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ORIGINAL ARTICLE

Evaluation of *IDH1* status in diffusely infiltrating gliomas by immunohistochemistry using anti-mutant and wild type IDH1 antibodies

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Abstract Glioma cells with the *isocitrate dehydrogenase* (*IDH*) *1* G395A mutation are strongly immunopositive for mIDH1^{R132H}, an antibody against mutant IDH1^{R132H} (clone H09). However, we encountered some gliomas which were ambiguously positive for mIDH1^{R132H} despite having the *IDH1* G395A mutation. The aim of this study was to establish an evaluation procedure of *IDH1* status by immunohistochemistry. Forty-three diffusely infiltrating gliomas were studied, and four of eight anaplastic oligoastrocytomas with the *IDH1* G395A mutation were modestly or weakly positive for both the mIDH1^{R132H} and an antibody against wild type IDH1, RcMab-1. Based on our staining results, the IDH1 expression of both wild and mutated types seemed to be codominant and also to be evenly suppressed under a certain condition. We propose a procedure for determining *IDH1* status. If a glioma is weakly positive for mIDH1^{R132H}, immunohistochemistry for RcMab-1 should be performed. If the tumor cells are strongly positive for RcMab-1, the *IDH1* G395A mutation is judged to be absent on the grounds that IDH1 expression is not suppressed. If the tumor cells are weakly positive for both mIDH1^{R132H} and RcMab-1, then a conclusion should be made after DNA sequencing. This procedure is useful for practical evaluation of *IDH1* status.

Keywords Glioma · IDH1 · Immunohistochemistry

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Introduction

Isocitrate dehydrogenase (IDH) 1 or IDH2 genes are mutated in 50–80 % of World Health Organization (WHO) grade II and III diffuse gliomas, and secondary glioblastomas, suggesting that the *IDH1/2* mutation is an early event in the development of diffuse gliomas [1]. IDH1 is located in the cytoplasm and peroxisomes, and the main function is to catalyze oxidative decarboxylation of isocitrate into α-ketoglutarate (αKG) [2]. Mutant IDH1 acquires the ability to reduce αKG into D-2-hydroxyglutarate (D-2HG) in an NADPH-dependent manner [3]. It has been suggested that mutant IDH1 contributes to gliomagenesis by D-2HG production. One hypothesis is that D-2HG inhibits prolyl hydroxylase-mediated degradation of HIF-1α, resulting in induction of vascular endothelial growth factor expression [4]. Another possibility is that marked elevation of D-2HG results in CpG hypermethylation at a large number of loci, which results in what is termed a glioma-CpG island methylator phenotype (G-CIMP) [5, 6]. G-CIMP is considered to have down-regulation of target gene expression, some of which may act as tumor suppressors. The most frequent type of *IDH* mutation is G395A, causing the amino acid substitution of arginine for histidine (R132H) (89.7 %), whereas *IDH2* mutations are rare (4.4 %) [7].

In 2009, two anti-IDH1^{R132H} monoclonal antibodies were reported (mIDH1^{R132H}, clone H09 [8]; clone IMab-1

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[9]), and detection of *IDH1* mutations by immunohistochemistry was made possible. Glioma cells with the *IDH1* G395A mutation show strong cytoplasmic and weaker nuclear staining for mIDH1^{R132H} [8, 10]. However, we have encountered some anaplastic oligoastrocytoma cases which showed ambiguous positive staining for mIDH1^{R132H} throughout the tissue despite harboring the *IDH1* G395A mutation. This discrepancy between immunohistochemical and DNA sequencing results hampers precise diagnosis. Recently, molecular targeting therapy and immunotherapy against *IDH1/2* mutations have been developed [11–13]; therefore, the strict confirmation of *IDH* status is becoming more important.

As *IDH1/2* mutations in gliomas are usually restricted to one allele, wild type IDH1/2 is expressed even in *IDH*-mutated tumors. We hypothesized that the expression of both IDH1 mutant and wild types may be suppressed by an unknown mechanism. The aim of this study was to investigate the relation between mutant and wild type IDH1 expression in diffusely infiltrating gliomas and to propose an evaluation procedure of *IDH1* status by immunohistochemistry.

Materials and methods

Glioma cases

A total of 43 diffusely infiltrating glioma cases were included in this study (Table 1). Forty-one tissue specimens were obtained from the pathology archives of Gunma University Hospital and Gunma University Graduate School of Medicine. Two tissue specimens were provided by other institutions and have been reported elsewhere [14, 15]. The histological types were as follows: 4 diffuse astrocytomas, 4 anaplastic astrocytomas, 1 glioneuronal tumor with neuropil-like islands (GTNI) [14], 5 glioblastomas, 1 glioblastoma with oligodendroglioma component, 4 oligodendroglioma, 1 oligodendroglioma with prominent neuronal differentiation [15], 7 anaplastic oligodendrogliomas, 6 oligoastrocytomas, and 10 anaplastic oligoastrocytomas. All cases were reviewed and diagnosed according to the current WHO classification of the central nervous system [16]. Sections used for immunohistochemistry and genetic analyses were prepared from formalin-fixed paraffin-embedded tissue specimens. The study protocol was approved by the institutional ethics committee and conducted according to the principles of the Declaration of Helsinki.

Immunohistochemistry

Mutant IDH1^{R132H} and wild type IDH1 protein expression was determined immunohistochemically with paraffin-embedded tumor specimens. A streptavidin–biotin immunoperoxidase technique with anti-IDH1^{R132H} antibody (mouse monoclonal, clone H09; Dianova GmbH, Hamburg, Germany) [8, 17] and anti-wild type IDH1 antibody (rat monoclonal, clone RcMab-1; Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) [18, 19] was carried out. Histofine SAB-PO kit (Nichirei, Tokyo, Japan) and VECTASTAIN Elite ABC Rat IgG Kit (Vector Laboratories, Burlingame, CA, USA) were used for H09 and RcMab-1, respectively. Sections of 3-μm thickness were autoclaved in 10 mM citrate buffer (pH 6.0) for 10 min at 121 °C for antigen retrieval. After blocking, the sections were treated with optimally diluted primary antibodies (H09 1:50; RcMab-1 1:200) and then incubated overnight in a moist chamber at 4 °C. Immunoreactions were visualized with the staining kits mentioned above and 3,3′-diaminobenzidine solution.

Direct DNA sequencing of *IDH1* mutations

Genomic DNA was extracted from paraffin-embedded sections, as described previously [20]. Briefly, regions of interest identified in a consecutive H&E-stained section were manually scraped off from formalin-fixed, paraffin-embedded tissue sections with a dry-heat sterilized spatula. For case 9 and 20, DNA was extracted separately from areas of astrocytoma and oligodendroglioma, and from areas of oligodendroglioma and neuronal differentiation, respectively. After deparaffinization with xylene and dehydration with ethanol, the tissue samples were digested with proteinase K. The DNA was then amplified by PCR using two primer sets for exon 4 of the *IDH1* gene. The sequences of the primers used to detect *IDH1* mutations have been reported previously [21]. PCR products were then sequenced on a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) according to standard procedures.

Results

Clinical data, histological diagnosis, and the results of immunohistochemistry and genetic analyses are summarized in Table 1. There were 22 male and 21 female patients, ranging in age from 13 to 81 years (mean 47.0 years, median 48 years). All the gliomas were located supratentorially. Histology of diffuse astrocytomas (Fig. 1a), anaplastic astrocytomas (Fig. 1d), glioblastomas, a glioblastoma with oligodendroglioma component (Fig. 1g), oligodendroglioma (Fig. 1j), anaplastic oligodendrogliomas, oligoastrocytomas, and anaplastic oligoastrocytomas (Fig. 2a, d, g, j) was conventional. In all the

Table 1 Clinicopathological summary, immunohistochemical findings, and *IDH1* status of diffusely infiltrating gliomas

Case	Histological diagnosis	Age	Gender	Location	mIDH1^{R132H}	RcMab-1	*IDH1* mutation
1	Diffuse astrocytoma	36	F	Right frontal lobe	++	++	G395A
2	Diffuse astrocytoma	30	F	Right temporal lobe	++	++	G395A
3	Diffuse astrocytoma	38	M	Left temporal lobe	−	+	−
4	Diffuse astrocytoma	16	M	Left frontal lobe	−	++	−
5	Anaplastic astrocytoma	61	M	Right temporal lobe	−	+	−
6	Anaplastic astrocytoma	56	M	Right frontal lobe	−	++	−
7	Anaplastic astrocytoma	27	M	Left frontal lobe	−	++	−
8	Anaplastic astrocytoma	63	F	Splenium of corpus callosum	−	++	−
9	Glioneuronal tumor with neuropil-like islands	49	F	Left parietal lobe	Astrocytoma: + Oligodendroglioma: ++	Astrocytoma: +/− Oligodendroglioma: +	G395A
10	Glioblastoma	65	F	Trigone of the left lateral ventricle	−	++	−
11	Glioblastoma	71	M	Right temporal lobe	−	++	−
12	Glioblastoma	61	M	Right thalamus	−	++	−
13	Glioblastoma	55	M	Left frontal lobe	−	+	−
14	Glioblastoma	67	F	Left frontal lobe	++	++	G395A
15	Glioblastoma with oligodendroglioma component	13	F	Right temporal lobe	++	++	G395A
16	Oligodendroglioma	58	F	Right temporal lobe and insula	+	+	G395A
17	Oligodendroglioma	22	F	Right frontal lobe	++	+	G395A
18	Oligodendroglioma	40	F	Right frontal lobe	−	+	−
19	Oligodendroglioma	56	M	Right temporal lobe	+	+	G395A
20	Oligodendroglioma with prominent neuronal differentiation	46	F	Right frontal lobe	Oligodendroglioma: ++ Neuronal element: +/−	Oligodendroglioma: ++ Neuronal element: +/−	G395A
21	Anaplastic oligodendroglioma	62	M	Right frontal lobe	−	+	−
22	Anaplastic oligodendroglioma	81	F	Left frontal and parietal lobes	+	+	G395A
23	Anaplastic oligodendroglioma	46	F	Right frontal lobe	++	+	G395A
24	Anaplastic oligodendroglioma	74	F	Right temporal lobe	++	+	G395A
25	Anaplastic oligodendroglioma	54	F	Right cingulate gyrus	−	++	−
26	Anaplastic oligodendroglioma	45	F	Right frontal lobe	++	++	G395A
27	Anaplastic oligodendroglioma	48	M	Left frontal lobe	++	++	G395A
28	Oligoastrocytoma	48	M	Right frontal lobe	++	++	G395A
29	Oligoastrocytoma	57	M	Left and right frontal lobes	++	++	G395A
30	Oligoastrocytoma	34	M	Left frontal lobe and insula	++	++	G395A
31	Oligoastrocytoma	23	M	Left frontal lobe	++	++	G395A
32	Oligoastrocytoma	32	M	Right frontal lobe	++	++	G395A
33	Oligoastrocytoma	43	M	Right frontal lobe	++	++	G395A
34	Anaplastic oligoastrocytoma	16	F	Right frontal lobe	+	++	G395A
35	Anaplastic oligoastrocytoma	48	M	Left frontal and parietal lobes	+	+	G395A
36	Anaplastic oligoastrocytoma	34	M	Left and right frontal lobes	+/−	+/−	G395A

Table 1 continued

Case	Histological diagnosis	Age	Gender	Location	mIDH1^{R132H}	RcMab-1	*IDH1* mutation
37	Anaplastic oligoastrocytoma	42	M	Left temporal lobe	+	+	G395A
38	Anaplastic oligoastrocytoma	70	F	Left frontal lobe	++	++	G395A
39	Anaplastic oligoastrocytoma	55	F	Right frontal lobe	++	++	G395A
40	Anaplastic oligoastrocytoma	34	M	Left and right frontal lobes	++	++	G395A
41	Anaplastic oligoastrocytoma	67	F	Right insular gyrus	−	++	C394T
42	Anaplastic oligoastrocytoma	43	M	Left temporal lobe	−	++	−
43	Anaplastic oligoastrocytoma	39	F	Left frontal lobe	+	+	G395A

++, strongly positive; +, modestly positive; +/−, weakly positive; −, negative

anaplastic oligoastrocytomas, both oligodendroglial and astrocytic tumor cells were intimately mixed. The GTNI case comprises mostly glioma components with scant neuropil-like islands, and the former was anaplastic oligoastrocytoma (Fig. 3a, d), in which the astrocytic and oligodendroglial components abutted with a clear border. The case of oligodendroglioma with prominent neuronal differentiation was composed of a mixture of oligodendroglioma, gangliocytoma, and neurocytoma-like areas (Fig. 3g, j). The neurocytoma-like area consisted of round tumor cells with a neuropil-like fibrillary matrix.

Diffuse astrocytomas, a glioblastoma, a glioblastoma with oligodendroglioma component, oligodendrogliomas, anaplastic oligodendrogliomas, and oligoastrocytomas, all of which had been demonstrated to have the *IDH1* G395A mutation, were immunohistochemically positive for both mIDH1^{R132H} and RcMab-1 (Fig. 1b, c, h, i). On the other hand, all the diffuse gliomas other than anaplastic oligoastrocytomas without *IDH1* mutation were mIDH1^{R132H}-negative and RcMab-1-positive (Fig. 1e, f).

The anaplastic oligoastrocytomas showed variable results. Eight cases had the *IDH1* G395A mutation and three of them (case 38, 39, 40) showed strong staining for both mIDH1^{R132H} and RcMab-1 (Fig. 2b, c). One case (case 34) showed modest and strong positive staining for mIDH1^{R132H} and RcMab-1, respectively. Three cases (case 35, 37, 43) showed modest staining for both mIDH1^{R132H} and RcMab-1 (Fig. 2e, f). One case (case 36) showed ambiguous staining for both mIDH1^{R132H} and RcMab-1 (Fig. 2h, i). A case with the *IDH1* C394T mutation (case 41; Fig. 2k, l) and a case with no *IDH1* mutation (case 42) were negative for mIDH1^{R132H} but positive for RcMab-1.

In the GTNI case, the oligodendroglioma-like area had strong and modest staining for mIDH1^{R132H} and RcMab-1, respectively (Fig. 3b, c). In contrast, the astrocytoma-like area showed indistinct staining for mIDH1^{R132H} and RcMab-1 (Fig. 3e, f). On the other hand, the *IDH1* G395A mutation was detected in both the oligodendroglioma-like and astrocytoma-like areas. For the oligodendroglioma case with prominent neuronal differentiation, the oligodendroglia-like tumor cells were positive for both mIDH1^{R132H} and RcMab-1 (Fig. 3h, i). In contrast, the staining for mIDH1^{R132H} and RcMab-1 in the neurocytoma-like component was ambiguous (Fig. 3k, l). However, the *IDH1* G395A mutation was detected in both the oligodendroglioma and neurocytoma-like areas by DNA sequencing.

Discussion

In this study we analyzed mutated and wild type IDH1 protein expression and the *IDH1* gene status of 43 gliomas, which encompassed almost all types of diffusely infiltrating gliomas listed in WHO classification. Anaplastic oligoastrocytomas with the *IDH1* G395A mutation had variable staining intensity for mIDH1^{R132H} among the cases. This finding disproved our previous belief that gliomas with the *IDH1* G395A mutation must be diffusely and strongly positive for mIDH1^{R132H}. Interestingly, there was a tendency that cases that were strongly or weakly positive for mIDH1^{R132H} showed comparable immunoreaction to RcMab-1. This suggests that the expression of both mutant and wild type IDH1 is comprehensively regulated and a glioma should not be regarded as *IDH1*-mutation negative just because of ambiguous positive staining of mIDH1^{R132H}.

Although the anaplastic oligoastrocytomas were variably positive for mIDH1^{R132H}, intra-tumor heterogeneity was not detected in any of these cases. In contrast, the GTNI and oligodendroglioma case with prominent neuronal differentiation showed differential staining intensity for mIDH1^{R132H} throughout the tumor components, although the *IDH1* G395A mutation was detected in every component. The tumor components which were ambiguously or strongly positive for mIDH1^{R132H} showed a

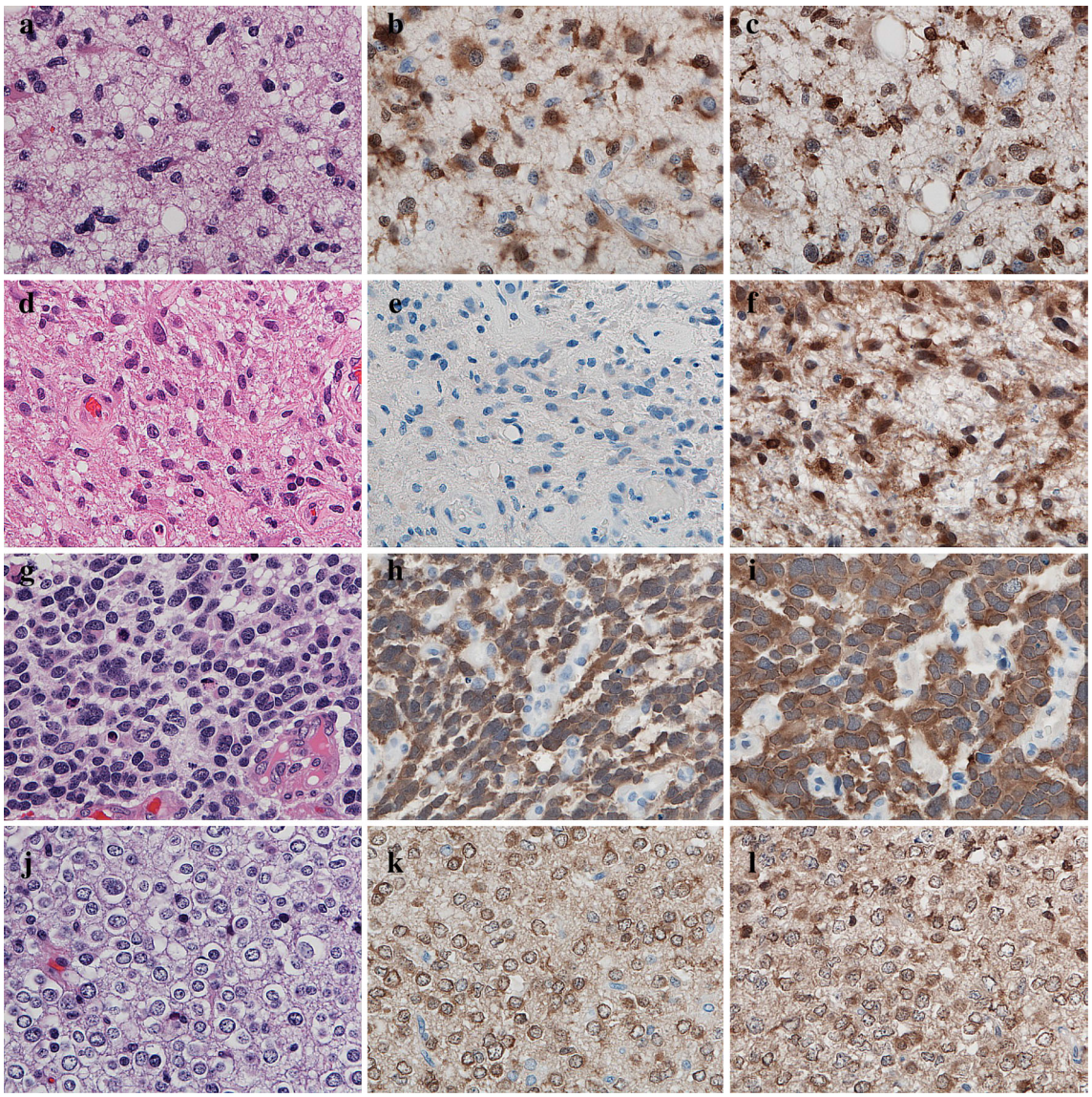

Fig. 1 Representative histological features and immunohistochemical staining of diffuse astrocytoma (**a**–**c** case 2), anaplastic astrocytoma (**d**–**f** case 6), glioblastoma with oligodendroglioma component (**g**–**i** case 15), and oligodendroglioma (**j**–**l** case 16). H&E stain (**a**, **d**, **g**, **j**), mIDH1^{R132H} (**b**, **e**, **h**, **k**), and RcMab-1 (**c**, **f**, **i**, **l**). Diffuse astrocytoma, glioblastoma with oligodendroglioma component, and oligodendroglioma are positive for both mIDH1^{R132H} and RcMab-1. Anaplastic astrocytoma is negative for mIDH1^{R132H} but positive for RcMab-1

corresponding similar degree of immunoreaction to RcMab-1. It is thought that IDH1 expression in each tumor component is regulated under different mechanisms.

The inconsistency between the weak mIDH1^{R132H}/RcMab-1 staining and presence of the *IDH1* G395A mutation in this study is difficult to explain. As tumor cells showed weaker staining for RcMab-1 than that of non-neoplastic cells such as endothelia (Figs. 2i, 3f, l), the suppression of whole IDH1 expression in the tumor cells would be assumed. The reason why this inconsistency was observed only in anaplastic oligoastrocytomas, GTNI, and oligodendroglioma with prominent neuronal differentiation was uncertain in this study.

To date, several monoclonal antibodies both specific and multi-specific for mutant and wild type IDH1/2 have been established [22]. Among them, the anti-wild type IDH1 antibody RcMab-1 contributed greatly to this study. Prior to this research, we compared three anti-wild type IDH1 antibodies: clone W09 [17], RMab-3 [23], and RcMab-1 [18, 19]. As a result, RcMab-1 showed the highest signal-to-background ratio (data not shown). Although direct sequencing keeps the best position in *IDH* mutation detection, we hereby emphasize the utility of immunohistochemistry. A previous report presented glioma cases that were determined to be *IDH1* wild type by DNA sequencing, nonetheless were

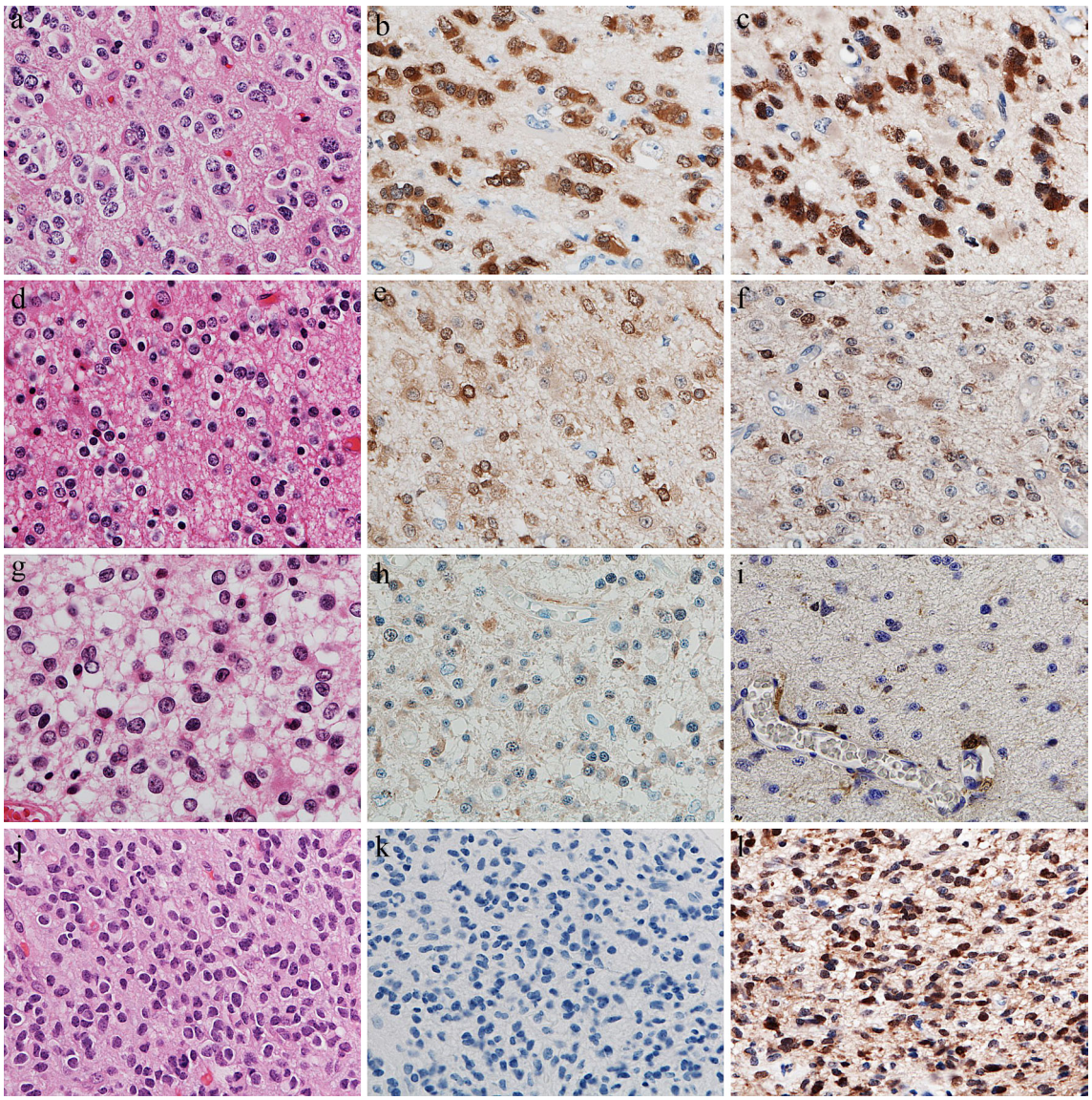

Fig. 2 Representative histological features and immunohistochemical staining of anaplastic oligoastrocytomas (**a**–**c** case 38, **d**–**f** case 43, **g**–**i** case 36, **j**–**l** case 41). H&E stain (**a**, **d**, **g**, **j**), mIDH1^{R132H} (**b**, **e**, **h**, **k**), and RcMab-1 (**c**, **f**, **i**, **l**). Staining of both mIDH1^{R132H} and RcMab-1 in cases 38, 43, and 36 are strongly, modestly, and ambiguously positive, respectively. Case 41 is negative for mIDH1^{R132H} but positive for RcMab-1

immunopositive for IMab-1 an antibody against mutant IDH1^{R132H}. The authors in that paper explained that DNA sequencing had not worked well with paraffin-embedded materials because of contaminated samples or low tumor cellularity [24]. Immunohistochemistry is a more readily available method and is useful even in small and hypocellular samples.

Taking all the discussion into consideration, we propose an evaluation procedure of *IDH1* status by immunohistochemistry in diffusely infiltrating gliomas. If tumor cells are strongly positive for mIDH1^{R132H}, the tumor is judged to have the *IDH1* G395A mutation. If tumor cells are negative for mIDH1^{R132H}, the *IDH1* G395A mutation is judged to be absent. If tumor cells are weakly positive for mIDH1^{R132H}, immunohistochemistry for RcMab-1 should be performed. If the tumor cells are strongly positive for RcMab-1, the *IDH1* G395A mutation is judged to be absent on the grounds that IDH1 expression is not suppressed. A false positive for the *IDH1* G395A mutation due to non-specific staining for mIDH1^{R132H} should also be considered. If the tumor cells are weakly positive for both mIDH1^{R132H} and RcMab-1, DNA sequencing should be

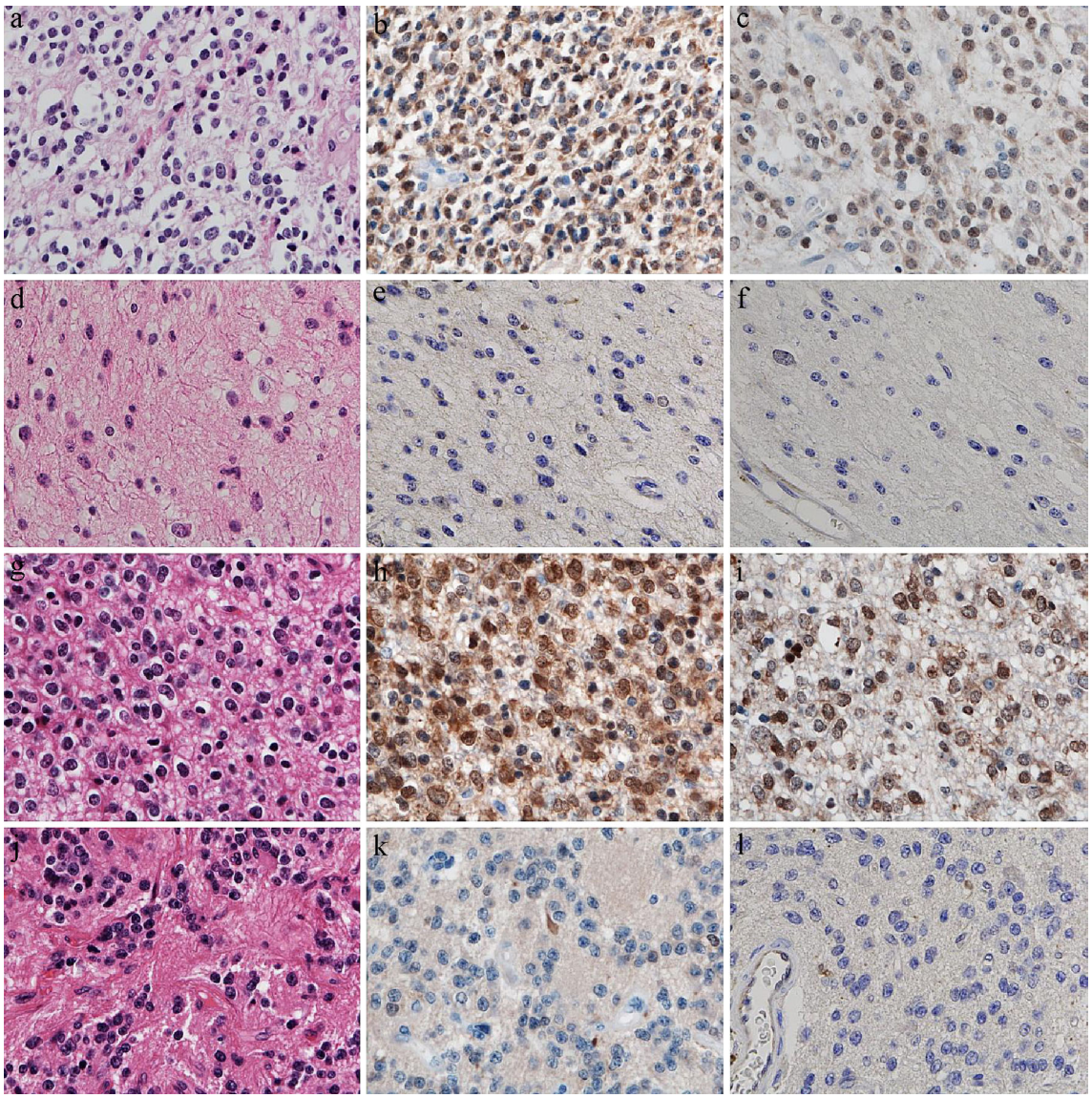

Fig. 3 Representative histological features and immunohistochemical staining of glioneuronal tumor with neuropil-like islands (GTNI) (**a–f** case 9) and oligodendroglioma with prominent neuronal differentiation (**g–l** case 20). H&E stain (**a**, **d**, **g**, **j**), mIDH1^{R132H} (**b**, **e**, **h**, **k**), and RcMab-1 (**c**, **f**, **i**, **l**). In the GTNI case, the oligodendroglioma-like area (**a–c**) is strongly and modestly positive for mIDH1^{R132H} and RcMab-1, respectively. In contrast, the astrocytoma-like area (**d–f**) shows indistinct staining for mIDH1^{R132H} and RcMab-1. In the oligodendroglioma with prominent neuronal differentiation case, the oligodendroglia-like area (**g–i**) is positive for both mIDH1^{R132H} and RcMab-1. In contrast, the staining for mIDH1^{R132H} and RcMab-1 in the neurocytoma-like component (**j–l**) is ambiguous

performed due to possible IDH1 suppression by unidentified regulatory mechanisms. This procedure will be useful for the practical evaluation of *IDH1* status.

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Conflict of interest The authors have no conflict of interest.

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