Mutation-specific IDH1 antibody differentiates oligodendrogliomas and oligoastrocytomas from other brain tumors with oligodendroglial-like morphology

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Abstract Isocitrate dehydrogenase 1 (IDH1) mutations are frequent in astrocytomas, oligoastrocytomas and oligodendrogliomas. We previously reported the generation of a mutation-specific antibody that specifically detects R132H mutated IDH1 protein (clone H09). Here, we investigate the feasibility of H09 immunohistochemistry to differentiate between oligodendrogliomas/oligoastrocytomas and other tumors with similar morphology. A total of 274 brain tumors presenting with focal or extensive clear cell morphology were investigated. High numbers of H09-positive cases were observed in adult grade II oligodendrogliomas (67 of 74, 91%), grade III oligodendrogliomas (65 of 69, 94%), grade II oligoastrocytomas (11 of 14, 79%) and grade III oligoastrocytomas (10 of 11, 91%). All cases of pediatric oligodendrogliomas (n = 7), neurocytomas (n = 41, 35 central, 4 extraventricular, 2 cerebellar liponeurocytomas), dysembryoplastic neuroepithelial tumors (n = 21), clear cell ependymomas (n = 8), clear cell meningiomas (n = 9) as well as 12 primary glioblastomas with oligodendrogial differentiation and 5 pilocytic astrocytomas with oligodendroglial-like differentiation were negative for H09 immunohistochemistry. Three oligodendrogliomas with neurocytic differentiation had evidence of IDH1/IDH2 mutations either by H09 immunohistochemistry or direct sequencing. We conclude that in tumors with an oligodendroglial-like morphology, binding of H09 is highly specific for oligodendrogliomas or oligoastrocytomas and substantially helps in the discrimination from other clear cell tumors. Negative H09 immunohistochemistry of an adult oligodendroglioma or oligoastrocytoma should prompt the consideration of other clear cell neoplasms. Further, our observations firmly assign oligodendrogliomas with neurocytic differentiation to the group of oligodendrogliomas and demonstrate that H09 is especially helpful for the difficult discrimination of such lesions from extraventricular neurocytomas.

Keywords Isocitrate dehydrogenase · IDH1 · R132H · Antibody · Oligodendroglioma · Clear cell

Introduction

Oligodendrogliomas represent a subset of diffusely infiltrating gliomas that are typically located in the cerebral hemispheres of adult patients and account for approximately 6–7% of all gliomas [24]. Well differentiated
oligodendrogliomas clinically correspond to WHO grade II (O II) and characteristically show uniformly distributed cells with monomorphic round nuclei, frequent calcifications and a delicate network of branching capillaries [17]. Anaplastic oligodendrogliomas WHO grade III (O III) additionally show increased pleomorphism, prominent mitotic activity, microvascular proliferation and occasionally necrosis [17]. Evidence of an additional astrocytic tumor cell component warrants the diagnosis of either oligoastrocytoma (OA II) or anaplastic oligoastrocytoma (OA III) [17]. Clear cell histology is a classic feature of all of these oligodendrogial lesions and is often integral to their diagnosis. This feature, characterized by a well-defined cell membrane, a clear cytoplasm and a central spherical nucleus is thought to represent autolytic cytoplasmic degeneration followed by acute cellular swelling. In line with this, clear cell morphology is generally not observed in frozen sections and in rapidly formalin-fixed specimens [17]. Various terms have been introduced to describe this typical histology, including perinuclear halo formation, “honey comb”, “fried egg” or “frog spawn” appearance. Although highly characteristic, clear cell morphology is not exclusive to oligodendrogliomas or oligoastrocytomas. Dysembryoplastic neuroepithelial tumors (DNT), central neurocytomas (central-NC), extraventricular neurocytomas (extraventricular-NC) and clear cell ependymomas (clear cell-E) may all exhibit prominent clear cell morphology and thus represent a differential diagnosis to oligodendroglioma or oligoastrocytoma [17]. Pilocytic astrocytoma may show a predominant oligodendroglial-like differentiation (PA-oligo) and then also constitutes a differential diagnosis [17]. Occasionally, primary glioblastomas contain foci that strongly resemble oligodendroglioma and may then be designated as glioblastoma with oligodendroglial differentiation (pGBM-oligo) [17]. These lesions may represent an important differential diagnosis to O III and OA III. Clear cell meningiomas (clear cell-M) often lack classic meningioma growth pattern and are characterized by the predominance of round to polygonal cells with clear cytoplasm [17]. Although several morphological features such as PAS positivity of the clear cells and the collagen-rich matrix may be helpful to differentiate these lesions from oligodendrogial tumors, primary brain tumors with clear cell morphology should be considered in the differential diagnosis.

Various immunohistochemical (IHC) markers have been studied for the differentiation of clear cell brain tumors. Preussser and colleagues [28] investigated a series of 60 oligodendrogial tumors and demonstrated widespread OLG2 expression in all cases, though equally strong expression in DNT and focal expression in 20% of both central-NC and clear cell-E limits its use as a differentiation marker. Prominent (>90%) NeuN expression has been proposed to distinguish central-NC from O II and clear cell-E [12], but focal and rarely even widespread NeuN expression has been documented in different series of oligodendrogliomas [29] and ependymomas [34]. In a study of 113 brain tumors, the oligodendrocytic marker Nogo-A was detected in 71% of oligodendrogliomas and 24% of glioblastomas, but was absent in astrocytomas and ependymomas [15]. Further markers helpful in the diagnosis of clear cell brain tumors are microtubule-associated protein-2, synaptophysin, epithelial membrane antigen or vimentin [3, 12]. While a combination of these markers may aid in the differentiation of most lesions, a highly specific and sensitive single IHC marker for oligodendrogliomas has not been found to date.

Recently high mutation rates of isocitrate dehydrogenase 1 (IDH1) have been reported in oligodendrogliomas, oligoastrocytomas and astrocytomas of WHO grades II and III [1, 9, 42, 45]. In these studies, IDH1 point mutations at codon 132 were found in 71–82% of O II, 67–86% of O III, 78–100% of OA II, and 66–100% of OA III. By far the most frequent mutation type results in an exchange of histidine for arginine (R132H). In one large study, this mutation type constituted 98 and 92% of all IDH1 mutations among O II and O III, respectively [9]. Oligodendrogliomas and oligoastrocytomas without IDH1 mutations are mutated in the analogous amino acid (R172) of the IDH2 gene in approximately one quarter of cases [9, 45]. In very rare cases mutations of codon 100 of IDH1 have also been detected in O III and diffuse astrocytoma WHO grade II [30]. Aside from acute myeloid leukemia [18], mutations of IDH1/IDH2 are highly specific for gliomas and have only been detected in single cases of other solid tumors [2, 10, 35].

We recently reported on the generation of a monoclonal antibody (clone H09; henceforth termed H09) specifically detecting IDH1 R132H mutated protein in routinely processed formalin-fixed paraffin-embedded tissue [4, 5]. In our previous study of H09 binding in brain tumors 16 of 16 O II and 14 of 16 O III were positive for H09 IHC [4]. Rates for OA II and OA III were comparably high. Here we investigate a large series of primary human brain tumors with clear cell morphology by H09 IHC. We confirm our previous observation of very high positive rates for oligodendrogliomas and oligoastrocytomas. We demonstrate that non-oligodendrogial tumors with clear cell morphology do not react with H09 and can thus be reliably differentiated from oligodendroglioma by H09 IHC. We further show that the determination of IDH1/IDH2 mutation status substantially helps in the difficult discrimination between oligodendrogliomas with neurocytic differentiation and extraventricular-NC.
Materials and methods

Human tumor specimens

A total of 274 formalin-fixed paraffin-embedded (FFPE) specimens of brain tumors presenting with focal or extensive clear cell morphology were retrieved from the Departments of Neuropathology of the Universities of Heidelberg, Tübingen, Münster, Zürich, and Frankfurt am Main. Initial diagnoses included O II, O III, OA II, AO III, pGBM-oligo, clear cell-E, DNT, central-NC, extraventricular neurocytic lesions, PA-oligo and clear cell-M. Of the specimens, 245 tumors derived from adult patients, while 29 were from pediatric patients (operated before the age of 18 years). All tissues were centrally reviewed as full sections hematoxylin and eosin (HE) stained slides and by additional IHC preparations as appropriate by an experienced investigator (D.C.) and diagnosed according to the 2007 WHO classification of central nervous system tumors [17]. All cases with discordant results to initial diagnosis or ambiguous histology were reviewed by a second experienced investigator (D.R.) and a consensus diagnosis was made. Pediatric oligodendrogliomas or oligoastrocytomas were defined as lesions operated before the age of 18 years. Due to low case numbers, pediatric oligodendrogliomas \( (n = 4) \) and pediatric oligoastrocytomas \( (n = 3) \) were combined in the group O-ped. All glioblastoma cases in this series presented as primary lesions and had at least focal clear cell morphology and were accordingly diagnosed as pGBM-oligo [17]. Some of these cases presented with combined oligoastrocytic differentiation and necrosis and were also classified as pGBM-oligo [17]. Cases with pGBM-oligo morphology and a confirmed lower grade precursor lesion (“secondary glioblastoma with oligodendroglial differentiation”) were not included in this study. Diagnosis of extraventricular neurocytic lesions was assisted by fluorescence in situ hybridization (FISH) for deletions of 1p/19q and NeuN IHC (methodological details below). Eighteen of the 25 oligoastrocytomas have been included in a previous series [4]. Review diagnoses and general patient characteristics are summarized in Table 1.

Immunohistochemistry

IHC for H09 (Dianova, Hamburg, Germany) was performed as previously described with slight adaptations [4]. In brief, sections cut to 4 μm were dried at 80°C for 15 min and further processed on a Ventana BenchMark XT® immunostainer (Ventana Medical Systems, Tucson, AZ, USA). After 60 min pretreatment with cell conditioner 2 (pH 6) the slides were incubated with 1:2 diluted H09 hybridoma supernatant at 37°C for 32 min. This dilution corresponds to a 1:30 dilution of the commercially available H09 antibody (Dianova). Antibody 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 chromogenic detection UltraView™Universal DAB Detection Kit (Ventana Medical Systems) was used. Slides were then removed from the immunostainer and mounted. A strong cytoplasmic immunoreaction product was scored positive. A weak diffuse staining and staining of macrophages were not scored positive. No chromogen was detected when primary antibody H09 was omitted from the procedure.

Automated IHC for neuronal nuclear protein (NeuN) and synaptophysin was performed by routine protocols on a Ventana BenchMark XT® immunostainer (NeuN: clone MAB377, dilution 1:100, 32 min incubation, Chemicon international, Temecula, USA; Synaptophysin: clone SY38, dilution 1:20, 32 min incubation, DakoCytomation, Glostrup, Denmark). For evaluation of NeuN, intensity \( (0 = \text{no staining}, 1–3 = \text{faintly, moderately, strongly positive}) \) was multiplied with the percentage of positive tumor cells \( (0 = \text{no positive cells}, 1 = \text{less than 10%}, 2 = 10–50\%, 3 = 51–80\% \) and \( 4 = \text{more than 80% positive cells} \) resulting in the immuno-reactive score (IRS) ranging from 0 to 12 as described by Remmele and Stegner [32]. An IRS score of three or higher is considered positive. Isolated perinuclear staining was not taken into account for this evaluation. Isolated staining of dysplastic neurons was also not scored positive, as this pattern could not reliably be differentiated from the staining of preexisting distorted neurons. For evaluation of synaptophysin IHC, cortical infiltration was carefully excluded, often with the aid of NeuN highlighting the cortex. A strong diffuse staining of the neuropil surrounding the tumor cells was considered positive. A perinuclear dot like pattern was not considered positive. Two-tailed Fisher’s exact test was used to examine the association of the presence or absence of H09 binding in oligodendrogial and non-oligodendrogial tumors.

Analysis of 1p and 19q losses by fluorescence in situ hybridization (FISH)

All H09-negative O II and O III, all extraventricular neurocytic lesions (extraventricular-NC and oligodendroglioma with neurocytic differentiation) as well as 6 central-NC and 6 O II for control were further analyzed by fluorescence in situ hybridization (FISH) for deletions of 1p and 19q on FFPE tissue. The two-color FISH assay was performed on 4-μm-thick sections using a mixed 1p36/1q25 dual color probe and 19p13/19q13 dual color probe set (ZytoLight SPEC, Cat. No Z-2075 and Z-2076, ZytoVision, Bremerhaven, Germany). For slide pretreatment, probe hybridization and post-hybridization processing, the Histology Accessory FISH Kit (Dako, Glostrup, Denmark)
was used. Nuclei were counterstained with DAPI/Antifade-Solution (ZytoVision) and fluorescent signals were enumerated with an Olympus BX50 fluorescent microscope with appropriate filters (Olympus, Hamburg, Germany). Samples showing sufficient FISH efficiency (~80% nuclei with signals) were evaluated. Signals were scored in at least 200 non-overlapping, intact nuclei. Deletions of 1p or 19q were defined by over 50% of tumor nuclei containing one signal, in hyperdiploid cases over 50% of tumor nuclei containing half the signals of the control probe.

PCR amplification and direct sequencing

DNA for direct sequencing was extracted from FFPE tumor tissue using Invisorb Genomic DNA Kit II (Invitek, Berlin, Germany). Tumor area was confirmed on a HE-stained slide of the respective paraffin block. When at least 80% of the area represented solid tumor 10 sections of 10 μm were directly cut into an Eppendorf tube and processed. If the total tumor area was smaller, 10-μm-thick empty slides were cut and relevant tumor areas were microdissected for DNA isolation. Primer design was based on accession number NM_005896 for IDH1 and NM_002168 for IDH2.

A fragment of 178 bp including codon 100 and 132 of IDH1 was amplified using 60 ng each of forward primer IDH1f TGATGAGAAGGGTTGAGGA and reverse primer IDH1r ATCCCCATAAGCAGCAGAC. A 173-bp fragment spanning codon 140 of IDH2 was amplified with 60 ng each of forward primer IDH2_140f QCTGCAG TGGGACCATATT and reverse primer IDH2_140r AATGGTGATGGGCTTGGTC. A 150-bp fragment spanning codon 172 of IDH2 was amplified with 60 ng each of forward primer IDH2_172f AGCCCATCATCTCGGAAA AAC and reverse primer IDH2_172r CTAGGGCAGGAG CTCCAGT. PCR was performed under standard buffer conditions, using 20 ng of tumor DNA and GoTaq® DNA Polymerase (Promega, Madison, WI, USA) and employed 35 cycles with denaturation at 95°C for 30 s, annealing at 56°C for 40 s and extension at 72°C for 50 s in a total volume of 15 μl.

Two microliters of the PCR amplification product were submitted to the sequencing reaction using the BigDye® Terminator v3.1 Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Twenty-five cycles were performed employing 12 ng of the sense primer IDH1f for IDH1 and the antisense primers IDH2_140r and IDH2_172r (see above) for IDH2, with denaturation at 95°C for 30 s, annealing at 56°C for 40 s and extension at 72°C for 50 s in a total volume.

In case of ambiguous readings, a second round of sequencing analysis was performed using the respective antisense primers (see above) and the same sequencing reaction conditions. Sequences were determined using the semiautomated sequencer (ABI® 3100 Genetic Analyzer, Applied Biosystems) and the Sequence Pilot version 3.1™ (JSI-Medisys, Kippenheim, Germany) software.

Results

H09 immunohistochemistry in adult oligodendroglialomas and oligoastrocytomas

Positive and negative scoring was unequivocal in all investigated cases. The majority of positive cases

<table>
<thead>
<tr>
<th>Tumor diagnosis</th>
<th>n</th>
<th>Location</th>
<th>Age median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O II</td>
<td>74</td>
<td>36 frontal, 11 temporal, 12 par-occ, 1 intraventricular, 1 cerebellar, 13 NA</td>
<td>44 (18–70)</td>
</tr>
<tr>
<td>O III</td>
<td>69</td>
<td>33 frontal, 4 temporal, 11 par-occ, 21 NA</td>
<td>46 (24–71)</td>
</tr>
<tr>
<td>OA II</td>
<td>14</td>
<td>7 frontal, 6 temporal, 1 par-occ</td>
<td>36 (26–66)</td>
</tr>
<tr>
<td>OA III</td>
<td>11</td>
<td>5 frontal, 4 temporal, 2 par-occ</td>
<td>41 (32–77)</td>
</tr>
<tr>
<td>O-NC</td>
<td>3</td>
<td>1 frontal, 1 par-occ, 1 NA</td>
<td>51 (48–73)</td>
</tr>
<tr>
<td>O-ped</td>
<td>7</td>
<td>2 frontal, 1 temporal, 1 par-occ, 3 NA</td>
<td>11 (2–17)</td>
</tr>
<tr>
<td>Central-NC</td>
<td>35</td>
<td>1 frontal, 1 temporal, 11 intraventricular, 22 NA</td>
<td>29 (6–73)</td>
</tr>
<tr>
<td>Extraventricular-NC</td>
<td>4</td>
<td>1 frontal, 1 temporal, 2 par-occ</td>
<td>28 (22–43)</td>
</tr>
<tr>
<td>Cerebellar liponeurocytoma</td>
<td>2</td>
<td>2 cerebellar</td>
<td>(22–49)</td>
</tr>
<tr>
<td>DNT</td>
<td>21</td>
<td>1 frontal, 4 temporal, 2 par-occ, 14 NA</td>
<td>21 (4–52)</td>
</tr>
<tr>
<td>Clear cell-E</td>
<td>8</td>
<td>2 frontal, 1 par-occ, 3 infratentorial, 2 NA</td>
<td>25 (7–78)</td>
</tr>
<tr>
<td>Clear cell-M</td>
<td>9</td>
<td>6 supratentorial, 1 infratentorial, 2 NA</td>
<td>35 (10–74)</td>
</tr>
<tr>
<td>pGBM-oligo</td>
<td>12</td>
<td>1 frontal, 7 temporal, 3 par-occ, 1 NA</td>
<td>61 (16–77)</td>
</tr>
<tr>
<td>PA-oligo</td>
<td>5</td>
<td>1 frontal, 1 temporal, 3 cerebellar</td>
<td>8 (4–33)</td>
</tr>
</tbody>
</table>

NA not available, par-occ parietal or occipital lobe
demonstrated a strong perinuclear cytoplasmic staining with additional weaker nuclear staining (Fig. 2a, b). In the infiltration zone, tumor cells with elongated positive processes and little perinuclear staining were readily identifiable in some positive cases, both in oligodendrogial and oligoastrocytic lesions. In cell-dense areas the staining was occasionally so intense that single cells were not discernible, while intratumoral vessels and adjoining CNS tissue remained negative (Fig. 2c, d). In our series of 74 O II, 67 tumors were scored positive (91%, Fig. 1). O III demonstrated an even higher rate of positive cases with 65 of 69 tumors positively labeled by H09 (94%, Fig. 1). Rates for OA II and OA III were also high, with 11 of 14 OA II (79%) and 10 of 11 OA III (91%) positive for H09 (Fig. 1). Histology of H09-negative oligodendrogliomas and oligoastrocytomas did not differ from that of positive cases.

Further characterization of H09-negative cases was performed by direct sequencing of IDH1/IDH2, 1p19q FISH analysis and additional IHC preparations. Sufficient material for DNA extraction was available for 14 of 15 H09-negative cases. Three of the four H09-negative O III harbored other mutations of either IDH1 (R132S) or IDH2 (R172K and R172M) while none of the H09-negative O II revealed mutations in these hotspots (Fig. 1; Table 2). Among the four H09-negative oligoastrocytomas, two OA II exhibited an IDH2 mutation (R172K and R172M, Fig. 1; Table 2). Combining the numbers of H09-positive cases with mutations detected by sequencing increases the rate of O III with evidence of IDH1/IDH2 mutations to almost 99% (68 mutated of 69) and the rate of OA II to 93% (13 mutated of 14). FISH analysis for deletions of chromosomal arms 1p or 19q was performed for H09-negative oligodendrogliomas (Table 2). Four of the 11 cases were not evaluable due to low FISH efficiency. Five of the remaining seven cases demonstrated combined deletions of 1p/19q, two cases had no 1p/19q losses. Two tumors with IDH1/IDH2 mutations other than R132H were evaluable by FISH and both had combined deletions of 1p/19q. Additional IHC was performed for the neuronal markers synaptophysin and NeuN (Table 2). All tumors were scored negative for synaptophysin and NeuN (all IRS 0). Clinical follow-up data was limited for these cases and revealed no unexpected clinical courses (Table 2).

H09 immunohistochemistry in brain tumors with oligodendroglioma-like morphology

In contrast to the high rates of positive cases among adult oligodendroglioma and oligoastrocytoma, all investigated cases of central-NC (n = 35, Fig. 3a, b), DNT (n = 21, Fig. 3c, d), clear cell-E (n = 8, Fig. 3e, f), PA-oligo (n = 5, Fig. 3g, h), O-ped (n = 7), pGBM-oligo (n = 12), clear cell-M (n = 9) and cerebellar liponeurocytoma (n = 2) were negative for H09 IHC (Fig. 1). Statistical analysis with two-tailed Fisher’s exact test demonstrated that H09 is highly significantly associated with oligodendroglioma/oligoastrocytoma when compared with the combined group of non-oligodendroglioma clear cell brain tumors (p < 0.0001). Corresponding results were obtained when only adult tumors were taken into analysis. The overall sensitivity of positive H09 staining for adult oligodendrogial/oligoastrocytic tumors was 91% (153 positive of 169 oligodendroglial/oligoastrocytic tumors), the specificity was 100% (76 negative of 76 non-oligodendroglial tumors).

Fig. 1 H09 immunohistochemistry and IDH1/2 sequencing of clear cell brain tumors. All cases were analyzed by IDH1 R132H mutation-specific antibody H09. In tumor types with at least one H09-positive case, all negative tumors were sequenced for other mutations of IDH1 or IDH2 (for details of mutations see Tables 2 and 3). Percentages are given for tumor types with at least 10 analyzed cases.
Seven extraventricular neurocytic lesions were also investigated (Table 3). As known for such lesions, histology of this tumor group was heterogeneous and ranged from classical neurocytoma morphology with frequent neuropil islands (Case 53308, Fig. 4a) to less cellular cases with frequent ganglioid cells (Cases 52082, 52074, 53342) or tumors with monomorphous cytology, single neuropil islands and Homer Wright rosettes (Cases 53042, 53512, Fig. 4b) or one case with monomorphous neurocytic cytology, frequent mitotic figures and microvascular proliferation (Case 53510). All cases were strongly positive for synaptophysin (Table 3). IHC for NeuN revealed that

### Table 2 Characterization of H09 negative oligodendrogliomas and oligoastrocytomas

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Diagnosis</th>
<th>Age</th>
<th>Gen</th>
<th>Location</th>
<th>Syn</th>
<th>NeuN</th>
<th>FISH 1p/19q</th>
<th>IDH1 (100/132)</th>
<th>IDH2 (140/172)</th>
<th>Follow-up</th>
</tr>
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<tbody>
<tr>
<td>52072</td>
<td>O II</td>
<td>39</td>
<td>f</td>
<td>r. frontal</td>
<td>neg</td>
<td>neg</td>
<td>del/del</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>10.1 years, DOD</td>
</tr>
<tr>
<td>53310</td>
<td>O II</td>
<td>33</td>
<td>f</td>
<td>r. central</td>
<td>neg</td>
<td>neg</td>
<td>ret/ret</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>NA</td>
</tr>
<tr>
<td>41388</td>
<td>O II</td>
<td>18</td>
<td>f</td>
<td>r. par-temp-occ</td>
<td>neg</td>
<td>neg</td>
<td>ret/ret</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>5.8 years, no rec</td>
</tr>
<tr>
<td>53272</td>
<td>O II</td>
<td>67</td>
<td>f</td>
<td>l. frontal</td>
<td>neg</td>
<td>neg</td>
<td>ND/ND</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>NA</td>
</tr>
<tr>
<td>53270</td>
<td>O II</td>
<td>49</td>
<td>f</td>
<td>r. central</td>
<td>neg</td>
<td>neg</td>
<td>del/del</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>1.4 years, no rec</td>
</tr>
<tr>
<td>53500</td>
<td>O II</td>
<td>49</td>
<td>f</td>
<td>r. temporal</td>
<td>neg</td>
<td>neg</td>
<td>ND/ND</td>
<td>ND/ND</td>
<td>ND/ND</td>
<td>NA</td>
</tr>
<tr>
<td>41082</td>
<td>O II</td>
<td>49</td>
<td>m</td>
<td>r. central</td>
<td>neg</td>
<td>neg</td>
<td>del/del</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>NA</td>
</tr>
<tr>
<td>51994</td>
<td>OA II</td>
<td>32</td>
<td>m</td>
<td>l. frontal</td>
<td>neg</td>
<td>neg</td>
<td>NP</td>
<td>wt/wt</td>
<td>wt/R172 K</td>
<td>4.2 years, rec</td>
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<tr>
<td>51996</td>
<td>OA II</td>
<td>37</td>
<td>m</td>
<td>r. frontal</td>
<td>neg</td>
<td>neg</td>
<td>NP</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>9.1 years, no rec</td>
</tr>
<tr>
<td>51360</td>
<td>OA II</td>
<td>40</td>
<td>f</td>
<td>l. temporal</td>
<td>neg</td>
<td>neg</td>
<td>NP</td>
<td>wt/wt</td>
<td>wt/R172 M</td>
<td>7 years, no rec</td>
</tr>
<tr>
<td>52076</td>
<td>O III</td>
<td>35</td>
<td>F</td>
<td>r. par-occ</td>
<td>neg</td>
<td>neg</td>
<td>ND/ND</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>NA</td>
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<tr>
<td>52066</td>
<td>O III</td>
<td>42</td>
<td>m</td>
<td>r. temp-occ</td>
<td>neg</td>
<td>neg</td>
<td>ND/ND</td>
<td>wt/wt</td>
<td>wt/R172 K</td>
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<tr>
<td>44826</td>
<td>O III</td>
<td>44</td>
<td>f</td>
<td>r. parietal</td>
<td>neg</td>
<td>neg</td>
<td>del/del</td>
<td>wt/R132S</td>
<td>wt/wt</td>
<td>1.3 years, no rec</td>
</tr>
<tr>
<td>45808</td>
<td>O III</td>
<td>52</td>
<td>f</td>
<td>l. frontal</td>
<td>neg</td>
<td>neg</td>
<td>del/del</td>
<td>wt/wt</td>
<td>wt/R172 M</td>
<td>3.7 years, rec</td>
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<tr>
<td>41252</td>
<td>OA III</td>
<td>77</td>
<td>m</td>
<td>l. par-occ</td>
<td>neg</td>
<td>neg</td>
<td>NP</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>2.6 years, no rec</td>
</tr>
</tbody>
</table>

**O** II oligodendroglioma WHO grade II, **O** III oligodendroglioma WHO grade III, **OA** II oligoastrocytoma WHO grade II, **OA** III oligoastrocytoma WHO grade III, **Gen** gender, **m** male, **f** female, **Syn** synaptophysin, **DOD** died of disease, **NA** not available, **NP** not performed, **ND** not determinable, **no rec** no recurrence, **rec** recurrence, **del** deletion, **ret** retention, **neg** negative, **IRS** immuno-reactive score

### IDH1 mutations in extraventricular neurocytic lesions

**Fig. 2** H09 immunohistochemistry of oligodendroglioma. Hematoxylin and eosin (HE) stained slide of grade II oligodendroglioma (a) and IHC with H09 of the same area showing strongly positive tumor cells (b). HE of grade III oligodendroglioma (c) and immunohistochemistry of the same area with very intense immunoreaction (d).

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three cases were negative (IRS 0–2) while the other four cases demonstrated a nuclear reaction in a fraction of oligoid appearing tumor cells ranging from approximately 20–60% (IRS 4–6) and were scored positive (Table 3). We additionally investigated a series of H09-positive O II and O III for NeuN IHC. All O II \((n = 39)\) and O III \((n = 34)\) were scored negative (71 cases IRS 0, 1 case IRS 1 and 1 case IRS 2, data not shown). FISH analysis of the seven putative extraventricular neurocytic lesions demonstrated combined deletions of 1p/19q in the three tumors negative for NeuN while the four cases positive for NeuN had no losses of 1p/19q (Table 3; Fig. 4g, h). The four cases with NeuN-positive cell compartment and no evidence of chromosomal deletions at 1p/19q were classified as extraventricular neurocytomas (extraventricular-NC); the three NeuN-negative cases with deletions of 1p/19q were
Table 3  Characterization of extraventricular neurocytic lesions

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Diagnosis</th>
<th>Age</th>
<th>Gen</th>
<th>Location</th>
<th>Syn</th>
<th>NeuN</th>
<th>FISH 1p/19q</th>
<th>IDH1 (100/132)</th>
<th>IDH2 (140/172)</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>52082</td>
<td>E-NC</td>
<td>43</td>
<td>m</td>
<td>r. parietal</td>
<td>pos</td>
<td>pos (IRS 4)</td>
<td>ret/ret</td>
<td>wt/wt</td>
<td>ND/wt</td>
<td>NA</td>
</tr>
<tr>
<td>52074</td>
<td>E-NC</td>
<td>22</td>
<td>m</td>
<td>r. temporal</td>
<td>pos</td>
<td>pos (IRS 4)</td>
<td>ret/ret</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>NA</td>
</tr>
<tr>
<td>53308</td>
<td>E-NC</td>
<td>28</td>
<td>f</td>
<td>r. par-occ</td>
<td>pos</td>
<td>pos (IRS 4)</td>
<td>ret/ret</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>21.8 years, no rec</td>
</tr>
<tr>
<td>53342</td>
<td>E-NC</td>
<td>28</td>
<td>m</td>
<td>r. frontal</td>
<td>pos</td>
<td>pos (IRS 6)</td>
<td>ND/ret</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>2.4 years, DOC</td>
</tr>
<tr>
<td>53042</td>
<td>O-NC</td>
<td>51</td>
<td>m</td>
<td>l. frontal</td>
<td>pos</td>
<td>neg (IRS 0)</td>
<td>del/del</td>
<td>wt/wt</td>
<td>wt/R172S</td>
<td>NA</td>
</tr>
<tr>
<td>53510</td>
<td>O-NC</td>
<td>73</td>
<td>m</td>
<td>r. parietal</td>
<td>pos</td>
<td>pos (IRS 0)</td>
<td>del/del</td>
<td>wt/R132H</td>
<td>ND</td>
<td>2.3 years, no rec</td>
</tr>
<tr>
<td>53512</td>
<td>O-NC</td>
<td>48</td>
<td>f</td>
<td>NA</td>
<td>pos</td>
<td>neg (IRS 2)</td>
<td>del/del</td>
<td>wt/R132H</td>
<td>ND</td>
<td>0.9 years, rec</td>
</tr>
</tbody>
</table>

E-NC extraventricular neurocytoma, O-NC oligodendroglioma with neurocytic differentiation, Gen gender, m male, f female, Syn Synaptophysin, NA not available, ND not determinable, pos positive, neg negative, del deletion, ret retention, no rec no recurrence, rec recurrence, DOC died of other causes, IRS immuno-reactive score

diagnosed as oligodendroglioma with neurocytic differentiation (O-NC). IHC with H09 marked 2 of 3 O-NC positive, while all extraventricular-NC were negative (Fig. 1). IDH1 and IDH2 direct sequencing of the H09-negative O-NC revealed an IDH2 mutation resulting in an arginine to serine substitution at codon 172 (R172S). Sequencing of the four extraventricular-NC did not reveal any IDH1/IDH2 mutation. Clinical follow-up for extraventricular neurocytic lesions was limited (Table 3). Of note, the extraventricular-NC with classical neurocytoma morphology (Case 53308) had a very long recurrence-free follow-up of 21.8 years. One of the H09-positive O-NC (Case 53512) had tumor recurrence within 1 year.

Additionally, FISH was performed for six central-NC and six H09-positive O II as control cases. Of the control cases, one central-NC was not evaluable due to low FISH efficiency. As expected, none of the remaining five central-NC cases exhibited a deletion of 1p or 19q while all six O II exhibited this genomic aberration (data not shown).

Discussion

Tumors with oligodendroglioma-like morphology have a diverse biological behavior ranging from benign WHO grade I lesions (e.g., pilocytic astrocytoma or DNT) to highly malignant WHO grade III or IV tumors. Correct diagnosis is paramount for optimal patient treatment and prediction of clinical course. In this study we investigated the application of IDH1 R132H mutation-specific antibody H09 in the differentiation between oligodendrogliomas or oligoastrocytomas and tumors with similar morphology.

Among oligodendrogliomas and oligoastrocytomas, H09 IHC demonstrated very high rates of cases with evidence of IDH1 R132H mutation. The rates are higher than in most reports relying on direct sequencing of IDH1 [1, 9, 42, 45]. Three reasons likely contribute to this difference. First, in the current study, adult and pediatric oligodendrogliomas were analyzed as separate tumor groups. This was done because there is evidence that despite similar histology, pediatric oligodendrogliomas represent a genetically distinct group without the “molecular signature” of 1p/19q co-deletion typical for adult oligodendrogliomas [14, 31]. In our preceding genetic study, 4 of 5 O-ped cases were IDH1/IDH2 wt [9]. In the study of Yan and colleagues [45] the median age of O II patients with IDH1 wt status was 13.5 years, indicating that the majority of tumors without mutation in this series were pediatric cases. Thus, high numbers of pediatric oligodendrogliomas in a tumor series bias towards IDH1/IDH2 wt status. In accordance with this, all seven O-ped of this study were negative for H09 IHC. The second reason for our relatively high mutation rates may relate to our past observation that direct sequencing bears the risk of underestimating the frequency of IDH1 mutations in diffuse gliomas [4]. In our previous series, IHC with H09 detected several IDH1 R132H mutations that were initially missed by direct sequencing but were confirmed after re-sequencing of additional tumor material. This demonstrates that suboptimal tumor sampling prior to DNA extraction may also bias a tumor series towards IDH1/IDH2 wt status. Third, prior to our analyses, all cases of this series received reference evaluation according to current WHO classification of brain tumors [17]. This resulted in change of diagnosis for several lesions. The most frequent change was from O III or OA III to pGBM-oligo (n = 6) followed by reclassification of two O II to clear cell-E and two O II to PA-oligo. All cases reclassified as non-oligodendrogial tumors by criteria of the current WHO classification were found to be negative for H09 IHC.

In accordance with previous studies [9, 45], three oligodendrogliomas and two oligoastrocytomas without IDH1 R132H mutation had other mutations of IDH1 or IDH2. The combination of H09 IHC and direct sequencing of IDH1/IDH2 results in a mutation rate of almost 99% for O III with only 1 case of 69 without evidence of a mutation. Due to these exceedingly high mutation rates future WHO classifications may consider including IDH1/IDH2
mutations in the definition of WHO grade III oligodendroglomas. For the present, O III without IHC or genetic evidence of IDH1/IDH2 mutations should be critically reviewed and other differential diagnoses, especially glioblastoma should be taken into consideration.

We recently observed a rare new type of heterozygous IDH1 mutation occurring at codon 100 [30]. The common consequence of all so far investigated IDH1 and IDH2 mutations is the production of 2-hydroxyglutarate [7, 41]. It is attractive to speculate that additional types of mutations either at other positions of IDH1/IDH2 or of other enzymes involved in similar enzymatic processes are still awaiting detection and that the production of 2-hydroxyglutarate is a prerequisite for the development of oligodendrogial tumors.

Our series included several cases of O II and OA II without evidence of IDH1/IDH2 mutations. All these cases...
had a typical histology and no indication of further differentiation. Three of five evaluable O II cases had combined losses of 1p/19q. Our method of investigating deletions of 1p/19q by FISH does not distinguish complete from incomplete deletions, so it is possible that these cases harbor incomplete deletions as recently indicated by a comparative genomic hybridization study [11, 16].

The very high IDH1 mutation rates among oligodendrogliomas and oligoastrocytomas offers great diagnostic potential for H09 IHC. Our study demonstrates that among clear cell brain tumors H09 immunoreaction is exclusive to oligodendrogliomas and oligoastrocytomas. This is in accordance with the very low mutation rates previously described for ependymomas, glioblastomas with oligodendroglial differentiation, pilocytic astrocytomas and DNT [1, 4, 10, 13, 36, 42]. The distinction between primary and secondary glioblastomas with oligodendroglial differentiation appears to be important [23]. In our experience IDH1/IDH2 mutation status is retained in all cases during malignant progression. We have observed only one single case with focal loss of an IDH1 mutation in secondary glioblastoma [30]. Thus, secondary glioblastomas with oligodendroglial differentiation that have developed from lower grade lesions are expected to have mutation rates equal to the high rates in WHO grade II and III oligodendroglioma/oligoastrocytoma. In contrast, the 12 primary glioblastomas with oligodendroglial differentiation of the present series did not have mutations, similar to the low rate of IDH1/IDH2 mutations in classical primary glioblastomas. Figure 5 gives an algorithm for the diagnosis of adult brain tumors with oligoid morphology based on H09 IHC.

Neurocytic lesions analyzed for H09 IHC included central-NC, cerebellar liponeurocytoma, extrventricular-NC and O-NC. Mutations of IDH1/IDH2 were detected in all O-NC, none were found in the other neurocytic lesions. The differentiation of extrventricular-NC and oligodendroglioma is a well-recognized diagnostic challenge [6, 19, 21, 38, 40]. In 2002, Perry et al. [27] proposed the diagnosis of oligodendroglioma with neurocytic differentiation and discussed a histogenetic link between oligodendroglioma and extrventricular-NC. Since then several cases have been reported that are consistent with this diagnosis [8, 20, 29, 39]. Indeed, the detection of neuronal markers in a subset of oligodendrogliomas [12, 29, 43, 44] as well as ultrastructural evidence of neuronal differentiation in some cases [22] indicates a spectrum of neuronal differentiation that may span from classical oligodendroglioma to the extreme end of oligodendroglioma with complete neurocytic morphology or even gangliocytic differentiation [25]. In line with our diagnostic approach, most studies classified these lesions primarily as oligodendrogial, based on the finding of deletions of 1p/19q in the majority of cases [8, 20, 27, 39]. The specificity of this genetic aberration as the "molecular signature" of oligodendrogial tumors has recently been challenged by the observation that approximately one quarter of extrventricular-NC also harbor co-deletions of 1p/19q [26, 33]. In this study we demonstrate that all extrventricular neurocytic lesions with 1p/19q co-deletions are further characterized by a second genetic marker typical for oligodendrogliomas (presence of IDH1/IDH2 mutation). The combination of 1p/19q co-deletion and IDH1/IDH2 mutation strongly supports the diagnosis of a primarily oligodendrogial lesion and firmly assigns O-NC to the group of oligodendrogliomas. Therefore, H09 IHC is a diagnostic tool that may substantially help in the challenging differentiation of O-NC from extrventricular-NC. Interestingly, all investigated oligodendrogliomas (H09 positive or negative) of this series were negative for NeuN, a marker generally positive in central-NC [37] and positive in all extrventricular-NC of this series. Thus in our hands, NeuN IHC was also able to differentiate O-NC.

![Fig. 5 Diagnostic algorithm for brain tumors with oligodendroglioma-like morphology based on H09 immunohistochemistry](image-url)
from extraventricular-NC and may be helpful as an additional marker besides H09 IHC. These results have to be interpreted with caution though, as previous studies have described several cases of NeuN-positive oligodendrogliomas [12, 29].

In conclusion, we demonstrate that the vast majority of adult oligodendrogliomas and oligoastrocytomas have mutations of either IDH1 or IDH2. IHC with the IDH1 R132H mutation-specific antibody H09 has great potential to identify oligodendrogliomas or oligoastrocytomas among clear cell brain tumors. Pediatric oligodendrogliomas elude this differentiation, as IDH1 mutations are frequently absent in these lesions. Our data further indicate that IHC with H09 may represent a powerful marker to differentiate extraventricular-NC from O-NC.

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Conflict of interest Under a licensing agreement between DIA-Nova GmbH, Hamburg, Germany, and the German Cancer Research Center in accordance with its conflict of interest policies.

References


