

Diagnostic revision of 206 adult gliomas (including 40 oligoastrocytomas) based on ATRX, IDH1/2 and 1p/19q status

Marta Mellai¹ · Laura Annovazzi¹ · Rebecca Senetta² · Carmine Dell'Aglio² ·
Marta Mazzucco¹ · Paola Cassoni³ · Davide Schiffer¹

Received: 19 April 2016 / Accepted: 9 October 2016 / Published online: 28 October 2016
© Springer Science+Business Media New York 2016

Abstract The diagnosis of 206 low and high grade adult gliomas, including 40 oligoastrocytomas, was revised based on the immunohistochemical reactivity for the ATRX protein, *IDH1/2* mutation status and 1p/19q chromosomal status. All oligodendrogliomas kept the initial diagnosis. Astrocytomas did not change diagnosis in 30 of 36 cases (83.3%); four of 36 (11.1%) cases were reclassified as oligodendroglioma, one (2.8%) as DNT and the other (2.8%) as reactive gliosis. Oligoastrocytomas changed diagnosis in 35 of 40 (87.5%) cases, being reclassified 22 of 40 (55%) as astrocytoma, 11 of 40 (27.5%) as oligodendroglioma and two of 40 (5%) as reactive gliosis. Four (10%) remained unclassifiable. In one case only (2.5%), the diagnosis of oligoastrocytoma could not be excluded since tumor astrocytes and tumor oligodendrocytes coexisted in mixed tumor areas. In the GBM tumor subgroup, GBMO disappeared because they were not substantiated by molecular genetics. Pilocytic astrocytomas retained ATRX expression. Loss of nuclear ATRX protein expression was strongly associated to *IDH1/2* mutations ($p=0.0001$) and mutually exclusive with total 1p/19q co-deletion ($p=0.0001$). In astrocytic tumors, loss of immunoreactivity for the ATRX protein was

significantly associated to the ALT phenotype ($p=0.0003$). The constitutive ATRX expression in microglia/macrophages may be misleading, especially in the identification of an oligodendroglial tumor infiltration. Of paramount importance in the recognition of oligodendroglial and astrocytic tumor cells were the double immunostainings for ATRX/GFAP, ATRX/*IDH1*^{R132H}, ATRX/Iba-1 and ATRX/CD68.

Keywords Oligoastrocytoma · ATRX · IDH · 1p/19q co-deletion · ALT

Introduction

The differential diagnosis between oligodendroglioma and astrocytoma has always been problematic. The diagnostic uncertainty was even more difficult for oligoastrocytoma (OA), recognized as mixed glioma with both an astrocytic and an oligodendroglial component [1]. Beside rare instances with the two clearly separate components, the two cell types usually appear intermingled. Diagnostic criteria were suggested, including at least 10% tumor oligodendrocytes in astrocytic gliomas [2] or 10% tumor astrocytes in oligodendroglial gliomas [3]. After the first description [4], the existence of OA was confirmed [5], although the possibility of an oligodendroglioma with reactive astrocytes was suggested [6]. The failure in the distinction between reactive and tumor astrocytes, as well as the occurrence of minigemistocytes [7], glial fibrillary oligodendrocytes (GFOC) [8–10] and real gemistocytes, greatly contributed to the indefinite histologic aspect.

Recently, somatic *alpha-thalassemialmental retardation syndrome X-linked (ATRX)* gene mutations, resulting in ATRX protein loss, have been found to be restricted

Electronic supplementary material The online version of this article (doi:10.1007/s11060-016-2296-5) contains supplementary material, which is available to authorized users.

✉ Davide Schiffer
davide.schiffer@unito.it

¹ Research Center, Policlinico di Monza Foundation, Via Pietro Micca, 29, 13100 Vercelli, Italy

² Unit of Pathology, Candiolo Cancer Institute, FPO, IRCCS Candiolo, Turin, Italy

³ Department of Medical Sciences, University of Turin, Turin, Italy

to *isocitrate dehydrogenase 1 and 2 (IDH1/2)* mutant low grade astrocytic gliomas [11–17]. ATRX belongs to the H3.3-ATRX-DAXX chromatin remodelling pathway involved in chromatin stabilization [18]. ATRX and its binding factor DAXX incorporate the histone protein H3.3 into the nucleosome at telomeres and pericentric heterochromatin [19, 20]. Alterations of this function cause loss of structural integrity at telomeres leading to tumorigenesis. ATRX or DAXX protein loss is associated to the alternative lengthening of telomeres (ALT), a telomerase-independent mechanism of telomere lengthening [11–13, 15, 21].

In a recent study, the retained nuclear ATRX expression, associated to *IDH1/2* mutations and 1p/19q co-deletion, allowed to reclassify 31 of 43 (72.1 %) OAs as oligodendrogliomas; 11 of 43 (25.6%), with concurrent ATRX protein loss and *TP53* mutations, were reclassified as astrocytomas. In one case (<1%), the astrocytes within the tumor were proved to be reactive [22]. These results were confirmed in a large collection of cases [23] and led to the disappearance of OA as a separate tumor entity. As ATRX is ubiquitously expressed in normal cells (endothelial cells, reactive astrocytes, microglia/macrophages and infiltrating lymphocytes), the crucial point is to ascertain the occurrence of ATRX-negative and *IDH1*^{R132H}-positive astrocytes in the tumor.

Molecular genetics became increasingly important for the definition of tumor entities and, to date, is an essential tool. The integration of molecular data (*ATRX* and *IDH1/2* mutation and 1p/19q chromosomal status) into the diffuse glioma diagnosis was definitively confirmed in the recent “International Society of Neuropathology (ISN)-Haarlem Consensus” guidelines for brain tumor classification [24].

The aim of this study was to apply the integrated diagnosis on a series of 206 adult gliomas of different histologic type and malignancy grade, including 40 OAs. The immunohistochemical reactivity for the ATRX protein has been used as surrogate for the *ATRX* mutation status.

Materials and methods

Brain tumor specimens

The study has been carried out on 154 low grade and 52 high grade adult gliomas from our collection. The initial histologic diagnosis according to the current World Health Organization (WHO) guidelines [1] is reported in Table 1. In the glioblastoma (GBM) tumor subgroup, tumors were classified as primary (pGBM) or secondary GBM (sGBM) based on progression from a documented grade II or III glioma. In presence of areas with the “honeycomb”

appearance, tumors were classified as GBM with oligodendroglial component (GBMO).

Patients underwent partial or total resection at different Italian Neurosurgery Units. Surgical tumor samples were formalin fixed, paraffin embedded (FFPE) and cut in 5- μ m thick sections.

Immunohistochemistry (IHC)

After haematoxylin and eosin (H&E) staining, IHC was performed with the primary antibodies listed in the supplementary Table S1 on a Ventana Full BenchMark® XT automated immunostainer (Ventana Medical Systems Inc., Tucson, AZ, USA). The ultraView™ Universal DAB Detection Kit was the revelation system (Ventana). Heat-induced epitope retrieval (HIER) was obtained with Tris–EDTA, pH 8 (Ventana). Double immunostainings for ATRX/GFAP, ATRX/*IDH1*^{R132H}, ATRX/Iba-1 and ATRX/CD68 were performed with the ultraView™ Universal Alkaline Phosphatase Red Detection Kit (Ventana).

The Ki-67/MIB-1 labelling index (LI) was calculated as the average percentage of positive nuclei on the total number of nuclei in five microscopic fields, at 1000 \times magnification.

Microglia/macrophages were quantified by cell counting as the average number of positive cells on Iba-1 and CD68-stained sections, respectively, in five microscopic fields, at 400 \times magnification. Reactive astrocytes were quantified in the same way on GFAP-stained sections.

A threshold of 5% of positive tumor nuclei was used to assign immunopositivity for ATRX.

Molecular genetics

Genomic DNA (gDNA) was isolated from FFPE tumor samples using the QIAamp DNA Mini Kit (Qiagen NV, Venlo, The Netherlands).

The 1p/19q chromosomal status was assessed by Multiplex Ligation-dependent Probe Amplification (MLPA) using the SALSA-MLPA Kit P088-B2 (lot numbers 0608-0112) (MRC-Holland, Amsterdam, The Netherlands), according to the manufacturer’s instructions. Threshold values to detect losses or gains in tumor samples were set at 0.75 and 1.4, respectively [25]. Fragment analysis was performed on an ABI® 3130 Genetic Analyzer (Life Technologies, Foster City, CA, USA) and data were collected by the GeneMapper v4.0 software (Life Technologies). Data were analyzed using Coffalyser v9.4 software (MRC-Holland). Total 1p/19q co-deletion was assigned when all consecutive probes on the chromosomal arm 1p showed a ratio <0.75. Partial loss on 1p was defined as evidence of telomeric or interstitial deletions interesting at least two consecutive probes.

Table 1 Immunoreactivity for the ATRX protein with regard to *IDH1/2* and 1p/19q status according to the initial histologic diagnosis

Initial diagnosis	Cases <i>n</i>	ATRX expression		IDH1/2 mutations		Total 1p/q19 co-deletion		Partial 1p/19q co-deletion		Intact 1p/19q		Gain on 1p/19q		
		<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	
PA	12	Retention	9	75	1/9	11.1	0/9	0	2/9	22.2	7/9	77.8	0/9	0
		Loss	3	25	0/3	0	0/2	0	1/2	50	1/2	50	0/1	0
DA	24	Retention	2	8.3	0/2	0	0/1	0	1/1	100	0/1	0	0/1	0
		Loss	22	91.7	14/21	66.7	0/11	0	3/11	27.3	8/11	72.7	0/1	0
AA	12	Retention	7	58.3	1/7	14.3	1/7	14.3	5/7	71.4	0/7	0	1/7	14.3
		Loss	5	41.7	3/5	60	0/3	0	2/3	66.7	1/3	33.3	0/3	0
OA	31	Retention	6	19.4	3/5	60	1/3	33.3	0/3	0	1/3	33.3	1/3	33.3
		*	4	12.9	3/4	75	0/3	0	3/3	100	0/3	0	0/3	0
		Loss	21	67.7	19/20	95	0/19	0	9/19	47.4	10/19	52.6	0/19	0
AOA	9	Retention	7	77.8	2/6	33.3	2/7	28.6	5/7	71.4	0/7	0	0/7	0
		*	1	11.1	0/0	0	0/1	0	1/1	100	0/1	0	0/1	0
		Loss	1	11.1	1/1	100	0/1	0	1/1	100	0/1	0	0/1	0
O	39	Retention	39	100	27/37	73	19/34	55.9	10/34	29.4	5/34	14.7	0/34	0
AO	27	Retention	27	100	12/23	52.2	7/22	31.8	12/22	54.5	3/22	13.6	0/22	0
pGBM	32	Retention	27	84.4	1/27	3.7	1/17	5.9	13/17	76.5	3/17	17.6	0/17	0
			5	15.6	1/5	20	0/2	0	1/2	50	0/2	0	0/2	0
sGBM	1	Loss	1	100	1/1	100	0/0	0	1/1	100	0/1	0	0/1	0
Gliosarcoma	12	Retention	10	83.3	0/10	0	1/10	10	1/10	10	8/10	80	0/10	0
		Loss	2	16.7	0/2	0	0/2	0	0/2	0	2/2	100	0/2	0
GBMO	7	Retention	6	85.7	0/5	0	0/4	0	1/4	25	3/4	75	0/4	0
		Loss	1	14.3	0/1	0	0/1	0	0/1	0	1/1	100	0/1	0

PA pilocytic astrocytoma, DA diffuse astrocytoma, AA anaplastic astrocytoma, OA oligoastrocytoma, AOA anaplastic oligoastrocytoma, O oligodendroglioma, AO anaplastic oligodendroglioma, pGBM primary glioblastoma, sGBM, secondary glioblastoma, GBMO glioblastoma with oligodendroglial component

*Occurrence of ATRX-positive and ATRX-negative tumor cells

Search for somatic point mutations at the hot-spot codons *IDH1* Arg132 (exon 4) (GenBank sequence NM_005896) and *IDH2* Arg172 (exon 4) (GenBank sequence NM_002168) was performed by Sanger direct sequencing on an ABI® 3130 Genetic Analyzer (Life Technologies), as published [26]. The BigDye Terminator v1.1 Cycle Sequencing Kit (Life Technologies) was used. Data were collected by the Sequencing Analysis v.5.3.1 software (Life Technologies).

Telomere Fluorescence In Situ Hybridization (FISH) and ALT assessment

In a subgroup of 14 gliomas, including eight grade II astrocytomas, three grade II oligodendrogliomas and three GBMs, as well as in one sample of normal nervous tissue (NNT), the presence of the ALT phenotype was assessed using the Telomere PNA FISH Kit/FITC kit (Dako, Glostrup, Denmark). The analysis was carried out on FFPE tissue sections prepared according to standard techniques using the Histology FISH Accessory kit (Dako). FISH was

analyzed using a Olympus BX61 microscope (Olympus Corporation, Tokyo, Japan) and the CytoVision software (Leica Biosystems, Newcastle Ltd, UK). An average of 500 nuclei was analyzed at five different target areas using H&E-stained sections as histotopographic reference. Areas exhibiting necrosis were excluded from consideration. ALT-positive cases were identified by large and very bright intra-nuclear FISH signals (ALT-associated telomeric *foci*) in tumor cells. Thus, cases were classified as ALT-positive if >1 % of tumor cells showed ultra-bright FISH signals. The ultra-bright FISH signals were considered as ALT-*foci* only when their intensities were > 10-fold that of the average signal intensities for individual benign stromal nuclei within the same case [11]. The signal intensities of ALT-associated telomeric *foci* were calculated using the CytoVision software (Leica Biosystems).

Statistical methods

Associations between categorical variables were evaluated by use of 2×2 contingency tables and the Chi square (χ^2)

or the two-tailed Fisher's exact test, as appropriate. Analysis was carried out by SPSS v23.0 software (SPSS Inc., Chicago, IL, USA).

P values <0.05 were considered as statistically significant.

Results

IHC

The immunoreactivity for the ATRX protein in the different tumor types is described in Table 1. The diagnostic revision was based on the following immunohistochemical findings (Fig. 1).

1. ATRX was ubiquitously expressed in endothelial cell, infiltrating lymphocyte and reactive astrocyte nuclei.
2. ATRX immunopositivity was retained in tumor oligodendroglial nuclei while it was typically lost in tumor astrocytic nuclei (more in diffuse than in anaplastic astrocytomas).
3. ATRX was constitutively expressed in microglia/macrophage nuclei. Iba-1 and CD68 IHC has been performed on tumor serial sections. The frequency of microglia/macrophage cells was on average 50–130 cells *per* field at 400× magnification (personal data). The occurrence of ATRX-positive tumor oligodendroglial nuclei could only be verified by the double immunostainings for ATRX/Iba-1 and ATRX/CD68 that specifically identify the microglia/macrophage content.
4. *IDH1/2* mutations and IDH1^{R132H} immunoreactivity in tumor cell components occurred in 70–90 % of low grade gliomas, with a higher frequency in oligodendroglial than in astrocytic gliomas [26, 27].
5. Although the IDH1^{R132H} immunoreactivity indicates the tumor nature of the cells, a minor percentage of *IDH1/2* wild type oligodendroglial or astrocytic tumor cells could not be detected.
6. The double immunostaining for ATRX/GFAP allowed to distinguish reactive from tumor astrocytes and tumor oligodendrocytes from tumor astrocytes.
7. Normal oligodendrocytes did not express ATRX; they expressed Cyclin D1 that, conversely, was not expressed by tumor oligodendrocytes [28].
8. Minigemistocytes, GFOC and gemistocytes of oligodendroglial origin were distinguished from large tumor astrocytes for their retained ATRX expression. When they were very frequent, the tumor could mimic a gemistocytic astrocytoma. However, the retained nuclear ATRX immunopositivity was helpful in recognizing the oligodendroglial nature.
9. Neurons of the normal cortex expressed ATRX whereas their satellites showed loss of nuclear ATRX expression. In the infiltrated cortex, perineuronal satellitosis was composed of an increasing number of ATRX-positive satellites.
10. Primary GBMs and gliosarcomas retained nuclear ATRX expression whereas sGBMs followed the immunohistochemical pattern of diffuse astrocytomas. GBMO preserved ATRX expression as pGBMs.
11. Pilocytic astrocytomas retained nuclear ATRX immunoreactivity.

Molecular genetics

The *IDH1/2* mutation rate and the 1p/19q chromosomal status in the different tumor types, according to the initial diagnosis and with respect to the ATRX immunoreactivity, are reported in Table 1.

Loss of nuclear ATRX expression was significantly associated to *IDH1/2* mutations in the whole astrocytic glioma group ($p=0.0001$) and in the tumor subgroups of low grade astrocytomas ($p=0.002$) and OAs ($p=0.0274$).

ATRX loss was mutually exclusive with the total 1p/19q co-deletion ($p=0.0001$) (Table 1).

The spectrum of activating mutations in the *IDH1/2* genes is illustrated in the supplementary Table S2.

ALT phenotype

The ALT phenotype was detected in all the eight astrocytoma cases exhibiting loss of nuclear ATRX protein expression ($p=0.0003$). All of them displayed the *IDH1* c.395G > A (p.R132H) mutation and, accordingly, IDH1^{R132H} immunoreactivity ($p=0.0003$). The percentage of ALT-positive tumor cells varied from 2.3 to 14 (supplementary Table S3).

Oligodendrogliomas and GBMs with retained ATRX expression did not show the ALT phenotype, as NNT (Fig. 2).

Change of tumor category after the integrated diagnosis

The diagnostic change of the glial tumor series is illustrated in the Figs. 3, 4.

In the astrocytic tumor subgroup, the initial diagnosis of astrocytoma was confirmed in 30 of 36 (83.3 %) cases (16 *IDH1/2* mutant and 14 wild type); four cases were reclassified as oligodendroglioma, one as dysembryoplastic neuroepithelial tumor (DNT) and the other as reactive gliosis. In the oligodendroglial tumor subgroup, the initial diagnosis was confirmed in all 66 cases (Fig. 3).

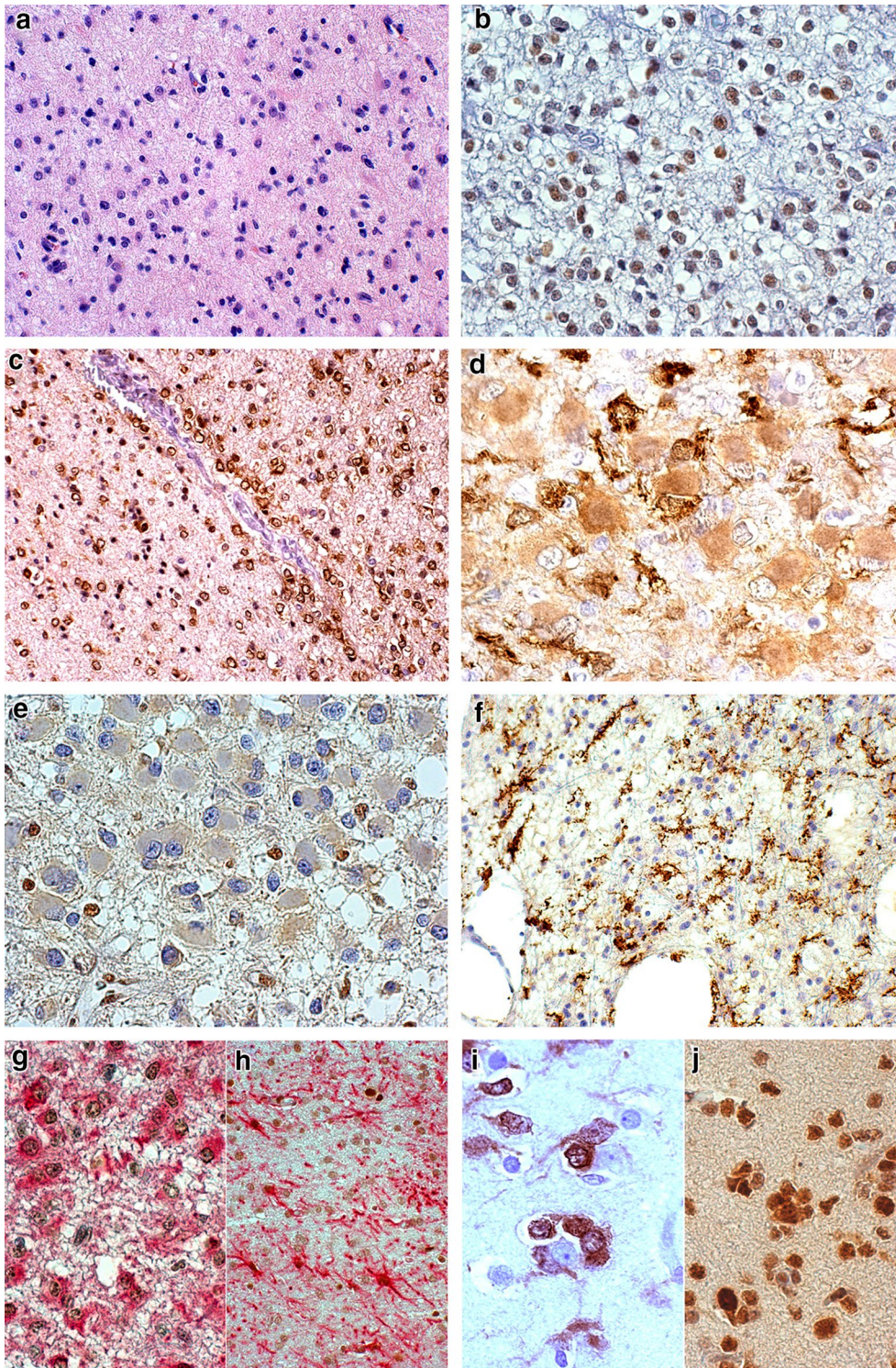


Fig. 1 Immunohistochemical reactivity for the ATRX protein. **a** Oligoastrocytoma with intermingled oligodendroglial and astrocytic cells, H&E, 200×. **b** Oligodendroglioma, ATRX-positive nuclei, DAB, 400×. **c** *Id*, pericapillary crowding of IDH1^{R132H}-positive tumor oligodendrocytes, DAB, 200×. **d** Gemistocytic astrocytoma, IDH1^{R132H}-positive tumor astrocytes, DAB, 400×. **e** *Id*, ATRX-negative tumor astrocytes, DAB, 400×. **f** *Id*, Iba-1-positive microglia/macrophage nuclei, DAB, 200×. **g** *Id*, ATRX-negative and GFAP-positive

tumor astrocytes, double immunohistochemistry (IHC) with DAB and *Alkaline Phosphatase Red*, respectively, 400×. **h** Oligodendroglioma, reactive astrocytes with GFAP-positive thick cytoplasm and long processes and ATRX-positive nuclei, double IHC with *Alkaline Phosphatase Red* and DAB, respectively, 400×. **i** *Id*, IDH1^{R132H}-positive perineuronal satellites, DAB, 400×. **j** *Id*, ATRX-positive perineuronal satellites, DAB, 400×. DAB, 3,3'-Diaminobenzidine

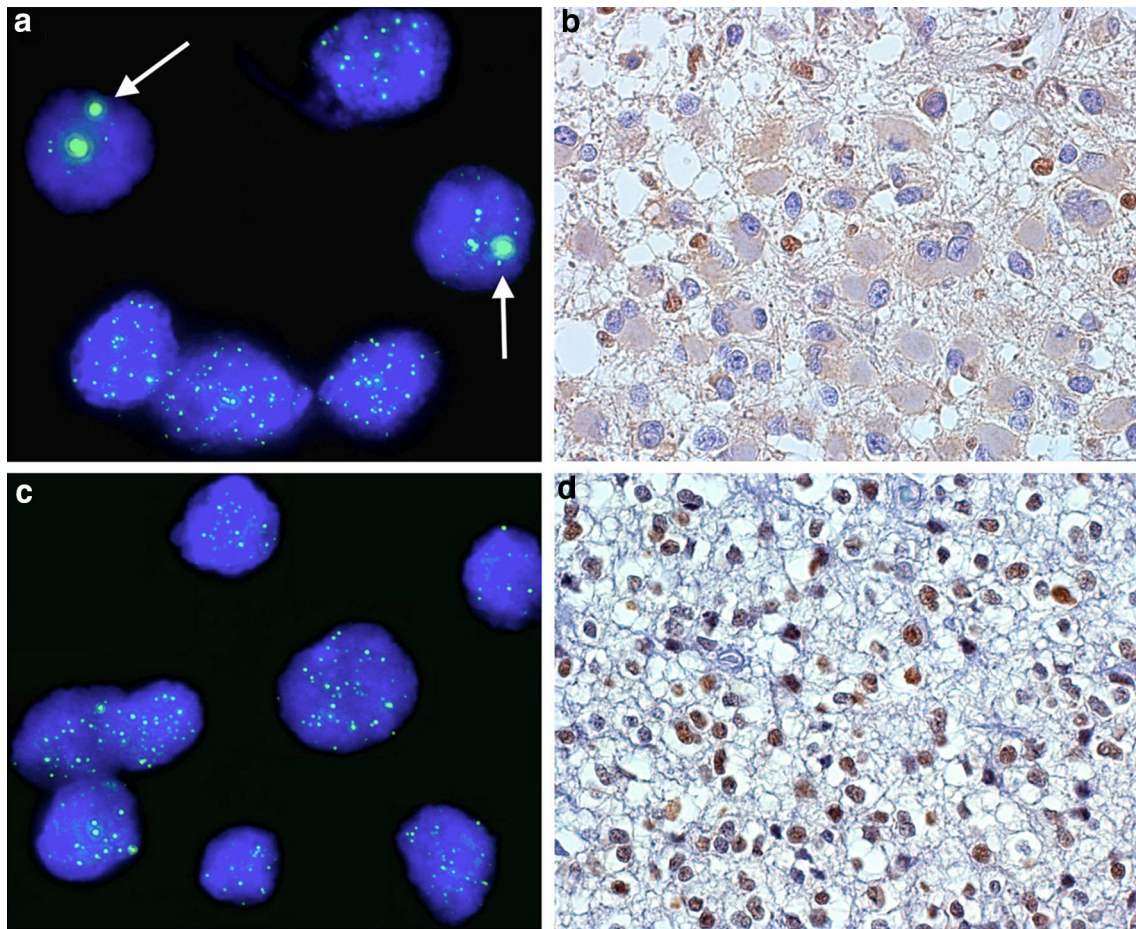


Fig. 2 ALT assessment in glioma specimens by telomere-specific FISH analysis. **a** Astrocytoma showing the ALT phenotype as indicated by large and ultra-bright telomere FISH signals (white arrows), original magnification 100 \times . **b** *Id*, Loss of immunohistochemical reactivity for the ATRX protein in astrocytic tumor nuclei, DAB,

400 \times . **c** Oligodendroglioma, absence of the ALT phenotype, original magnification 100 \times . **d** *Id*, Retained ATRX protein expression in oligodendroglial tumor nuclei, DAB, 400 \times . DAB, 3,3'-Diaminobenzidine

In the tumor subgroup of OA (Fig. 4), the initial diagnosis changed in 35 of 40 (87.5%) cases: 22/40 (55%) were reclassified as astrocytoma due to the absence of a total 1p/19q co-deletion, occurrence of *IDH1/2* mutations and loss of ATRX expression in GFAP-positive phenotypically looking tumor astrocytes; 11/40 (27.5%) were reclassified as oligodendroglioma due to retained ATRX expression in all cases and *IDH1/2* mutations in seven of them. Total 1p/19q co-deletion was detected in 1/11 (9.1%) cases whereas partial 1p or 19q deletions in 6/11 (54.5%) cases; three cases showed intact 1p/19q and one was unsuitable. In 2/40 (5%) cases, both wild type for *IDH1/2* genes, the initial diagnosis changed into reactive gliosis due to retained ATRX expression in GFAP-positive reactive astrocytes and to the lack of ATRX-positive tumor oligodendrocytes. Among the five remaining cases (12.5%), in only one with partial 1p/19q co-deletion, ATRX-negative and GFAP-positive astrocytes coexisted with a number of ATRX-positive

and GFAP-negative oligodendrocytes; both cell components showed *IDH1*^{R132H} immunopositivity by the double immunostaining for ATRX/*IDH1*^{R132H}. The diagnosis of OA in this case was not excluded. In two *IDH1/2* mutant cases, one with partial 1p/19q co-deletion and the other with intact 1p/19q, the diagnosis of OA could not be maintained because of the technical impossibility to perform *IDH1*^{R132H} IHC. One *IDH1/2* wild type case with partial 1p/19q co-deletion and heterogeneous ATRX expression was regarded as ambiguous since the tumor nature of the two cell components could not be ascertained. The last one remained unclassifiable due to the lack of molecular data.

In the GBM tumor subgroup, the initial diagnosis was confirmed in all cases whereas GBMO were reclassified as pGBMs due to the absence of a total 1p/19q co-deletion and of *IDH1/2* mutations.

Pilocytic astrocytomas showed a peculiar molecular profile with ATRX retention and wild type *IDH1/2* genes.

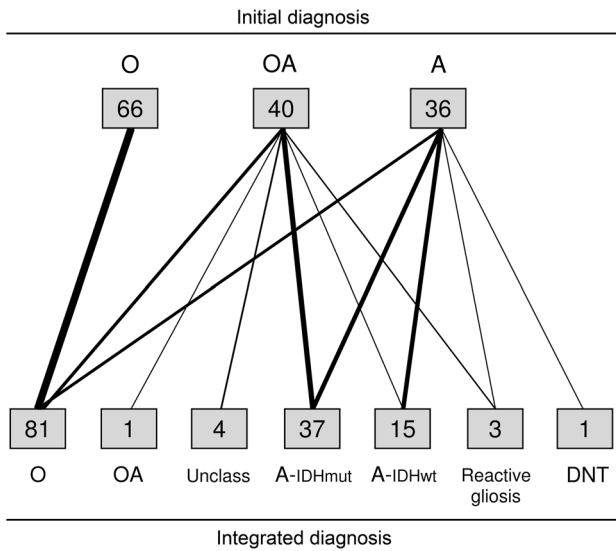


Fig. 3 Changes from the initial histologic diagnosis to the integrated diagnosis in 142 low grade astrocytic and oligodendroglial gliomas. Width of bars indicates relative proportions of the diagnostic changes into the different tumor categories. A astrocytoma, OA oligoastrocytoma, O oligodendroglioma, IDH isocitrate dehydrogenase, DNT dysembryoplastic neuroepithelial tumor, Unclass unclassifiable

Discussion

The change of tumor category from the initial histologic diagnosis to the molecular diagnosis mainly depends on the criteria used in the initial tumor definition. The integrated diagnosis demonstrates that molecular genetics greatly improves the differential diagnosis between oligodendroglial and astrocytic gliomas.

In this regard, the immunohistochemical ATRX expression, as surrogate for the ATRX mutation status, is of paramount importance. In the present study, we demonstrate that the retention of ATRX expression in all oligodendroglial tumor nuclei strongly supports the diagnosis of oligodendroglioma. Conversely, its loss in tumor astrocytic nuclei favors the diagnosis of astrocytoma. As a matter of fact, ATRX protein loss occurs in 92% of diffuse astrocytomas and in 41.7% of anaplastic astrocytomas. The lower frequency in the latter is in line with the literature, reporting loss of ATRX expression in 45–89% of anaplastic astrocytomas [13–16, 23].

In our series, the initial histologic diagnosis was confirmed in all oligodendroglial tumors and in 83.3% of astrocytic low grade gliomas. Eleven per cent of the latter were reclassified as oligodendroglioma due to a retained ATRX expression in tumor nuclei associated to IDH1/2 mutations and total 1p/19q co-deletion. The remaining cases were reclassified as DNT or reactive gliosis. In contrast, in the tumor subgroup of OA, most tumors (87.5%) were reclassified into different tumor categories. Fifty-five

per cent of them were reclassified as astrocytoma and 27.5% as oligodendroglioma based on the ATRX protein expression and molecular data for IDH1/2 mutation and 1p/19q chromosomal status. Five per cent of the cases were reclassified as reactive gliosis since the reactive nature has been demonstrated. For one case only, the diagnosis of OA could not be excluded due to the demonstration of both ATRX-positive tumor oligodendrocytes and ATRX-negative tumor astrocytes in mixed tumor areas. Our findings are in line with the literature [17, 22, 23] and support the disappearance of OA as a separate tumor entity. However, rare instances of clearly biphasic OA exhibiting morphologic and molecular heterogeneity, including heterogeneous ATRX immunoreactivity, have been described [29, 30]. These findings indicate that tumor cells could differentiate in oligodendroglial and astrocytic lineage or, alternatively, arise from progenitors before the differentiation in the two lineages.

The distinction between reactive or tumor astrocytes has always been a crucial point in the diagnosis of OA. Reactive astrocytes occur in almost all gliomas and are difficult to be identified since their number, size and process production are variable. After the discovery of ATRX mutations, reactive astrocytes can be unequivocally distinguished from tumor astrocytes by the double immunostaining for ATRX/GFAP, due to their nuclear ATRX retention. In presence of a IDH1 c.395G > A (p.R132H) mutation, the IDH1^{R132H} immunoreactivity contributes to reveal the tumor nature of the cells. However, in the 10–30% of diffuse astrocytomas without IDH1/2 mutations, tumor astrocytes may remain undetectable.

Three critical points must be emphasized: (1) IDH1/2 mutations occur at undifferentiated progenitors in 70–90% of low grade gliomas [31–33], with a higher mutation rate in oligodendroglomas than in astrocytomas [26, 27]; (2) total 1p/19q co-deletion and ATRX retention characterizes oligodendroglioma excluding astrocytoma [34, 35]; (3) ATRX mutations/ATRX protein loss occur at the astrocytic progenitors and, therefore, when associated to TP53 mutations, are the genetic alterations supporting the diagnosis of astrocytoma and sGBM [36].

In our series, a total 1p/19q co-deletion was detected in only 25/54 (46.3%) oligodendroglial tumors (classified initially on the typical morphology of “honeycomb” appearance and “chicken wire” vessel distribution); on the other hand, partial 1p and/or 19q deletions were present in 21/54 (38.9%) of them and in 11/20 (55%) astrocytic tumors. It is widely accepted, therefore, that partial deletions may also occur in astrocytic gliomas in relation to malignant transformation [37–39]. It remains to be clarified how tumors with classic oligodendroglial phenotype and lacking in total 1p/19q co-deletion might be classified. This is known to be more frequent in pediatric oligodendroglial tumors

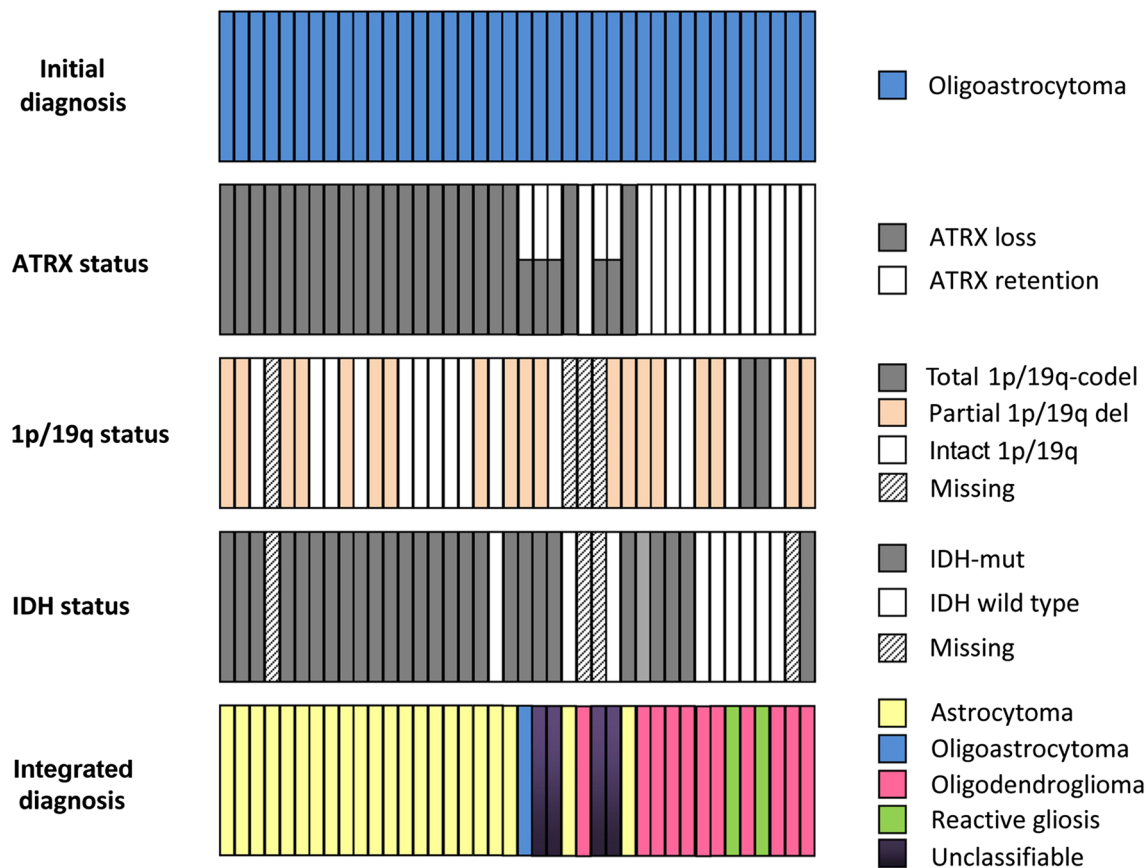


Fig. 4 Overview of the genetic aberrations in the initial series of 40 oligoastrocytomas. Each column corresponds to a single case. Change of tumor category from the initial to the integrated diagnosis

with regard to the immunohistochemical reactivity for the ATRX protein, *IDH1/2* mutation status and 1p/19q chromosomal status

[40–42]. However, in our series, the average patient age for tumors with intact 1p/19q, irrespective of the *IDH1/2* mutation status, was 48 years (range 33–56 years). The problem is even complicated by the possibility to find a false “honeycomb” appearance mimicking the perinuclear “halo” of oligodendroglial tumors containing ATRX-positive nuclei. This morphologic aspect could recognize other sources due to either microglia/macrophage contamination or a water disturb/edema of unspecified nature, as it may happen, for instance, in pilocytic astrocytoma [43].

The occurrence of minigemistocytes, GFOC or even of real gemistocytes could be misleading in the differential diagnosis between oligodendroglioma and astrocytoma, especially when in large number. However, their nuclear ATRX immunoreactivity resolves the problem.

The distinction between pGBMs and sGBMs is preserved because it coincides with the *IDH1/2* mutation status. Primary GBMs and gliosarcomas retain ATRX expression, demonstrating their origin from earlier glial progenitor cells or stem cells and not from the astrocytic lineage. In contrast, sGBMs follow the ATRX immunohistochemical

pattern observed in diffuse astrocytomas. GBMO, with intact 1p/19q and retained nuclear ATRX expression, would disappear. It remains to be reconsidered the significance of partial 1p/19q deletions in gliomas.

The retained nuclear ATRX expression in most pilocytic astrocytomas demonstrates their origin from a precursor preceding the separation of the astrocytic and oligodendroglial lineages. As a matter of fact, they also carry wild type *IDH1/2* genes. ATRX mutations should, therefore, hit the astrocytic lineage after its origin from a common ancestor.

Astrocytic tumors with loss of nuclear ATRX protein expression and *IDH1* c.395G > A (p.R132H) mutation/*IDH1*^{R132H} immunoreactivity exhibit the ALT phenotype for the telomere maintenance. Our data are in agreement with previous ones [11–15, 21, 44].

Finally, a crucial point is the great influence of microglia/macrophages on the recognition of tumor oligodendrocytes since they constitutively express ATRX in their nuclei. Microglia/macrophages can be extremely frequent in diffuse gliomas; when abundant, their ATRX immunoreactivity could be assigned to oligodendroglial tumor

cells and lead to a misleading diagnosis between oligodendroglioma and astrocytoma. In order to recognize an oligodendroglial tumor infiltration, which often results from a biopsy, the double immunostainings for ATRX/Iba-1 and ATRX/CD68 are mandatory to recognize the occurrence of ATRX-positive and Iba-1/CD68-negative tumor oligodendroglial nuclei (personal data). ATRX-negative nuclei in infiltration areas correspond to normal oligodendrocytes that can be revealed by the double immunostaining for ATRX/Iba-1, beside their Cyclin D1 expression. In IDH1^{R132H} mutant cases, the double immunostaining for ATRX/IDH1^{R132H} unequivocally identifies oligodendroglial tumor cells. The coexistence of normal and tumor oligodendrocytes can be demonstrated through the ATRX IHC in perineuronal satellitosis: in its initial phases, ATRX-positive and ATRX-negative nuclei can be found around neurons; in later phases, all nuclei retain ATRX immunopositivity. Perineuronal satellitosis can be definitely considered as due to tumor oligodendrocytes arising from the white matter and disposing around neurons rather than to a tumor transformation of normal satellites.

The relationship of the ATRX status with the patient overall survival or time to treatment failure in astrocytic gliomas could not be established due to the low number of IDH1/2 mutant cases with retained ATRX expression.

In the last years, molecular genetics greatly contributed to refine histopathology confirming to be an essential tool for the diagnosis of gliomas [45]. A typical example is the almost disappearance of OA practically based on the ATRX mutation status.

Acknowledgments Marta Mazzucco was supported by a fellowship from Fondazione Cassa di Risparmio di Vercelli (Vercelli, Italy).

Compliance with ethical standards

Conflict of interest Authors declare no potential conflict of interest.

References

- Louis DN, Ohgaki H, Wiestler OD, Cavenee WK (2007) WHO classification of tumours of the central nervous system, 4th edn. IARC Press, Lyon, pp 1–309
- Coons SW, Johnson PC, Scheithauer BW, Yates AJ, Pearl DK (1997) Improving diagnostic accuracy and interobserver concordance in the classification and grading of primary gliomas. *Cancer* 79:1381–1393
- Krouwer HG, van Duinen SG, Kamphorst W, van der Valk P, Algra A (1997) Oligoastrocytomas: a clinicopathological study of 52 cases. *J Neurooncol* 33:223–238
- Cooper ERA (1935) The relation of oligocytes and astrocytes in cerebral tumours. *J Pathol* 41:259–266
- Hart MN, Petito CK, Earle KM (1974) Mixed gliomas. *Cancer* 33:134–140
- Daumas-Duport C, Varlet P, Tucker ML, Beuvon F, Cervera P et al (1997) Oligodendrogliomas. Part I: patterns of growth, histological diagnosis, clinical and imaging correlations: a study of 153 cases. *J Neurooncol* 34:37–59
- de Armond SJ, Eng LF, Rubinstein LJ (1980) The application of glial fibrillary acidic (GFA) protein immunohistochemistry in neurooncology. A progress report. *Pathol Res Pract* 168:374–394
- van der Meulen JD, Houthoff HJ, Ebels EJ (1978) Glial fibrillary acidic protein in human gliomas. *Neuropathol Appl Neurobiol* 4:177–190
- Herpers MJ, Budka H (1984) Glial fibrillary acidic protein (GFAP) in oligodendroglial tumors: gliofibrillary oligodendroglioma and transitional oligoastrocytoma as subtypes of oligodendroglioma. *Acta Neuropathol* 64:265–272
- Choi BH, Kim RC (1984) Expression of glial fibrillary acidic protein in immature oligodendroglia. *Science* 223:407–409
- Heaphy CM, de Wilde RF, Jiao Y, Klein AP, Edil BH et al (2011) Altered telomeres in tumors with ATRX and DAXX mutations. *Science* 333:425. doi:10.1126/science.1207313
- Kannan K, Inagaki A, Silber J, Gorovets D, Zhang J et al (2012) Whole-exome sequencing identifies ATRX mutation as a key molecular determinant in lower-grade glioma. *Oncotarget* 3:1194–1203
- Jiao Y, Killela PJ, Reitman ZJ, Rasheed AB, Heaphy CM et al (2012) Frequent ATRX, CIC, FUBP1 and IDH1 mutations refine the classification of malignant gliomas. *Oncotarget* 3:709–722
- Liu XY, Gerges N, Korshunov A, Sabha N, Khuong-Quang DA et al (2012) Frequent ATRX mutations and loss of expression in adult diffuse astrocytic tumors carrying IDH1/IDH2 and TP53 mutations. *Acta Neuropathol* 124:615–625. doi:10.1007/s00401-012-1031-3
- Wiestler B, Capper D, Holland-Letz T, Korshunov A, von Deimling A et al (2013) ATRX loss refines the classification of anaplastic gliomas and identifies a subgroup of IDH mutant astrocytic tumors with better prognosis. *Acta Neuropathol* 126:443–451. doi:10.1007/s00401-013-1156-z
- Cryan JB, Haidar S, Ramkissoon LA, Bi WL, Knoff DS et al (2014) Clinical multiplexed exome sequencing distinguishes adult oligodendroglial neoplasms from astrocytic and mixed lineage gliomas. *Oncotarget* 5:8083–8092
- Leeper HE, Caron AA, Decker PA, Jenkins RB, Lachance DH et al (2015) IDH mutation, 1p19q codeletion and ATRX loss in WHO grade II gliomas. *Oncotarget* 6:30295–30305. doi:10.18632/oncotarget.4497
- Clynes D, Higgs DR, Gibbons RJ (2013) The chromatin remodeler ATRX: a repeat offender in human disease. *Trends Biochem Sci* 38:461–466. doi:10.1016/j.tibs.2013.06.011
- Goldberg AD, Banaszynski LA, Noh KM, Lewis PW, Elsaesser SJ et al (2010) Distinct factors control histone variant H3.3 localization at specific genomic regions. *Cell* 140:678–691. doi:10.1016/j.cell.2010.01.003
- Lewis PW, Elsaesser SJ, Noh KM, Stadler SC, Allis CD (2010) Daxx is an H3.3-specific histone chaperone and cooperates with ATRX in replication-independent chromatin assembly at telomeres. *Proc Natl Acad Sci USA* 107:14075–14080. doi:10.1073/pnas.1008850107
- Nguyen DN, Heaphy CM, de Wilde RF, Orr BA, Odia Y et al (2013) Molecular and morphologic correlates of the alternative lengthening of telomeres phenotype in high-grade astrocytomas. *Brain Pathol* 23:237–243. doi:10.1111/j.1750-3639.2012.00630
- Sahm F, Reuss D, Koelsche C, Capper D, Schittenhelm J et al (2014) Farewell to oligoastrocytoma: in situ molecular genetics favor classification as either oligodendroglioma or astrocytoma. *Acta Neuropathol* 128:551–559. doi:10.1007/s00401-014-1326-7
- Reuss DE, Sahm F, Schrimpf D, Wiestler B, Capper D et al (2015) ATRX and IDH1-R132H immunohistochemistry with subsequent copy number analysis and IDH sequencing as a

- basis for an “integrated” diagnostic approach for adult astrocytoma, oligodendroglioma and glioblastoma. *Acta Neuropathol* 129:133–146. doi:[10.1007/s00401-014-1370-3](https://doi.org/10.1007/s00401-014-1370-3)
24. Louis DN, Perry A, Burger P, Ellison DW, Reifenberger G et al (2014) International Society Of Neuropathology-Haarlem consensus guidelines for nervous system tumor classification and grading. *Brain Pathol* 24:429–435. doi:[10.1111/bpa.12171](https://doi.org/10.1111/bpa.12171)
 25. Weller M, Berger H, Hartmann C, Schramm J, Westphal M et al (2007) Combined 1p/19q loss in oligodendroglial tumors: predictive or prognostic biomarker? *Clin Cancer Res* 13:6933–6937
 26. Mellai M, Piazzi A, Caldera V, Monzeglio O, Cassoni P et al (2011) IDH1 and IDH2 mutations, immunohistochemistry and associations in a series of brain tumors. *J Neurooncol* 105:345–357. doi:[10.1007/s11060-011-0596-3](https://doi.org/10.1007/s11060-011-0596-3)
 27. Mellai M, Caldera V, Annovazzi L, Schiffer D (2013) The distribution and significance of IDH mutation in gliomas. In: Lichter T (ed) *Evolution of the molecular biology of brain tumors and therapeutic implications*. Rijeka, Croatia, pp. 299–342
 28. Bosone I, Cavalla P, Chiadò-Piat L, Vito ND, Schiffer D (2001) Cyclin D1 expression in normal oligodendroglia and microglia cells: its use in the differential diagnosis of oligodendrogliomas. *Neuropathology* 21:155–161
 29. Wilcox P, Li CC, Lee M, Shivalingam B, Brennan J et al (2015) Oligoastrocytomas: throwing the baby out with the bathwater? *Acta Neuropathol* 129:147–149. doi:[10.1007/s00401-014-1353-4](https://doi.org/10.1007/s00401-014-1353-4)
 30. Huse JT, Diamond EL, Wang L, Rosenblum MK (2015) Mixed glioma with molecular features of composite oligodendroglioma and astrocytoma: a true “oligoastrocytoma”? *Acta Neuropathol* 129:151–153. doi:[10.1007/s00401-014-1359-y](https://doi.org/10.1007/s00401-014-1359-y)
 31. Sanson M, Marie Y, Paris S, Idhah A, Laffaire J et al (2009) Isocitrate dehydrogenase 1 codon 132 mutation is an important prognostic biomarker in gliomas. *J Clin Oncol* 27:4150–4154. doi:[10.1200/JCO.2009.21.9832](https://doi.org/10.1200/JCO.2009.21.9832)
 32. Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA et al (2009) IDH1 and IDH2 mutations in gliomas. *N Engl J Med* 360:765–773. doi:[10.1056/NEJMoa0808710](https://doi.org/10.1056/NEJMoa0808710)
 33. Hartmann C, Meyer J, Balss J, Capper D, Mueller W et al (2009) Type and frequency of IDH1 and IDH2 mutations are related to astrocytic and oligodendroglial differentiation and age: a study of 1,010 diffuse gliomas. *Acta Neuropathol* 118:469–474. doi:[10.1007/s00401-009-0561-9](https://doi.org/10.1007/s00401-009-0561-9)
 34. Reifenberger J, Reifenberger G, Liu L, James CD, Wechsler W et al (1994) Molecular genetic analysis of oligodendroglial tumors shows preferential allelic deletions on 19q and 1p. *Am J Pathol* 145:1175–1190
 35. Cairncross JG, Ueki K, Zlatescu MC, Lisle DK, Finkelstein DM et al (1998) Specific genetic predictors of chemotherapeutic response and survival in patients with anaplastic oligodendrogliomas. *J Natl Cancer Inst* 90:1473–1479
 36. Thon N, Eigenbrod S, Kreth S, Lutz J, Tonn JC et al (2012) IDH1 mutations in grade II astrocytomas are associated with unfavorable progression-free survival and prolonged postrecurrence survival. *Cancer* 118:452–460. doi:[10.1002/ncr.26298](https://doi.org/10.1002/ncr.26298)
 37. von Deimling A, Bender B, Jahnke R, Waha A, Kraus J et al (1994) Loci associated with malignant progression in astrocytomas: a candidate on chromosome 19q. *Cancer Res* 54:1397–1401
 38. Hartmann C, Johnk L, Kitange G, Wu Y, Ashworth LK et al (2002) Transcript map of the 3.7-Mb D19S112-D19S246 candidate tumor suppressor region on the long arm of chromosome 19. *Cancer Res* 62:4100–4108
 39. Barbashina V, Salazar P, Holland EC, Rosenblum MK, Ladanyi M (2005) Allelic losses at 1p36 and 19q13 in gliomas: correlation with histologic classification, definition of a 150-kb minimal deleted region on 1p36, and evaluation of CAMTA1 as a candidate tumor suppressor gene. *Clin Cancer Res* 11:1119–1128
 40. Raghavan R, Balani J, Perry A, Margraf L, Vono MB et al (2003) Pediatric oligodendrogliomas: a study of molecular alterations on 1p and 19q using fluorescence in situ hybridization. *J Neuro-pathol Exp Neurol* 62:530–537
 41. Kreiger PA, Okada Y, Simon S, Rorke LB, Louis DN et al (2005) Losses of chromosomes 1p and 19q are rare in pediatric oligodendrogliomas. *Acta Neuropathol* 109:387–392
 42. Rodriguez FJ, Tihan T, Lin D, McDonald W, Nigro J et al (2014) Clinicopathologic features of pediatric oligodendrogliomas: a series of 50 patients. *Am J Surg Pathol* 38:1058–1070. doi:[10.1097/PAS.0000000000000221](https://doi.org/10.1097/PAS.0000000000000221)
 43. Prayson RA (2016) Oligodendroglioma look-alikes: the histopathologic differential diagnosis. In: Reeves C (ed) *Oligodendrogliomas*. Nova Science Publishers, Hauppauge, pp 83–118
 44. Sturm D, Bender S, Jones DT, Lichter P, Grill J et al (2014) Paediatric and adult glioblastoma: multifactorial (epi)genomic culprits emerge. *Nat Rev Cancer* 14:92–107. doi:[10.1038/nrc3655](https://doi.org/10.1038/nrc3655)
 45. Foote MB, Papadopoulos N, Diaz LA Jr (2015) Genetic classification of gliomas: refining histopathology. *Cancer Cell* 28:9–11. doi:[10.1016/j.ccell.2015.06.014](https://doi.org/10.1016/j.ccell.2015.06.014)