Brain Tumor Pathology (2018) 35:106–113
https://doi.org/10.1007/s10014-018-0312-5

ORIGINAL ARTICLE

Immunohistochemical ATRX expression is not a surrogate for 1p19q codeletion

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Received: 23 February 2018 / Accepted: 7 March 2018 / Published online: 17 March 2018

Abstract
The IDH-mutant and 1p/19q co-deletion (1p19q codel) provides significant diagnostic and prognostic value in lower-grade gliomas. As *ATRX* mutation and 1p19q codel are mutually exclusive, ATRX immunohistochemistry (IHC) may substitute for 1p19q codel, but this has not been comprehensively examined. In the current study, we performed ATRX-IHC in 78 gliomas whose *ATRX* statuses were comprehensively determined by whole exome sequencing. Among the 60 IHC-positive and 18 IHC-negative cases, 86.7 and 77.8% were *ATRX*-wildtype and *ATRX*-mutant, respectively. *ATRX* mutational patterns were not consistent with ATRX-IHC. If our cohort had only used IDH status and IHC-based ATRX expression for diagnosis, 78 tumors would have been subtyped as 48 oligodendroglial tumors, 16 IDH-mutant astrocytic tumors, and 14 IDH-wildtype astrocytic tumors. However, when the 1p19q codel test was performed following ATRX-IHC, 8 of 48 ATRX-IHC-positive tumors were classified as "1p19q non-codel" and 3 of 16 ATRX-IHC-negative tumors were classified as "1p19q codel"; a total of 11 tumors (14%) were incorrectly classified. In summary, we observed dissociation between ATRX-IHC and actual 1p19q codel in 11 of 64 IDH-mutant LGGs. In describing the complex IHC expression of *ATRX* somatic mutations, our results indicate the need for caution when using ATRX-IHC as a surrogate of 1p19q status.

Keywords Glioma · ATRX · Immunohistochemistry · Whole exome sequencing · 1p19q codel

Introduction

Gliomas are common brain tumors. Although grade II and III gliomas (lower-grade gliomas, LGGs) are less aggressive than glioblastoma multiforme, most result in recurrence and eventual fatality [1, 2]. In the revised 4th edition of the classification of tumors of the central nervous system of the World Health Organization in 2016, in addition to histology, genetic markers were included to define tumor entities [3]; accordingly, the status of the *IDH* mutation and 1p19q codeletion (1p19q codel) became essential for the diagnosis and prognosis of gliomas, particularly for LGGs [4–8].

A variety of methods [9] for analyzing the 1p19q codel such as fluorescence in situ hybridization [6, 7, 10], comparative genomic hybridization [11], and multiplex ligation-dependent probe amplification [12] have been developed, but have limitations in routine clinical practice because of their complexity and cost. Accordingly, molecular surrogate markers for the 1p19q codel are needed for LGGs.

ATRX mutation is thought to occur at an early stage of glioma tumorigenesis [1] and influence its development [13–15], thereby playing a potential role as a glioma classification marker. However, *ATRX* mutation shows various patterns (i.e., non-recurrent mutations) [16] and sequencing is complicated and time-consuming. *ATRX* mutation and 1p19q codel are mutually exclusive [1, 8]. On the other hand, the presence of *ATRX*-wildtype does not necessarily indicate the presence of 1p19q codel (Table 1). However, assuming that *ATRX* mutation prevents the expression of ATRX [17], ATRX negativity in immunohistochemistry

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Table 1 Mutual exclusiveness of 1p/19q codeletion and *ATRX* mutation in three cohorts

	Current study		JPN		TCGA	
	1p19q codel (+)	1p19q codel (−)	1p19q codel (+)	1p19q codel (−)	1p19q codel (+)	1p19q codel (−)
Grades II/III gliomas						
ATRX-mutant	3	19	5	91	3	157
ATRX-wildtype	40	16	143	96	135	127
IDH-mutant grade II/III gliomas						
ATRX-mutant	3	16	5	85	3	156
ATRX-wildtype	40	5	143	28	135	48
		$p < 0.00001$		$p < 0.00001$		$p < 0.00001$

Each genetic status was determined as described previously [1, 26, 27]

JPN Japanese Cohort, *TCGA* The Cancer Genome Atlas Cohort

(IHC) is a possible substitute for detection of 1p19q codel. Some studies demonstrated that ATRX immunonegativity in IHC coincided with *ATRX* mutation [18–23]. Moreover, some reports proposed that ATRX-IHC should be used for clinical classification without assessment of the 1p19q codel [19, 23–25]. However, few reports have examined the sensitivity and specificity of ATRX-IHC for *ATRX* mutation and 1p19q codel in a relatively large cohort.

In this study, we investigated whether ATRX loss in IHC is consistent with *ATRX* mutation and 1p19q codel identified by whole-exome sequencing (WES) in 78 glioma samples.

Materials and methods

Mutual exclusivity of *ATRX* mutation and 1p19q codel in two large cohorts

To investigate mutual exclusivity of *ATRX* mutation and 1p19q codel, we used data sets from two independent cohorts of LGG patients from Japan (JPN) ($n = 335$) and The Cancer Genome Atlas (TCGA, $n = 422$) [26] (Table 1). Mutations in *IDH1, IDH2*, and *ATRX* were investigated using WES and/or targeted deep sequencing of custom bait library or PCR-amplified fragments as previously described. The 1p19q codel status was determined, as described previously [1, 27].

Tumor samples and gene mutation analysis

All 78 patients were diagnosed with grade II or III gliomas independently by three pathologists. With informed consent, all tumor samples were collected at the time of surgery at Nagoya University between 2003 and 2012. All *ATRX*-mutant samples were collected during initial surgery without pretreatment. WES of these samples was performed as part of a large cohort in a previous report [1]. Mutations in *IDH1*/2 and *ATRX* and the 1p19q codel were analyzed using deep sequencing for all samples.

Immunohistochemistry

For the primary reaction, two anti-ATRX antibodies and an anti-IDH1 R132H antibody were used for immunohistochemistry. Because ATRX is expressed in normal cells, to avoid incorrect assignment of ATRX immunopositivity to non-tumor cells, adjacent section was stained using an anti-IDH1-R132H antibody. AMab-6 (Wako, Osaka, Japan) [28] is a monoclonal anti-ATRX antibody produced in mice and HPA001906 is a polyclonal anti-ATRX antibody (Sigma-Aldrich, St. Louis, MO, USA) produced in rabbits. HMab-2 (Wako) [29] is an anti-IDH1 R132H monoclonal antibody produced in mice. The tumor samples were fixed with 10% formalin and embedded in paraffin. Sections (5-μm thick) were prepared using a microtome (REM710, Yamato Kohki Industrial, Saitama, Japan). After deparaffinization and hydration, the sections were incubated in retrieval solution (Tris-EDTA buffer pH 9.0 for HMab-2 and HPA001906, and citrate acid buffer pH 6.0 for AMab-6) for 20 min at 100 °C in an electric pod. After antibody retrieval, subsequent procedures were performed using a Histostainer automatic immunostaining machine (HS36A, Nichirei Bioscience Inc., Tokyo, Japan). The sections were blocked with antibody diluent (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) at room temperature for 10 min and incubated for 1 h with each primary antibody diluted to 1 μg/ml. An appropriate second labeled polymer from the EnVision HRP kit (Dako) was used and the sections were incubated for 30 min. The Histostainer was used until incubation of second antibody and subsequent washing, and substrate-chromogen solution from the DAB Substrate Kit (Vector Laboratories, Burlingame, CA, USA) was applied for 10 min. After washing, the sections were counter-stained

with hematoxylin and mounted in Multi-Mount (Matsunami Glass Inc., Kishiwada, Japan).

Multiple fluorescence immunohistochemistry

We carefully classified the tumors by staining ATRX and IDH1-R132H in 2 consecutive sections or used multi-colored co-immunofluorescent histology to confirm that ATRX expression was derived from tumor cells, rather than ATRX-positive normal cells. Fluorescence IHC (fIHC) was performed using the Opal 4-color Manual IHC kit (PerkinElmer, Inc., Waltham, MA, USA). HMab-2 and HPA001906 were used as primary antibodies in the same sections. A similar procedure as for normal immunohistochemistry was used until incubation and washing HMab-2 as the first primary antibody. After washing HMab-2, the secondary antibody labeled HRP was applied and incubated for 10 min. After washing, Opal 570 reagent was applied and incubated for 10 min. After washing, the sections were re-incubated in antigen retrieval solution for 15 min at 100 °C in an electric pod with blocking. HPA001906 antibody was applied as a second primary antibody and incubated for 1 h. After washing, a second polymer labeled HRP was applied and incubated for 10 min, Opal 520 reagent was applied and incubated for 10 min. The sections were counter-stained with DAPI and mounted in Vectorshield mounting medium (Vector Laboratories). Fluorescence images were captured using a fluorescence microscope (BZ9000; Keyence, Osaka, Japan).

Statistical analysis

The statistical significance of the differences between two groups was determined using Student's *t* test and Fisher's exact test. p values < 0.05 were considered significant.

Results

Patient characteristics

IDH-mutant and *IDH*-wildtype diffuse astrocytomas, *IDH*-mutant and *IDH*-wildtype anaplastic astrocytomas, oligodendrogliomas, and anaplastic oligodendrogliomas (*IDH*-mutant, 1p19q codel) accounted for 16, 5, 5, 9, 30, and 13 cases, respectively (Table 2). The proportion of cases with/without 1p19q codel or with/without *ATRX* mutation in this study was similar to that in the 2 large cohorts (see also Table 1). Progression-free survival and overall survival times were 2.6 and 3.7 years, respectively. Before sampling, 21 patients (27%) received either or both chemo- and radiotherapy.

Table 2 Patients' characteristics

The number of cases	78
PFS (years)	2.6
OS (years)	3.7
Gender	
Female	34
Male	44
Age (years, mean)	41.1
(Range)	(17.6–72.9)
WHO classification	
Diffuse astrocytoma, IDH-mutant	16
Diffuse astrocytoma, IDH-wildtype	5
Anaplastic astrocytoma, IDH-mutant	5
Anaplastic astrocytoma, IDH-wildtype	9
Oligodendroglioma, IDH-mutant and 1p/19q codeleted	30
Anaplastic oligodendroglioma, IDH-mutant and 1p/19q codeleted	13
Pretreatment	
None	60
Radiotherapy	13
Chemotherapy	1
Chemo-radiotherapy	4

PFS progression-free survival, *OS* overall survival, *WHO* World Healthcare Organization

Table 3 Correlation between loss of ATRX expression and *ATRX* mutations

	ATRX-wildtype	*ATRX*-mutant	Total
IHC-positive	52	8	60
IHC-negative	4	14	18
	56	22	78
			$p < 0.00001$

IHC immunohistochemistry

Correlation between loss of ATRX expression and *ATRX* mutations

We performed IHC using 2 anti-ATRX antibodies with different epitopes. The results were consistent in all samples.

Of the 78 cases, 60 showed ATRX expression (IHC-positive) and 18 showed loss of ATRX expression (IHC-negative) in tumor cells (Table 3). Among the 60 IHC-positive cases, 52 (86.7%) were *ATRX*-wildtype and 8 (13.3%) were *ATRX*-mutant by WES. Among the 18 IHC-negative cases, 14 (77.8%) were *ATRX*-mutant and 4 (22.2%) were *ATRX*-wildtype by WES. All *ATRX*-mutant tumors did not exhibit specific mutation patterns such as splicing, frameshifts, or single-nucleotide variants by WES (Table 4).

Table 4 *ATRX* mutation patterns by whole-exome sequence

	IHC-negative	IHC-positive	Total	*p* value
Number of cases	14	8	22	
Mutation pattern				
Splicing	4	0	4	0.2536
Frameshift insertion	1	2	3	0.5273
Frameshift deletion	4	1	5	0.613
Nonsynonymous SNV	2	3	5	0.3089
Stopgain SNV	3	2	5	1
	IHC-negative	IHC-positive	Total	
Number of cases	14	8	22	
Mutation pattern				
Missense	2	3	5	
Indel + nonsense + splice site	12	5	17	
			$p = 0.3089$	

Does ATRX loss in immunohistochemical analysis serve as a surrogate for 1p19q codeletion?

First, as previously reported, *ATRX* mutation was mutually exclusive with 1p19q codel (Table 1). Most IDH-mutant LGGs with *ATRX* mutation did not have 1p19q codel (84, 94, and 98% in this study, the JPN cohort, and the TCGA cohort, respectively), and 93–98% of IDH-mutant LGGs with 1p19q codel harbored *ATRX*-wildtype. In contrast, 52–71% of *ATRX*-wildtype tumors displayed 1p19q codel (Table 1). Second, a tumor with *ATRX* mutation did not always exhibit loss of ATRX expression, as described above (Table 3).

We next evaluated whether ATRX expression in IHC serves as a surrogate for 1p19q codel. A spreadsheet of the results of IDH status, 1p19q codel status, ATRX-IHC, and *ATRX* sequencing in 78 cases is shown in Fig. 1. Of total, 11 cases with IDH mutation (Cases 41–51 in Fig. 1) showed discrepancy between IHC-based ATRX expression and actual 1p19q status. Cases #41 and #42, for instance, did not show ATRX expression in tumor cells, while normal endothelial cells and normal brain cells expressed ATRX. These tumors were initially diagnosed as astrocytoma; however, genetic investigation of 1p19q and *ATRX* revealed that they harbored 1p19q codel and wild-type *ATRX*, and thus the final diagnosis was oligodendroglioma, IDH-mutant, and 1p19q codel (Fig. 2a, b).

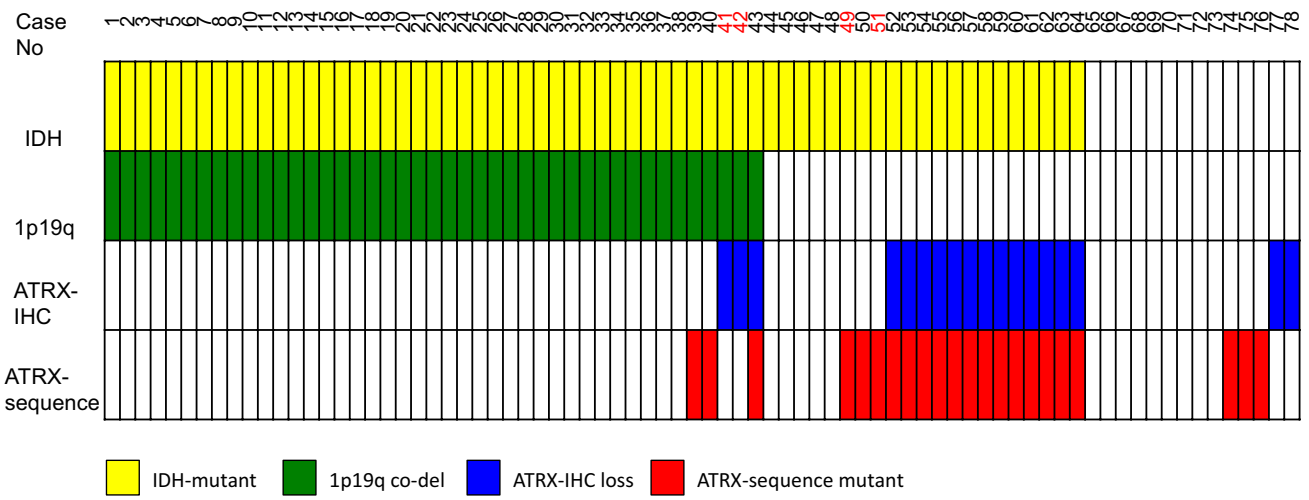

Fig. 1 Spreadsheet of IDH status, 1p19q codel, ATRX-IHC, and *ATRX* sequencing in each case. For the cases indicated in red, the pictures of their pathology are shown in Figs. 2 and 3

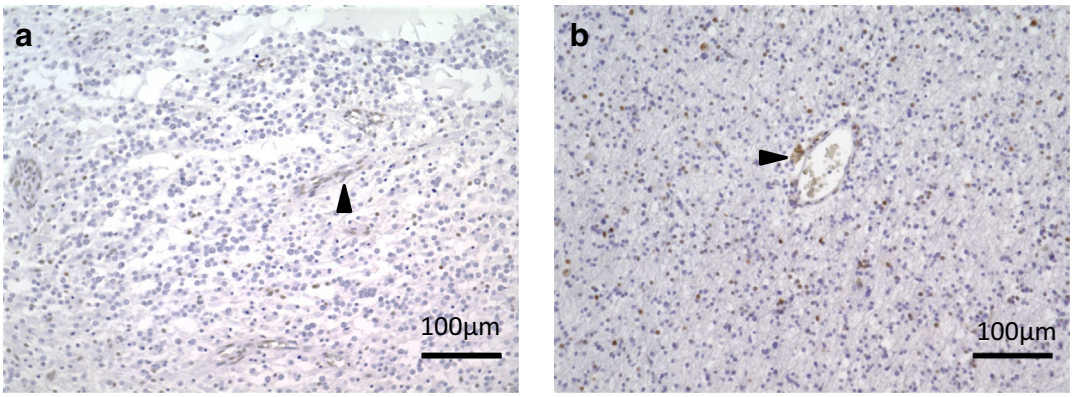

Fig. 2 Cases #41 (**a** Oligodendroglioma, IDH-mutant, 1p19q codeleted, Grade II) and #42 (**b** Anaplastic Oligodendroglioma, IDH-mutant, 1p19q codeleted, Grade III) did not show ATRX expression in tumor cells, while normal endothelial cells and normal brain cells expressed ATRX. The diagnosis of these tumors was initially astrocytoma; however, following genetic investigation of 1p19q and *ATRX*, they were found to harbor 1p19q codel and wildtype *ATRX*. Thus, the final diagnosis was oligodendroglioma, IDH-mutant, 1p19q codel. **c** In Case #42, the multicolored co-immunofluorescent histology with anti-ATRX and IDH1-R132H antibodies revealed that abundant IDH-R132H-positive tumor cells did not merge with ATRX-positive cells

Additionally, multicolored co-immunofluorescent histology with anti-ATRX and IDH1-R132H antibodies confirmed that abundant IDH1-R132H-positive tumor cells did not merge with ATRX-positive cells (Fig. 2c). Cases #49 and #51 were ARTX-IHC positive, and thus based on pathological diagnosis were oligodendroglioma (Fig. 3a, b). However, as tumor DNA did not display 1p19q codel, the diagnosis changed to astrocytoma, IDH-mutant. Co-immunofluorescent histology showed co-expression of ATRX and IDH1-R132H (Fig. 3c).

If our cohort had only been diagnosed based on IDH status and IHC-based ATRX expression, 78 tumors would have been subtyped as 48 oligodendroglial tumors, 16 IDH-mutant astrocytic tumors, and 14 IDH-wildtype astrocytic tumors (Fig. 4). However, when the 1p19q codel test was performed following ATRX-IHC, 8 of 48 ATRX-IHC-positive tumors were classified as "1p19q non-codel" and 3 of 16 ATRX-IHC-negative tumors were classified as "1p19q codel"; a total of 11 tumors (14%) were incorrectly classified (Fig. 4).

Discussion

The current study suggests that ATRX-IHC does not have reliable sensitivity as a surrogate for 1p19q codel. We found that diagnostic accuracy was 82.8% in 64 IDH-mutant LGGs when only ATRX-IHC was used to examine 1p19q codel.

Among the 48 tumors showing positive ATRX, 8 (16.6%) were found to be 1p19q non-codel. Similarly, 3 (18.7%) of 16 tumors with negative ATRX had 1p19q codel. Thus, 11 of 78 tumors required reclassification.

We demonstrated that *ATRX* mutation was not well-correlated with the loss of expression in IHC. In contrast, intratumor heterogeneity and sampling should be considered. Purkait et al. reported that ATRX-IHC was matched to *ATRX* sequencing in only 50% of cases (3 of 6) [30]. They performed *ATRX* sequencing of microdissected sections showing a mosaic or negative pattern for ATRX-IHC. Their results revealed that both mutational and

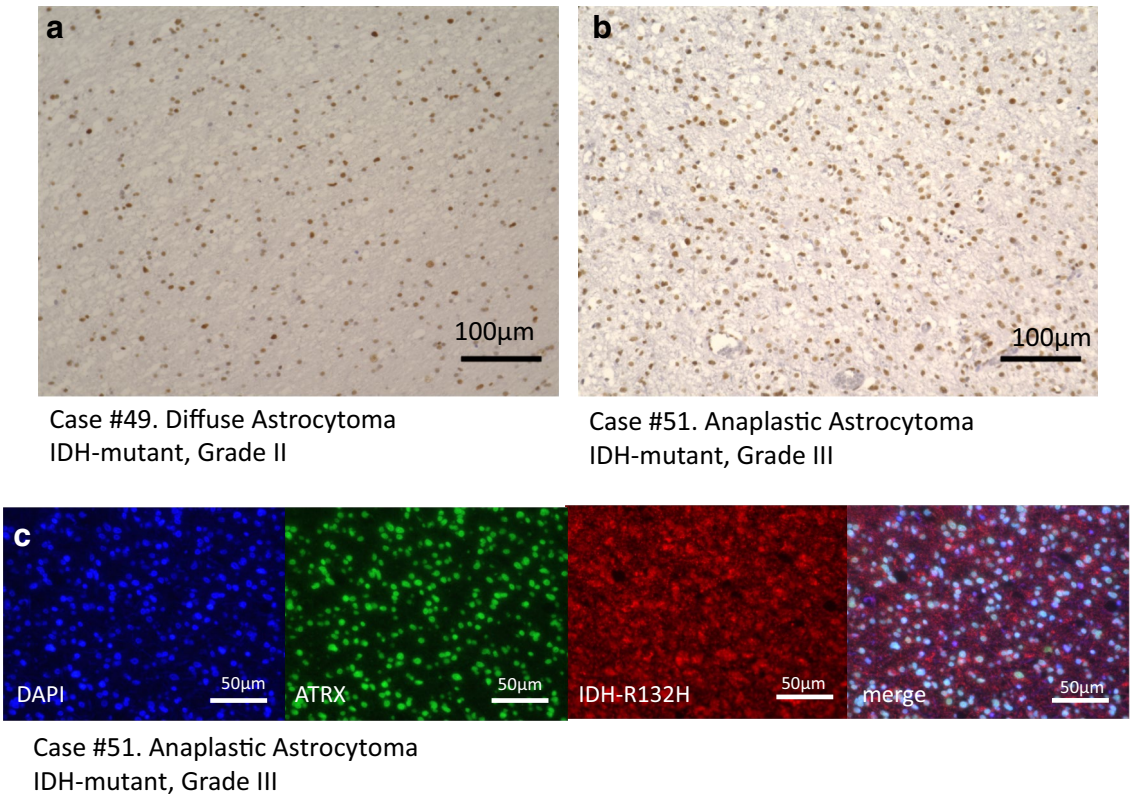

Fig. 3 Cases #49 (**a** Diffuse astrocytoma, IDH-mutant, Grade II) and #51 (**b** Anaplastic astrocytoma, IDH-mutant, Grade III) are ATRX-IHC-positive, which led to the pathological diagnosis of oligodendroglioma. However, as the tumor DNA did not display 1p19q codel, the diagnosis was changed to astrocytoma, IDH-mutant. **c** In Case #51,co-immunofluorescent histology showed co-expression of ATRX and IDH1-R132H

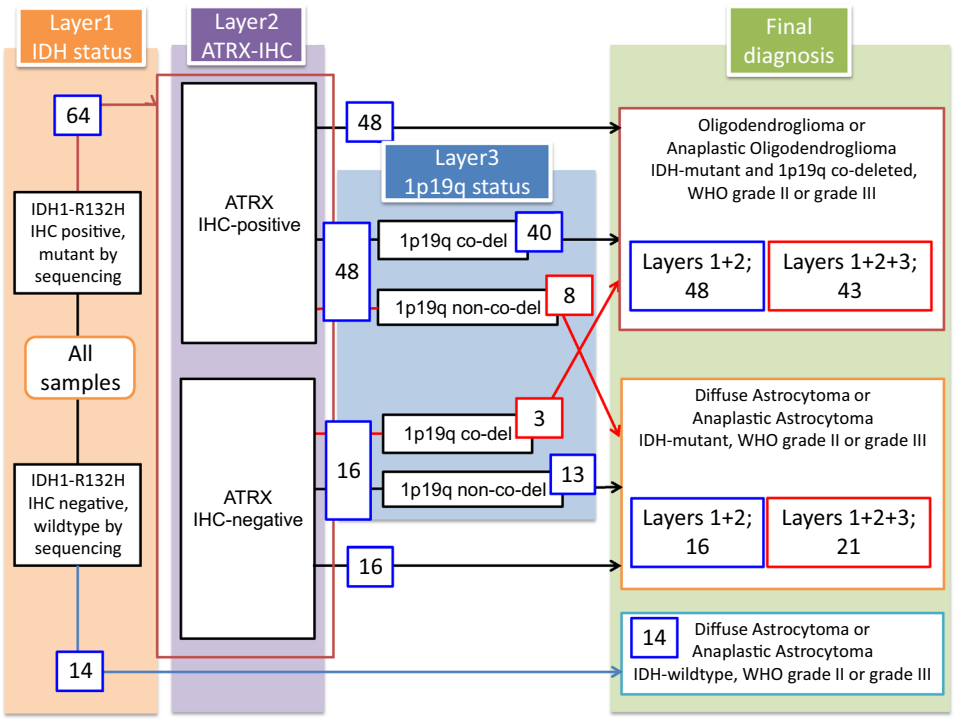

Fig. 4 Flowchart of LGG diagnosis in our cohort. If our cohort had only been diagnosed based on IDH status and IHC-based ATRX expression, 78 tumors would have been subtyped as 48 oligodendroglial tumors, 16 IDH-mutant astrocytic tumors, and 14 IDH-wild-type astrocytic tumors. However, when the 1p19q codel test was performed following ATRX-IHC, 8 of 48 ATRX-IHC-positive tumors were classified as "1p19q non-codel" and 3 of 16 ATRX-IHC-negative tumors were classified as "1p19q codel"; a total of 11 tumors (14%) were incorrectly classified

expressional statuses showed diverse patterns when sampling was performed from different sites, even within the same tumor. Such intratumor heterogeneity may influence the discrepancy between the results using ATRX-IHC and WES. In contrast, non-recurrent *ATRX* mutation in glioma may not lead to the loss of ATRX expression. Our study showed that only splicing of ATRX resulted in IHC negativity, but frameshifts and single-nucleotide variants did not affect the results of ATRX-IHC (Table 4). Although we used two different antibodies for distinct epitopes in this study, they may not be universally applicable for such different mutation patterns.

Whether ATRX-IHC can be used as a surrogate of 1p19q codel remains unclear. Ikemura et al. demonstrated that ATRX-IHC was 100% concordant with *ATRX* sequencing in 19 LGGs as well as 1p19q status in 53 LGGs [31]. In contrast, Purkait et al. and Reuss et al., respectively, reported that among 30 and 72 tumors with 1p19q codel, 3 (10%) and 2 (3%) did not show ATRX expression [19, 30]. In European Association for Neuro-Ongology guideline mentioned that ATRX-IHC might facilitate diagnosis but is not a substitute for 1p19q codel testing [32]. On the other hand, cIMPACT-NOW update2 suggests that 1p19q testing can be skipped in cases showing ATRX-IHC negativity [33]. However, in our cohort, 3 (19%) of 16 IDH-mutant and ATRX-negative tumors harbored 1p/19q codel, suggesting that the utility of ATRX-IHC as a surrogate of 1p19q codel may be still debatable.

In summary, we observed a dissociation between ATRX-IHC positivity and the presence of actual 1p19q codel in 11 of 64 IDH-mutant LGGs. In describing the complex IHC expression of *ATRX* somatic mutations, our results indicate the need for caution when using ATRX-IHC as a surrogate of 1p19q status.

Acknowledgements This research was supported by a Grant-in-Aid for Scientific Research on Innovative Areas "Chemistry for Multimolecular Crowding Biosystems" (A.N.) (JSPS KAKENHI Grant no. 17928985) and Platform Project for Supporting Drug Discovery and Life Science Research [Basis for Supporting Innovative Drug Discovery and Life Science Research (BINDS)] from AMED under Grant no. JP17am0101078 (Y.K.). The authors thank Drs. Ichiro Ito and Reiko Watanabe for their second pathological review.

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