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ORIGINAL PAPER

Adult *IDH* wild type astrocytomas biologically and clinically resolve into other tumor entities

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Abstract *IDH* wild type (*IDH*wt) anaplastic astrocytomas WHO grade III (AA III) are associated with poor outcome. To address the possibilities of molecular subsets among astrocytoma or of diagnostic reclassification, we analyzed a series of 160 adult *IDH*wt tumors comprising 120 AA III and 40 diffuse astrocytomas WHO grade II (A II) for molecular hallmark alterations and established methylation and copy number profiles. Based on molecular profiles and hallmark alterations the tumors could be grouped into four major sets. 124/160 (78 %) tumors were diagnosed as the molecular equivalent of conventional glioblastoma (GBM), and 15/160 (9 %) as GBM-*H3F3A* mutated (GBM-H3). 13/160 (8 %) exhibited a distinct methylation profile that was most similar to GBM-H3-K27, however, lacked the *H3F3A* mutation. This group was enriched for tumors of infratentorial and midline localization and showed a trend towards a more favorable prognosis. All but one of the 120 *IDH*wt AA III could be assigned to these three groups. 7 tumors recruited from the 40 A II, comprised a variety of molecular signatures and all but one were reclassified into distinct WHO entities of lower grades. Interestingly, *TERT* mutations were exclusively restricted to the molecular GBM (78 %) and associated with poor clinical outcome.

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However, the GBM-H3 group lacking *TERT* mutations appeared to fare even worse. Our data demonstrate that most of the tumors diagnosed as *IDH*wt astrocytomas can be allocated to other tumor entities on a molecular basis. The diagnosis of *IDH*wt diffuse astrocytoma or anaplastic astrocytoma should be used with caution.

Keywords *IDH1* · *IDH2* · Astrocytoma · Glioblastoma · Classification · *TERT* · *H3F3A*

Introduction

Classification of diffuse astrocytic tumors

The classification of astrocytomas according to WHO 2007 predominantly relies on the evaluation of histopathology and immunohistochemistry [12]. In recent years molecular parameters have been developed and proven powerful astrocytoma classifiers. In fact, the previously well accepted and quite harmonious WHO classification scheme for astrocytomas has experienced challenges by molecular findings that are not reflected in morphology. The presence of isocitrate dehydrogenase 1 (*IDH1*) and isocitrate dehydrogenase 2 (*IDH2*) mutations in the majority of diffuse astrocytomas WHO grade II (A II) and anaplastic astrocytomas WHO grade III (AA III) and their association with a more favorable course, especially in AA III are strong evidence that heterogeneous tumors have been lumped together under a single diagnosis. There is increasing evidence from multiple studies that the poor clinical outcome of *IDH*wt AA III is a result of a considerable proportion of unrecognized glioblastomas (GBM) in this group [2, 4, 22, 26]. Such evidence is based on an overlap of distinct molecular lesions in *IDH*wt AA III and GBM and on similar clinical courses. It remains to be tested how many *IDH*wt A II or *IDH*wt AA III cannot be allotted to other tumor entities by molecular analyses.

Frequency of *IDH1* and *IDH2* mutations in WHO 2007 classified and graded astrocytomas

Upon recognition of *IDH* mutations in a series of GBM, predominantly secondary GBM [16], several studies demonstrated *IDH1* or *IDH2* mutations in A II and AA III. The percentages of *IDH* mutations given for A II ranged from 59 to 90 % and those for AA III from 52 to 78 % [1, 5–7, 25, 28]. Interestingly, the frequency of *IDH* mutations in all studies was higher in A II than in AA III.

Aim and design of the study

The present study was designed to interrogate the hypothesis that the group of IDH*wt* astrocytoma is in fact composed of distinctive pathobiological entities. A series of 160 adult *IDH*wt A II or *IDH*wt AA III was analyzed for molecular parameters consistent with other brain tumor entities. The overall survival (OS) of these subgroups was compared to that of the brain tumor entity in question.

Materials and methods

Tissue collection

For the Heidelberg series, paraffin blocks containing tissue of adult patients (18 years of age and older) with *IDH*wt A II or AA III were collected from the archives of Neuropathology departments of Universities of Heidelberg, Münster and Zürich, the UCL Institute of Neurology (08/H0716/16) and from the German Glioma Network (GGN). Tumors with 1p/19q co-deletion were excluded. One reference set was extracted from a recently published series by the TCGA ($n = 231$) [2]. Therefore, the results here are in part based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/.

Determination of copy number alterations and G-CIMP phenotype by 450k array analysis

The Illumina Infinium HumanMethylation450 BeadChip (450k) array was used to determine the DNA methylation status of 482,421 CpG sites (Illumina, San Diego, USA) according to the manufacturer's instructions at the

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Genomics and Proteomics Core Facility of the DKFZ. The array data was used to calculate a low-resolution copy number profile (CNP) as previously described [21]. Further, the data were analyzed as previously described to allot the tumors to either a G-CIMP or a non-G-CIMP cluster [27].

IDH1/IDH2, *H3F3A* and *TERT* promoter mutation analyses

Primer design for sequencing was based on accession numbers NM_005896 for *IDH1*, NM_002168 for *IDH2* and NM_002107.4 for *H3F3A* (http://www.ncbi.nlm.nih.gov). Primers for *IDH1*: forward 5′-TGATGAGAAGAGGGTTG AGGA-3′; reverse 5′-GCAAAATCACATTATTGCCAAC-3′ and for *IDH2*: forward 5′-CTCCACCCTGGCCTACCT-3′; reverse 5′-GCTGCAGTGGGACCACTATT-3′.PCR and sequencing were performed as previously described [5]. Primers for *H3F3A*: forward 5′-CATGGCTCGTACAAAG CAGA-3′; reverse 5′-CAAGAGAGACTTTGTCCCATTTT T-3′. PCR and sequencing were performed as previously described [20]. A 163 bp fragment of the TERT promoter region spanning the hotspot mutations at positions 1,295,228 and 1,295,250 was amplified using GoTaq G2 Hot Start Polymerase (Promega, Madison, USA) and the primers hTERT-short-for 5′-CAGCGCTGCCTGAAACTC-3′ and hTERT-short-rev, 5′-GTCCTGCCCCTTCACCTT-3′ as previously described [9]. Sequences were determined using a semi-automated sequencer (ABI 3100 Genetic Analyzer, Applied Biosystems, Foster City) and Sequence Pilot version 3.1 software (JSI-Medisys, Kippenheim, Germany).

Immunohistochemistry

Immunohistochemistry was conducted on 4 µm thick formalin-fixed, paraffin-embedded (FFPE) tissue sections mounted on StarFrost Advanced Adhesive slides (Engelbrecht, Kassel, Germany) followed by drying at 80 °C for 15 min. Immunohistochemistry was performed on a BenchMark Ultra immunostainer (Ventana Medical Systems, Tucson, AZ, USA). Sections were stained with anti-IDH1-R132H antibody H09 (Dianova, Hamburg, Germany) as previously described [3]. ATRX immunohistochemistry was performed as previously described [19]. In brief, after deparaffinization, slides were pretreated at 95 °C in Cell Conditioning 1 buffer (Ventana) for 90 min. The sections were incubated with primary antibody HPA001906 (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:200 for 2 h. Standard Ventana signal amplification was used. For BRAFV600E staining V600E-specific clone VE1 was used. After pretreatment with cell conditioner 1 (pH 8) for 64 min, sections were incubated with VE1 hybridoma supernatant (monoclonal, dilution 1:5) at 37 °C for 32 min. Antibody incubation was followed by OptiView HQ Universal Linker for 12 min, incubation with OptiView HRP Multimer for 12 min, and signal amplification including the Ventana OptiView Amplification Kit (Ventana, catalogue number 760-099).

Statistics

The Kaplan–Meier plots and log-rank were conducted by JMP statistics software (version 9.0.0; SAS Institute, Cary, NC, USA). The 450k data was processed with the Bioconductor package minfi (version 1.12). For the unsupervised hierarchical clustering only CpGs with a standard deviation (SD) greater than 0.2 across the beta values were selected. The samples were clustered with Ward's linkage method and the pairwise similarity was calculated using the Euclidean distance. For the hierarchical clustering of the CpGs average linkage and the Euclidian distance were applied. R version 3.1.3 and Bioconductor version 3.0 were used.

Results and discussion

Study and reference groups

To compare tumors diagnosed as *IDH*wt astrocytoma with other entities, a study and a reference set were formed. The study set contained 160 *IDH*wt astrocytomas with 40 being diagnosed as diffuse A II and 120 as AA III. The reference set contained 132 *IDH* mutated (*IDH*mut) astrocytomas, 100 *IDH*wt GBM and 21 *H3F3A* mutated gliomas (GBM-H3-K27 and GBM-H3-G34). All tumors of both study and reference groups were subjected to 450k methylation analysis and low-resolution copy number profiles were calculated from the methylation data. For comparison of OS a TCGA control cohort of 105 *IDH*mut anaplastic astrocytomas was used.

Molecular hallmark lesions in *IDH*mut and *IDH*wt astrocytic tumors of the reference cohort associate with distinct methylation patterns

Unsupervised hierarchical clustering of the tumors in the reference set revealed two main methylation clusters perfectly matching the *IDH* status (suppl. Fig. 1). Given the dominant hypermethylation phenotype of *IDH*mut tumors [14, 23] we then re-clustered the *IDH*wt tumors separately to achieve a more refined differentiation (suppl. Fig. 2).

The *IDH*wt tumors segregated into two main clusters. One of these clusters was composed only of GBM cases which again formed two subclusters reminiscent of the "mesenchymal" and "classic" methylation groups [21]. The second main cluster was enriched for *H3F3A*-mutant

cases. The G34 and K27 mutant cases formed homogenous subclusters within this cluster confirming the reported distinctiveness of methylation profiles associated with both mutations [21]. A third subcluster composed of GBM cases reminiscent of the previously reported "RTK1" methylation cluster was also present [21]. Of note, two of the GBM reference cases formed a small subcluster adjacent to the *H3F3A*-K27 mutant cluster. Both cases were sequenced and found to be wild type for *H3F3A*.

In line with previous reports, [8] *TERT* promoter mutations were found in 88 % of all GBM cases irrespective of the methylation cluster but in only 2/132 (1 %) *IDH*mut cases and in none of the *H3F3A* mutant cases. *TERT* mutation is therefore considered a hallmark lesion for GBM. Analysis of CNPs confirmed 7p gain/10q loss to be highly characteristic for GBMs. In 88 % of such cases the whole chromosomes were affected. 7p gain/10q loss was present in 75 % of cases in the GBM cluster and in 20 % of GBM-H3-G34 but absent in all *IDH*mut and GBM-H3-K27 cases. *EGFR* amplification was detected in 38 % of GBMs but in only 1/132 *IDH*mut cases. *EGFR* amplification was differently distributed between the various GBM methylation groups with the highest frequency in the "classic" cluster. The combination of loss of the entire arm 10q combined with partial or complete losses on 13q and 14q was recognized as a distinct pattern present in 20 % of GBM but was absent in other groups. Therefore, 7p gain/10q loss, *EGFR* amplification and the combination of loss of the entire arm 10q combined with losses on 13q and 14q were also considered hallmark lesions for GBM. At least one of these hallmark alterations was present in 96 % of GBM cases. The distribution of molecular lesions in the reference sets is given in suppl. Table 1. Typical examples for CNPs are provided in Fig. 1.

H3F3A mutated gliomas exhibit morphological features of both, GBM and AA III. Clinically the presence or absence of necrosis has not been shown to be of prognostic relevance, and the clinical course of these patients is similar to those with GBM. For these reasons we pooled these tumors under the provisional designation GBM *H3F3A* mutated (GBM-H3). GBM-H3 frequently showed loss of chromosomal material or complex alterations on 3q (48 %) and frequently was accompanied by gain of 17q (33 %). In GBM-H3-K27 there was frequently a gain (45 %) or occasionally an amplification of *MDM2* (9 %), often with a co-gain of *CDK4* (45 %), a feature shared with a minority of GBMs (9 %) but virtually absent in all other cases. Interestingly, the two *H3F3A* wild type GBM reference cases clustering adjacent to *H3F3A-K27* mutant GBM lacked conventional GBM hallmark alterations and exhibited CNP reminiscent to *H3F3A* mutant GBMs.

ATRX loss was present in 96 % of *IDH*mut astrocytomas, in 5/5 GBM-H3-G34, in 5/6 GBM-H3-K27, but only in 3/94 (3 %) GBM. A compilation of the alterations in the respective reference sets is provided in supplementary Table 1.

Establishing "integrated" diagnoses for *IDH* wild type astrocytomas and comparison with clinical outcomes

For the analysis of the study set, composed exclusively of *IDH*wt astrocytomas we used the same clustering strategy as for the reference set. First, the *IDH*wt astrocytomas were clustered with the reference set to identify possible cases with a G-CIMP phenotype. Two tumors (1 %) exhibited a G-CIMP phenotype and mapped to the *IDH*mut cluster despite showing *IDH1/2* wild type sequences in Sanger sequencing and were provisionally designated astrocytoma-IDH-like. The remaining cases were then re-clustered without the *IDH*mut reference set. Based on the distribution of the molecular findings, the *IDH*wt astrocytoma set could be subdivided in four major groups (Fig. 2a). OS of the four groups is given in Fig. 3a.

All tumors were assessed for distinct molecular lesions. The integrated diagnosis of GBM was assigned to 124/160 (78 %) tumors. 115 tumors segregated to a GBM cluster and showed at least one of the GBM hallmark alterations *TERT* promoter mutation, 7p gain/10q loss, *EGFR* amplification or combined 10q/13q/14q deletion. Additional 6 tumors segregated to the GBM cluster and showed complex CNPs with gains and losses involving several chromosomes or gene amplifications. Therefore, these tumors were also considered to be GBMs. Two additional cases that clustered adjacent to GBM-H3-K27 cases but showed *TERT* promoter mutations were classified as GBMs. A single case which clustered together with GBM-H3-G34 cases but was *H3F3A* wild type and showed a complex CNP with *EGFR* amplification was also classified as GBM.

Accounting for 78 % of cases this was by far the most numerous group. Median survival in this group was 19.4 months—very well matching survival seen in GBM patients and thus further justifying the diagnosis of GBM. Presence or absence of *TERT* promoter mutations did not affect survival in this group.

15 (9 %) tumors exhibited a methylation profile typical of *H3F3A* mutated GBM and indeed harbored hotspot mutations; 12 were GBM-H3-K27 and three GBM-H3-G34. This group of 15 patients predominantly containing *H3F3A*-K27 mutations exhibited a median survival of 16.9 months matching the observation of very poor clinical courses in pediatric glioma with *H3F3A*-K27 mutations [21, 24]. Comparison of OS of this group with that of a control cohort of *IDH*mut astrocytoma is shown in Fig. 3b. *H3F3A* mutations were highly associated with loss of nuclear expression of ATRX (8/11 cases, 73 %) which further emphasizes that this group represents a clearly

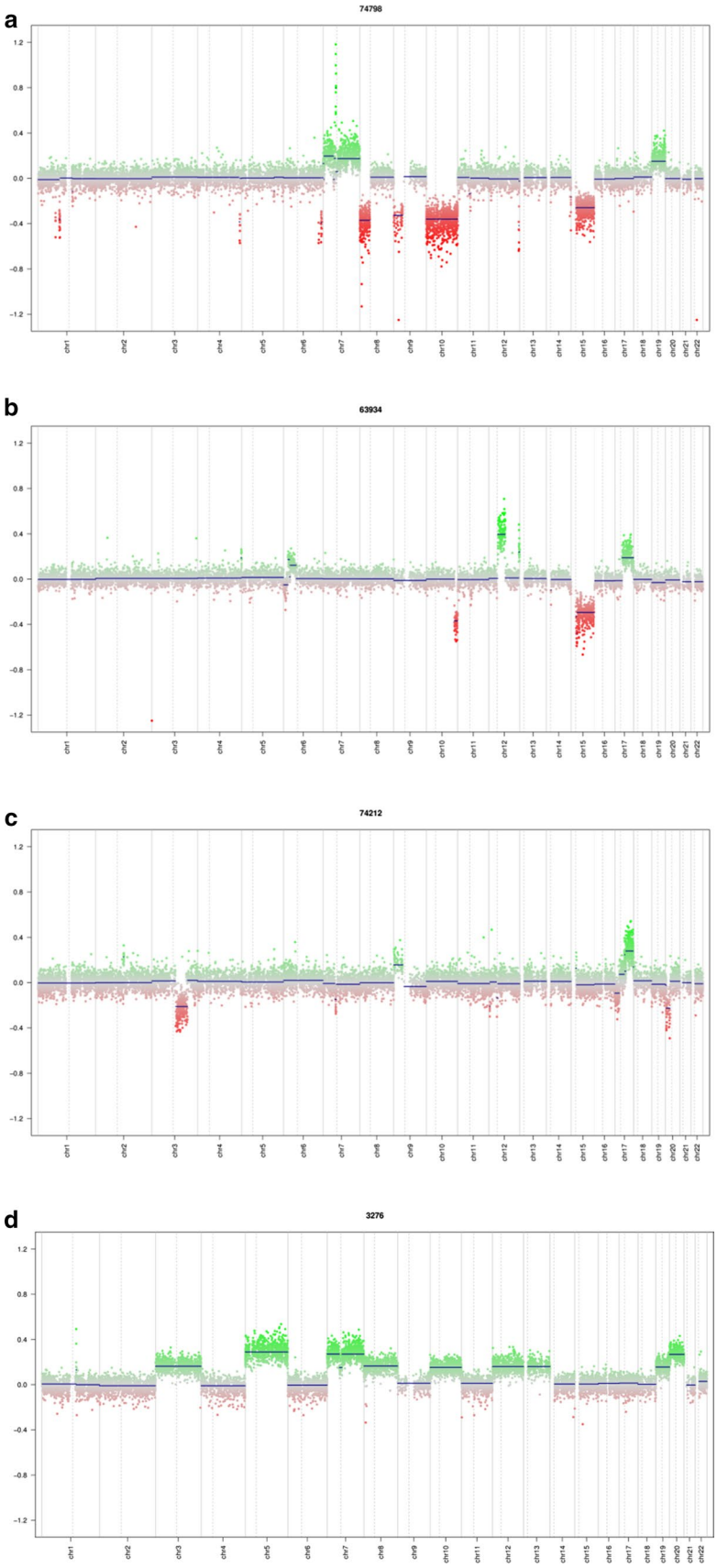

Fig. 1 From *top* to *bottom*: **a** typical copy number profile of GBM with gain of 7, loss of 10, *CDKN2A* deletion and *EGFR* amplification; **b** CNP of GBM-H3-K27 with co-gain of *MDM2/CDK4* and 17q gain; **c** CNP of a MID-HGG with a deletion on 3q and a 17q gain; **d** typical profile of ganglioglioma with several whole chromosomal gains

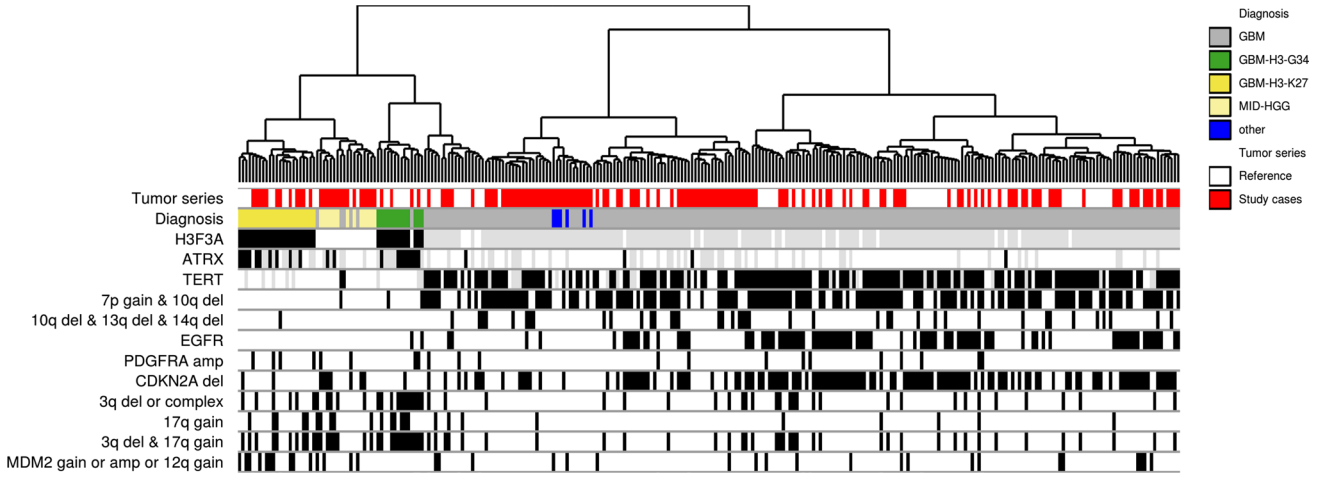

Fig. 2 Molecular profiling of non-CIMP *IDH*wt astrocytomas and GBM and GBM-H3 reference cases. The dendrogram depicts the results of unsupervised hierarchical clustering of methylation levels of the top 18856 most variant probes (SD > 0.20). The row "Tumor series" indicates study cases in *red* and reference cases in *white*. Diagnoses of reference cases were set prior to analysis and remained unchanged. Integrated diagnosis of study cases are indicated by *different colors*: *GBM* glioblastoma (*gray*), *GBM-H3-G34* glioblastoma H3F3A-G34 mutated (*green*), *GBM-H3-K27* glioblastoma H3F3A-K27 mutated (*yellow*), *MID-HGG* midline high grade glioma (*light yellow*), other (*blue*). For each sample associated results of *TERT* and *H3F3A* hotspot sequencing, ATRX immunohistochemistry and selected chromosomal copy number variants are indicated: *black* indicated presence of the alteration, *white* indicates absence of the alteration, *gray* indicates unknown status

defined GBM. Likewise, the lack of *TERT* mutations further separates GBM-H3 from classical GBM.

Thirteen tumors (8 %) with a distinct methylation profile clustering adjacent to GBM-H3-K27 lacked *H3F3A* mutations. These tumors did not exhibit the hallmark alterations of GBM but showed complex CNP with overlaps to both G34 and K27 H3F3A mutant GBMs. This group was enriched for tumors of midline localization and has not previously been described. The median survival of 54.7 months may indicate a more favorable course than that of GBM patients, and, therefore this group may qualify as a second astrocytoma subgroup apart from *IDH*mut astrocytoma (Fig. 3c). Since OS still is shorter than that of *IDH*mut astrocytoma, we suggest that grouping with malignant astrocytic tumors may be appropriate (Fig. 3c). Therefore, this tumor group was provisionally termed 'midline high grade glioma' (MID-HGG).

6/160 (5 %) cases assigned to a common subcluster within the large GBM cluster lacked all hallmark alterations and demonstrated a CNP that was either completely balanced or showed only minor chromosomal alterations or trisomies. These cases were considered to most likely comprise an assortment of other lower grade neuroepithelial tumors. Case 3276 exhibited a CNP with several whole chromosomal gains including chromosomes 5 and 7. This pattern is typical for low grade glioneuronal tumors and is absent in diffuse astrocytomas [17]. Furthermore, this case harbored a *BRAF*V600E mutation strongly suggestive of ganglioglioma [10]. Case 68924 showed a similar CNP with trisomies of chromosomes 5, 7 and 20. Histologic re-evaluation demonstrated an Alcian blue-positive tumor matrix and an astrocytic as well as an oligodendroglial appearance of the tumor cells. Even though "floating neurons" were not found in this small biopsy a dysembryoplastic neuroepithelial tumor (DNT) seems the most likely diagnosis. A similar histological constellation was present in case 72220 which exhibited a completely balanced CNP. Similarly, case 72274 did not show any chromosomal aberrations and histological re-evaluation revealed an atypical ganglion cell component in this tumor consistent with ganglioglioma. Histological re-evaluation of case 49164 revealed tumor areas with bipolar tumor cells and Rosenthal fibers suggestive of pilocytic astrocytoma. Case 50133 had a unique methylation profile and a copy number profile showing gain of chromosome 12 and losses of chromosomes 13 and 22q. Histological re-evaluation revealed an overall low but perivascular accentuated GFAP expression and a "dot-like" EMA expression. It was concluded that this spinal tumor should be re-diagnosed as ependymoma.

Thus, molecular analyses completely resolved this group of morphologically diagnosed *IDH*wt astrocytomas into established entities. A compilation on the molecular alterations of *IDH*wt astrocytomas is provided in Table 1. The data for each individual tumor are listed in suppl. Table 2. The changes from the initial WHO diagnosis to an integrated diagnosis are shown in Fig. 4.

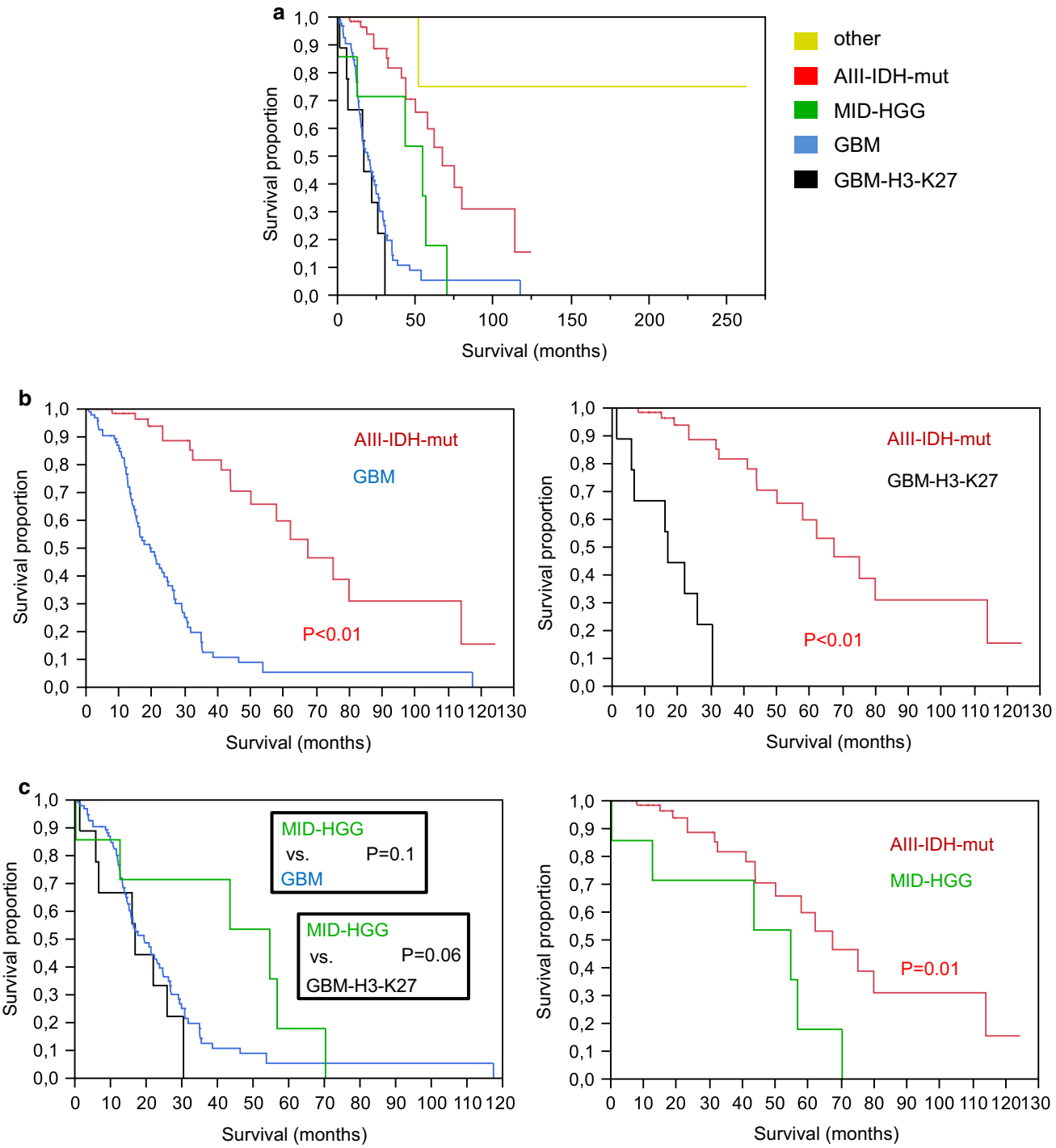

Fig. 3 Survival of patients: **a** overview over survival of the four *IDH*wt groups compared with AIII-*IDH*mut; **b** survival of patients with integrated diagnoses of GBM (*left*) or GBM-H2F3A-K27-mut (*right*) compared with patients diagnosed with *IDH*mut anaplastic astrocytoma; **c** survival of patients of the MID-HGG group compared with GBM and GBM-H3-K27 (*left*) and AIII-*IDH*mut (*right*)

In conclusion, these data demonstrate that 139/160 (87 %) of *IDH*wt astrocytomas on molecular and clinical grounds are indistinguishable from GBM or GBM-H3. Interestingly, upon separation of GBM from GBM-H3, the latter having the least favorable prognosis, the presence of *TERT* mutations in the GBM group did not influence survival (suppl. Fig. 3). 6/160 tumors were misdiagnosed other lower grade gliomas. Most interestingly, a group of 13 tumors emerged with a distinct methylation profile most similar to GBM-H3-K27 and enriched for midline tumors

Table 1 Overview of 160 *IDH*wt astrocytomas divided into four distinct molecular groups

Designation	GBM	GBM-H3	MID-HGG	Others
n/(%)	124 (78 %)	15 (9 %)	13 (9 %)	8 (7 %)
Methylation profile	GBM (121)	H3	Distinct	Variant
H3F3A-mutant	0	15	0	0
TERT-mutant	84/108 (78 %)	0	0	0
ATRX loss (IHC)	2/82 (2 %)	8/11 (73 %)	2/10 (20 %)	0/6 (0 %)
7p gain/10q loss	83/124 (67 %)	0	0	0
EGFR amp	54/124 (44 %)	0	0	0
10q-/13q-/14q-	21/124 (17 %)	1 (7 %)	0	0
OS median (months)	19.4	16.9	54.7	Not reached
AA III ($n = 120$)	96 (80 %)	12 (10 %)	10 (8 %)	1 (1 %)
A II ($n = 40$)	28 (70 %)	3 (7 %)	4 (10 %)	7 (17 %)
Age median	54	51.5	54	49

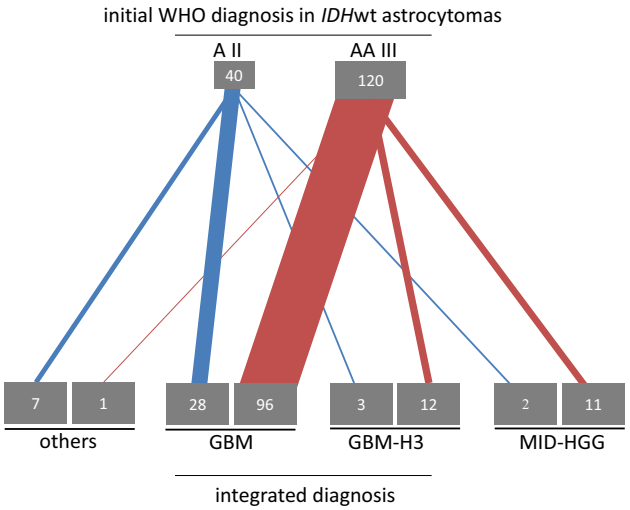

Fig. 4 Changes from initial WHO to integrated diagnosis in 160 patients with *IDH*wt astrocytoma. *Width of bars* indicates relative proportions of the initial tumor groups. *A II* diffuse astrocytoma WHO grade II, *AA III* anaplastic astrocytoma WHO grade III, *GBM* glioblastoma, *GBM-H3* glioblastoma *H3F3A* mutated, *MID-HGG* midline high grade glioma

but lacked a *H3F3A* mutation. This group showed a trend towards better survival than GBMs.

Implication for future diagnostic approach to diffuse and anaplastic astrocytomas

In-depth genome wide molecular genetic analysis is available only in few diagnostic institutions. Even more common analysis methods, such as fluorescent in situ hybridization or gene sequencing are not affordable to all departments and access to immunohistochemistry can be limited in countries with emerging markets. That said, even with the absence of immunohistochemical and molecular genetic analyses tumors must be classifiable to provide a basis for therapy. In a recent meeting under sponsorship of the International Society of Neuropathology (ISN) held in Haarlem, the Netherlands, the consensus "ISN-Haarlem" guidelines were developed [13]. One notable proposition was the introduction of a "not otherwise specified" (NOS) category for tumors which could not be analyzed by IHC and/or molecular genetics in a desired way. While this is an important and pragmatic approach, it must be made very clear that the diagnoses of astrocytoma NOS, of astrocytoma *IDH*mut and of astrocytoma *IDH*wt refer to patients with very different characteristics: The diagnosis astrocytoma NOS inevitably will contain *IDH*mut astrocytomas and a considerable proportion of GBM, and less frequently other tumor entities as well as some oligodendrogliomas. The *IDH*mut astrocytoma group can be expected to be quite homogenous, however will contain some oligodendrogliomas which do require 1p/19 analysis for exclusion. Most problematic is the group of *IDH*wt astrocytomas because, as we show in this study, this set contains a variety of entities with defined molecular patterns. In the present series, 83 % of *IDH*wt astrocytomas exhibited clear molecular and clinical traits of GBM or GBM-H3. Related to this group are astrocytomas negatively scoring with the IDH1-R132H specific antibody. Because no more than 10 % of all *IDH* mutations in diffuse glioma are other than the IDH1-R132H type, this group can be expected to consist predominantly of GBM or GBM-H3. A schematic summary of this concept problem is given in Fig. 5.

Astrocytoma NOS is reserved for cases in which molecular characterization is not available. Most cases in which the IDH-R132H mutation has been excluded by immunohistochemistry, but which have not been sequenced for rare mutations, will be *IDH*wt and thus are best separated from the NOS designation.

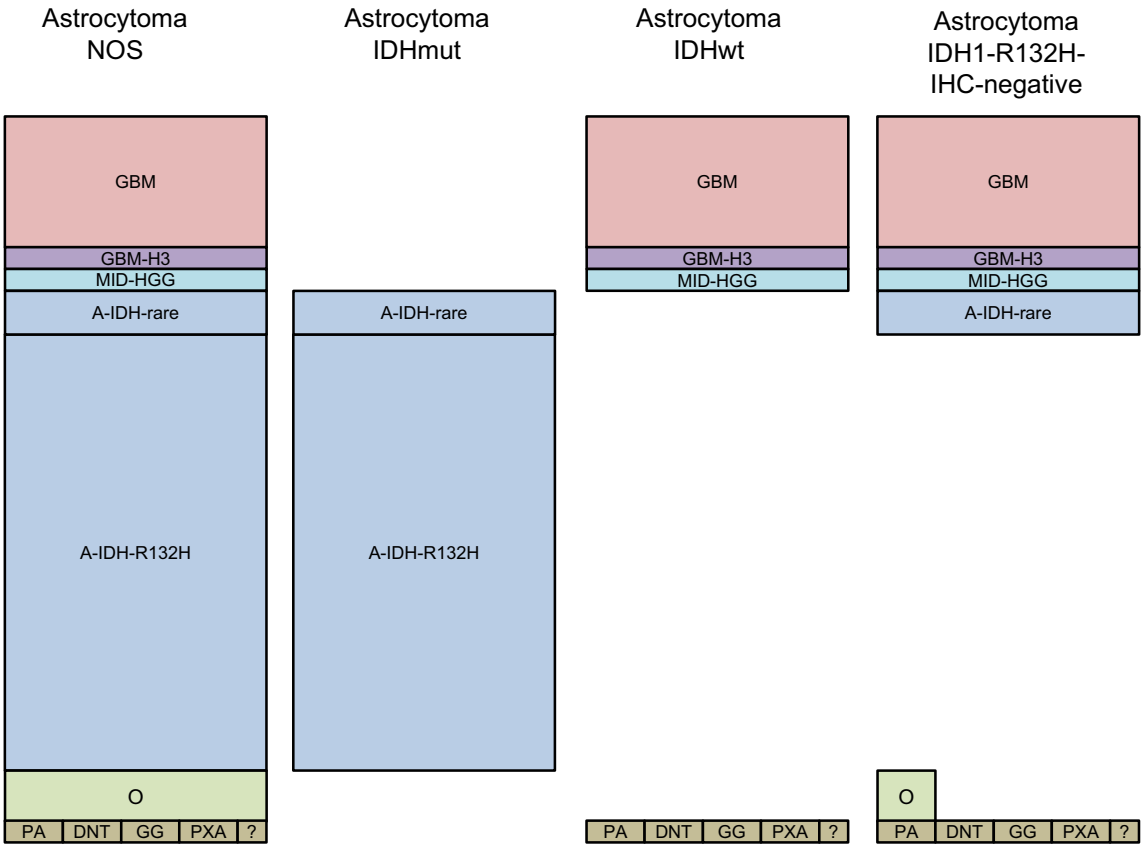

Fig. 5 Different sets of tumors are included in the classification categories of astrocytoma NOS, *IDH*mut, *IDH*wt, and IDH1-R132H-IHC-negative. From *left* to *right*: Astrocytoma NOS includes different tumors with the histopathological features of astrocytomas (*left*). Astrocytoma *IDH*mut constitutes a homogenous group (*middle left*), astrocytoma *IDH*wt (*middle right*) is a mixed bag with different tumors excluding astrocytoma *IDH*mut and astrocytoma IDH1-R132H-IHC-negative (*right panel*) is similar but contains those astrocytomas and oligodendrogliomas with rare *IDH1* and *IDH2* mutations. *Size of boxes* approximates the incidence in % and varies between institutions. *PA* pilocytic astrocytoma, *DNT* dysembryoplastic neuroepithelial tumor, *GG* ganglioglioma, *PXA* pleomorphic xanthoastrocytoma, *O* oligodendroglioma

The diagnosis of *IDH*mut astrocytoma can be considered to define a molecular and clinical homogenous entity. In fact, recent evidence points to only minor differences in clinical characteristics between patients with *IDH*mut diffuse A II and A III [2, 15, 18, 22]. We strongly recommend to use every possibility to come to this diagnosis by IHC with IDH1-R132H specific antibodies and, if required by sequencing exons 4 of *IDH1* and *IDH2*.

In the face of the steadily increasing number of molecular parameters of potential diagnostic relevance, neuropathological practice must aim at reducing additional assays to the necessary. In our experience, a feasible approach further refined from a previous report [19] towards the diagnosis of diffuse gliomas starts with the analysis of ATRX and IDH1-R132H (H09) immunohistochemistry. A large group consisting of astrocytomas exhibiting nuclear ATRX loss and positively staining with IDH1-R132H antibody does not require further analysis. Tumors with nuclear ATRX loss and lack of H09 binding need to be sequenced for rare *IDH* mutations and *H3F3A* mutations: *IDH* mutations render these tumors astrocytoma *H3F3A* mutations place them into the GBM-H3 group. *IDH*mut tumors with nuclear ATRX expression require testing for the complete loss of 1p/19q. All tumors with 1p/19q co-deletion represent oligodendrogliomas. This will also identify those *IDH*mut astrocytomas lacking both ATRX mutations and 1p/19q co-deletions. Further, all tumors of proven *IDH*wt need not be tested for 1p/19q co-deletion, as the latter alteration in practice always is associated with *IDH* mutations [11, 19, 29]. *IDH*wt tumors most likely represent GBM or misinterpreted other low grade glial or glioneuronal lesions. If diagnostically necessary, the analysis of *TERT* promoter mutations is helpful in distinguishing between these groups with *TERT* mutations exclusively present in the GBM group. The MID-HGG group currently cannot be distinguished without methylation profiling. A flow diagram of a possible approach is provided in Fig. 6.

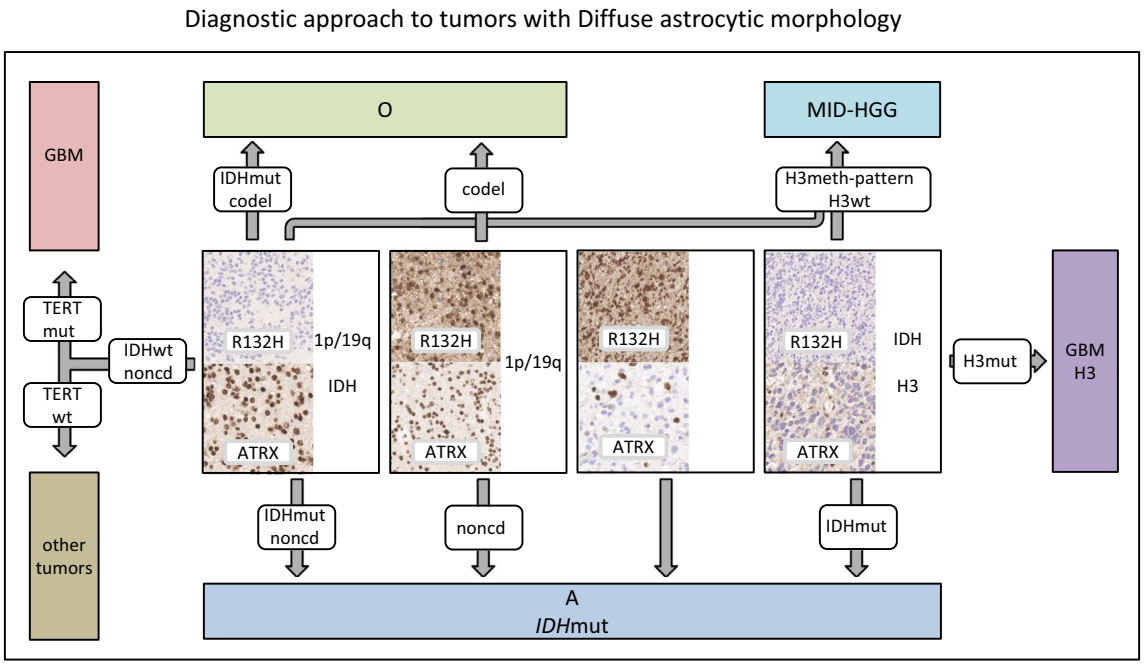

Fig. 6 Diagnostic approach to tumors with diffuse astrocytic morphology starting with IDH1-R132H and ATRX immunohistochemistry. Following this diagnostic algorithm, an integrated diagnosis can be obtained with reduced involvement of molecular analyses. The MID-HGG group currently cannot be distinguished without methylation profiling. While the assessment of *TERT* promoter mutations is not required for distinguishing between distinct groups of malignant glioma (GBM, GBM-H3, MID-HGG), it may be helpful in separating *IDH*wt GBM from other *IDH*wt lower grade tumors

Conclusions

Our data demonstrate that approximately 80 % of *IDH*wt astrocytomas in fact represent underdiagnosed GBM or GBM-H3 and a smaller fraction represents misclassified lower grade tumors such as pilocytic astrocytomas, pleomorphic astrocytomas, DNTs or gangliogliomas. A further 8 % of the *IDH*wt astrocytomas emerge as a new midline high grade glioma subset.

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