Acta Neuropathol
DOI 10.1007/s00401-010-0770-2

ORIGINAL PAPER

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

David Capper · David Reuss · Jens Schittenhelm · Christian Hartmann · Juliane Bremer · Felix Sahm · Patrick N. Harter · Astrid Jeibmann · Andreas von Deimling

Received: 31 August 2010 / Revised: 26 October 2010 / Accepted: 26 October 2010
© Springer-Verlag 2010

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 oligodendroglial 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 oligodendroglioma-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

D. Capper · D. Reuss · C. Hartmann · F. Sahm · A. von Deimling (✉)
Department of Neuropathology, Institute of Pathology, Ruprecht-Karls-Universität Heidelberg, Im Neuenheimer Feld 220/221, 69120 Heidelberg, Germany
e-mail: andreas.vondeimling@med.uni-heidelberg.de

D. Capper · D. Reuss · C. Hartmann · A. von Deimling
Clinical Cooperation Unit Neuropathology, German Cancer Research Center, Heidelberg, Germany

J. Schittenhelm
Institute of Brain Research, University Hospital Tuebingen, Tübingen, Germany

J. Bremer
Institute of Neuropathology, University Hospital of Zürich, Zürich, Switzerland

P. N. Harter
Institute of Neurology (Edinger Institute), Goethe University School of Medicine, Frankfurt am Main, Germany

A. Jeibmann
Institute of Neuropathology, University Hospital Münster, Münster, Germany

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

Published online: 11 November 2010

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 oligodendroglial 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 oligodendroglial 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. Preusser and colleagues [28] investigated a series of 60 oligodendroglial tumors and demonstrated widespread OLIG2 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-oligodendroglial 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 section 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 = no staining, 1–3 = faintly, moderately, strongly positive) was multiplied with the percentage of positive tumor cells (0 = no positive cells, 1 = less than 10%, 2 = 10–50%, 3 = 51–80% and 4 = 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 perinecrotic nuclear 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 oligodendroglial and non-oligodendroglial 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 (Zyto*Light* 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)

Table 1 Review diagnoses and general patient characteristics

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

NA not available, *par-occ* parietal or occipital lobe

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 ($\sim$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 TGATGAGAAGAGGGTTGAGGA and reverse primer IDH1r ATCCCCATAAGCATGACGAC. A 173-bp fragment spanning codon 140 of *IDH2* was amplified with 60 ng each of forward primer IDH2_140f QCTGCAG TGGGACCACTATT 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 AGCCCATCATCTGCAAA AAC and reverse primer IDH2_172r CTAGGCGAGGAG 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 15 s and extension at 60°C for 240 s. 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 oligodendrogliomas and oligoastrocytomas

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

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 oligodendroglial 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 und 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 oligodendroglial/oligoastrocytic tumors was 91% (153 positive of 169 oligodendroglial/oligoastrocytic tumors), the specificity was 100% (76 negative of 76 non-oligodendroglial tumors).

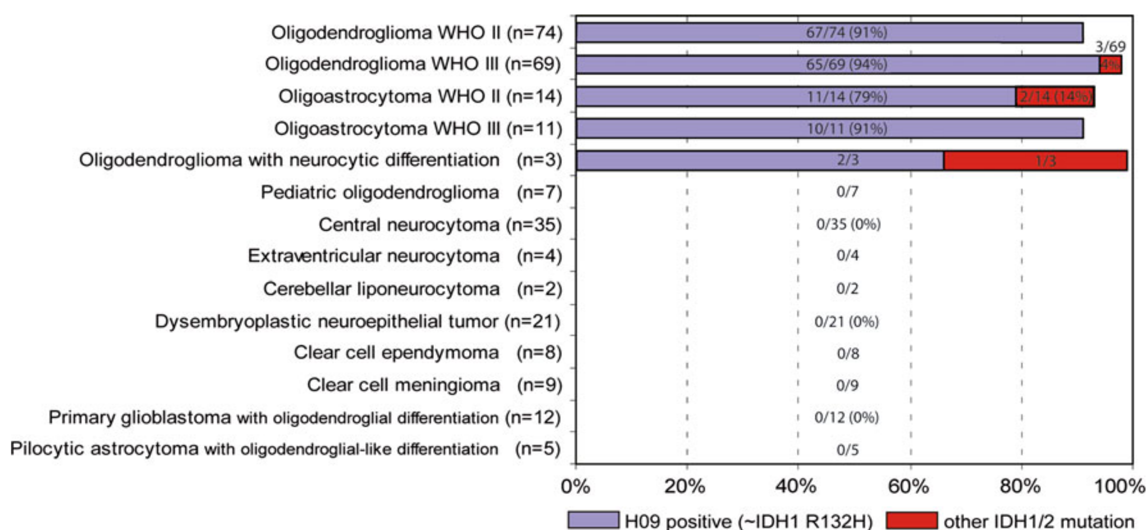

Fig. 1 H09 immunohistochemistry and IDH1/2 sequencing of clear cell brain tumors. All cases were analyzed by IDH1 R132H mutation-specific antibody H09. In tumor types with at least one H09-positive case, all negative tumors were sequenced for other mutations of IDH1 or IDH2 (for details of mutations see Tables 2 and 3). Percentages are given for tumor types with at least 10 analyzed cases

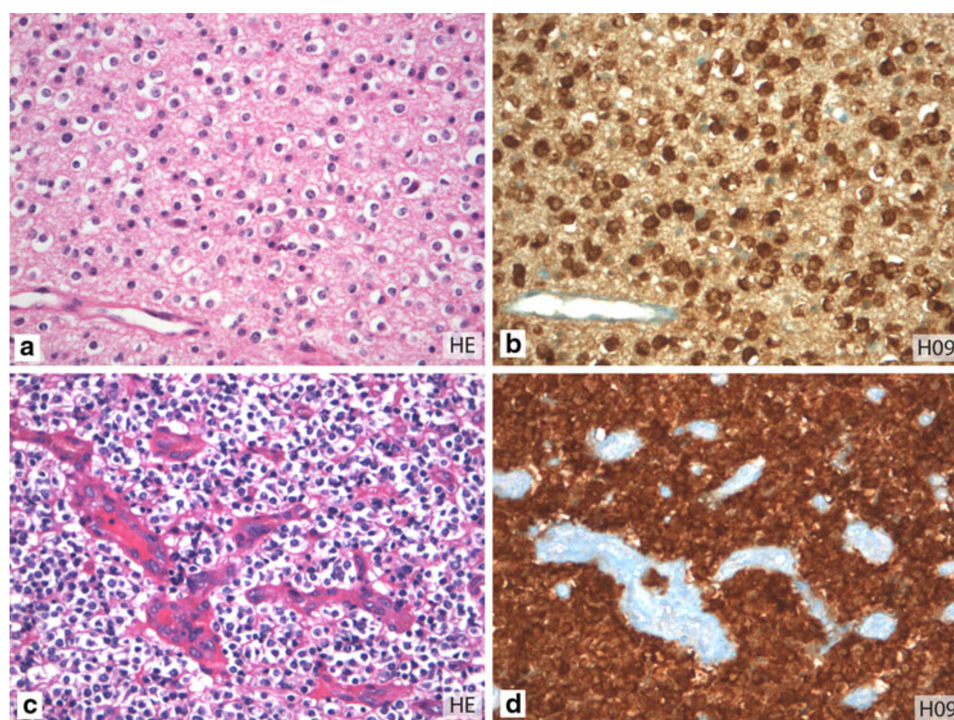

Fig. 2 H09 immunohistochemistry of oligodendroglioma. Hematoxylin and eosin (*HE*) stained slide of grade II oligodendroglioma (**a**) and IHC with H09 of the same area showing strongly positive tumor cells (**b**). HE of grade III oligodendroglioma (**c**) and immunohistochemistry of the same area with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

Table 2 Characterization of H09 negative oligodendrogliomas and oligoastrocytomas

Case no.	Diagnosis	Age	Gen	Location	Syn	NeuN	FISH 1p/19q	IDH1 (100/132)	IDH2 (140/172)	Follow-up
52072	O II	39	f	r. frontal	neg	neg (IRS 0)	del/del	wt/wt	wt/wt	10.1 years. DOD
53310	O II	33	f	r. central	neg	neg (IRS 0)	ret/ret	wt/wt	wt/wt	NA
41388	O II	18	f	r. par-temp-occ	neg	neg (IRS 0)	ret/ret	wt/wt	wt/wt	5.8 years, no rec
53272	O II	67	f	l. frontal	neg	neg (IRS 0)	ND/ND	wt/wt	wt/wt	NA
53270	O II	49	f	r. central	neg	neg (IRS 0)	del/del	wt/wt	wt/wt	1.4 years, no rec
53500	O II	49	f	r. temporal	neg	neg (IRS 0)	ND/ND	ND	ND	NA
41082	O II	49	m	r. central	neg	neg (IRS 0)	del/del	wt/wt	wt/wt	NA
51994	OA II	32	m	l. frontal	neg	neg (IRS 0)	NP	wt/wt	wt/R172 K	4.2 years, rec
51996	OA II	37	m	r. frontal	neg	neg (IRS 0)	NP	wt/wt	wt/wt	9.1 years no rec
51360	OA II	40	f	l. temporal	neg	neg (IRS 0)	NP	wt/wt	wt/R172 M	7 years no rec
52076	O III	35	F	r. par-occ	neg	neg (IRS 0)	ND/ND	wt/wt	wt/wt	NA
52066	O III	42	m	r. temp-occ	neg	neg (IRS 0)	ND/ND	wt/wt	wt/R172 K	NA
44826	O III	44	f	r. parietal	neg	neg (IRS 0)	del/del	wt/R132S	wt/wt	1.3 years, no rec
45808	O III	52	f	l. frontal	neg	neg (IRS 0)	del/del	wt/wt	wt/R172 M	3.7 years, rec
41252	OA III	77	m	l. par-occ	neg	neg (IRS 0)	NP	wt/wt	wt/wt	2.6 years, no rec

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

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

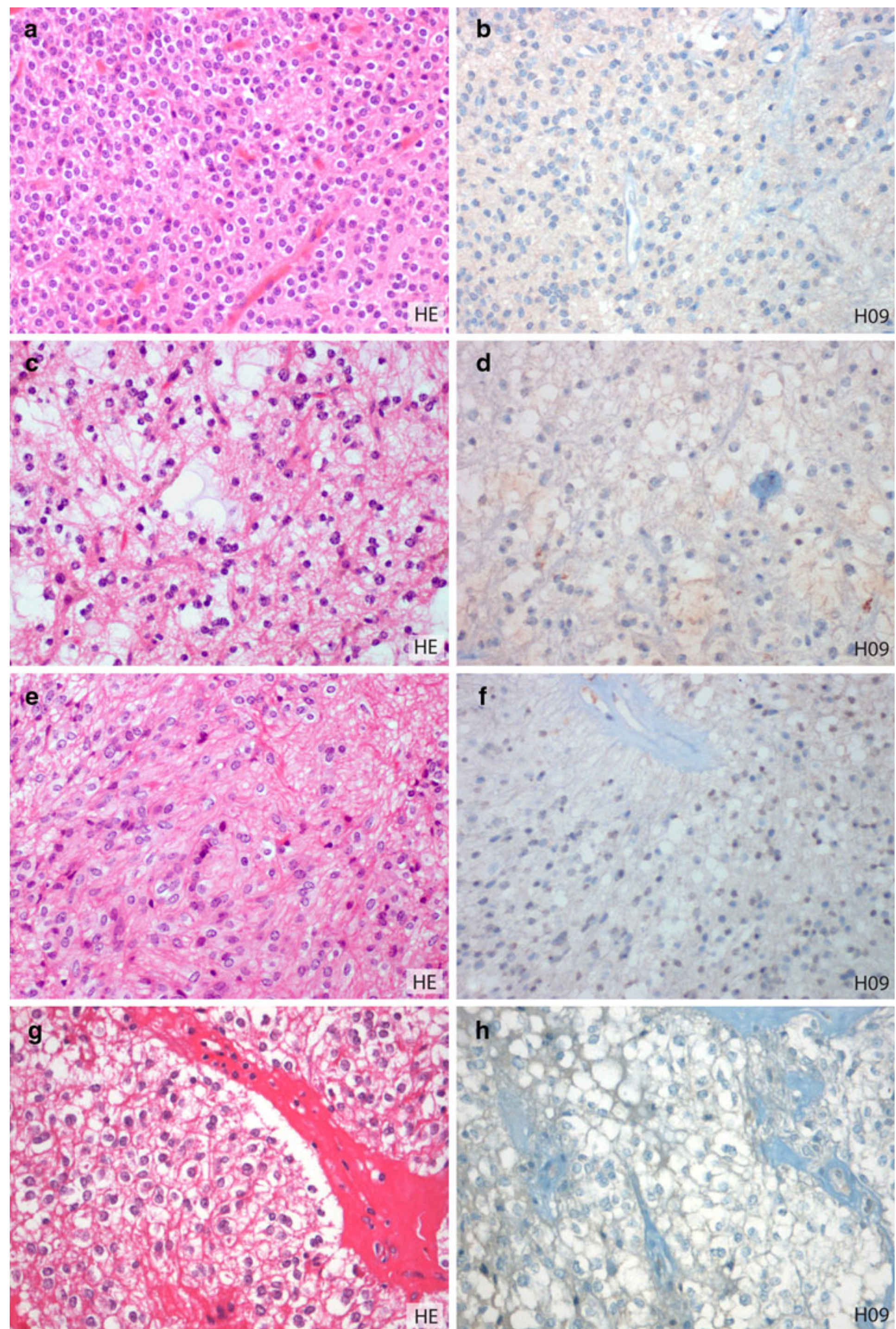

Fig. 3 H09 immunohistochemistry of non-oligodendroglioma clear cell brain tumors. Central neurocytoma with pronounced clear cell morphology (**a**) negative for H09 (**b**). Dysembryoplastic neuroepithelial tumor with oligoid cells and single floating neurons (**c**) negative for H09 (**d**). Clear cell ependymoma (**e**) negative for H09 (**f**). Pilocytic astrocytoma with predominant oligodendroglial-like differentiation (**g**) negative for H09 (**h**)

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

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

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-oligodendroglial 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*

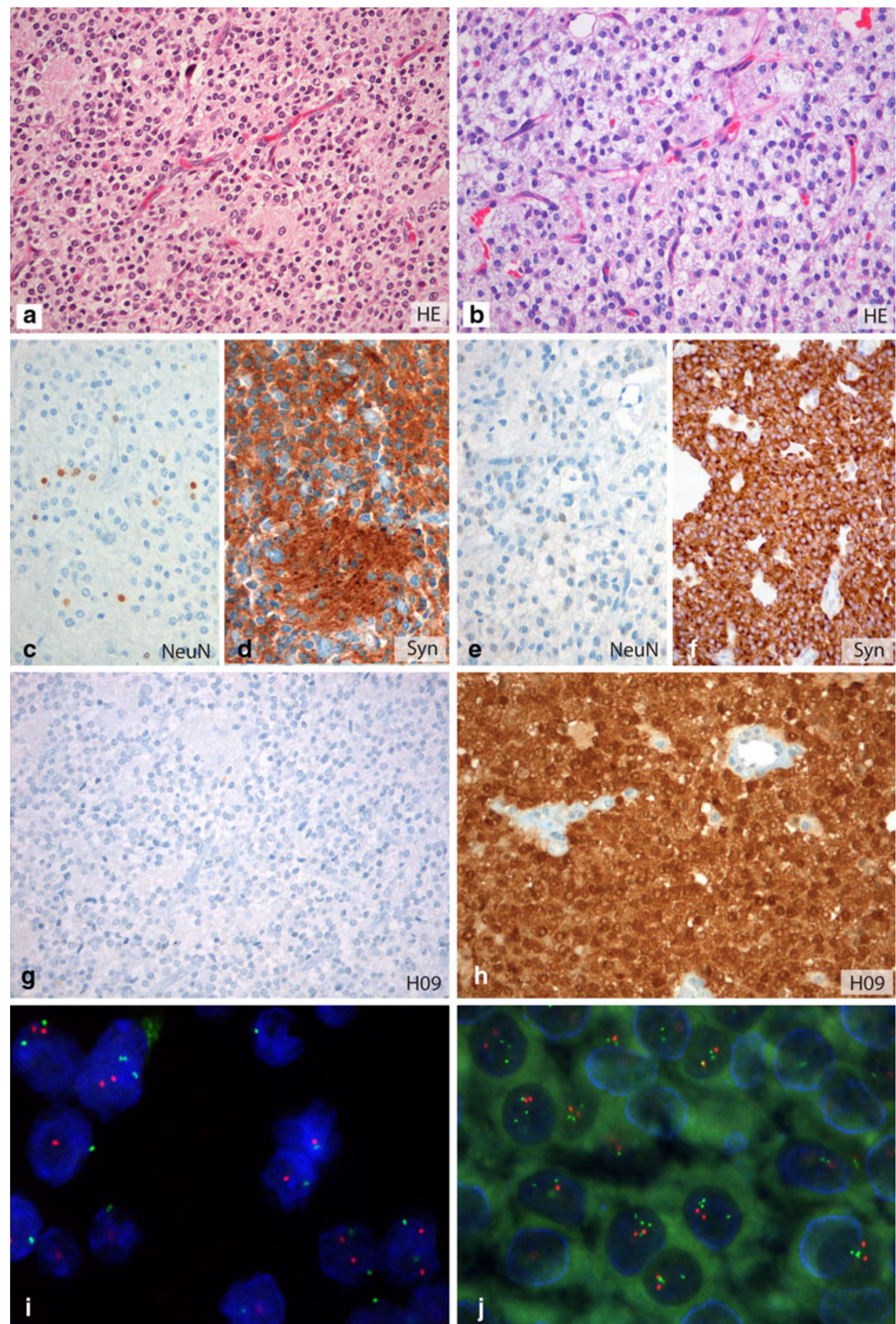

Fig. 4 H09 differentiates extraventricular neurocytoma from oligodendroglioma with neurocytic differentiation. On the *left* extraventricular neurocytoma (Case 53308) with frequent neuropil islands (**a**), scattered NeuN-positive tumor cells corresponding to immuno-reactive score 4 (**c**) and strong positivity for synaptophysin (**d**). The tumor is negative for H09 immunohistochemistry (IHC) (**g**). On the *right*, oligodendroglioma with neurocytic differentiation (Case 53512, **b**). The tumor shows scattered weakly NeuN-positive tumor cells corresponding to immuno-reactive score 2 (**e**) and is strongly positive for synaptophysin (**f**). The tumor is also strongly positive for H09 (**h**). Fluorescence in situ hybridization (FISH) of extraventricular neurocytoma. Compared to control probe (1q36 *green*), probe for 1p36 (*red*) detects an equal number of DNA elements indicating retention of chromosome arm 1p (**i**). FISH of oligodendroglioma with neurocytic differentiation (Case 53042) demonstrating polysomy of chromosome 1 (*green*) and relative deletion of 1p (*red*) (**j**)

mutations in the definition of WHO grade III oligodendrogliomas. 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 oligodendroglial 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, extraventricular-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 extraventricular-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 extraventricular-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 oligodendroglial, 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 oligodendroglial tumors has recently been challenged by the observation that approximately one quarter of extraventricular-NC also harbor co-deletions of 1p/19q [26, 33]. In this study we demonstrate that all extraventricular 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 oligodendroglial 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 extraventricular-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 extraventricular-NC of this series. Thus in our hands, NeuN IHC was also able to differentiate O-NC

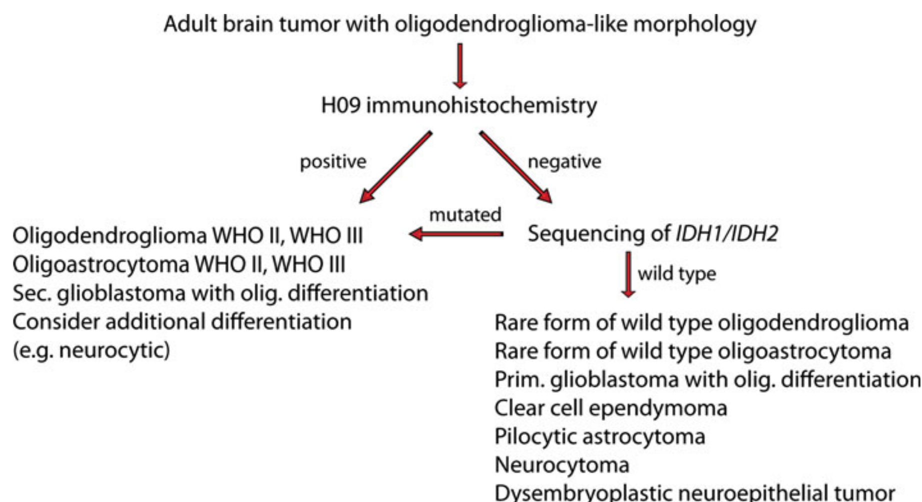

Fig. 5 Diagnostic algorithm for brain tumors with oligodendroglioma-like morphology based on H09 immunohistochemistry

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.

Acknowledgments We would like to thank Jochen Meyer and Franziska Moessler for excellent technical assistance. This work was supported by the Bundesministerium für Bildung und Forschung (BMBF–01ES0730 and 01GS0883).

Conflict of interest Under a licensing agreement between DIANOVA GmbH, Hamburg, Germany, and the German Cancer Research Center, Dr. Capper, Dr. Hartmann and Dr. von Deimling are entitled to a share of royalties received by the German Cancer Research Center on the sales of H09 antibody. The terms of this arrangement are being managed by the German Cancer Research Center in accordance with its conflict of interest policies.

References

1. Balss J, Meyer J, Mueller W et al (2008) Analysis of the IDH1 codon 132 mutation in brain tumors. Acta Neuropathol 116:597–602
2. Bleeker FE, Lamba S, Leenstra S et al (2009) IDH1 mutations at residue p.R132 (IDH1(R132)) occur frequently in high-grade gliomas but not in other solid tumors. Hum Mutat 30:7–11
3. Blumcke I, Muller S, Buslei R et al (2004) Microtubule-associated protein-2 immunoreactivity: a useful tool in the differential diagnosis of low-grade neuroepithelial tumors. Acta Neuropathol 108:89–96
4. Capper D, Weissert S, Balss J et al (2010) Characterization of R132H mutation-specific IDH1 antibody binding in brain tumors. Brain Pathol 20:245–254
5. Capper D, Zentgraf H, Balss J et al (2009) Monoclonal antibody specific for IDH1 R132H mutation. Acta Neuropathol 118:599–601
6. Chou S, Varikatt W, Dexter M et al (2010) Extraventricular neurocytoma with atypical features and ganglionic differentiation. J Clin Neurosci 17:920–922
7. Dang L, White DW, Gross S et al (2009) Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. Nature 462:739–744
8. Fujisawa H, Marukawa K, Hasegawa M et al (2002) Genetic differences between neurocytoma and dysembryoplastic neuroepithelial tumor and oligodendroglial tumors. J Neurosurg 97:1350–1355
9. Hartmann C, Meyer J, Balss J et al (2009) Type and frequency of IDH1 and IDH2 mutations are related to astrocytic and oligodendroglial differentiation and age: a study of 1,010 diffuse gliomas. Acta Neuropathol 118:469–474
10. Ichimura K, Pearson DM, Kocialkowski S et al (2009) IDH1 mutations are present in the majority of common adult gliomas but are rare in primary glioblastomas. Neuro Oncol 11:341–347
11. Idbaih A, Marie Y, Pierron G et al (2005) Two types of chromosome 1p losses with opposite significance in gliomas. Ann Neurol 58:483–487
12. Koperek O, Gelpi E, Birner P et al (2004) Value and limits of immunohistochemistry in differential diagnosis of clear cell primary brain tumors. Acta Neuropathol 108:24–30
13. Korshunov A, Meyer J, Capper D et al (2009) Combined molecular analysis of BRAF and IDH1 distinguishes pilocytic astrocytoma from diffuse astrocytoma. Acta Neuropathol 118:401–405
14. Kreiger PA, Okada Y, Simon S et al (2005) Losses of chromosomes 1p and 19q are rare in pediatric oligodendrogliomas. Acta Neuropathol 109:387–392
15. Kuhlmann T, Gutenberg A, Schulten HJ et al (2008) Nogo-a expression in glial CNS tumors: a tool to differentiate between oligodendrogliomas and other gliomas? Am J Surg Pathol 32:1444–1453
16. Labussiere M, Idbaih A, Wang XW et al (2010) All the 1p19q codeleted gliomas are mutated on IDH1 or IDH2. Neurology 74:1886–1890
17. Louis DN, Ohgaki H, Wiestler OD et al (2007) WHO classification of tumors of the central nervous system. IARC, Lyon
18. Mardis ER, Ding L, Dooling DJ et al (2009) Recurring mutations found by sequencing an acute myeloid leukemia genome. N Engl J Med 361:1058–1066
19. Mrak RE, Yasargil MG, Mohapatra G et al (2004) Atypical extraventricular neurocytoma with oligodendroglioma-like spread and an unusual pattern of chromosome 1p and 19q loss. Hum Pathol 35:1156–1159
20. Mueller W, Lass U, Veelken J et al (2006) 45-year-old male with symptomatic mass in the frontal lobe. Brain Pathol 16:89–90, 93
21. Mut M, Guler-Tezel G, Lopes MB et al (2005) Challenging diagnosis: oligodendroglioma versus extraventricular neurocytoma. Clin Neuropathol 24:225–229
22. Ng H, Ko H, Tse C (1994) Immunohistochemical and ultrastructural studies of oligodendrogliomas revealed features of neuronal differentiation. Int J Surg Pathol 2:47–56
23. Nobusawa S, Watanabe T, Kleihues P et al (2009) IDH1 mutations as molecular signature and predictive factor of secondary glioblastomas. Clin Cancer Res 15:6002–6007
24. Ohgaki H, Kleihues P (2005) Population-based studies on incidence, survival rates, and genetic alterations in astrocytic and oligodendroglial gliomas. J Neuropathol Exp Neurol 64:479–489
25. Perry A, Burton SS, Fuller GN et al (2010) Oligodendroglial neoplasms with ganglioglioma-like maturation: a diagnostic pitfall. Acta Neuropathol 120:237–252
26. Perry A, Fuller CE, Banerjee R et al (2003) Ancillary FISH analysis for 1p and 19q status: preliminary observations in 287 gliomas and oligodendroglioma mimics. Front Biosci 8:a1–a9
27. Perry A, Scheithauer BW, Macaulay RJ et al (2002) Oligodendrogliomas with neurocytic differentiation. A report of 4 cases with diagnostic and histogenetic implications. J Neuropathol Exp Neurol 61:947–955
28. Preusser M, Budka H, Rossler K et al (2007) OLIG2 is a useful immunohistochemical marker in differential diagnosis of clear cell primary CNS neoplasms. Histopathology 50:365–370
29. Preusser M, Laggner U, Haberler C et al (2006) Comparative analysis of NeuN immunoreactivity in primary brain tumours: conclusions for rational use in diagnostic histopathology. Histopathology 48:438–444
30. Pusch S, Sahm F, Meyer J et al (2010) Glioma IDH1 mutation patterns off the beaten track. Neuropathol Appl Neurobiol
31. Raghavan R, Balani J, Perry A et al (2003) Pediatric oligodendrogliomas: a study of molecular alterations on 1p and 19q using

fluorescence in situ hybridization. J Neuropathol Exp Neurol 62:530–537
32. Remmele W, Stegner HE (1987) Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. Pathologe 8:138–140
33. Rodriguez FJ, Mota RA, Scheithauer BW et al (2009) Interphase cytogenetics for 1p19q and t(1;19)(q10;p10) may distinguish prognostically relevant subgroups in extraventricular neurocytoma. Brain Pathol 19:623–629
34. Rodriguez FJ, Scheithauer BW, Robbins PD et al (2007) Ependymomas with neuronal differentiation: a morphologic and immunohistochemical spectrum. Acta Neuropathol 113:313–324
35. Sahm F, Capper D, Meyer J et al (2010) Immunohistochemical analysis of 1844 human epithelial and hematopoietic tumors and sarcomas for IDH1R132H mutation. Histopathology
36. Sanson M, Marie Y, Paris S et al (2009) Isocitrate dehydrogenase 1 codon 132 mutation is an important prognostic biomarker in gliomas. J Clin Oncol 27:4150–4154
37. Soylemezoglu F, Onder S, Tezel GG et al (2003) Neuronal nuclear antigen (NeuN): a new tool in the diagnosis of central neurocytoma. Pathol Res Pract 199:463–468
38. Takeuchi H, Kubota T, Kitai R et al (2009) Chromosome 1p and 19q deletions in malignant glioneuronal tumors with oligodendroglioma-like component. J Neurooncol 91:33–38
39. Vajtai I, Arnold M, Vassella E (2005) Oligodendroglioma with neurocytic differentiation and characteristic loss of heterozygosity on chromosomes 1p and 19q. Acta Neuropathol 110:520–522
40. Vallat-Decouvelaere AV, Gauchez P, Varlet P et al (2000) So-called malignant and extra-ventricular neurocytomas: reality or wrong diagnosis? A critical review about two overdiagnosed cases. J Neurooncol 48:161–172
41. Ward PS, Patel J, Wise DR et al (2010) The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. Cancer Cell 17:225–234
42. Watanabe T, Nobusawa S, Kleihues P et al (2009) IDH1 mutations are early events in the development of astrocytomas and oligodendrogliomas. Am J Pathol 174:1149–1153
43. Wharton SB, Chan KK, Hamilton FA et al (1998) Expression of neuronal markers in oligodendrogliomas: an immunohistochemical study. Neuropathol Appl Neurobiol 24:302–308
44. Wolf HK, Buslei R, Blumcke I et al (1997) Neural antigens in oligodendrogliomas and dysembryoplastic neuroepithelial tumors. Acta Neuropathol 94:436–443
45. Yan H, Parsons DW, Jin G et al (2009) IDH1 and IDH2 mutations in gliomas. N Engl J Med 360:765–773