

Farewell to oligoastrocytoma: in situ molecular genetics favor classification as either oligodendroglioma or astrocytoma

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Received: 29 April 2014 / Revised: 23 July 2014 / Accepted: 23 July 2014 / Published online: 21 August 2014
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Abstract Astrocytoma and oligodendroglioma are histologically and genetically well-defined entities. The majority of astrocytomas harbor concurrent *TP53* and *ATRX* mutations, while most oligodendrogliomas carry the 1p/19q co-deletion. Both entities share high frequencies of *IDH* mutations. In contrast, oligoastrocytomas (OA) appear less clearly defined and, therefore, there is an ongoing debate whether these tumors indeed constitute an entity or whether they represent a mixed bag containing both astrocytomas and oligodendrogliomas. We investigated 43 OA diagnosed

in different institutions employing histology, immunohistochemistry and in situ hybridization addressing surrogates for the molecular genetic markers *IDH1*R132H, *TP53*, *ATRX* and 1p/19q loss. In all but one OA the combination of nuclear p53 accumulation and *ATRX* loss was mutually exclusive with 1p/19q co-deletion. In 31/43 OA, only alterations typical for oligodendroglioma were observed, while in 11/43 OA, only indicators for mutations typical for astrocytomas were detected. A single case exhibited a distinct pattern, nuclear expression of p53, *ATRX* loss, *IDH1* mutation and partial 1p/19q loss. However, this was the only patient undergoing radiotherapy prior to surgery, possibly contributing to the acquisition of this uncommon combination. In

Electronic supplementary material The online version of this article (doi:10.1007/s00401-014-1326-7) contains supplementary material, which is available to authorized users.

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OA with oligodendroglioma typical alterations, the portions corresponding to astrocytic part were determined as reactive, while in OA with astrocytoma typical alterations the portions corresponding to oligodendroglial differentiation were neoplastic. These data provide strong evidence against the existence of an independent OA entity.

Keywords Mixed glioma · Oligoastrocytoma · 1p/19q · ATRX · TP53 · IDH1

Introduction

According to the World Health Organization (WHO) classification of central nervous system tumors diffuse astrocytomas of grades II and III share infiltrative growth with astrocytic differentiation and the most frequent molecular alteration in these tumors is *TP53* mutation which is observed in 60–70 % [25]. More recent findings include mutations in the *isocitrate-dehydrogenases 1* and *2* (*IDH1*;*IDH2*) as well as in the *alpha-thalassemialmental retardation syndrome X-linked* gene (*ATRX*) in the majority of the cases [1, 16, 18, 19, 29, 35, 46]. Oligodendrogliomas of grades II and III share a typical morphological fried egg pattern and combined losses of chromosomal arms 1p and 19q. Likewise, recent studies reported a high frequency of *IDH1* and *IDH2* mutations of about 80–88 % also in oligodendroglioma [16, 46]. The WHO classification also recognizes the tumor entity oligoastrocytoma (OA) also termed mixed gliomas of grades II and III. However, this tumor entity is poorly defined merely by the presence of both tumor cells with astrocytic and oligodendroglial differentiation. No guidelines are given regarding the minimal percentages of either part required for the diagnosis of OA. This is good practice in light of modern surgical procedures applying aspiration and coagulation, thus resulting in submission of only a minor tumor fraction for neuropathological examination. Further aggravation to the diagnostician derives from the diversion of material to biobanks. In consequence, the pathologist is left clueless on the representative nature of the material finally submitted. Therefore, it is not surprising to see the diagnosis of OA made with extraordinary varying frequencies in different institutions [10, 14, 30].

Originally, the term OA was coined in 1935 by Eugenia Cooper in the seminal work “The relation of oligocytes and astrocytes in cerebral tumors” [11]. The analysis describes for the first time mixed gliomas with astrocytic and oligodendroglial cells either intermingled or regionally

separated. Intriguingly, the author already considered whether the astrocytic component might be purely reactive. In 1974, Hart and colleagues expanded on this study in their work “Mixed Gliomas” [15], coining the different distributions of cell compartments as of the diffuse and of the compact type. Cooper’s consideration that astrocytic portions could also be reactive has later on been expanded in the classification system by Daumas–Duport, culminating in the hypothesis that all diffuse gliomas represent oligodendrogliomas with reactive astrocytosis [12].

Molecular genetic analyses early on casted doubt on the entity of OA. Analyses of small series of OA detected 1p/19q losses in neither or both astrocytic and oligodendroglial portions. Further, OA without 1p/19q loss frequently harbored *TP53* mutations again in both tumor portions [27]. Another study addressing 1p/19q status and other molecular aberrations by in situ hybridization supported the finding that the majority of cells in mixed gliomas homogeneously share aberrations and concluded that polyclonal origin is rather unlikely. However, the authors state that this does not abrogate the possibility that the cells might cytologically differentiate along either astrocytic or oligodendroglial lineage [14].

Moreover, emergent clinical data contribute to the debate on the existence of OA: recent results from clinical studies demonstrated the overwhelming importance of 1p/19q loss for therapy prediction and prognosis which was independent of the histological diagnoses of OA and oligodendroglioma [5, 39, 41]. These findings obviously reduce the relevance for the morphological distinction of the entity OA.

Recent developments in neuropathology now provide tools to resolve this discrepancy. The major genetic hallmarks for oligodendroglioma and astrocytoma can nowadays be identified in tissue sections on the single-cell level. This includes H09 staining demonstrating the *IDH1*R132H mutation [8, 9], p53 upregulation associated with *TP53* mutation [24, 26, 33], loss of ATRX expression indicating *ATRX* mutation [23] and in situ hybridization for 1p/19q providing information on chromosome copy numbers.

To systematically address the question toward the existence of OA molecularly distinct from both, oligodendroglioma or astrocytoma, we collected a series of 43 tumors diagnosed at different institutions and assessed the hallmark genetic aberrations in tissue section in the context with morphology.

Materials and methods

Tissue

Formalin-fixed paraffin-embedded tissue of 43 oligoastrocytomas diagnosed between 1989 and 2013 was obtained

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from the Departments and Institutes of Neuropathology Muenster (12 cases), Tuebingen (11 cases) and Heidelberg (20 cases) in accordance with local ethical approval. All cases were centrally reviewed at the Dept. of Neuropathology Heidelberg. For inclusion the cases had to meet two criteria: presence of IDH1R132H mutation allowing for the unequivocal identification of tumor cells and clearly demarcated areas of oligodendroglial and astrocytic differentiation, or intermingled histology, respectively, of at least 1 cm².

Immunohistochemistry

Sections cut to 3 µm were incubated and processed on a Ventana BenchMark XT[®] immunostainer (Ventana Medical Systems, Tucson, AZ, USA). Antibodies were anti-human IDH1R132H (H09, Dianova, Hamburg, Germany), anti-human ATRX (1:400, Sigma, HPA001906), and anti-human p53 (1:50, Novocastra, DO-1). The Ventana staining procedure included pretreatment with cell conditioner 2 (pH 6) for 60 min or cell conditioner 1 (pH 8) for 60 min for Ki67, ATRX and p53, respectively, followed by incubation with primary antibody at 37 °C for 32 min. Incubation was followed by Ventana standard signal amplification, UltraWash, counterstaining with one drop of hematoxylin for 4 min and one drop of bluing reagent for 4 min. For visualization, ultraView[™] Universal DAB Detection Kit (Ventana Medical Systems) was used.

IDH1R132H and ATRX staining were evaluated as previously described [7, 42]. Staining of >5 % of nuclei for p53 was considered “positive”. IDH1R132H, p53 and ATRX were evaluated separately for areas with rather astrocytic and rather oligodendroglial morphology as determined on corresponding H&E sections.

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) analysis was performed on FFPE tissue as previously described [21]. In brief, tissue was cut at 5 mm. Tissue preparation for dual-probe hybridization was facilitated by ZytoLight FISH-tissue implementation kit according to manufacturers' instructions (ZytoVision, Bremerhaven, Germany). Dual-color probes, ZytoLight SPEC 1p36/1q25 and ZytoLight SPEC 19q13/19p13, were used for locus-specific 1p and 19q analysis, respectively, following manufacturers' instructions (ZytoVision). Nuclei were counterstained with 4,6-diamidino-2 phenylindole (DAPI).

Copy number profiling

The Illumina Infinium HumanMethylation450 (450 k) array was used to obtain the DNA methylation status of

482,421 CpG sites (Illumina, San Diego, USA) according to the manufacturer's instructions at the Core Facility of the DKFZ. The array data were used to calculate a low-resolution copy number profile as previously described [37].

Sequencing

Fragments spanning all 37 exons of *ATRX* and exons 5–8 of *TP53* were amplified using 20 ng each of the respective forward and reverse primer. Primer design was based on accession number NM_000489.4 and NM_000546.5, respectively, as previously described [34]. For PCR, 100 ng of DNA and HotStar 2× PCR Master Mix (Qiagen, Hilden, Germany) were employed. PCR was performed in a total volume of 30 µl, and included initial denaturation at 95 °C for 180 s, followed by 35 cycles with denaturation at 95 °C for 30 s, annealing at 56 °C for 25 s and extension at 72 °C for 40 s. Two microliters of the amplification product were submitted to bidirectional sequencing using the BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequences were determined using a ABI 3500 Genetic Analyzer (Applied Biosystems) and the Sequence Pilot version 3.1 (JSI-Medisys, Kippenheim, Germany) software.

Results and discussion

The presence of cytoplasmic staining with H09 for IDH1R132H, with intense nuclear staining of DO-1 for p53, with loss of HPA001906 binding for ATRX or with FISH status demonstrating combined 1p/19q loss, was taken as evidence for the neoplastic nature of the respective cells. Based on the wide and generally accepted body of literature, 1p/19q loss was taken as molecular evidence for an oligodendroglial, while ATRX loss and p53 accumulation were taken as molecular evidence for astrocytic nature of the respective tumor cells. By selection bias all 43 OA in our series contained an *IDH1R132H* mutation allowing for the unequivocal identification of tumor cells. To support the criteria for neoplastic versus reactive nature beyond IDH1R132H staining in these 43 tumors, we also considered a neoplastic state for cells in the absence of IDH1R132H if the presence of one of the other indicators, i.e., nuclear p53 accumulation, ATRX loss or combined 1p/19q loss was demonstrated individually. This is also prompted by previous studies reporting cases with loss of mutant IDH protein in tumor cells or differing molecular status in microdissected tissue [22, 31]. However, we did not observe such a constellation in a single instance. This strongly supports the assumption of having identified all tumorous cells by identification of mutant IDH protein. In 43 OA, we identified 30 cases with IDH1R132H protein

Table 1 Distribution of molecular alterations in areas of astrocytic and oligodendroglial appearance

n	IDH1R132H		p53		ATRX		1p		19q	
	Astrocytic	Oligodendroglial	Astrocytic	Oligodendroglial	Astrocytic	Oligodendroglial	Astrocytic	Oligodendroglial	Astrocytic	Oligodendroglial
30 ^a	Neg	Pos	Neg	Neg	Pos	Pos	Ret	DEL	Ret	DEL
1 ^a	Pos	Pos	Neg	Neg	Pos	Pos	DEL	DEL	DEL	DEL
11 ^b	Pos	Pos	Pos	Pos	Neg	Neg	Ret	Ret	Ret	Ret
1 ^c	Pos	Pos	Pos	Pos	Neg	Neg	pDEL	pDEL	pDEL	pDEL

Initially, all tumors were diagnosed as oligoastrocytomas

neg Negative, *pos* positive, *ret* retained chromosomal arms, *DEL* deleted chromosomal arm, *pDEL* partially deleted chromosomal arm

Upon reevaluation ^a were classified as oligodendrogliomas and ^b as astrocytomas. ^c Is a recurrent and previously treated tumor with partial deletion of chromosomal arms 1p and 19q (ID 63214)

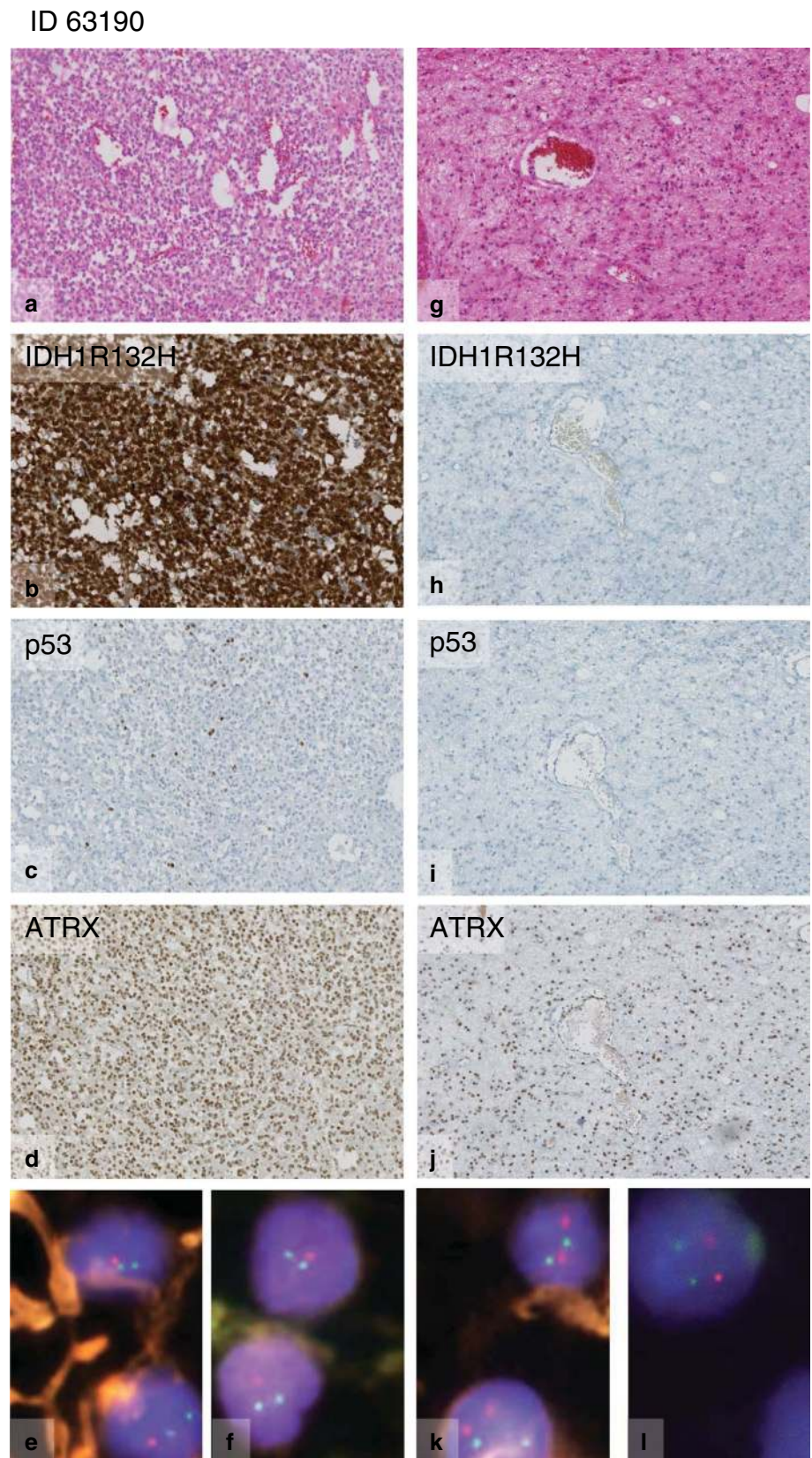
exclusively in cells with morphologically oligodendroglial differentiation. The tumor areas assumed to represent the astrocytic portion of OA in the initial diagnosis were entirely devoid of all, IDH1R132H expression, nuclear p53 accumulation, loss of ATRX expression and 1p/19q loss. In contrast, areas harboring mutant IDH1R132H protein exhibited 1p/19q loss. Thus, in 70 % of OA, the portion of astrocytic differentiation did not contain the molecular alterations assessed for and, therefore, was interpreted as reactive. In conclusion, all cells of unequivocal neoplastic nature carried mutations typical for oligodendroglioma, and, consequently, the tumors were allotted to the entity oligodendroglioma (Table 1). A representative case is shown in Fig. 1; additional examples are given in supplementary Figs. 1, 8 and 9.

The remaining cases were attributed to two groups characterized by one of the following constellations: (1) the four molecular parameters were identical in both tumor areas. (2) There were differences in the presence of the molecular alterations in both tumor areas.

Constellation (1) would strongly argue for a clonal origin of the entire tumor reducing the astrocytic and oligodendroglial differentiation to a merely morphological variation without a genetic basis. This was observed in 12 of the 13 remaining tumors. Interestingly, 11/12 tumors exhibited ATRX loss and p53 accumulation. In contrast, none of these 11 tumors carried 1p/19q loss. Thus, all tumor cells in these tumors carried the molecular fingerprints typical for astrocytoma (Table 1). A representative case is shown in Fig. 2; additional examples are given in supplementary Figs. 3, 4, 5 and 10. The single remaining of these 12 cases with homogeneous distribution of all markers in the entire lesion showed retained ATRX expression, no p53 accumulation but co-deletion of 1p/19q in both morphologically astrocytic and oligodendroglial compartments. Thus, this case was interpreted as oligodendroglioma (Suppl. Fig. 2). Constellation (2) was not observed in a single tumor of our series. Thus, we were not able to detect a single case with clear positive evidence for portions exhibiting molecular alterations typical for oligodendroglioma or astrocytoma in a mutually exclusive distribution.

The last case did not fit in one of the categories defined above. As in all the other tumors histological analysis revealed both tumor portions reminiscent of astrocytoma and oligodendroglioma. However, molecular analysis demonstrated the combination of 1p/19q loss in all, ATRX loss in all and nuclear p53 expression in the majority of tumor cells. Thus, this tumor posed as a “molecular hybrid” with genetic alterations typical for oligodendroglioma as well as astrocytoma. Morphological and molecular genetic findings are demonstrated in Fig. 3. However, this tumor was a recurrent lesion and the only tumor in our series known to previously have been irradiated. A history of irradiation

Fig. 1 Histology of oligoastrocytoma of the compact type. Areas of oligodendroglial (*left column, a–d*) and astrocytic (*right column, e–f*) differentiation. **a, e** HE $\times 100$; **b, f** expression of mutant IDH1R132H is confined to the oligodendroglial portion. **c, g** p53 upregulation only in single cells of oligodendroglial portion. **d, j** ATRX expression is retained. The oligodendroglial fraction revealed 1p/19q co-deletion (**e, f**) whereas the astrocytic portion had retained alleles (**k, l**)



ID 43056

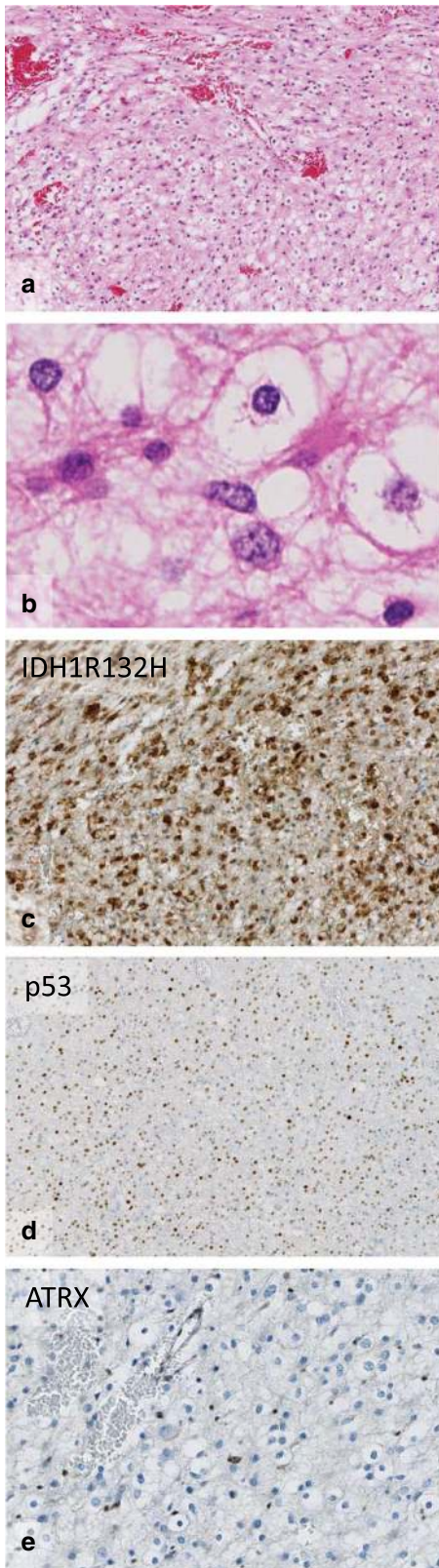
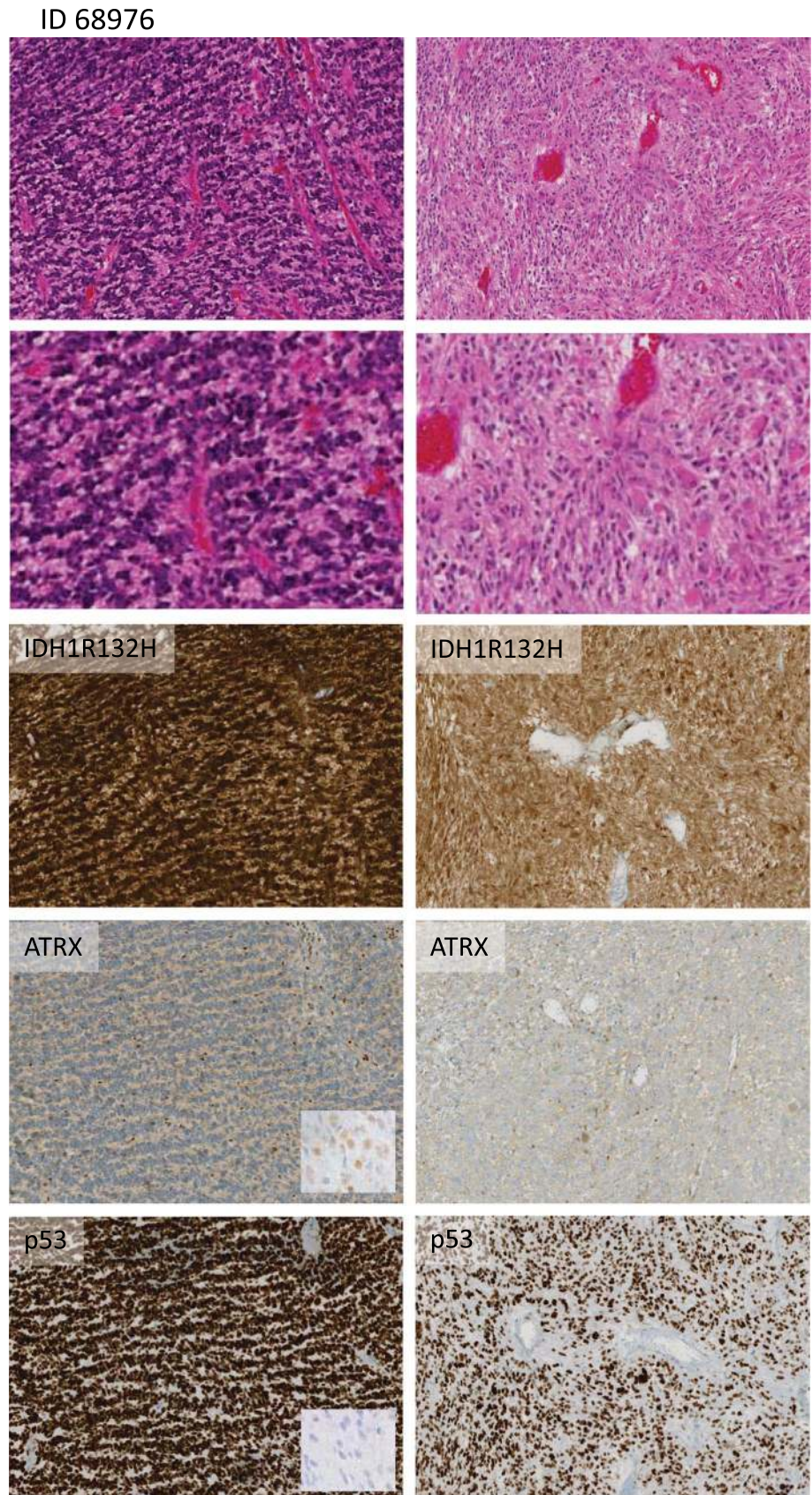


Fig. 2 Histology of oligoastrocytoma of the diffuse type. **a** HE $\times 100$, **b** HE $\times 600\times$; **c** expression of mutant IDH1R132H. **d** p53 upregulation in the majority of tumor cells. **e** ATRX expression is lost in the tumor cells. No 1p/19q co-deletion was detected (data not shown)

has been associated with nuclear p53 accumulation and may well explain this finding [17]. Moreover, reduction of ATRX expression due to such treatment has been reported in cell lines [2], but not documented in tissue. Indeed, assessment of the primary manifestation revealed retained ATRX expression and no p53 accumulation (insets Fig. 3). Since this was the only case in which the in situ evaluation of IDH1R132H, p53, ATRX and 1p/19q status did not unequivocally resolve the designation as astrocytoma or oligodendroglioma, we also performed Sanger sequencing for exons 5–8 of TP53 and all coding exons of ATRX. Neither TP53 nor ATRX mutations were detected. Further, we examined a copy number profile derived from Illumina 450 k methylation analysis of this case (Suppl. Fig. 7). Losses on 1p and 19q turned out to affect only parts of the respective chromosomal arms. Thus the molecular hallmark of oligodendroglioma, combined loss of the entire arms 1p and 19q was not present. Therefore, the primary tumor manifestation of this patient may group with rarer tumors lacking combined 1p/19q loss but also exhibiting retained ATRX expression [18, 19, 35].

Apart from morphological aspects, another finding in favor of the diagnosis of OA was the observation of survival differing in astrocytoma, OA and oligodendroglioma with oligodendroglioma exhibiting best, astrocytoma most unfavorable and OA a somewhat intermediate outcome. This has been demonstrated by several studies [3, 13, 36]. However, in the “post 1p/19q era”, these differences vanished upon stratification for the presence of this alteration. The NOA-04 prospective trial on anaplastic gliomas reported virtually identical outcomes for patients with oligodendroglioma or OA. For patients with 1p/19q co-deleted OA this prompts a diagnosis of an oligodendroglioma. The non-1p/19q co-deleted OA still had a prognosis not distinguishable from oligodendroglioma, despite their molecular behavior as astrocytoma [41]. It was the discovery of ATRX loss, which not only signifies astrocytic tumors, but also defines a subgroup of astrocytoma with a better prognosis. Hence, OA without 1p/19q co-deletion in the NOA-04 trial mainly harbored an ATRX loss and could therefore be grouped as astrocytoma with favorable prognosis [42]. Recent analyses proved the 450 k array to be more reliable for copy number profiling of chromosomal arms 1p/19q than the previously applied FISH and multiplex ligation probe assay in equivocal cases [44]. Reassessment of the

Fig. 3 Irradiated recurrent tumor with ribboning architecture (*left column*) and other areas with spindle-shaped cells and fibrillary matrix. Both compartments show IDH1R132H mutation, ATRX loss of expression and p53 upregulation. The initial lesion presented with retained ATRX and no p53 accumulation (*insets*). Chromosomal arms 1p and 19q showed only partial deletion (Suppl. Fig. 7)



NOA-04 cases with 450 k array now identified two prognostically distinct groups of anaplastic *IDH* mutant gliomas with favorable survival of 1p/19q co-deleted cases irrespective of histology [43]. The recently presented EORTC 26951 trial including anaplastic oligodendrogliomas and OAs also detected unique features of tumors with 1p/19q co-deletion, however, without stratifying for diagnosis anymore [39]. Taken together, upon separating OA into groups with and without 1p/19q loss the co-deleted tumors clinically fare like oligodendroglioma while the non-deleted tumors clinically group with astrocytomas. Thus, the clinical evidence for a tumor entity OA has lost its basis.

The molecular findings and the clinical study data strongly argue against OA. This is of significant relevance given the varying frequency of this diagnosis made in different institutions accompanied by wide interobserver variation [10]. We propose to refrain from diagnosing OA and classifying these tumors either as oligodendroglioma or astrocytoma. A practical approach includes *IDH1/2* immunohistochemistry and/or *IDH1/2* sequencing analysis, 1p/19q analysis by FISH or other methods and *ATRX* immunohistochemistry. Although *TP53* mutation is a hallmark of astrocytoma frequently associated with enhanced detection of intracellular p53 protein, the accumulation of p53 has also been described in reactive gliosis [6, 7, 20]. The entire loss of *ATRX* expression in tumor cells while vessels show positive staining might therefore be the more reliable marker for astrocytic neoplastic cells. Presence of *IDH1* mutation is among the strongest indicators for diffuse astrocytic or oligodendroglial gliomas. In oligodendroglial tumors, *IDH* mutations are highly associated with 1p/19q loss. Areas lacking these alterations that have an astrocytic appearance should alert to the possibility of reactive alterations mimicking tumor. This is easily recognized in presence of the most prevalent *IDH1R132H* mutation. Absence of *IDH1R132H* protein in the astrocytic portion argues for reactive gliosis and the lesion should be classified as oligodendroglioma. In absence of 1p/19q co-deletion, the loss of *ATRX* expression should prompt the diagnosis of astrocytoma. How should be dealt with tumors sharing molecular features of astrocytoma and oligodendroglioma? In such a rare instance, which was not observed in our series apart from the single (1/43, 2 %) irradiated case with initial *ATRX* expression and only in 4 of 379 (1.1 %) diffuse gliomas analyzed for *TP53* and 1p/19q status in a variety of studies [4, 28, 32, 38, 40, 45] the term “oligoastrocytoma” may be applied for the time being.

In conclusion, we provide evidence that OA segregates into two groups, genetically matching oligodendroglioma on one and astrocytoma on the other side. This requires *IDH*, 1p/19q and *ATRX* analyses as standard diagnostic routine. Our findings support parting with the diagnosis of OA.

Acknowledgments The study was supported by the Medical Faculty Heidelberg PostDoc Fellowship and the DKFZ Intramural Funding Program, Priority Topic Intratumoral Heterogeneity, to FS. We wish to thank Tanja Goeck and Katrin Kalis for skillful technical assistance.

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