finetek Japan) at −75°C. Immunostaining with R-IHC was applied on 3-μm frozen sections, and H&E staining was also performed on the serial sections. The frozen sections on slide glasses were air dried for 30 s and fixed by acetone at room temperature (RT) for 30 s. Endogenous peroxidase was quenched by 3% H_2O_2 for 1 min at RT. Subsequently, the sections were incubated with primary antibody under a high-voltage (4.0 kV, offset 2.4 kV), low-frequency (5 Hz) AC electric field for 5 min. The sections were washed 3 times with PBS (Muto Pure Chemicals, Tokyo, Japan) with 0.1% Tween20 (Tokyo Chemical Industry, Tokyo, Japan) and incubated with Histofine Simple Stain MAX PO (Nichirei Biosciences, Tokyo, Japan) for 5 min under the same electric field conditions. The AC electric field was obtained with R-IHC type A II (Akita Epson Corporation, Akita, Japan). Immunoproducts were visualized by diaminobenzidine (DAB) as a substrate at RT for 1 min, counterstained with hematoxylin, dehydrated, and mounted with coverslips. Thus, the R-IHC procedure took 20 min, in-

cluding counterstaining and mounting. Detailed mechanisms of the R-IHC procedure are described elsewhere[10].

Intraoperative R-IHC for specific mouse monoclonal antibodies against IDH1, GFAP, Olig2, and Ki-67/MIB-1 was also performed in each case (Table 2).

IHC for FFPE tissues

The specimens after intraoperative pathological diagnosis were fixed in 10% buffered formalin and embedded in paraffin. Three-micrometer-thick sections of FFPE samples were incubated with Paraffin Stretcher (Sakura Finetek Japan, Tokyo, Japan) at 50°C overnight, and immunohistochemical staining for IDH1-R132H, GFAP, Olig2, and Ki-67 was performed by the labeled streptavidin biotin method using Ventana BenchMark XT immunostainer (Ventana Medical Systems, Tucson, AZ, USA).

Evaluation of immunohistochemical staining

All the stained specimens were evaluated by 2 pathologists (Y.H. and H.N.) using a standard light microscope (Olympus BX50F4 ; Olympus Corporation, Tokyo, Japan). Immunohistochemical results of IDH1-R132H, GFAP, or Olig2 expression, positive or negative, with cut-off values of 5% positivity in tumor cells, were determined. Faint staining was judged to be negative. The labeling index (LI) for Ki-67/MIB-1 was calculated by positively immunostained tumor cells in more than 100 tumor cells.

DNA extraction

FPPE specimens of CNS tumors were used for *IDH1* mutation analysis. The target tumor lesion was identified by an experienced pathologist (M.T.) under a standard light microscope (Olympus BX50F4) and marked on a couple of 10-μm-thick unstained sections using sterile toothpicks. Each area was excised with sterile disposable scalpels (Feather Safety Razor, Osaka, Japan), and DNA was isolated using WaxFree DNA extraction kits (Trimgen Corporation, Sparks, MD, USA) according to the manufacturer's instructions. DNA concentration and purity were evaluated by the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

PCR amplification and sequencing of an IDH1 fragment

A 129-bp fragment encoding a catalytic domain of IDH1 including codon 132 was amplified by PCR using the sense primer IDH1(f) 5´-CGGTCTTCAGAGAAGCCATT and the antisense primer IDH1(r) 5´-GCAAAATCACATTATTGCCAAC[15,16]. The reaction volume for PCR included 500 ng of DNA extracted from each sample, 0.4 μM of each primer, 0.2 mM dNTP mix, 1.5 mM $MgSO_4$, and 1 U of KOD plus DNA polymerase. The amplification conditions were as follows : denaturation for 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 56°C for 40 s, and extension at 72°C for 40 s. The final extension step at 72°C was extended to 7 min. PCR products were electrophoresed on 1.5% agarose gels (Agarose LO3 ; Takara, Tokyo, Japan) and photographed. For the control of the reliability of PCR, additional sense primer IDH1(f) 5´-ACCAAATGGCACCATACGA and antisense primer IDH1(r) 5'-TTCATACCTTGCTTAATGGGTGT for generating a 254-bp fragment were used in each DNA sample under the same PCR conditions. Cleaned PCR products using the QIAquick Spin Purification procedure (Qiagen, Crawley, West Sussex, UK) were subjected to standard direct sequencing using the IDH1(f) primer.

Results

R-IHC for intraoperative pathological diagnosis using GFAP, Olig2, and Ki-67

R-IHC in this study was successfully performed as an effective tool for histopathological diagnosis. Table 1 summarizes the results of GFAP, Olig2, and Ki-67/MIB-1 staining by R-IHC and standard IHC for each case. The tumors were also histopathologically evaluated for necrosis and mitosis described in Table 1. The intraoperative and final pathological diagnoses of the tumors are also shown. All the 16 gliomas were found to be positive for GFAP by both R-IHC and standard IHC. The results of R-IHC for Olig2 were almost identical to those of standard IHC. The Ki-67/MIB-1 indices based on R-IHC using frozen sections were significantly correlated with those of paraffin-embedded sections. The indices cor-

Table 1. Patients' characteristics, pathological diagnoses,

			R-IHC results (Frozen)						
Case	Age/Sex	Frozen HE	GFAP	Olig2	Ki-67	IDH1 R-132H	Mitosis	Necrotic area	Frozen HE+R-IHC
1	29/M	II (Oligoastrocytoma with atypia)	(+)	(+)	15.0%	(+)	(−)	(−)	II or III (Oligoastrocytoma with atypia or anaplastic oligoastrocytoma)
2	32/F	II (Diffuse astrocytoma or oligoastrocytoma)	(+)	(−)	3.0%	(+)	(−)	(−)	II (Diffuse astrocytoma or oligoastrocytoma)
3	55/F	II (Oligoastrocytoma)	(+)	(+)	10.0%	(+)	(−)	(−)	II (Oligoastrocytoma)
4	47/F	II (Oligoastrocytoma)	(+)	(+)	12.4%	(+)	(−)	(−)	III (Oligoastrocytoma with partly grade III component)
5	38/M	III (Anaplastic oligoastrocytoma)	(+)	(+)	10.0%	(−)	(−)	(−)	III (Anaplastic oligoastrocytoma)
6	45/M	II (Oligodendroglioma)	(+)	(+)	4.0%	(+)	(−)	(−)	II (Oligodendroglioma)
7	78/M	III (Anaplastic oligoastrocytoma)	(+)	(−)	25.7%	(+)	(−)	(+)	III or IV (Anaplastic oligoastrocytoma or Glioblastoma)
8	12/M	I (Pilocytic astrocytoma)	(+)	Not done	1.0%	(−)	(−)	(−)	I (Pilocytic astrocytoma)
9	72/F	III or IV (Anaplastic astrocytoma or Glioblastoma)	(+)	(+)	40.0%	(−)	(+)	(−)	III or IV (Anaplastic astrocytoma or Glioblastoma)
10	76/M	III (Malignant glioma)	(+)	(+)	23.3%	(+)	(−)	(+)	III or IV (Anaplastic astrocytoma or Glioblastoma)
11	60/F	IV (Glioblastoma)	(+)	(−)	29.4%	(−)	(−)	(+)	IV (Glioblastoma)
12	76/M	IV (Glioblastoma)	(+)	(−)	13.1%	(−)	(−)	(+)	IV (Glioblastoma)
13	77/M	IV (Glioblastoma)	(+)	(−)	1.8%	(−)	(−)	(+)	IV (Glioblastoma)
14	57/M	IV (Glioblastoma)	(+)	(+)	40.0%	(−)	(+)	(+)	IV (Glioblastoma)
15	68/F	IV (Glioblastoma)	(+)	(+)	15.0%	(−)	(+)	(+)	IV (Glioblastoma)
16	78/F	IV (Glioblastoma)	(+)	(−)	23.8%	(+)	(−)	(+)	IV (Glioblastoma)

M, male ; F, female ; (+), positive ; (—), negative ; NA, not applicable ; R-IHC, rappid immunohistochemistry ; IHC, nase 1 ; GFAP, glial fibrillary acidic protein.

related positively to each other well ($r = 0.84$).

R-IHC for IDH1-R132H

Figures 1, 2 and 3 show the representative results of R-IHC for the patients with and without IDH1-R132H mutation. In Figure 1, the representative case was patient 6, with a final diagnosis of anaplastic oligodendroglioma in both frontal lobes. Both standard IHC and R-IHC revealed positive staining for GFAP, and the Ki-67/MIB-1 LI was 4.0% with R-IHC and 10.6% with standard IHC. Mutated IDH1 was mainly localized in the cytoplasm, and occasional nuclear staining was observed. Another representative case, as figure 2, was patient 4 with recurrent tumor of anaplastic oligoastrocytoma, receiving chemoradiation therapy before the current operation. IDH1 R-IHC revealed positive staining intraoperatively and could be detected as mutation positive at that time. Thus, out of 9 grade I-III tumors, 5 (55.6%) were positive both with R-IHC and standard IHC (cases 1, 2, 3, 4, 6) (Table 1). The tumor of patient 7 was characterized by necrotic areas, and partial positivity for IDH1-R132H was observed only by R-IHC. This was considered to be non-specific staining. In contrast, in 2 out of 7 glioblastomas (29%, patients 10 and 16), partial positivity for IDH1-R132H was observed only by R-IHC, which was considered non-specific staining. In case 16, vivid peri-cytoplasmic staining was observed with only IDH1 R-IHC, which made us misjudge it as mutation positive (Figure 3). Tumor cells were positive for GFAP and negative for Olig2 by both R-IHC and standard IHC, and the Ki-67 values were 23.8% and 11.6%, respectively. The sensitivity of IDH1 R-IHC was 83% (5/6) ; specificity, 70% (7/10) ; positive predictive value, 63% (5/8) ; negative predictive value, 88% (7/8) ; and accuracy, 75% (12/16).

***IDH1* mutational analysis**

IDH1 mutation was detected at codon 132 in 6 out of 9 grade I-III tumor cases (67%) (cases 1-6). All the mutations in codon 132 (from CGT to CAT) were heterozy-

immunohistochemical results, and IDH1 mutation status

Standard IHC results (FFPE)						
GFAP	Olig2	Ki-67 (after R-IHC)	Mitosis	IDH1-R132H	*IDH1* mutation	Final diagnosis
(+)	(+)	8.3%	(−)	(+)	(+)	III (Anaplastic oligoastrocytoma)
(+)	(−)	0% (12.7% in surgical speciens)	(−)	(+)	(+)	III (Anaplastic oligoastrocytoma)
(+)	(+)	13.4%	(−)	(+)	(+)	III (Anaplastic oligoastrocytoma)
(+)	(+)	12.8%	(−)	(+)	(+)	III (Anaplastic oligoastrocytoma)
(+)	(+)	10.8%	(−)	(+)	(+)	III (Anaplastic oligoastrocytoma)
(+)	(+)	10.6%	(−)	(+)	(+)	III (Anaplastic oligodendroglioma)
(+)	(−)	10.7%	(−)	(−)	(−)	III (Anaplastic oligoastrocytoma)
(+)	(−)	7.4%	(−)	(−)	(−)	I (Pilocytic astrocytoma)
(+)	(+)	45.7%	(+)	(−)	(−)	III (Anaplastic astrocytoma)
(+)	(+)	19.8%	(−)	(−)	(−)	IV (Glioblastoma)
(+)	(−)	21.3%	(−)	(−)	(−)	IV (Glioblastoma)
(+)	(−)	9.4%	(−)	(−)	(−)	IV (Glioblastoma)
(+)	(−)	2.4%	(−)	(−)	(−)	IV (Glioblastoma)
(+)	(+)	49.8%	(+)	(−)	(−)	IV (Glioblastoma)
(+)	(+)	24.8%	(+)	(−)	(−)	IV (Glioblastoma)
(+)	(−)	11.6%	(−)	(−)	(−)	IV (Glioblastoma)

immunohistochemistry ; FFPE, formali-fixed paraffin-embedded ; IDH1, isocitrate dehydroge-

gous and resulted in amino acid substitution from arginine to histidine (IDH1-R132H), the most common mutation in IDH1. Five out of 6 patients harboring *IDH1* mutation were positive for an anti-IDH1-R132H-specific monoclonal antibody by both R-IHC and standard IHC. One anaplastic oligoastrocytoma and 2 glioblastoma specimens with non-specific IDH1-R132H staining in R-IHC (patients 7, 10, and 16) without *IDH1* mutation contained necrotic lesions as mentioned above. In patient 5, IDH1 staining by R-IHC was negative despite being positive by standard IHC. In patient 2, the tumor was negative for Ki-67 by standard IHC, but the highest Ki-67 index in the surgical specimen was 12.7%, leading to a final diagnosis of anaplastic oligoastrocytoma.

Discussion

At present, the clinical course of CNS tumors can be predicted and the modality chosen according to the WHO histopathological grading[1]. Recently, glioblastoma with *IDH1* mutation was reported to have a better prognosis than anaplastic astrocytoma without the mutation[17]. Another study revealed that patients with low-grade gliomas without *IDH* mutation had shorter overall survival (median survival, 1.7 years) than did those with low-grade gliomas and glioblastoma with mutated *IDH*[18]. These reports indicate that, for such tumors, molecular testing is as useful as histopathological diagnosis. Furthermore, immunohistochemical IDH1 R-IHC staining may be even more important because enough surgical margins would be required if the tumor is found to be IDH1-negative. Capper et al. reported that glioma occasionally has mutated *IDH1* while gliosis does not[19]. In our study, patient 4 had recurrent tumor as mentioned above. In such a case, diagnosis based only on intraoperative frozen H&E staining might be difficult because of the existence of gliosis or degenerative change due to the posttherapeutic change[20]. Positive staining for IDH1 R-IHC and a high Ki-67/MIB-1 LI would be a helpful tool for deciding the tumor progression area intraop-

Table 2. Primary antibodies for frozen and FFPE specimens employed in the study.

Specificity	Clone	Source	Dilution
IDH1-R132H	H09	COSMO Bio (Tokyo, Japan)	1 : 20
GFAP	6F-2	Dako (Tokyo, Japan)	1 : 100
Olig2	Olig2	IBL (Gumma, Japan)	1 : 100
Ki-67	MIB-1	Dako (Tokyo, Japan)	1 : 1 (prediluted antibody)

Abbreviations : IDH1=isocitrate dehydrogenase 1 ; GFAP = glial fibrillary acidic protein

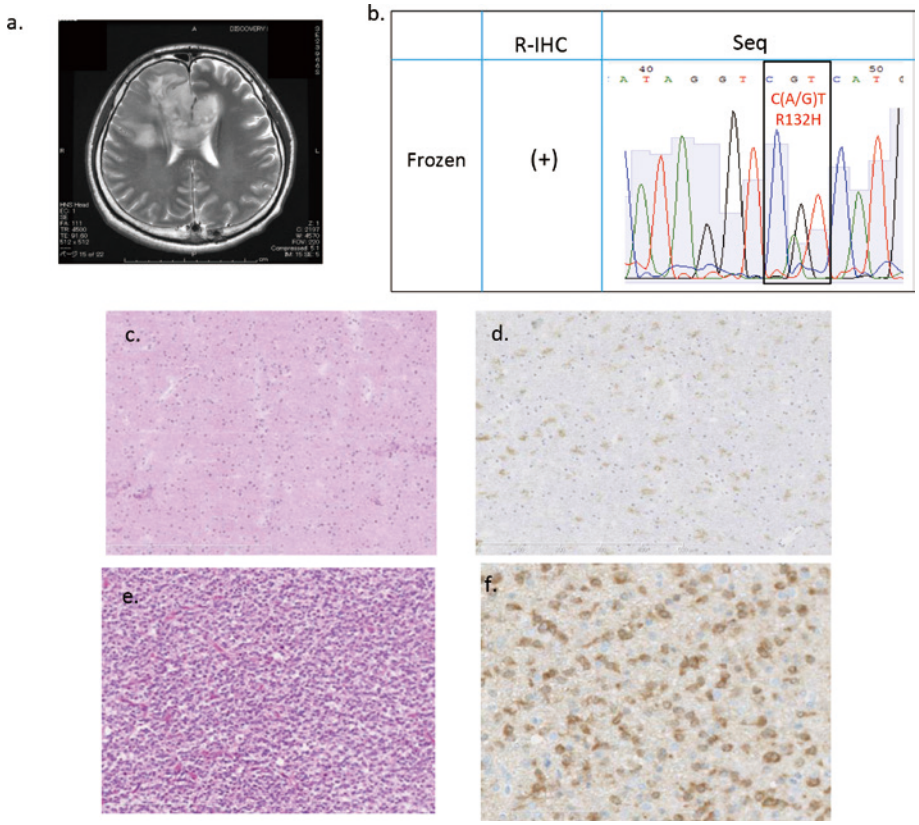

Fig. 1. Radiological and histological findings in patient 6.
a. MRI : magnetic resonance imaging revealing a heterogeneously enhanced tumor in both frontal lobes.
b. DNA sequencing data : mutation was observed in *IDH1* codon 132 (from CGT to CAT).
c. H&E staining in frozen specimen : photomicrograph showing a mildly cellular tumor composed predominantly of atypical glial cells. Scale bar : 400 μm.
d. IDH1-R132H staining by R-IHC in a frozen specimen : R-IHC analysis showing positive staining for a monoclonal antibody specific for IDH1 R-132H mutation. Scale bar : 500 μm.
e. H&E staining in a FFPE specimen : photomicrograph showing a highly cellular tumor composed predominantly of atypical glial cells. Scale bar : 200 μm.
f. IDH1-R132H staining by standard IHC in a FFPE specimen : standard IHC analysis showing positive staining for IDH1 mutation. Scale bar : 100 μm.

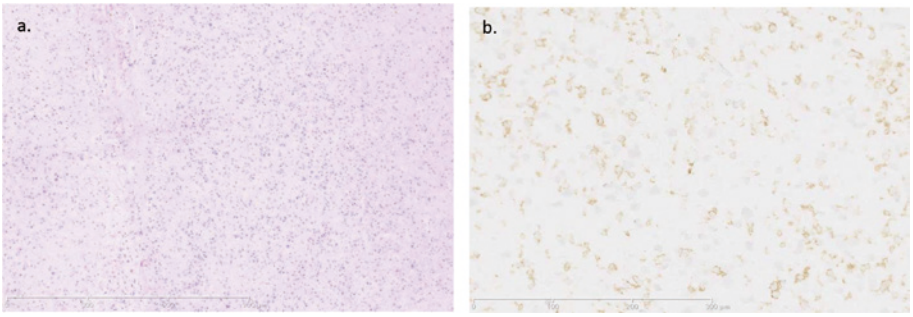

Fig. 2. Histological findings in patient 4.
a. H&E staining in frozen specimen : photomicrograph showing a mildly to moderately cellular tumor composed predominantly of atypical glial cells. Scale bar : 900 μm.
b. IDH1-R132H staining by R-IHC in a frozen specimen : R-IHC analysis showing positive staining for a monoclonal antibody specific for IDH1 R-132H mutation. Scale bar : 300 μm.

eratively in such situations.

Of 6 mutation-positive tumors, 5 revealed appropriate staining results, but in case 5, only IDH1 R-IHC showed a false negative result. This can be ascribed to insufficient activation of the antigen. In patients 7, 10, and 16, who showed false positive staining only by IDH1 R-IHC, the lesion contained necrotic areas as mentioned above, which might have led to non-specific staining. Thus, with current techniques, careful observation is required for detecting necrotic areas by H&E staining. More specific antibodies or more suitable staining conditions are required for lower non-specific staining, which would bestow the method with sufficient specificity for clinical use. As the number of available mutation-specific antibodies for IDH1 is limited, various staining conditions for IDH1 R-IHC have been tested before this study.

Several methods for R-IHC have been developed in the past decades. Our AC method is superior to them in terms of staining accuracy and rapidness[10,11]. The present condition also appears to be the best in terms of sensitivity, which would be of more importance in intraoperative diagnosis. Further studies are warranted to attain higher specificity of IDH1 R-IHC with stringent conditions. A combination of the results of IDH1 mutation, TERT mutation, and co-deletion of 1p/19q has been found to make a difference in the prognosis[21]. Inclusion of these assays may provide useful information to surgeons by reporting the results of R-IHC IDH1 staining intraoperatively.

Our approach has the advantage of examining the same samples for IDH1 expression by IHC and *IDH1* mutation by sequencing. One limitation, however, is that it has not been clarified if tumor heterogeneity exists for *IDH1* mutation in gliomas. Such heterogeneity would make intraoperative R-IHC unsuitable for detecting *IDH1* mutation in gliomas. However, in the present study, tumor heterogeneity was not found in any of the 6 cases with mutant *IDH1*, and both R-IHC and standard IHC showed homogenous staining for IDH1 in these cases. Second, it has not been clarified whether cancer stem cells in gliomas have *IDH1* mutation. Simultaneous evaluation of IDH1 and cancer stem cell markers such as CD133 by R-IHC could serve as indicators of the clinical course of gliomas. Besides, R-IHC is potentially applicable for the intraoperative detection of gliomas harboring druggable genetic alterations, such as *EGFR*, *PDGFR*, or *MET* alterations.

To conclude, our present results demonstrate the usefulness and reliability of applying IDH1 R-IHC as an intraoperative test. At present, the number of antibodies available for detecting genetic mutations is limited for R-IHC, but in the future, the development of more mutation-specific antibodies for *IDH1* and other genes would lead to superior intraoperative genetic diagnosis of CNS tumors by R-IHC.

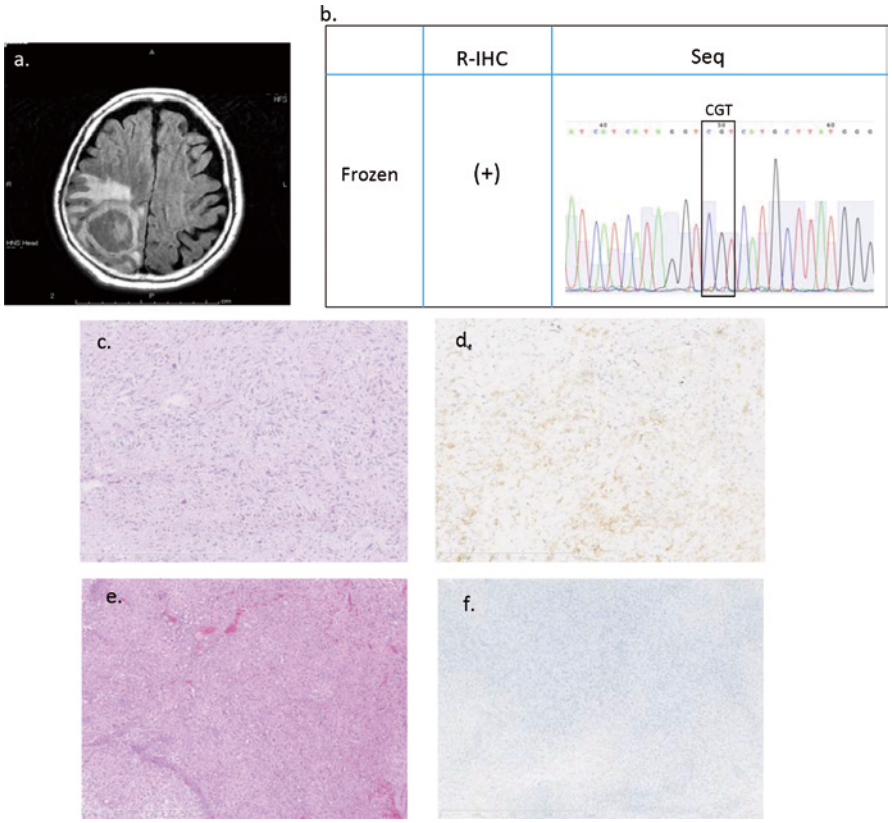

Fig. 3. Radiological and histological findings in patient 16.
a. MRI : magnetic resonance imaging revealing a ring-enhanced tumor in the right parietal lobe.
b. DNA sequencing data : no mutation was observed in *IDH1* codon 132.
c. H&E staining in a frozen specimen : photomicrograph showing an anaplastic and moderately to highly cellular tumor composed of pleomorphic astrocytic tumor cells. Scale bar : 500 μm.
d. IDH1-R132H staining by R-IHC in a frozen specimen : R-IHC analysis showing positive staining for a monoclonal antibody specific for IDH1 R-132H mutation. Scale bar : 500 μm.
e. H&E staining in a FFPE specimen : photomicrograph showing an anaplastic and highly cellular tumor composed of pleomorphic astrocytic tumor cells. Scale bar : 1 mm.
f. IDH1-R132H staining by standard IHC in a FFPE specimen : standard IHC analysis showing negative staining for IDH1 mutation. Scale bar : 1 mm.

References

1) Louis, D.N., Ohgaki, H., Wiestler, O.D., Cavenee, W.K., Burger, P.C., Jouvet, A., Scheithauer, B.W. and Kleihues, P. (2007) The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol*., **114**, 97-109.
2) Ichihara, T., Nakao, A., Suzuki, Y., Sakamoto, J., Nonami, T., Harada, A., Nagura, H. and Takagi, H. (1989) Improvement of the rapid immunoperoxidase staining method for intraoperative pathological diagnosis of pancreatic cancer using microwave irradiation. *J. Surg. Oncol*., **42**, 209-214.
3) Hatta, H., Tsuneyama, K., Kumada, T., Zheng, H., Cheng, C., Cui, Z., Takahashi, H., Nomoto, K., Murai, Y. and Takano, Y. (2006) Freshly prepared immune complexes with intermittent microwave irradiation result in rapid and high-quality immunostaining. *Pathol. Res. Pract*., **202**, 439-445.
4) Richter, T., Nährig, J., Komminoth, P., Kowolik, J. and

Werner, M. (1999) Protocol for ultrarapid immunostaining of frozen sections. *J. Clin. Pathol*., **52**, 461-463.

5) Kämmerer, U., Kapp, M., Gassel, A.M., Richter, T., Tank, C., Dietl, J. and Ruck, P. (2001) A new rapid immunohistochemical staining technique using the EnVision antibody complex. *J. Histochem. Cytochem*., **49**, 623-630.

6) Haapasalo, J., Mennander, A., Helen, P., Haapasalo, H. and Isola, J. (2005) Ultrarapid Ki-67 immunostaining in frozen section interpretation of gliomas. *J. Clin. Pathol*., **58**, 263-268.

7) Uzuka, T., Aoki, H., Natsumeda, M., Kakita, A., Takahashi, H. and Fujii, Y. (2011) Indication of intraoperative immunohistochemistry for accurate pathological diagnosis of brain tumors. *Brain Tumor Pathol*., **28**, 239-246.

8) Mönig, S.P., Luebke, T., Soheili, A., Landsberg, S., Dienes, H.P., Hölscher, A.H. and Baldus, S.E. (2006) Rapid immunohistochemical detection of tumor cells in gastric carcinoma. *Oncol. Rep*., **16**, 1143-1147.

9) Hatta, H., Tsuneyama, K., Kondo, T., Takano Y. (2010) Development of an ultrasound-emitting device for performing rapid immunostaining procedures. *J. Histochem. Cytochem.*, **58**(5), 421-428.

10) Toda, H., Minamiya, Y., Kagaya, M., Nanjo, H., Akagami, Y., Saito, H., Ito, M., Konno, H., Motoyama, S. and Ogawa, J. (2011) A novel immunohistochemical staining method allows ultrarapid detection of lymph node micrometastases while conserving antibody. *Acta. Histochem. Cytochem*., **44**, 133-139.

11) Tanino, M., Sasajima, T., Nanjo, H., Akesaka, S., Kagaya, M., Kimura, T., Ishida, Y., Oda, M., Takahashi, M., Sugawara, T., Yoshioka, T., Nishihara, H., Akagami, Y., Goto, A., Minamiya, Y., Tanaka, S., R-IHC Study Group. (2015) Rapid immunohistochemistry based on alternating current electric field for intraoperative diagnosis of brain tumors. *Brain Tumor Pathol*., **32**, 12-19.

12) Yan, H., Parsons, D.W., Jin, G., McLendon, R., Rasheed, B.A., Yuan, W., Kos, I., Batinic-Haberle, I., Jones, S., Riggins, G.J., Friedman, H., Friedman, A., Reardon, D., Herndon, J., Kinzler, K.W., Velculescu, V.E., Vogelstein, B. and Bigner, D.D. (2009) IDH1 and IDH2 mutations in gliomas. *N. Engl. J. Med*., **360**, 765-773.

13) Capper, D., Weissert, S., Balss, J., Habel, A., Meyer, J., Jäger, D., Ackermann, U., Tessmer, C., Korshunov, A., Zentgraf, H., Hartmann, C. and von Deimling, A. (2010) Characterization of R132H mutation-specific IDH1 antibody binding in brain tumors. *Brain Pathol*., **20**, 245-254.

14) Kato, Y. (2015) Specific monoclonal antibodies against IDH1/2 mutations as diagnostic tools for gliomas. *Brain Tumor Pathol*., **32**, 3-11.

15) Watanabe, T., Nobusawa, S., Kleihues, P. and Ohgaki, H. (2009) IDH1 mutations are early events in the development of astrocytomas and oligodendrogliomas. *Am. J. Pathol*., **174**, 1149-1153.

16) Horbinski, C., Kofler, J., Kelly, L.M., Murdoch, G.H. and Nikiforova, M.N. (2009) Diagnostic use of IDH1/2 mutation analysis in routine clinical testing of formalin-fixed, paraffin-embedded glioma tissues. *J. Neuropathol. Exp. Neurol*., **68**, 1319-1325.

17) Hartmann, C., Hentschel, B., Wick, W., Capper, D., Felsberg, J., Simon, M., Westphal, M., Schackert, G., Meyermann, R., Pietsch, T., Reifenberger, G., Weller, M., Loeffler, M. and von Deimling, A. (2010) Patients with IDH1 wild type anaplastic astrocytomas exhibit worse prognosis than IDH1-mutated glioblastomas, and IDH1 mutation status accounts for the unfavorable prognostic effect of higher age : implications for classification of gliomas. *Acta. Neuropathol*., **120**, 707-718.

18) Cancer Genome Atlas Research Network. (2015) Comprehensive, integrative genomic analysis of diffuse lower-grade gliomas. *N. Engl. J. Med*., **372**, 2481-2498.

19) Capper, D., Sahm, F., Hartmann, C., Meyermann, R., von Deimling, A. and Schittenhelm, J. (2010) Application of mutant IDH1 antibody to differentiate diffuse glioma from nonneoplastic central nervous system lesions and therapy-induced changes. *Am. J. Surg. Pathol*., **34**, 1199-1204.

20) Ishikawa, E., Yamamoto, T., Satomi, K., Matsuda, M., Akutsu, H., Shibuya, M., Nakai, K., Sakamoto, N., Takano, S. and Matsumura, A. (2014) Intraoperative pathological diagnosis in 205 glioma patients in the pre-BCNU wafer era : retrospective analysis with intraoperative implantation of BCNU wafers in mind. *Brain Tumor Pathol*., **31**, 156-161.

21) Eckel-Passow, J.E., Lachance, D.H., Molinaro, A.M.,

et al. (2015) Glioma groups based on 1p/19q, IDH, and TERT promoter mutations in tumors. *N. Engl. J. Med.*, **372**, 2499–2508.