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Oligoastrocytoma: A Vanishing Tumor Entity

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Abstract

Oligoastrocytoma (OA) was a glioma recognized in the current World Health Organization (WHO) classification of the central nervous system (CNS) tumors as a mixed tumor with an astrocytic and an oligodendroglial component. Its definability was, however, poor so that its prevalence varies in the various collections. A series of contributions of the literature and the “International Society of Neuropathology (ISN) – Haarlem Consensus” recently denied its existence as a tumor entity on the basis of 1p/19q, isocitrate dehydrogenase (IDH) and α -thalassemia/mental retardation syndrome X-linked (ATRX) status. Most tumors previously diagnosed as OAs were, therefore, reclassified as either oligodendrogliomas or astrocytomas.

We revised 40 OAs from our glioma series initially diagnosed with stringent histologic criteria. After the revision based on the above mentioned molecular markers, most of them changed diagnosis falling into the categories of oligodendroglioma or astrocytoma. Only one fulfilled the stringent criteria of the current classification system, whereas two cases remained undefined.

Since ATRX is constitutively expressed in microglia/macrophages, their number in the histologic sections has a paramount importance in recognizing the oligodendroglial component. The double ATRX/GFAP, ATRX/IDH1^{R132H} and ATRX/Iba-1 immunostainings greatly conditions the recognition of the oligodendroglial and astrocytic tumor cells.

Keywords: oligoastrocytoma, 1p/19q co-deletion, IDH, ATRX, prognosis

1. Introduction

In the 2007 World Health Organization (WHO) classification of the central nervous system (CNS) tumors [1], oligoastrocytoma (OA) was considered as a mixed glioma with an astrocytic and an oligodendroglial component [2, 3]. It could be easily recognized if the two components were clearly separated, as it rarely happened. Usually, the two cell types were found intermingled and the differential diagnosis towards astrocytomas and oligodendrogliomas was difficult and, frequently, it remained undefined. Since its first recognition [4] and confirmation [5], its definability was poor and the tumor was even considered as an oligodendroglioma with reactive astrocytes [6]. The poor tumor definability explains why the prevalence of OAs in the various collections of the literature has been so variable and it accounted for the need to establish criteria useful for the recognition of the tumor. It was suggested to rely on the occurrence of at least 10% neoplastic oligodendrocytes in astrocytic gliomas [7] or 10% neoplastic astrocytes in oligodendroglial gliomas [8], provided that oligodendrocytes were neoplastic and not normal, and that astrocytes were tumor and not reactive. These criteria, however, were too vague in the clinical practice and the definition of the tumor remained a subjective matter. In particular, the recognition of normal from tumor oligodendrocytes, especially in slightly infiltrating or diffuse growth where the cell density is low, remained unsolved [9].

As a matter of fact, the diagnostic uncertainties of OA were also due to the lack of a reliable marker for tumor oligodendrocytes in tissue sections. The prevailing diffuse and infiltrating growth of both oligodendrogliomas and the oligodendroglial component of OAs led to find the coexistence in the same tumor area of tumor and normal oligodendrocytes to the point that it was even difficult to establish, in some cases, whether a tumor infiltration existed or not. On the other hand, the difficulty to recognize a mild oligodendroglial infiltration in oligodendrogliomas themselves was already known, even exploiting the occurrence of abnormal nuclei or crowding of tumor cells along capillaries or around neurons.

Another critical point was the difficulty to distinguish reactive from tumor astrocytes and to recognize the real nature of minigemistocytes [10], glial fibrillary oligodendrocytes (GFOC) [11–13], real gemistocytes, and perineural satellites.

The origin of the tumor was referred to a progenitor stage preceding the astrocytic and oligodendroglial differentiation and, therefore, tumor suppressor protein p53 (TP53) mutations were searched for in the astrocytic component and the 1p/19q co-deletion in the oligodendroglial one and a genetic analysis was suggested [14]. Anyway, the variability of OA prevalence in the various collections remained high. It was observed that the absence of 1p/19q co-deletion, typical of oligodendrogliomas, entailed the occurrence of TP53 mutations [15], but also that it could be present or absent in both tumor components and that all tumor cells seemed to share the same genetic aberrations [14].

2. Epidemiology and demographics

2.1. Incidence

OA was the third most common glioma. It accounted for 1% of all brain tumors and 5–10% of all glial neoplasms. The incidence of OA was approximately 0.03 *per* 100,000 individuals in the United States. Young and middle-aged adult population was affected. The median age of diagnosis was 42 years. Males were more commonly affected than females; the male to female ratio was approximately 1.43–1. OA usually affected individuals of the Caucasian race with a higher incidence rate in developed countries [1].

2.2. Etiology

Common risk factors in the development of OA included family history of brain tumors, ionizing radiation, and allergic diseases.

2.3. Location

OA preferentially developed in the cerebral hemispheres with a frequency that corresponded to the relative size of the cerebral lobes (frontal, temporal, parietal, and occipital) [1]. It commonly arose in the supratentorial regions. Occasional locations were insula, diencephalon, and spinal cord whereas cerebellar location was very uncommon.

2.4. Clinical features

The most common symptoms were seizure, headache, and personality changes.

2.5. Neuroradiological features

On magnetic resonance imaging (MRI), OA was described as characterized by a mass which is typically hypointense on T1-weighted images and hyperintense on T2-weighted images. No enhancement is observed on Gadolinium enhanced T1-weighted images (**Figure 1A, B**).

Figure 1. Magnetic resonance imaging (MRI) of 20-years old woman. A – OA, T1-weighted sequence after gadolinium contrast enhancement (arrow); B – Id, hyperintensity in T2-weighted sequence.

3. Histopathology

According to the current WHO classification system [1], OA was classified in two subtypes: grade II OA (OAI) and grade III OA (OAI).

3.1. Macroscopic appearance

On gross pathology, OA was characterized by a soft, well-defined, grey-tan, mucoid or hemorrhagic, calcified mass with or without necrosis that may expand the gyrus, and cause blurring of the grey white matter junction.

3.2. Microscopic appearance

On histopathologic analysis, OA was characterized by highly cellular lesions composed of both tumor astrocytes and oligodendrocytes that could be separated or intermingled [5], *i.e.* the tumor could be defined as “biphasic” (**Figure 2A–C**) or “diffuse” (**Figure 2D**). Astrocytic tumor cells scattered within oligodendroglial cells had to be recognized as neoplastic and not reactive/hypertrophic astrocytes.

Figure 2. Histopathologic features of OA. A – OAI with separated astrocytic and oligodendroglial components, x100; B – Id, astrocytic component, x400; C – Id, oligodendroglial component, x400; D – OAI with intermingled astrocytic and oligodendroglial cells, x200; E – OAI, x200. All hematoxylin and eosin (H&E).

OAI. The tumor showed a moderate cellularity with no or low mitotic activity. Microcalcifications and microcystic degeneration could occur.

Reactive astrocytes are present in all gliomas, OA included; in the latter, their distinction from tumor astrocytes was the most important problem since the protean appearance of reactive

astrocytes, with large cytoplasm, and thick and long processes or with small cytoplasm with short processes and in variable number, did not allow a clear-cut distinction from tumor astrocytes.

An important bias was the occurrence of minigemistocytes, GFOC, and true gemistocytes. Both minigemistocytes and GFOC were regarded as either transitional forms between oligodendrocytes and astrocytes, corresponding to a bipotential glial progenitor cell [16], or as glial fibrillary acidic protein (GFAP) expressing oligodendrocytes [12], remnants of myelin forming glia of the developmental period [13].

A high frequency of minigemistocytes could confer an astrocytic aspect to the tumor.

OAIII. The tumor was mainly characterized by a significant or brisk mitotic activity (≥ 6 mitoses *per* 10 high power field [HPF]) and a high Ki-67/MIB-1 proliferation index, nuclear atypia, necrosis, and apoptotic cells (**Figure 2E**). The malignant transformation was considered as proceeding either from the one or the other cell component. In the differential diagnosis towards glioblastoma (GBM), the occurrence of circumscribed necroses was decisive; the presence of microvascular proliferations (MVPs) would indicate the grade III when occurring in the oligodendroglial part and the grade IV when in the astrocytic one.

3.3. Immunohistochemistry (IHC)

IHC was practically based only on GFAP expression. No specific immunohistochemical marker was available for oligodendrocytes [17, 18], although MAP2, OLIG2, Cyclin D1, and alpha-internexin (INA) immunopositivity could be found in the oligodendroglial component. Approximately one third of OAs showed nuclear p53 accumulation, more commonly in the astrocytic cells [19].

4. Molecular genetics

As in all gliomas, the origin of the tumor proceeds from the step-wise accumulation of genetic/epigenetic alterations. Thirty-fifty percent of OAs exhibited loss of heterozygosity (LOH) on chromosomes 1p and 19q [20, 21], while approximately 30% of them harboured TP53 mutations. In particular, OAs of the temporal lobe more frequently exhibited TP53 mutations, and less commonly, 1p and 19q losses [22, 23].

OAI typically exhibited the type and distribution of genetic alterations observed in grade II gliomas [22]. OAIII showed genetic alterations commonly involved in the progression of astrocytic and oligodendroglial tumors, including loss of 9p with homozygous deletion of the cyclin-dependent kinase inhibitor 2A (CDKN2A) (p14^{ARF}) gene, allelic loss on chromosome 10q and epidermal growth factor receptor (EGFR) gene amplification [24].

5. Treatment and prognosis

OAs responded less favourably to chemotherapy (CHT) due to the chemoresistance of their astrocytic components [25]. Studies have shown that the standard of care for 1p/19q co-deleted oligodendroglial tumors should be the combination of CHT and radiotherapy (RT). In OA, a favourable prognosis was associated to young age, grade II and extent of resection [26].

Compared to astrocytomas, OAs shared with oligodendrogliomas a more favourable prognosis and an improved response to adjuvant therapy. The NOA-04 prospective trial on anaplastic gliomas reported virtually identical outcomes for patients with oligodendrogliomas or OAs [27].

6. New criteria for glioma diagnosis after the “ISN-Haarlem Consensus”

Our knowledge on the nature of OA underwent a profound change after the “International Society of Neuropathology (ISN)-Haarlem *Consensus*” guidelines led to the official recognition of the indispensability of the genetic analysis in order to obtain an “integrated” diagnosis of gliomas [28]. Referred to grade II and III adult gliomas, this new approach would involve a combination of histologic and molecular data based on the 1p/19q, isocitrate dehydrogenase (IDH) 1/2 and α -thalassemia/mental retardation syndrome X-linked (ATRX) status.

6.1. 1p/19q chromosomal status

The genetic hallmark of oligodendroglial tumors is a combined chromosomal deletion of the short arm of the chromosome 1 (1p) and the long arm of the chromosome 19 (19q). Combined 1p and 19q losses were described in 80–90% of grade II and in 50–70% of grade III tumors [22, 24].

The 1p/19q chromosomal status was recognized as an important diagnostic biomarker in the clinical practice. The 1p/19q co-deletion was reported in 60–70% of oligodendrogliomas with a classical histologic phenotype (perinuclear “halo” and “chicken wire” vascular pattern). Partial 1p or 19q deletion occurred in approximately 75% of the cases [29, 30]. Oligodendroglial tumors with 1p/19q co-deletion were observed to typically arise at an extra-temporal location, whereas tumors with intact 1p/19q at the temporal lobe [22]. In contrast, childhood oligodendrogliomas only rarely exhibited chromosomal abnormalities.

Importantly, the occurrence of 1p/19q co-deletion supports the diagnosis of oligodendroglioma, especially when histology is atypical. However, its absence does not exclude this diagnosis, leaving unsolved the question of oligodendrogliomas with intact 1p/19q.

In OA, the frequency of the 1p/19q co-deletion was approximately 50% [22]. Virtually, it was mutually exclusive with LOH of chromosome 17p13 and TP53 mutations, both typical of astrocytic tumors. In OA, the 1p/19q co-deletion was referred to the oligodendroglial component, whereas TP53 mutations to the astrocytic one.

6.1.1. Mechanism of the combined loss of the chromosomes 1p and 19q

The mechanism of the combined loss of the two chromosomal arms is an unbalanced t(1;19)(q10;p10) translocation of 19p to 1q. A centromeric or pericentromeric translocation of chromosomes 1 and 19 results in two derivative chromosomes, der(1;19)(p10;q10) and der(1;19)(q10;p10), followed by the loss of the derivative chromosome containing the short arm of chromosome 1 and the long arm of chromosome 19 [31, 32].

The extent of the 1p/19q co-deletion has important diagnostic and prognostic implications. The chromosomal arm 1p is entirely deleted only in pure oligodendroglial tumors and the whole-arm 1p deletion has a strong favorable prognostic significance. Small telomeric (1p36) or interstitial 1p deletions are frequent as well, but with an opposite prognostic significance; they associate neither with deletion on the chromosomal arm 19q [33] nor with response to CHT [34].

While the total 1p/19q co-deletion is almost completely exclusive of oligodendrogliomas, partial 1p deletions are frequent in GBMs and isolated 19q loss in mixed and astrocytic gliomas in relation to the malignant transformation [35, 36].

Currently, there is a general agreement to diagnose the classical oligodendroglioma only in presence of a whole-arm 1p/19q co-deletion [37, 38].

Most oligodendrogliomas with 1p/19q co-deletion harbor IDH1/2, telomerase reverse transcriptase (TERT), homolog of the *Drosophila capicua* (CIC) and far upstream element-binding protein 1 (FUBP1) somatic mutations, and O⁶-methylguanine-DNA methyltransferase (MGMT) or CDKN2A (p14^{ARF}) promoter hypermethylation [37, 39–41].

6.1.2. Methods for the detection of 1p/19q chromosomal status

Different methods for the detection of the 1p/19q chromosomal status are employed in the routine diagnostics. Fluorescent *in situ* hybridization (FISH) is a single-locus technique, limited to the 1p36 locus and thus not regarded as a suitable tool due to the high risk of false-positive results [34, 37, 42, 43]. On the other hand, LOH analysis is generally carried out with a low number of microsatellite markers covering only a small chromosomal region.

In contrast, multi-locus techniques, as comparative genomic hybridization (CGH) or multiplex ligation-dependent probe amplification (MLPA) detect gene copy number changes on the whole chromosome and distinguish whole-arm from partial 1p deletion [44]. Additionally, they can reveal putative gain of functions on both 1p and 19q chromosomes [45, 46].

In particular, MLPA has been validated as a high-resolution gene dosage assay for the screening of large deletions and duplication/amplification events in human cancers. In gliomas, three independent studies validated MLPA to assess the 1p/19q status by comparing MLPA data with CGH data obtained on the same tumor series, mainly composed of oligodendroglial tumors [47–49]. In the authors' experience, MLPA is a reliable and powerful tool to assess the 1p/19q status on formalin fixed and paraffin embedded tumor samples.

6.2. IDH1/2 mutations

IDHs catalyze the oxidative decarboxylation of isocitrate to α -ketoglutarate with production of NADH/NADPH and they are involved in the Krebs cycle.

Recurrent somatic point mutations affect the arginine (Arg) residue at codon 132 in the IDH1 gene on chromosome 2q33.3. Less frequently, they occur at the homolog Arg (R) residue at codon 172 in the IDH2 gene on chromosome 15q26.1. The IDH1/2 mutation rate is in the range of 70–80% in OII and OIII [50–53] and less in OAI and OAII, with a higher frequency in 1p/19q co-deleted tumors [37]. IDH1 mutations prevail in astrocytic tumors whereas IDH2 mutations are more common in oligodendroglial tumors [51, 54].

In low grade gliomas, they are prognostic favorable factors [53, 55, 56].

The c.395G>A (p.R132H) mutation can be easily detected by a anti-IDH1^{R132H} mutation-specific antibody by immunohistochemical techniques [53, 57]. Tumor cells show IDH1^{R132H} immunopositivity in their cytoplasm, whereas reactive astrocytes and normal glia cells are negative. This is particularly evident in the picture of cortical perineuronal satellitosis where positive (tumor) and negative (normal) satellites can be found, at the beginning of invasion.

6.2.1. ATRX mutations

The ATRX gene is located on chromosome Xq21.1, contains 35 exons, and encodes a 2,492 amino acid protein. ATRX belongs to the H3.3-ATRX-DAXX chromatin remodeling pathway, involved in chromatin stabilization [58]. ATRX and its binding factor death-associated protein 6 (DAXX) incorporates the histone protein H3.3 into the nucleosome at telomeres and pericentric heterochromatin [59, 60]. Alterations of this function lead to loss of structural integrity at telomeres leading to tumorigenesis. In fact, ATRX or DAXX protein loss is associated to the alternative lengthening of telomeres (ALT), a telomerase-independent mechanism of telomere lengthening [61–66].

Germline ATRX mutations give rise to a syndrome characterized by severe mental retardation [67] and to α -thalassemia.

Somatic ATRX mutations occur in gliomas of different types and histologic grades [38, 61–65, 68–70]. They are more frequent in grade II (67%) and in grade III (73%) astrocytic tumors and in secondary GBMs (57%), as well as in mixed gliomas (25% in grade II and 27–53.8% in grade III tumors) [64, 65, 69, 70]. In contrast, they are rare in primary GBMs (4%), pediatric GBMs (20%) and in pure oligodendroglial tumors (<10%) [63, 64, 68]. Very importantly, ATRX mutations do not affect pilocytic astrocytomas [64].

ATRX mutations occur in 70% of IDH mutant and intact 1p/19q low grade gliomas [62, 64, 65]. Restricted to IDH mutant tumors, they are significantly associated to TP53 mutations and nuclear p53 overexpression and to astrocytic differentiation; they are mutually exclusive with 1p/19q co-deletion [71, 72]. ATRX and IDH1/2 mutations occur in association and they may represent early genetic alterations in the development of gliomas affecting progenitors before their differentiation along the two lineages.

In pediatric gliomas, all ATRX mutations cluster near the C-terminal helicase domains; in adult tumors, they are evenly distributed across the gene, mainly as frameshift mutations leading to truncated proteins [63, 64, 69].

The relatively large size of the ATRX gene makes the mutation analysis difficult to be applied in the routine diagnostic procedures. The immunohistochemical evaluation of the ATRX protein expression could represent an alternative method to assess the ATRX status. Although studies reported concordant results between the mutation analysis and IHC [38, 72], tumor heterogeneity in the ATRX expression and concurrent normal non-tumor cells with constitutive ATRX expression may explain possible discrepancy. As a matter of fact, ATRX mutations/ATRX protein loss characterizes astrocytic gliomas, whereas retained ATRX immunoreactivity characterizes oligodendroglial gliomas. Referred to OA, the former is typical of the astrocytic component while the latter of the oligodendroglial one [71].

6.2.2. ATRX and prognosis

Patients harboring ATRX mutations would show a better outcome [65]. ATRX has important prognostic implications in anaplastic gliomas [65]. ATRX loss is a prognostic factor in IDH mutant and non 1p/19q co-deleted low grade gliomas [62, 65]. In GBMs, ATRX loss affects younger patients [72].

7. Nosographic position of OA after the “ISN-Haarlem Consensus”

The “integrated” diagnosis provided for grade II and III adult gliomas covers the diagnostic uncertainties between astrocytoma and oligodendroglioma and, mainly, of OA.

It has been found that OAs more frequently exhibited the molecular signature of either pure oligodendroglioma (IDH1/2, 1p/19q co-deletion, CIC, FUB1, and TERT promoter mutations) or pure astrocytoma (IDH1/2, TP53, and ATRX mutations) with almost total exclusivity [71]. A recent study proved that most low grade gliomas (including 74 OAs) with IDH1/2 mutations and intact 1p/19q harbored a high frequency of TP53 mutations (94%) and ATRX mutations (86%) [73].

In 31 of 43 OAs (72.1%), the absence of ATRX mutations, associated with IDH1/2 mutations and total 1p/19q co-deletion, reclassified them as oligodendrogliomas; 11 of 43 (25.6%), with concurrent ATRX protein loss and TP53 mutations were reclassified as astrocytomas. The astrocytes within the tumor were, therefore, interpreted as reactive [71]. These results were similar to a previous one [64] and were confirmed in a large collection of cases [38]. The conclusion is that OA should be removed from the WHO classification as a distinct tumor entity, although rare instances of clearly biphasic OAs exhibiting morphologic and molecular heterogeneity have been described. Mixed areas of the tumor could show heterogeneous ATRX immunoreactivity with positive reactive astrocytes and negative tumor astrocytes [74, 75]. As ATRX is ubiquitously expressed in normal cells (endothelial cells, reactive astrocytes, microglia cells, and lymphocytes) [38, 71], the real problem in the diagnosis of OA according

to the 2007 WHO classification seems to ascertain the occurrence of ATRX-negative and IDH1^{R132H}-positive astrocytes in the tumor.

The astrocytic and oligodendroglial components have been found to share the same molecular signature, but with a sheer cell differentiation [71]. They should be regarded as “morphologically ambiguous” rather than “mixed” tumors, as conventionally referred. 1p/19q co-deletion and TP53 mutations represent distinct mechanisms of oncogenesis but they do not provide evidence for a genetic signature specifically related to OA [76].

The “ISN-Haarlem Consensus” suggested considering OA or tumors with ambiguous histology as diffuse astrocytoma when harboring IDH1/2 mutations, intact 1p/19q, and ATRX loss; as oligodendroglioma when harboring IDH1/2 mutations, 1p/19q co-deletion, and intact ATRX and as diffuse astrocytoma when harboring wild type IDH. In the absence of molecular data, the tumor should be diagnosed as oligodendroglioma or diffuse astrocytoma not otherwise specified (NOS). Finally, the denomination of OA would be only maintained when molecular testing does not solve tumor diagnosis [27].

Anyway, there is no doubt on the usefulness of the ATRX IHC [72] and of the double ATRX/IDH1^{R132H} immunostaining [77] in the diagnosis of adult diffuse gliomas. Based on molecular data from the above mentioned markers on 54 OAs, it has been concluded that OA represents a morphological grey zone rather than a group of truly “mixed” or “intermediate” gliomas [78]. Importantly, it remains unsolved how to explain IDH mutant diffuse gliomas with ATRX expression and intact 1p/19q (neither merely astrocytic nor oligodendroglial lesions) or, more rarely, cases with loss of ATRX protein expression and total 1p/19q co-deletion (both astrocytic and oligodendroglial lesions).

8. Observations on personal OA series

Our analysis started from the established principles that 1p/19q co-deletion is typical of oligodendrogliomas, but that it occurs in a low percentage only of tumors with a classical oligodendroglial phenotype (“honeycomb” appearance and “chicken wire” vessels). Our 40 OA cases had been initially diagnosed according to stringent histologic criteria, so that their number is slightly lower compared to others’ series. The current revision has been carried out on the basis of 1p/19q chromosomal status, as detected by MLPA, IDH1/2 mutation status by IHC and sequencing analysis, and ATRX expression by IHC. The key points were: 1) the retained expression of ATRX by tumor oligodendroglial nuclei and by reactive astrocyte, microglia/macrophage, endothelial cell, lymphocyte nuclei, and the ATRX protein loss in tumor astrocytic nuclei; 2) IDH1/2 mutations, present in low grade gliomas with a higher frequency in oligodendrogliomas than astrocytomas [52, 79] (**Figure 3A–D**); 3) therefore, although they reveal the tumor nature of cells, IDH1/2 mutations can lack both in oligodendroglial and astrocytic tumor cells. As, beside reactive astrocytes, ATRX is expressed in microglia/macrophage nuclei, Iba-1, CD68, CD16, and CD163 IHC has been performed on parallel serial tumor sections, as well as the double ATRX/GFAP, ATRX/Iba-1, and ATRX/IDH1^{R132H} immunostaining. Microglia/macrophages can reach in the tumor sections a frequen-

cy of 50–130 $\times$ 40 HPF (personal data); ATRX expressing nuclei could be, therefore, referred to tumor oligodendrocytes only if their number encompasses the number of microglia/macrophage nuclei. The occurrence of microglia/macrophages is, in our experience, the main bias to recognize the oligodendroglial component in OA (**Figure 4A–H**). Normal oligodendrocytes do not express ATRX and they can be distinguished in this way from tumor oligodendrocytes; moreover, they express Cyclin D1 that, in contrast, is not expressed by tumor oligodendrocytes unless they are cycling cells (**Figure 5A–D**) [80].

Figure 3. Immunohistochemistry (IHC). A – Oligodendroglioma, ATRX-positive cells, DAB, x200; B – Id, IDH1^{R132H} - positive perinuclear rim in tumor cells, DAB, x200. C – Gemistocytic astrocytoma, ATRX-negative and GFAP-positive tumor astrocytes, double IHC with DAB and *Fast Red*, respectively, x400; D – Id, IDH1^{R132H} -positive cells, DAB, x400. Anti-IDH1^{R132H} mouse monoclonal antibody (clone H09, Dianova GmbH, Hamburg, Germany) and anti-ATRX rabbit polyclonal antibody (HPA001906, Sigma Aldrich Co., St. Louis, MO, USA). DAB, 3,3'-Diaminobenzidine.

Figure 4. Immunohistochemistry (IHC). A – Diffuse astrocytoma, GFAP-positive cells, x200; B– Id, scattered ATRX-positive nuclei, the frequency of which corresponds to the frequency of CD68 positive-cells (C), both x200; D – Diffuse astrocytoma, apparent OA with GFAP-positive astrocytes and possible oligodendroglial nuclei, x200; E – Id, scattered ATRX-positive nuclei, x200; F – Id, Iba-1-positive cells covering the number of ATRX-positive nuclei, x200; G – Oligodendroglial infiltration, ATRX-positive and ATRX-negative nuclei, x200; H – Id, Iba-1-positive cells, x200 in 40 $\times$ high power field. All 3,3'-Diaminobenzidine (DAB).

Figure 5. Immunohistochemistry (IHC). A – Oligodendroglioma, mild infiltration with several ATRX-negative normal oligodendrocytes and few ATRX-positive nuclei (tumor nuclei or microglia/macrophage cells?), DAB, x200; B – Id, Cyclin D1-positive normal oligodendrocytes, DAB, x200; C – Oligodendroglioma, ATRX-positive endothelial cells, DAB, x400; D – Gemistocytic astrocytoma, microglia/macrophage cells, Iba-1- and ATRX-positive cells, double IHC with *Fast Red* and DAB, respectively, x400. DAB, 3,3'-Diaminobenzidine.

The diagnosis of the 40 OA cases changed in 87.5% of them (35/40): 22/40 (55%) were reclassified as astrocytomas due to the absence of total 1p/19q co-deletion, the occurrence of IDH1/2 mutations, and the loss of ATRX expression in GFAP-positive, phenotypically looking tumor astrocytes (**Figure 6A–D**); 11/40 (27.5%) were reclassified as oligodendrogliomas due to IDH1/2 mutations, retained ATRX expression, total 1p/19q in 2/11 (18.2%) cases and partial 1p or 19q deletions in 9/11 (81.8%) cases. In 2/40 (5%) cases the diagnosis changed into reactive gliosis due to retained ATRX expression in GFAP-positive reactive astrocytes and to the lack of ATRX-positive oligodendrocytes (**Figure 6E–F**). Among the remaining five cases, in one case with partial 1p/19q co-deletion, ATRX-negative and GFAP-positive astrocytes co-existed with a number of ATRX-positive and GFAP-negative oligodendrocytes; both cell components showed IDH^{R132H} immunopositivity by double ATRX/IDH1^{R132H} immunostaining. Importantly, by the double ATRX/Iba-1 immunostaining, it was possible to verify that the number of ATRX-positive oligodendroglial nuclei was higher than the number of Iba-1-positive cells. The diagnosis of OA in this case was thus confirmed (**Figure 7A**). Two wild type IDH cases without total 1p/19q co-deletion and with heterogeneous ATRX expression were regarded as ambiguous since the tumor nature of the two cell components could not be ascertained. In the other two cases, one with partial 1p/19q deletion and one with intact 1p/19q, the diagnosis of OA could not be maintained since it was technically impossible to perform IDH1^{R132H} IHC.

Figure 6. Immunohistochemistry (IHC). A – Gemistocytic astrocytoma, GFAP-positive cells, DAB, x200; B – Id, ATRX-negative cells, DAB, x200; C – Anaplastic astrocytoma, GFAP-positive cells, DAB, x200; D – Id, ATRX-negative cells, DAB, x200; E – Oligodendroglioma, reactive astrocytes with GFAP-positive thick cytoplasm and long processes and ATRX-positive nuclei, double IHC with *Fast Red* and DAB, respectively, x400. F – Reactive gliosis, reactive astrocytes with GFAP-positive large cytoplasm and short processes and ATRX-positive nuclei, double IHC with *Fast Red* and DAB, respectively, x400. DAB, 3,3'-Diaminobenzidine.

Figure 7. Immunohistochemistry (IHC). A – OA, ATRX- and IDH1^{R132H}-positive astrocytes, double IHC with DAB and *Fast Red*, respectively, x630; B – Oligodendroglial minigemistocytes with ATRX-positive nuclei and GFAP-positive cytoplasm, double IHC with DAB and *Fast Red*, respectively, x1000; C – Oligodendroglioma, satellitosis with ATRX-positive nuclei, DAB, x200; D – Normal cortex, ATRX-positive neurons with ATRX-negative satellites, DAB, x400; E – Oligodendroglial cortical infiltration, ATRX-positive neurons with ATRX-positive and ATRX-negative satellites, DAB, x400; F – Id, ATRX-positive pericapillary tumor cells, DAB, x200. DAB, 3,3'-Diaminobenzidine.

The distinction of reactive from tumor astrocytes has always been a crucial point in the diagnosis of OA. Their recognition as reactive is a point of reference in denying the existence of such tumor category. Reactive astrocytes retain nuclear ATRX protein expression, as tumor oligodendrocytes, while tumor astrocytes lack ATRX expression.

By comparing our results with those of the literature, it is noteworthy that the change of diagnosis from the initial to the current analyses of cases, largely depends on the criteria used in the initial recognition of OAs. Upon the reclassification of the 35 cases as astrocytoma, oligodendroglioma, or reactive gliosis, only one could deserve the dignity of OA among the remaining five cases.

It must be incidentally remark that total 1p/19q co-deletion occurred in 43/113 (38.1%) of our oligodendroglioma series (selected by the typical morphology of honeycomb appearance and chicken wire vessel distribution); partial 1p and/or 19q deletions occurred in 36/82 (43.9%) of oligodendrogliomas and in 12/24 (50%) astrocytomas. Referring to the 40 cases with initial diagnosis of OA, two had total 1p/19q co-deletion, 14 partial 1p/19q deletion, 12 intact 1p/19q, and one a gain of function on the chromosome 19q. For the remaining 11 cases, the 1p/19q status was not available. It is widely accepted that partial 1p/19q deletions may occur in other gliomas, but one wonders how tumors with an oligodendroglial phenotype with partial deletions or intact 1p/19q can be classified.

9. Conclusions

By applying the “integrated” approach to the revision of our OA series, we conclude that, OA could no longer be regarded as a separate tumor entity. However, rare cases might retain the OA denomination [74, 75, our case], indicating that tumors can differentiate in oligodendroglial and astrocytic sense, even maintaining the same molecular genetics [71], or that they can arise from progenitors before differentiation in the two lineages.

Two points must be emphasized: one is the importance of the double immunostaining to recognize the astrocytic nature of tumor cells, especially the association between IDH and ATRX expression, considering that IDH1/2 mutations prevail in oligodendrogliomas in comparison to astrocytomas [52, 79]. In supposed mixed tumors with the two separate components, it has been observed that these share the same molecular asset and that astrocytes in the tumor are reactive cells [71]. However, in tumors with the two cell types intermingled, tumor cells can be distinguished from reactive astrocytes by the double ATRX/GFAP immunostaining. It must be remarked that in rare instances 1p/19q co-deletion can be associated with ATRX mutations/ATRX protein loss [64] and that, since a quota of astrocytomas do not harbor IDH1/2 mutations, tumor astrocytes with wild type IDH may be found.

Minigemistocytes show definitely the oligodendroglial nature due to their ATRX-positive nuclei (**Figure 7B**).

The other point is the great influence that microglia/macrophage occurrence may exert in the recognition of tumor oligodendrocytes due to their nuclear ATRX positivity. In cases where

the number of microglia/macrophages is very high to reach the number of ATRX-positive nuclei, if tumor astrocytes are demonstrable, the tumor should be reclassified as astrocytoma. In order to recognize an oligodendroglial tumor infiltration, often result of a biopsy, it is still mandatory that the number of ATRX-positive nuclei in a given area encompasses the number of microglia/macrophages (personal data). ATRX-negative nuclei in infiltration areas correspond to normal oligodendrocytes. Only the double ATRX/Iba-1 immunostaining can reveal the occurrence of normal oligodendrocytes, beside their Cyclin D1 expression. In IDH1^{R132H} mutant cases, the double ATRX/IDH1^{R132H} immunostaining unequivocally identifies oligodendroglial tumor cells. The coexistence of normal and tumor oligodendrocytes by ATRX IHC can be demonstrated in perineuronal satellitosis: in its initial phases, ATRX-positive and ATRX-negative nuclei are found around neurons; in later phases, all nuclei are ATRX-positive (**Figure 7C–E**). The same can be said for pericapillary satellitosis (**Figure 7F**).

The last point to be discussed is the possibility to find a false “honeycomb” appearance mimicking the typical perinuclear “halo” with ATRX-positive nuclei. This aspect cannot be interpreted as suggestive of an oligodendroglial origin of the tumor, being expressions of a water disturb/edema of unspecified nature, as it may happen for instance, in pilocytic astrocytoma [81].

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Authors declare no potential conflicts of interest.

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