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# Anaplastic pilocytic astrocytoma - an IDH-wildtype diffuse glioma characterized by a distinct DNA methylation profile and combined alterations in MAPK pathway genes, CDKN2A/B and ATRX

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| **Abstract:** | Tumors with histological features of pilocytic astrocytoma (PA), but with increased mitotic activity and additional high-grade features (particularly microvascular proliferation and palisading necrosis) are often designated anaplastic pilocytic astrocytomas (APAs). The status of APA as a separate entity has not yet been clarified and molecular features have only been partially characterized. We performed DNA methylation profiling of 102 tumors with the histological differential diagnosis of APA. T-distributed stochastic neighbor embedding (t-SNE) and hierarchical clustering analysis of these 102 cases against 158 reference cases from 12 glioma reference DNA methylation classes revealed that a subset of 83 of these tumors form a DNA methylation class distinct from the reference classes. These 83 tumors were thus defined as DNA methylation class APA (MC APA). The 19 remaining tumors were distributed amongst the reference classes. Among these, in 11 tumors the molecular diagnosis could be further confirmed by additional analyses, whereas 8 remained non-classifiable. Median age of patients with MC APA was 41.5 years. The most frequent localization was the posterior fossa (74%). Deletions of CDKN2A/B (66/83, 80%), MAPK pathway gene alterations (49/65, 75%, most frequently affecting NF1, followed by BRAF and FGFR1) and mutations of ATRX or loss of ATRX expression (33/74, 45%) were the most common molecular alterations. All tumors were IDH1/2 wildtype. The MGMT promoter was methylated in 38/83 tumors (45%). Outcome analysis confirmed an unfavorable clinical course compared with PA, but better than IDH wildtype glioblastoma (GBM). In conclusion, we show that APAs carry a distinct DNA methylation profile, frequent alterations in MAPK pathway genes (particularly NF1) in combination with alterations of CDKN2A/B and ATRX, affect patients who are on average older than those with PA and have an intermediate clinical outcome. |

# Anaplastic pilocytic astrocytoma – an *IDH*-wildtype diffuse glioma characterized by a distinct DNA methylation profile and combined MAPK pathway, *CDKN2A/B* and *ATRX* alterations

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## Abstract

Tumors with histological features of pilocytic astrocytoma (PA), but with increased mitotic activity and additional high-grade features (particularly microvascular proliferation and palisading necrosis) are often designated anaplastic pilocytic astrocytomas (APAs). The status of APA as a separate entity has not yet been clarified and molecular features have only been partially characterized. We performed DNA methylation profiling of 102 tumors with the histological differential diagnosis of APA. T-distributed stochastic neighbor embedding (t-SNE) and hierarchical clustering analysis of these 102 cases against 158 reference cases from 12 glioma reference DNA methylation classes revealed that a subset of 83 of these tumors form a DNA methylation class distinct from the reference classes. These 83 tumors were thus defined as DNA methylation class APA (MC APA). The 19 remaining tumors were distributed amongst the reference classes. Among these, in 11 tumors the molecular diagnosis could be further confirmed by additional analyses, whereas 8 remained non-classifiable. Median age of patients with MC APA was 41.5 years. The most frequent localization was the posterior fossa (74%). Deletions of *CDKN2A/B* (66/83, 80%), MAPK pathway gene alterations (49/65, 75%, most frequently affecting *NF1*, followed by *BRAF* and *FGFR1*) and mutations of *ATRX* or loss of ATRX expression (33/74, 45%) were the most common molecular alterations. All tumors were *IDH1/2* wildtype. The *MGMT* promoter was methylated in 38/83 tumors (45%). Outcome analysis confirmed an unfavorable clinical course compared with PA, but better than *IDH* wildtype glioblastoma (GBM). In conclusion, we show that APAs carry a distinct DNA methylation profile, frequent alterations in MAPK pathway genes (particularly *NF1*) in combination with alterations of *CDKN2A/B* and *ATRX*, affect patients who are on average older than those with PA and have an intermediate clinical outcome.

## Key words

anaplastic pilocytic astrocytoma; pilocytic astrocytoma with anaplasia; methylation profile based classification; panel sequencing; ATRX; BRAF; NF1; FGFR1; MGMT; CDKN2A/B; IDH-wildtype; molecular characterization; DNA copy number alterations

## Introduction

PA accounts for approximately 5% of gliomas across all age groups and for approximately 25% of all brain tumors encountered in pediatric neurosurgical practice [9, 48], thereby representing the most common primary brain tumor in patients of 0 to 19 years of age. In contrast, the majority of gliomas in adults are either diffuse gliomas with *IDH1* mutation (either 1p19q co-deleted or 1p19q intact) or highly malignant GBMs [52]. Where gross total resection is feasible, clinical outcome of PA is usually favorable with 10 year overall survival rates of around 95% [9, 10, 14, 42]. The vast majority of PAs harbor single genetic alterations in genes encoding proteins of the mitogen-activated protein kinase (MAPK) pathway. The most frequent molecular findings in PAs are fusions of *BRAF* with *KIAA1549* or rarely other genes, BRAF V600E point mutation or alterations of *NF1*, *FGFR1* or *NTRK* family genes [24]. Different localizations of PA are associated with different frequencies of molecular alterations with posterior fossa tumors harboring a *BRAF* fusion in up to 80-90% of cases and supratentorial lesions showing this alteration in only about 60% of cases [21, 22]. Of further interest is the reported age association of *BRAF* fusions that seem to be less frequent in the adult population (e.g. 30% of patients of 31-40 years and 7% of patients older than 40 years of age) [17, 22]. To date, BRAF gene alterations are considered a molecular hallmark of WHO grade I PA with a typically favorable outcome [18, 21, 44]. Rare cases of PA, particularly in older patients, can have a more aggressive clinical behavior [4, 6, 41, 42, 48, 55]. The tumors of these patients may show anaplastic histological features including increased nuclear atypia, increased mitotic activity, prominent endothelial proliferation and/or palisading necrosis and have been discussed to represent a separate entity or subentity [42]. In the WHO classification 2016, this glioma subgroup is designated pilocytic astrocytoma with anaplasia. The identification of this particular subset of more aggressively behaving tumors is challenging, as the histological features described in the WHO classification do not exclude the diagnosis of conventional PA WHO grade I [9, 42], while histological overlap with pleomorphic xanthoastrocytoma/anaplastic pleomorphic xanthoastrocytoma and GBM has also been described. Moreover, according to the WHO classification, grading and nomenclature of pilocytic astrocytoma with anaplasia are still to be defined [1]. One study on the molecular characterization of clinically aggressive/recurrent or histologically defined APA revealed a heterozygous *PTEN*/10q loss in 6 of 19 (32%) and a homozygous *CDKN2A/B* deletion in 3 of 15 (20%) cases. In this cohort, *BRAF* fusions were identified in 63% of cerebellar lesions [41]. A different study observed an association with neurofibromatosis type I in 28% of APA [16]. Nevertheless, more comprehensive approaches are necessary to establish a molecular profile.

Recently, DNA methylation profiling became an important adjunct tool for tumor classification and identification of molecular subclasses [19, 30, 32, 33, 43, 46, 49]. To further characterize

APA, we analyzed a retrospective series of 102 cases with histological features of APA by DNA methylation profiling, DNA copy number analysis, next generation gene panel sequencing as well as by histological characterization and correlated these data with clinical information. Herein, we report that a major subset (83/102, 81%) of these cases form a distinct DNA methylation class, harbor frequent MAPK pathway gene alterations and have additional molecular and clinical features that clearly distinguish them from WHO grade I PAs as well as from other adult diffuse gliomas - thereby warranting their classification as a separate entity.

## Materials and Methods

### Tissue samples

Formalin fixed and paraffin embedded tissue of 102 retrospective cases with histological features compatible with the diagnosis of APA were retrieved from the archives of several large Neuropathology departments: the Department of Neuropathology, University Hospital Heidelberg; the Department of Neurosurgery, University Hospital of Mannheim; the Department of Neuropathology, Charité Universitätsmedizin Berlin; the Department of Neuropathology, University of Bonn; the Department of Neuropathology, Heinrich Heine University Duesseldorf; the Institute for Neuropathology, University of Essen; the Institute for Neuropathology, University of Freiburg; the Institute of Neuropathology, University of Giessen; the Department of Neuropathology, Hannover Medical School; the Department of Neuropathology, University of Cologne; the Department of Neuropathology, Leipzig University; the Institute for Neuropathology, University of Münster; the Institute for Pathology and Neuropathology, University of Tübingen; the Department of Neuropathology, University of Würzburg; the Institute for Neuropathology, the Department of Neurology, University Hospital and University of Zurich; the Institute of Pathology, University of Bern; the Division of Neuropathology of the Johns Hopkins School of Medicine, Baltimore, Maryland, USA; the Mayo Clinic, Rochester, Minnesota, USA; the Division of Neuropathology, UCL Institute of Neurology in London; the Department of Cellular Pathology, Queen's Hospital, Romford. In addition, we obtained material via the German Glioma Network. Tissue collection and processing as well as data collection were performed in compliance with local ethics regulations and approval.

### Clinical data

The following clinical data were acquired, when possible: referred histological diagnosis, patient gender, age at diagnosis of APA, localization, manifestation (primary lesion or recurrent

lesion), history of a precursor lesion, history of irradiation, presence of a tumor predisposition syndrome, progression-free survival and overall survival.

### Histological examination

Hematoxylin and eosin (HE)-stained slides of 74 tissue samples were systematically reviewed for morphological criteria. These histological features were: general morphological growth pattern, cellularity, nuclear pleomorphism, mitoses, necrosis, vascular proliferation, eosinophilic granular bodies or Rosenthal fibers and infiltration pattern.

### Immunohistochemistry

For cases with available tissue, immunohistochemistry with antibodies binding to ATRX (n=54), H3 K27M (n=47), IDH1 R132H (n=45) or BRAF V600E (n=51) was performed on a Ventana BenchMark XT Immunostainer (Ventana Medical Systems, Tucson, Arizona, USA) using established protocols. For dilutions and antibody details, see Supplementary Table 1.
Immunostaining with antibodies against BRAF V600E, IDH1 R132H and H3 K27M was scored as either positive or negative. For all three antibodies, nonspecific staining of macrophages, eosinophilic granular bodies and calcified deposits was excluded from analysis. Staining of vessels or reactive glia was also not considered. Loss of nuclear ATRX expression was scored as specific, if over 80% of tumor cell nuclei showed loss of expression, while nuclei of non-neoplastic cells, such as endothelia, microglia, lymphocytes and reactive astrocytes, were positive. Of note, weak to moderate staining of tumor cell cytoplasm was occasionally seen and was considered as non-specific [40]. Slides were scanned on a NanoZoomer Digital Slide Scanner (Hamamatsu, Hamamatsu, Japan) and photographed using Aperio ImageScope software (v11.0.2.725, Aperio Technologies, Vista, California, USA).

### DNA extraction and quantification

DNA was extracted from FFPE tissue using the automated Maxwell system (Promega, Fitchburg, Massachusetts, USA) according to the manufacturer's instructions. DNA concentration was determined using the Qubit dsDNA BR Assay kit (Invitrogen, Carlsbad, California, USA) following the producer's guidelines.

### Generation of DNA methylation array data, copy number profile calculation, determination of *MGMT* promoter methyltation status

From each tissue sample, 200 to 500 ng of DNA were processed for DNA methylation analysis. The Infinium HumanMethylation450 Bead-Chip (450k) array (Illumina, Carlsbad, California,

USA) was used to determine the DNA methylation status of 482421 CpG sites according to the manufacturer's instructions at the Genomics and Proteomics Core Facility of the German Cancer Research Center (DKFZ). A copy number profile (CNP) was calculated from the methylation array data as previously described [20] using the 'conumee' package in R (http://bioconductor.org/packages/release/bioc/html/conumee.html). *MGMT* promoter methylation status was determined from the methylation array data as described [5]. Amplifications in DNA copy number profile were defined as focal regions of copy number gain with a notably higher amplitude than regions of suspected single-copy gains.

### *H3F3A, BRAF, IDH1, IDH2* and *TERT* promoter mutation analysis by Sanger sequencing

Primer design and sequencing were performed according to standard protocols. Primer sequences and NM accession numbers for the respective genes are listed in Supplementary Table 2. For the amplification reaction, the following reagents were used: 12,5 µl of Go Taq G2 DNA polymerase (Promega), 1.25 µl forward primer (10 pmol/µl), 1.25 µl reverse primer (10 pmol/µl), 8.0 µl nuclease free water and 2 µl template DNA (approximately 25 ng/µl). PCR conditions are listed in Supplementary Table 3.

### *MGMT* promoter pyrosequencing

For cases with indeterminable *MGMT* promoter methylation status by 450k methylation analysis, additional pyrosequencing was performed using the therascreen® MGMT Pyro® kit (QIAGEN®) and the PyroMark® Q24 system (QIAGEN®) according to the manufacturer's protocol. Bisulfite conversion was done with the EpiTect fast DNA bisulfite kit (QIAGEN®). According to a studies published by Quillien et al., Felsberg et al. and Reifenberger et al. [12, 36, 38], the cutoff value for *MGMT* promoter methylation status was set as follows: a mean *MGMT* promoter methylation percentage < 8% across the investigated CpG sites was considered as non-methylated and a value ≥ 8% was considered as methylated.

### Gene panel sequencing

Gene panel sequencing was performed and resulting data were analyzed as previously described [45]. In brief, extracted DNA was sheared on a M220 Focused-ultrasonicator™ (Covaris®, Woburn, Massachusetts, USA). DNA integrity and fragment size were determined by the Bioanalyzer 2100 (Agilent, Santa Clara, California, USA). Sequencing was performed on a NextSeq 500 instrument (Illumina, Carlsbad, USA) with a mean coverage of 645 reads and a standard deviation of 455 reads. Gene panel sequencing data were automatically annotated using annovar software that integrates information from databases such as dbSNP, the 1000 Genomes Project and COSMIC, as well as with SIFT and PolyPhen2 scores to infer

the possible biological relevance of an alteration [53]. Gene panel sequencing data were then filtered applying the following criteria: firstly, exonic and splicing variants were selected. From these, synonymous and stoploss variants were not further considered. Thereafter, variants with a frequency not exceeding 1% in the healthy population as well as undescribed variants were selected according to the 1000 Genomes Project database. Variants described as known polymorphisms in the Single Nucleotide Polymorphism database (dbSNP, version 138: https://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi?view+summary=view+summary&build_id=138) were not considered. Insertions and deletions were filtered for exonic frameshift changes that were not yet detected in the healthy population according to the 1000 Genomes Project database and that were not present in the Single Nucleotide Polymorphism database (dbSNP). The remaining items (nonsynonymous, stopgain or splicing site variants, frameshift insertions and frameshift deletions) were evaluated for their potential clinic-pathological relevance using the COSMIC database (http://cancer.sanger.ac.uk/cosmic) and the ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar/) and categorized with regard to the probable consequences on protein function: damaging, possibly damaging, likely not relevant or no applicable information. Variants assessed as damaging or possibly damaging were considered as mutations. Variants assessed as likely not relevant or with lack of information about clinical relevance were not considered. For determining the MAPK pathway status, we considered the following genes: *NF1, BRAF, FGFR1, KRAS, NRAS, HRAS, NTRK2* and *PTPN11*. A full list of the genes represented in the applied gene panel is provided in Supplementary Table 4.

## Reference datasets

Reference datasets for t-SNE, clustering and copy number analyses: for t-SNE and hierarchical clustering, the following glioma reference classes were included: diffuse midline glioma, *H3 K27* mutant (DMG K27; 14 cases); high-grade neuroepithelial tumor, *H3 G34* mutant (GBM G34; 11 cases); GBM of the midline (GBM MID; 10 cases); GBM, mesenchymal subtype (GBM MES; 15 cases); GBM, RTK I subtype (GBM RTK I; 15 cases); GBM, RTK II subtype (GBM RTK II; 8 cases), pleomorphic xanthoastrocytoma (PXA; 17 cases), PA of the supratentorial hemispheres (PA SUP; 12 cases), PA of the posterior fossa (PA PF; 15 cases), PA of the midline (PA MID; 18 cases), dysembryoplastic neuroepithelial tumor (DNET; 13 cases) and diffuse leptomeningeal glioneuronal tumor (DLGNT; 10 cases).

Detailed descriptions of the reference methylation classes used in this study are outlined under the following link: https://www.molecularneuropathology.org/mnp/classifier/2

Reference sets for the Kaplan Meier analysis: Outcome data were available for 41 of the MC APA patients. For patients with an initial diagnosis of a lower-grade glioma, the date of the first histological diagnosis of anaplasia was considered as starting point for the outcome analysis.

Kaplan Meier analysis was performed in comparison to survival data from additional 281 glioma patients of 5 reference classes: 82 PAs WHO grade I, 18 PXAs, 26 anaplastic astrocytomas, IDH mutant (AIII *IDH* mut), 90 GBMs, IDH mutant (GBM *IDH* mut) and 56 GBMs, *IDH* wildtype (GBM *IDH* wt).

### Statistical analyses

The DNA methylation array data were processed with the R/Bioconductor package minfi (version 1.20) [3]. For unsupervised hierarchical clustering of APAs and reference samples, we selected the 20,000 most variably methylated CpG sites across the dataset according to median absolute deviation. Pairwise similarity of samples was calculated using Euclidean distance. Clusters were then linked according to the Ward's linkage method. t-SNE plot was computed via the R package Rtsne [28] using the 20,000 most variable CpG sites according to standard deviation, 2,000 iterations and a perplexity value of 20. To define the DNA methylation class APA, cutoffs for the X and the Y values were deduced from the t-SNE plot (Fig 1) as follows: $-10 < Y < 10$ and $-20 < X < 2$. Survival data of APA patients and reference group patients were evaluated via Kaplan-Meier analysis using the JMP software (SAS Institute, Cary, North Carolina, USA). To verify differences of the survival distributions between the respective patient groups, the Log-rank test was applied.

## Results

### APA form a distinct DNA methylation class

We performed DNA methylation analysis of 102 gliomas with a histological differential diagnosis of APA. t-SNE analysis of their DNA methylation profiles against 158 reference cases from 12 different established glioma DNA methylation classes revealed a distinct DNA methylation class comprising 83 of these 102 tumors (81%; Fig 1). Subgroups did not become apparent in the t-SNE analysis. The 19 remaining tumors were congruent with or close to one of the reference classes. Two of these showed a high similarity to high-grade glioma reference classes (one to DMG K27 and the other to GBM MID), two to the PXA reference class and 15 cases were similar to the low-grade glioma reference classes comprising PAs and DNETs. The 83 tumors in the APA group, henceforth designated as DNA methylation class APA (MC APA), were further characterized by including clinical and molecular features. Of note, the MC APA also contained two tumors with a H3 K27M mutation. Indeed, these lesions were located near the midline (one in the third and lateral ventricle and the other in the cerebellum), but did not otherwise show histological features of DMG K27, as both cases had a piloid morphology.

In addition, we performed clustering analysis of the cohort previously analyzed by t-SNE (Supplementary Fig 1). This analysis revealed a cluster of 81/83 APAs which were congruent with the tumors of the MC APA identified by t-SNE confirming the assignment as a distinct molecular entity. Within this methylation cluster, two subclusters (a and b, see Supplementary Fig 1) were putatively identified. There was, however, no high consistency in repeated clustering analyses and no obvious clinical, histological or molecular differences were seen between the two subclusters.

## Re-classification of tumors with histological features of APA, but divergent DNA methylation profile

t-SNE and clustering analysis revealed that 19 of the 102 tumors with histological features of APA displayed a DNA methylation profile that differed from the MC APA (Fig 1 and Supplementary Fig 1). In keeping with our experience with other series [50] (Capper et al.: "DNA methylation-based classification of human central nervous system tumours", under revision), the majority of these tumors could be re-classified on the basis of re-evaluation of histology and orthogonal molecular findings (immunohistochemistry for BRAF V600E, H3 K27M, ATRX, copy number profile analysis): six tumors were downgraded to PA WHO grade I, one case was re-classified as PXA, one as diffuse midline glioma with H3 K27M mutation, one as GBM, *IDH* wildtype and two as low grade gliomas, not otherwise specified (NOS). The remaining eight cases could not be concretely classified. Supplementary Table 5 and Supplementary Fig 2 and 3 give details and show examples of re-classified and non-classifiable cases.

## Clinical characteristics of the MC APA

The tumors in the MC APA (n=83) were preferentially located in the posterior fossa (74%), most frequently in the cerebellum (63%). 17% were located supratentorially, two cases (2%) had a supra- as well as infratentorial localization and 5 cases (7%) were located in the spinal compartment (Fig 2a). Median patient age at resection was 41.5 years with only 8 out of 76 tumors (11%) occurring in patients below the age of 20 years (Fig 2b). The distribution of male and female patients was balanced (male female ratio of 1.1; n=83). Of 67 patients with available data, 42 patients (63%) presented as primary diagnosis without previously known lesions, whereas the investigated tumors of 25 patients (37%) represented recurrences (Fig 2c) from either low-grade primary tumors (8 cases, 12%), primary APA (3 cases, 4%) or primary tumors with unknown initial grade (14 cases, 17%). Among the 8 patients with an initially low-grade tumor, 6 had an initial diagnosis of PA, while the remaining two patients were initially diagnosed as oligodendroglioma WHO grade II and diffuse astrocytoma WHO grade II. For the 25 recurrent lesions, relapses occurred within 10 years in 18 cases (72%), whereas time to relapse was longer than 10 years in four cases (16%) and was unknown for another

three cases (12%). Median overall survival after primary diagnosis with APA was 24 months (n=41). Median time to progression was 1.2 years with a wide variation ranging from 66 days up to 28.3 years. For patients with a low-grade primary tumor, median time to recurrence was 3.2 years (n=6), whereas in patients with a high-grade primary tumor, median time to progression was only 0,9 years (n=3). For only 5% (4 cases) of the patients in the whole cohort, previous irradiation was reported. A diagnosis of neurofibromatosis type 1 was clinically known in one patient and clinically suspected in a second patient. Other hereditary tumor predisposition syndromes were not reported.

### Histological and immunohistochemical findings in APA

We were able to comprehensively evaluate 74 cases of the MC APA histologically including the assessment of growth and infiltration pattern, cellularity, nuclear pleomorphism, mitotic count, presence of necrosis, vascular proliferation and presence of eosinophilic granular bodies and/or Rosenthal fibers. Results are summarized in Fig 3. Examples for histological features of the MC APA are shown in Fig 4a, 4b and 4c.

Immunohistochemical analysis included assessment of the glioma markers ATRX, BRAF V600E, H3 K27M and IDH1 R132H. In 44% (24/54) of APAs nuclear ATRX expression was lost in the tumor cells (Fig 4d). BRAF immunohistochemistry revealed only one positive case (1/51, 2%). By H3 K27M immunohistochemistry and confirmatory Sanger sequencing analysis, one case (1/47, 2%) with a *H3 K27M* mutation was identified (Supplementary Fig 4). For all the remaining cases tested by immunohistochemistry, H3 K27M (Fig 4e) and BRAF V600E were negative. Notably for this group of non-GBM diffuse gliomas, IDH1 R132H immunohistochemistry was negative in all cases investigated (45/45) (Fig 4f).

### Molecular characteristics of the MC APA - *CDKN2A/B* deletion represents the most frequent structural aberration of APA

Copy number profile analysis of the molecular APA series disclosed numerous, partially complex chromosomal alterations: in 73 of 83 cases (88%), more than three structural aberrations were found, whereas a small subset of 10 cases (12%) exhibited three or fewer alterations. Fig 5a illustrates a representative low-grade posterior fossa PA copy number profile, whereas in Fig 5b, an example of a copy number profile for a tumor of the MC APA is shown. Fig 5c and d show the averaged copy number profiles of 45 PA reference cases (comprising 15 cases of posterior fossa PA, 18 cases of midline PA and 12 cases of supratentorial PA) and from all 83 tumors of the MC APA. The most frequent aberration in the MC APA (66/83 cases, 80%) was a deletion of *CDKN2A/B*. This was frequently associated with broader deletions or complex changes on chromosome arm 9p (41/83 cases, 49%). A further significant finding was a *BRAF* fusion indicated by a focal peak on chromosome 7q in

15 out of 74 assessed cases (20%) (Fig 5b, 5d, 6). Other recurrent copy number aberrations were gains and/or complex changes on chromosome arms 12q (27%) and 17q (33%) as well as deletions on chromosomes 1p (19%), 8p (23%) and 19q (22%). A *CDK4* amplification was detected in seven cases (8%) and this was associated with a concomitant *MDM2* amplification in four cases (5%). Other recurrent amplifications more commonly seen in GBM (e.g. of *MDM4, PDGFRα, EGFR, CDK6, MET*) were not observed.

### APAs harbor mutations in MAPK pathway genes

Alterations affecting genes encoding members of the mitogen-activated protein kinase (MAPK) pathway have previously been found to occur in up to 100% of WHO grade I PAs [22-24]. By combining immunohistochemistry, panel sequencing and copy number analysis, we were able to comprehensively examine the status of MAPK pathway gene alterations for 65 of 83 tumors of the MC APA (Fig 6). In 49 out of these 65 cases (75%), at least one characteristic MAPK pathway gene alteration was detected. In notable contrast to typical PAs, the most frequently affected gene was *NF1,* being altered in 20 of 67 lesions (30%). In total, five tumors exhibited a deletion, 10 cases harbored a mutation (including one clinically known NF1 syndrome patient) and three cases showed both, a deletion in the copy number profile and a mutation of the NF1 gene. The remaining two cases showed immunohistochemical loss of NF1 protein expression and a balanced *NF1* gene dosage in the copy number profile. *BRAF* was the second most frequently altered MAPK pathway gene: 15 of 74 cases (20%) harbored a *BRAF* fusion and one case (1%) a *BRAF* V600E mutation. In 12 out of 64 cases (19%) an *FGFR1* alteration was detected. Among these, 11 of 64 tumors (17%) had a point mutation at one of the known hotspots (K656E/N, N546D/K) and one case (2%) exhibited an *FGFR1*/*TACC1* fusion. In another two of 64 cases (3%) a *KRAS* point mutation (Q61H in one and V14A in the other) was detected (Fig 6). MAPK pathway gene alterations were mutually exclusive with the exception of one case harboring both an *NF1* and an *FGFR1* alteration (Fig 6).

### APAs frequently display *ATRX*, but rarely *TERT* alterations

Alterations in mechanisms of telomere maintenance are well known drivers of tumor progression in a variety of entities. In particular, *TERT* promoter and *ATRX* mutations are frequently described in diffuse gliomas and have been established as useful markers for their classification and prognostication [35, 39, 40]. Unexpectedly, we found a high rate of *ATRX* alterations in the MC APA: in 15 of 64 tumors (23%) mutant *ATRX* was detected by panel sequencing and 24 of 54 tumors (44%) showed an immunohistochemical loss of nuclear ATRX expression. For 21 of these 24 cases, panel sequencing data were also available: in these, a relevant *ATRX* mutation was confirmed in 12 cases (57%). In the 18 cases with retained nuclear ATRX expression and with available panel sequencing data, no *ATRX* mutations were detected. The sensitivity of our gene panel sequencing approach to predict *ATRX* protein loss

was thus 57%, the specificity being 100%. In a study on IDH mutant gliomas, *ATRX* gene alterations were also not found in a subset of cases with a loss of nuclear ATRX expression. It appears likely that other mechanisms than exonic mutations may also result in a functional (and immunhistochemical) loss of ATRX protein [56].

*ATRX* alterations and *TERT* promoter mutations are known to occur in a mutually exclusive manner in gliomas [25, 40]. To determine the rate of *TERT* promoter mutations in the MC APA and to explore, whether these are mutually exclusive with *ATRX* alterations in this newly defined tumor class, we performed Sanger sequencing of the *TERT* promoter region of 74 tumors of the MC APA from which 31 cases carried an *ATRX* alteration (Fig 6). Two tumors (3%) with a *TERT* promoter mutation, both with the nucleotide exchange C228T, were identified. Both tumors did not harbor an *ATRX* alteration. While gain of chromosome 5p was observed in approximately 10% of APAs, no focal TERT amplifications were seen (Fig. 5c).

### *MGMT* promoter hypermethylation is a frequent finding in APAs

In 38 out of 83 tumors of the MC APA (46%), the *MGMT* promoter was hypermethylated, another 38 tumors (46%) showed a non-methylated *MGMT* promoter and in seven tumors (8%), the *MGMT* promoter methylation status could not be determined (Fig 6).

### Comparison of the MC APA with the molecular subtypes of glioma previously described by Ceccarelli et al.

A subset of adult diffuse *IDH* wildtype gliomas showing a DNA methylation profile distinct from the classic-like and mesenchymal-like GBM defined by Sturm et al. [51] has recently been described by a pan-glioma analysis of The Cancer Genome Atlas (TCGA) data [8]. This subset of tumors has been shown to be further subdivided into two DNA methylation subclasses, from which one shows similarity to GBM and the other to PA. These DNA methylation subclasses have been designated “LGm6-GBM” and “PA like low-grade gliomas” (LGG) [8]. DNA methylation profiles of the MC APA and the PA-like LGG subset of tumors from the TCGA series were re-analyzed together with the reference classes described above by t-SNE (Figure 7). While 3 of 27 and 14 of 27 TCGA tumors clustered more closely with our GBM and our low-grade glioma reference classes, the DNA methylation profiles of a subset of 9 of 27 TCGA cases showed high similarity to the MC APA. Interestingly, these tumors were also enriched for *NF1*- or *BRAF* alterations, *CDKN2A/B* loss and/or *ATRX* mutation [8] further confirming that these tumors may likely represent a distinct molecular entity.

### Outcome analyses

Outcome data were available for 41 of the 83 patients with a MC APA tumor: at the date of the last follow-up, 18 patients (44%) had deceased, and 23 patients (56%) were alive. Median overall survival was 720 days (approximately 24 months). Kaplan Meier analysis of these 41

patients against five glioma reference classes showed a survival probability inferior to patients with conventional PA, PXA and IDH mutant anaplastic astrocytoma and comparable to patients with IDH mutant GBM, but superior to patients with IDH wildtype GBM (Fig 8a). Of note, univariate outcome analysis of patients of the MC APA with a proven characteristic MAPK pathway gene alteration compared with patients without such an alteration showed a significantly worse prognosis for the latter (p=0.032, Fig 8b), suggesting that non-canonical alterations may be associated with more aggressive clinical behavior. Kaplan-Meier analysis for each individual MAPK pathway gene alteration alone was also performed and showed no significant differences of survival probabilities between patients with *BRAF, NF1 or FGFR1* altered tumors and patients negative for a characteristic MAPK pathway gene alteration (Supplementary Fig 5). No significant outcome differences were seen in patients with *MGMT* methylated versus non-methylated tumors (p=0.922) as well as in patients with *ATRX* altered versus *ATRX* wildtype tumors (p=0.685) (Fig 8c, d). Furthermore, outcome analysis dependent on histological criteria (presence/absence of necrosis, mitotic count) was performed (n=38). No significant difference in survival probability between tumors with *versus* without necrosis (p=0,468) or with 1-2 *versus* more than 2 mitoses (p=0,383) was evident (see Supplementary Fig 6).

## Discussion

In this study we report a novel class of glial tumors defined by a characteristic DNA methylation profile and harboring many cases with a morphological overlap with PA with anaplasia as described in the WHO classification 2016 [1]. However, detailed histological evaluation shows a wide range of morphological features that would occasionally be more in keeping with other tumor entities (especially high-grade glioma/GBM). Thus, as with a growing number of CNS tumors, the tumor class of APA as defined here by DNA methylation profiling (MC APA) is not exactly congruent with PA with anaplasia as defined in the current WHO classification [1].

Initially, t-SNE and cluster analysis of the DNA methylation profiles were performed and consistently revealed a distinct DNA methylation cluster denoted MC APA. The closest resemblance of the DNA methylation profiles of these tumors was evident with the reference DNA methylation class of diffuse leptomeningeal glioneuronal tumor (DLGNT) (Fig 1, Supplementary Fig 1). Indeed, these tumors may be closely related to APA, since they also frequently harbor BRAF fusions in conjunction with other alterations not found in classical PA (e.g. 1p loss) [7].

Our further investigations focused on the clinical, histological and molecular characterization of the MC APA. As it has previously been reported for a series of histologically defined APAs [42], we observed a higher median patient age in the MC APA than in conventional PA [6]. In

fact, the gliomas belonging to the MC APA mostly arise in adults. Only 8% of these tumors occurred in patients less than 18 years of age. The most frequent tumor localization in our series was the posterior fossa (74%) with 63% of the tumors originating from the cerebellum. Compared to a previously reported series of histologically defined APAs [42], the posterior fossa location seems even more frequent among gliomas of the MC APA. Importantly, clinical history of radiotherapy was reported in only 5% (4/83) of the patients. Thus, the role of irradiation in the progression to APA, as reported previously, may require additional investigation [2, 42].

As shown in Fig 3, we observed a wide spectrum of histological features in our series: most of the tumors in the MC APA showed a piloid or GBM-like general morphological pattern, were moderately cellular, had moderate nuclear pleomorphism, at least 1 mitosis per 10 HPF and microvascular proliferation. Around one third of the tumors exhibited areas of necrosis and one third showed eosinophilic granular bodies or Rosenthal fibers, respectively. In summary, morphological characteristics of the MC APA were not particularly specific and may overlap with other low and high-grade gliomas. Despite a small subset of cases showing typical histological features of anaplasia, our investigations revealed that the histology of the majority of cases in our series appears rather inconspicuous. Thus, molecular analysis may be required in order to come to a final, integrated diagnosis. With regard to immunohistochemistry, the most interesting finding was a loss of nuclear ATRX expression. Comparison of immunohistochemical ATRX staining of tumors in the MC APA and a control cohort of conventional PAs showed that ATRX loss is restricted to the former. In keeping with our results, Ebrahimi et al. did not detect ATRX alterations in conventional PAs, but identified one case of APA and one case of BRAF-fused cerebellar GBM with loss of ATRX expression [11].

*MGMT* promoter methylation status has been shown to have predictive and prognostic value, particularly for patients with GBM [15, 27, 31]. This study revealed that *MGMT* promoter hypermethylation was present in approximately half of APAs, whereas in a control cohort of conventional PAs, the *MGMT* status was either non-methylated in most of the cases or intermediate in a small subset of tumors. These data indicate that *MGMT* promoter methylation status may be of diagnostic relevance in terms of distinguishing conventional PA from APA. However, the present data do not provide any evidence for an association of MGMT promoter methylation status with OS amongst the MC APA. Admittedly, the prognostic value of this outcome analysis is limited, as clinical information about a previous chemotherapy and the applied pharmaceuticals (e.g. temozolomide) were incomplete.

Another important finding of this study was that the distribution of MAPK pathway gene alterations in the MC APA clearly differed from that known for conventional PA. For instance, *BRAF* fusions are known to be the most abundant molecular alteration in conventional PAs

with a frequency of up to 75%, depending on tumor localization and patient age. The second most common alterations in approximately 7% of PAs affect the NF1 gene, followed by activating *BRAF* mutations (5%) and *FGFR1* hotspot mutations (5%). Rarely (about 1 up to 2% each) *NTRK2* fusions, *PTPN11* mutations and *RAF1* fusions were also found [22, 24]. In contrast, with a frequency of only 20%, *BRAF* fusions are surprisingly rare in the MC APA, whereas NF1 turned out as the most frequently affected gene being altered in 30% of the tumors. Furthermore, a substantial fraction (16%) of tumors harbored a *FGFR1* hotspot mutation and in one tumor, a *FGFR1/TACC1* fusion was detected, as previously reported in pediatric low-grade as well as in high-grade gliomas. This aberration has also been shown to transform primary astrocytes into highly proliferating glial tumors [47, 57]. *NTRK2* fusions, *PTPN11* mutations or *RAF1* fusions were not found and a *KRAS* mutation was detectable in only two cases (2%). *FGFR1* alterations have been described to be preferentially found in extra-cerebellar and especially in midline gliomas [9, 24]. However, in the present series, the majority of *FGFR1* mutant cases were located in the posterior fossa (Fig 6). Hence, these data indicate that *FGFR1* alterations may not be restricted to extracerebellar or midline locations. *BRAF* alterations have been described to be less frequent (36% of cases) in adult compared to juvenile PAs [34]. In a study conducted by Hasselblatt et al., comparable results were obtained with a fraction of *BRAF* fusions of 30% in patients aged 31-40 years and of 7% in patients older than 40 years [17]. As also the MC APA proved to be characterized by a higher patient age, the age distribution of *BRAF* fusions may explain the comparably low fraction of tumors positive for a *BRAF* fusion in the present series. We furthermore observed that *CDKN2A/B* deletion in a *BRAF* fusion positive astrocytic glioma is highly suggestive for the diagnosis of APA and makes the diagnosis of WHO grade I PA less likely.

Aberrant activation of the MAPK pathway in PA, particularly by *BRAF* or *RAS* activation and *NF1* inactivation, is thought to trigger oncogene-induced senescence (OIS) via engagement of the cyclin dependent kinase inhibitors $p16^{Ink4a}$ and/or $p21^{Waf1}$. As these proteins trigger cell cycle arrest, they are known as tumor suppressors and are supposed to be associated with the indolent biological and clinical behavior of conventional PAs [22]. Rodriguez et al. observed a loss of p16 in a subset of PAs with anaplastic features [41]. Another study revealed that PA patients with immunohistochemical loss of p16 expression had a shorter overall survival than PA patients with retained p16 expression [37]. These findings raised the question, whether the prognosis of patients with PA/APA may be associated with the presence or absence of p16. Approximately half of the cases comprising the MC APA displayed a MAPK pathway gene alteration in combination with a CDKN2A/B deletion. For these lesions, impairment of OIS may be assumed. We hypothesized that impaired OIS may at least in part contribute to the adverse clinical course of APA. From 26 patients with MAPK pathway gene altered and *CDKN2A/B* deleted tumors and with available survival data, 10 (38%) were indicated as deceased and 16

(62%) as alive with a median overall survival of 604 days. Independent of MAPK status, patients with CDKN2A/B deleted tumors had a median overall survival of 618 days (n=37), whereas patients with tumors with balanced CDKN2A/B copy number had a median overall survival of 1072 days (n=4). Further studies with a higher case number are required to particularly determine the impact of *CDKN2A/B* deletions on survival of (MAPK pathway gene altered) APAs.

Existence of anaplastic features in PA has previously been found to be associated with decreased overall survival. In one series outcomes of APA patients were indeed less favorable in comparison to conventional PA patients. Survival data were stated as comparable to WHO grade II and III diffusely infiltrative astrocytomas, but still better than grade WHO IV GBMs [42]. Another study suggested that APA patients may have a better prognosis than other high-grade gliomas in adult patients [13]. However, Kaplan Meier analysis of patients with molecularly-defined APAs (MC APA) in the present series revealed a survival probability worse than patients with IDH mutant anaplastic astrocytoma, WHO grade III and comparable to patients with IDH mutant GBM, WHO grade IV (although better than patients with IDH wildtype GBM, WHO grade IV). Median overall survival of approximately 24 months (720 days) was consistent with the results of other authors [42]. Notably, the comparability of the outcome data discussed above may be limited, as cases for previously performed analyses were selected by histological criteria, whereas for the present series, molecular data were additionally taken into account and may have led to a higher discrimination selectivity with respect to other glioma entities. Another notable finding of this study was that patients with tumors harboring a characteristic MAPK pathway gene alteration were found to survive significantly longer than patients with tumors carrying no characteristic MAPK pathway gene alteration. Further analyses, such as RNA and whole-exome sequencing, are required to investigate alternative alterations in the latter tumors and, when indicated, to reappraise, whether the absence of MAPK pathway gene alterations in APA may indeed be associated with a worse outcome.

In conclusion, APA, PA WHO grade I, PXA and to some extent GBM may show overlapping histological and/or molecular features. Importantly, PXAs are also known to show alterations in *BRAF* as well as *CDKN2A/B* [26, 54] and PAs may also harbor *BRAF* fusions [17, 21, 22]. Hence, the distinction between these three entities may become challenging [29]. Fig 9 provides a suggestion of a diagnostic algorithm in case APA is suspected. As indicated, immunohistochemical and single parameter molecular analysis may at least help to exclude other glioma classes. Nonetheless, comprehensive molecular investigations (such as DNA methylation profiling and/or gene panel sequencing) are recommended to definitely confirm this diagnosis. As defined in the present study, APA is characterized by a distinct DNA methylation profile and alterations of NF1, BRAF or FGFR1 together with CDKN2A/B and

ATRX loss as well as MGMT promoter hypermethylation. Further investigations will be required to compile a set of molecular markers to underpin the diagnosis of APA, in order to cement its position in brain tumor classifications and enable, for example, reliable stratification of clinical trials.

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figure

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# Fig 1

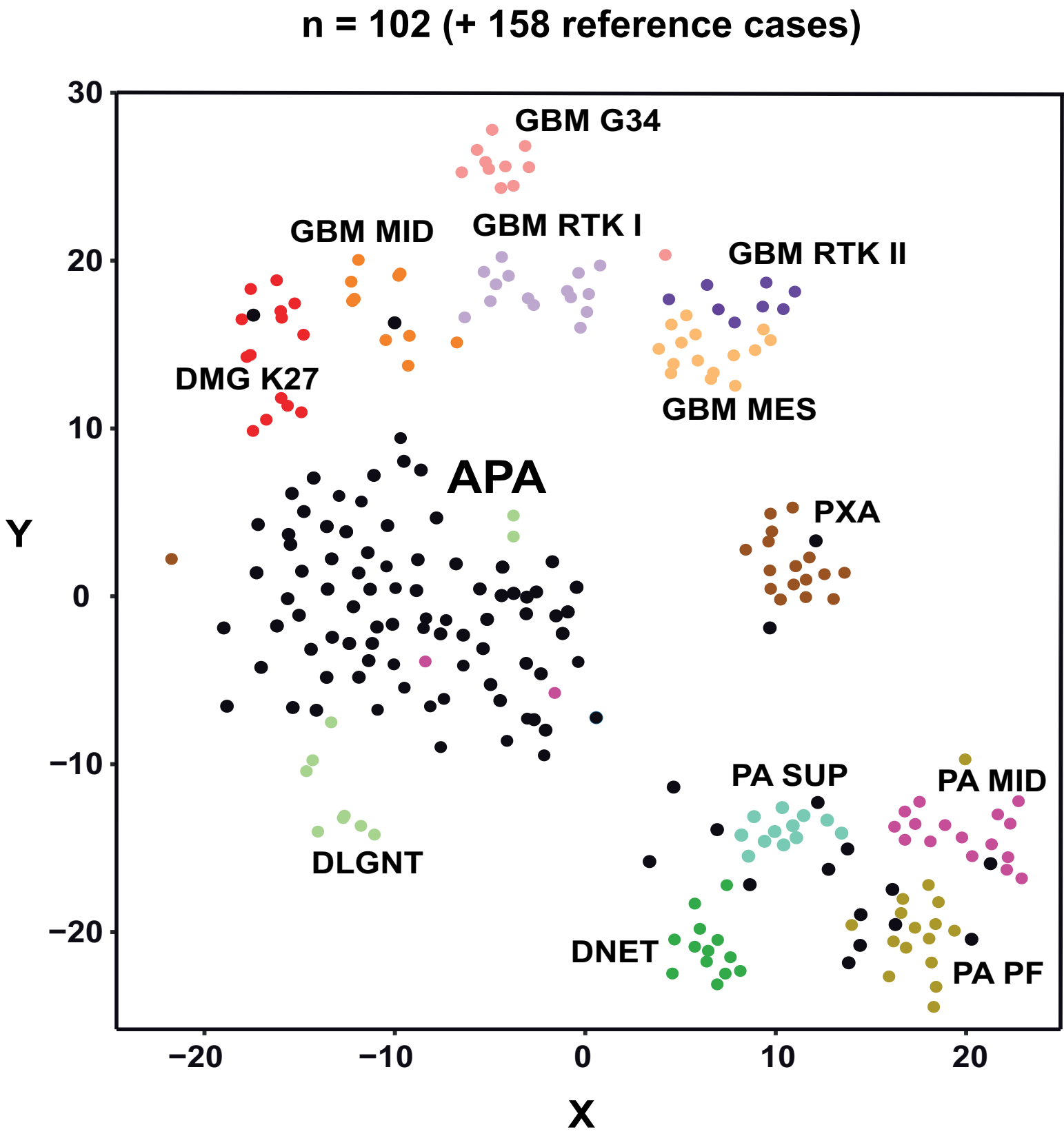

figure **Fig 2**

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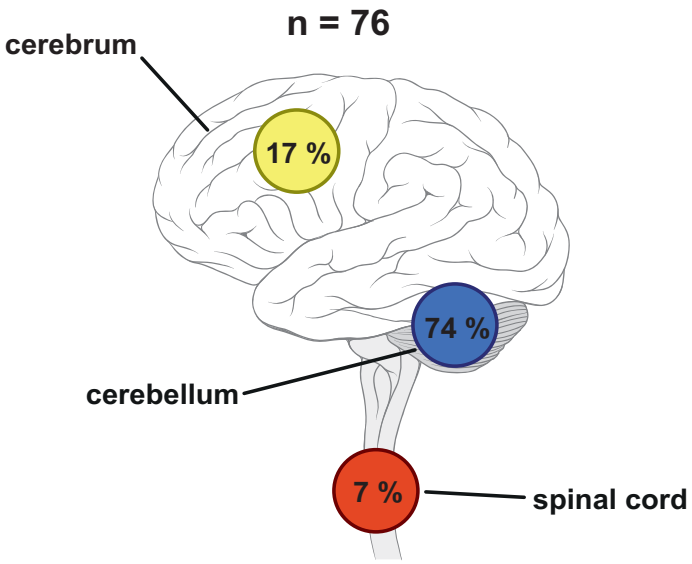
**a**

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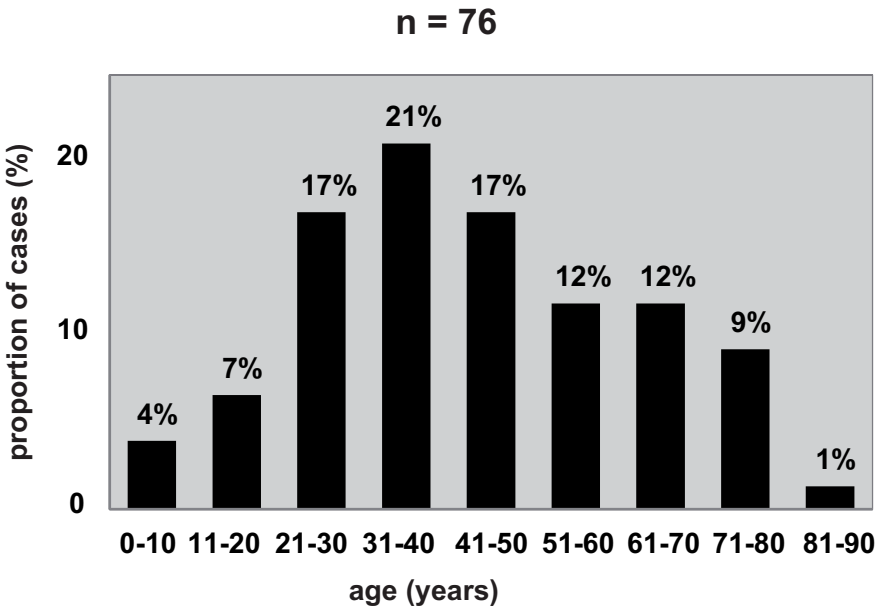


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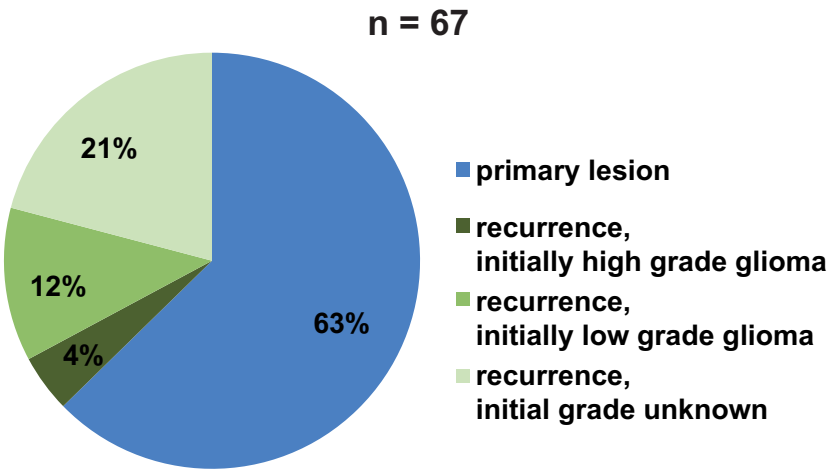

figure

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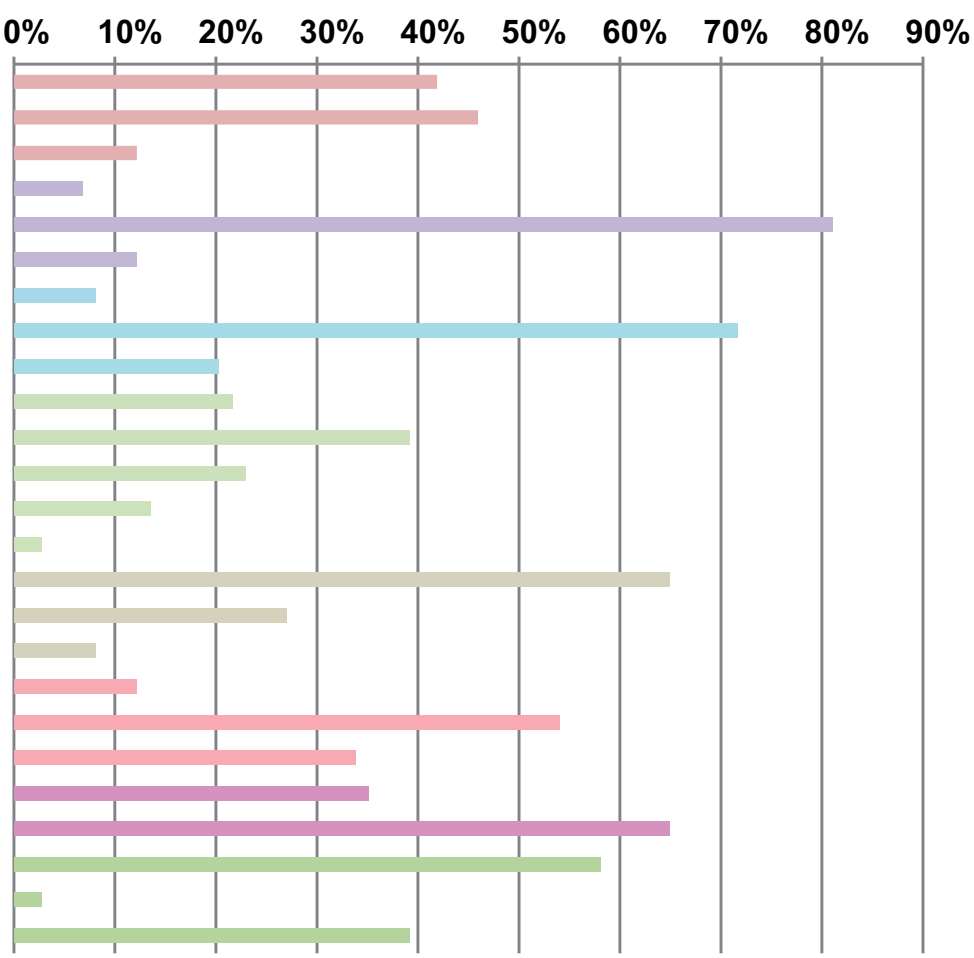
# Fig 3

n = 74

| histological criterion | specification | # cases |
|---|---|---|
| morphology | mainly piloid | 31 |
| | at least focally GBM like | 34 |
| | at least focally PXA like | 9 |
| cellularity | low | 5 |
| | moderate | 60 |
| | high | 9 |
| nuclear pleomporphism | slight | 6 |
| | moderate | 53 |
| | bizarre nuclei | 15 |
| mitoses | none | 16 |
| | 1-2 per 10 HPF | 29 |
| | 3-5 per 10 HPF | 17 |
| | 6-20 per 10 HPF | 10 |
| | > 20 per 10 HPF | 2 |
| necrosis | none | 48 |
| | non-palisading | 20 |
| | palisading | 6 |
| vascular changes | none | 9 |
| | hypertrophy/multilayering | 40 |
| | glomeruloid endothelia | 25 |
| eosinophilic granular bodies or Rosenthal fibers | present | 26 |
| | absent | 48 |
| infiltration pattern | diffuse | 43 |
| | obvious demarcation | 2 |
| | not evaluable | 29 |



figure

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Fig 4

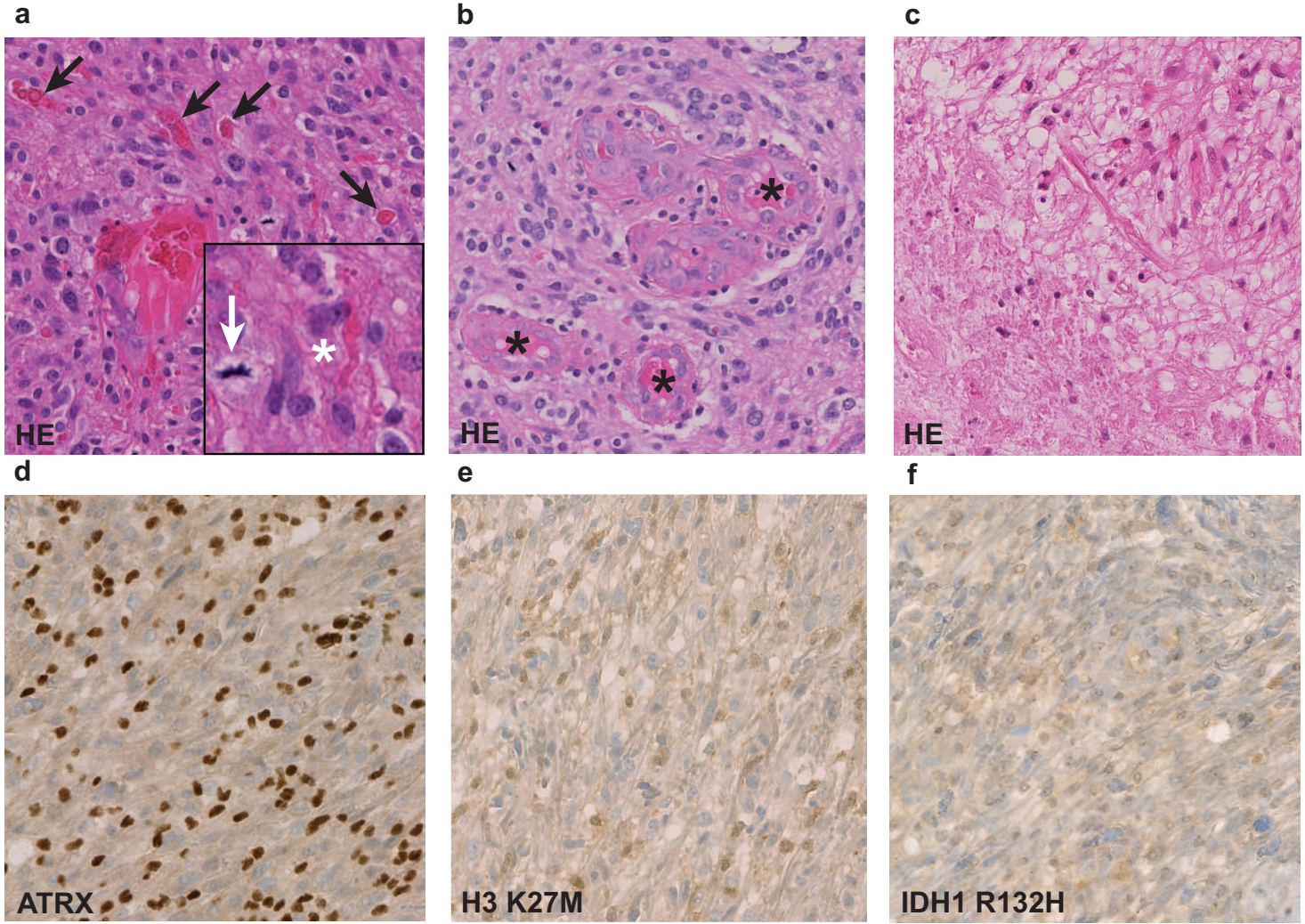
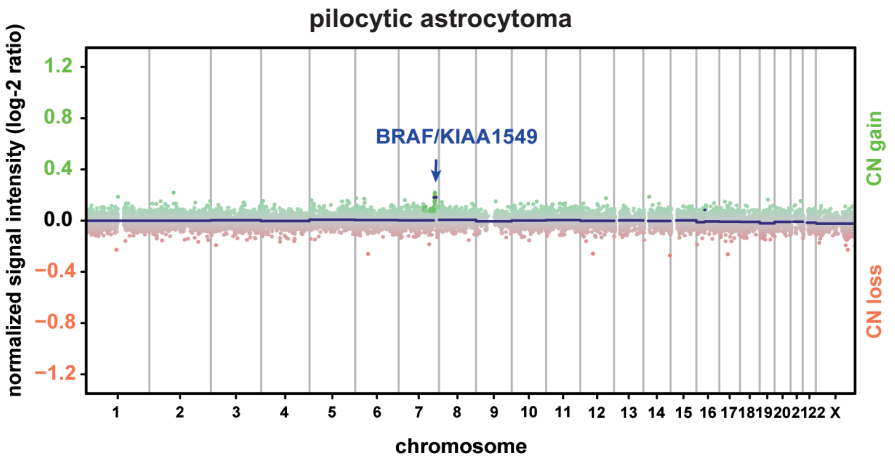


figure Fig 5



a

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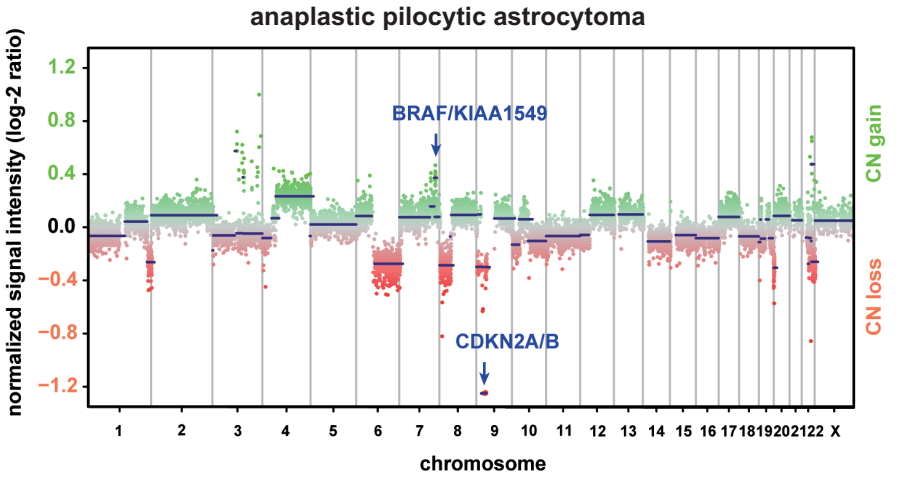


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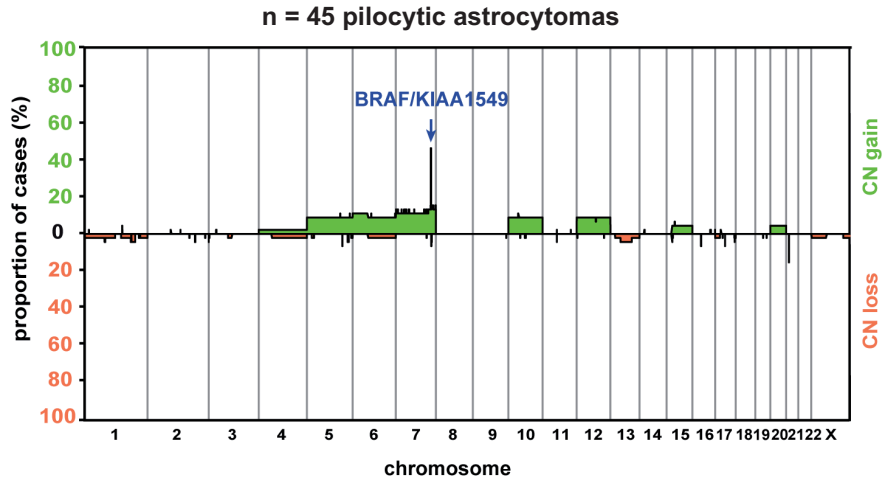


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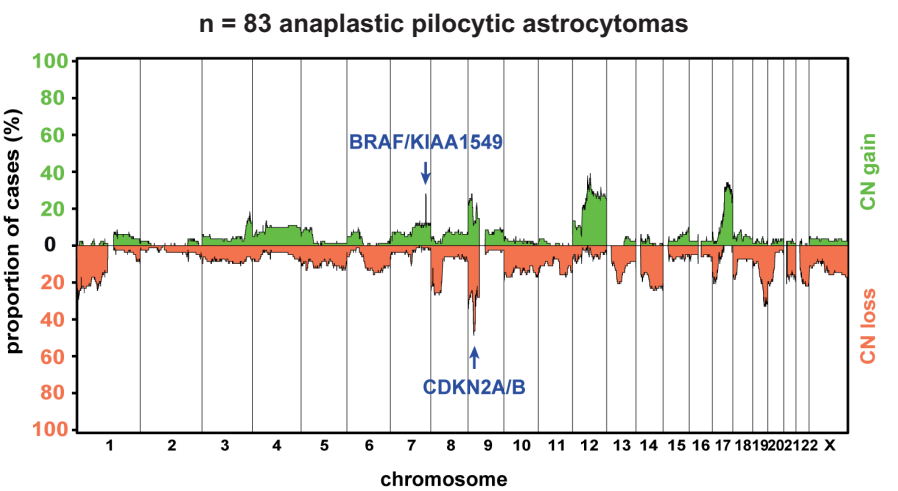

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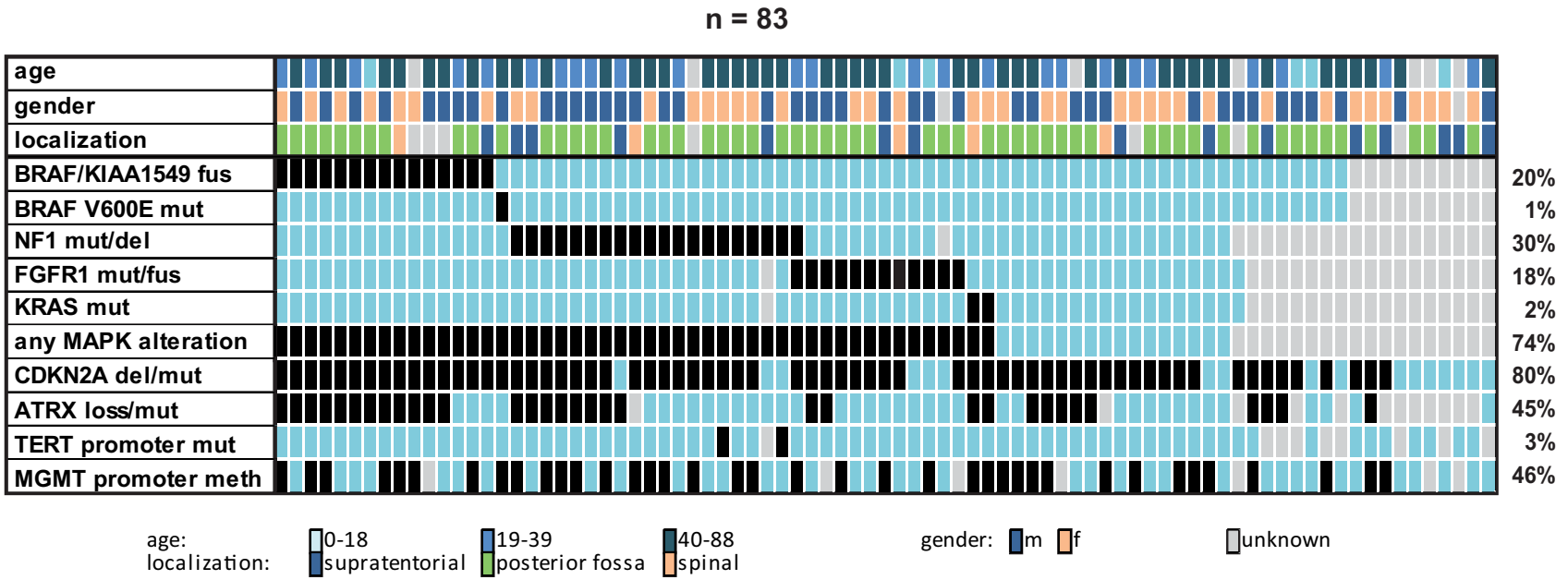
**Fig 6**



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# Fig 7

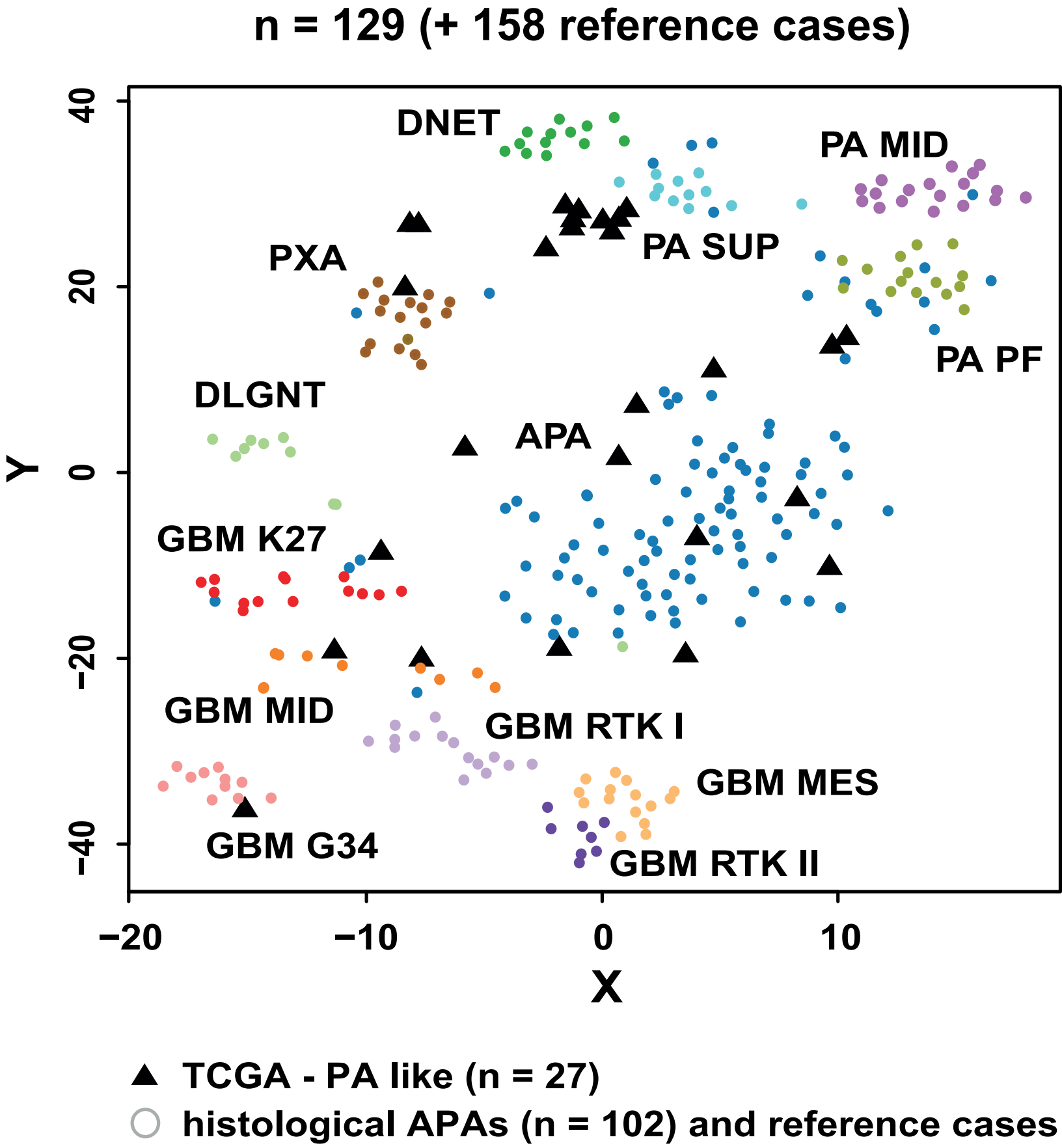

Fig 8

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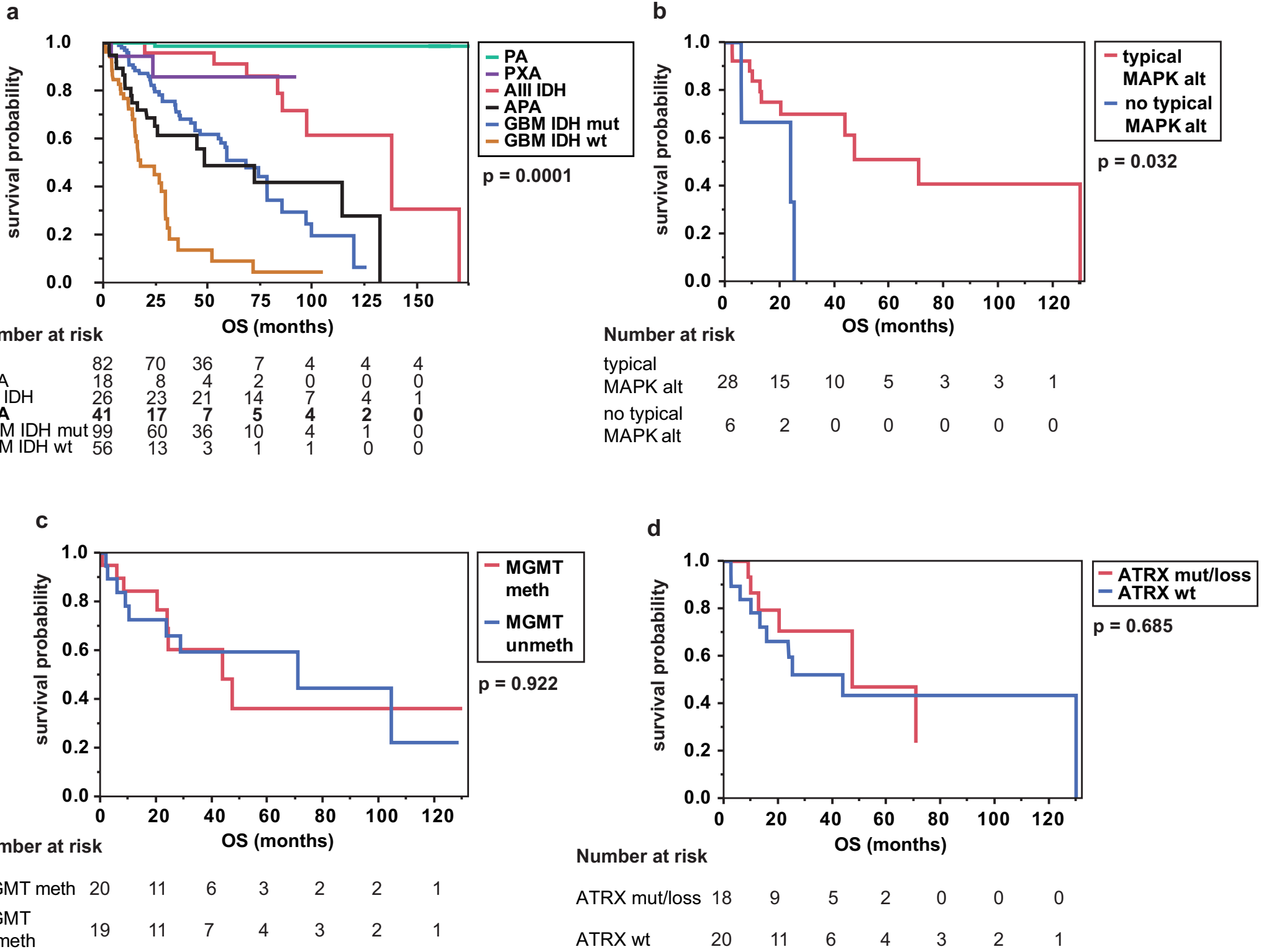


figure
**Fig 9**



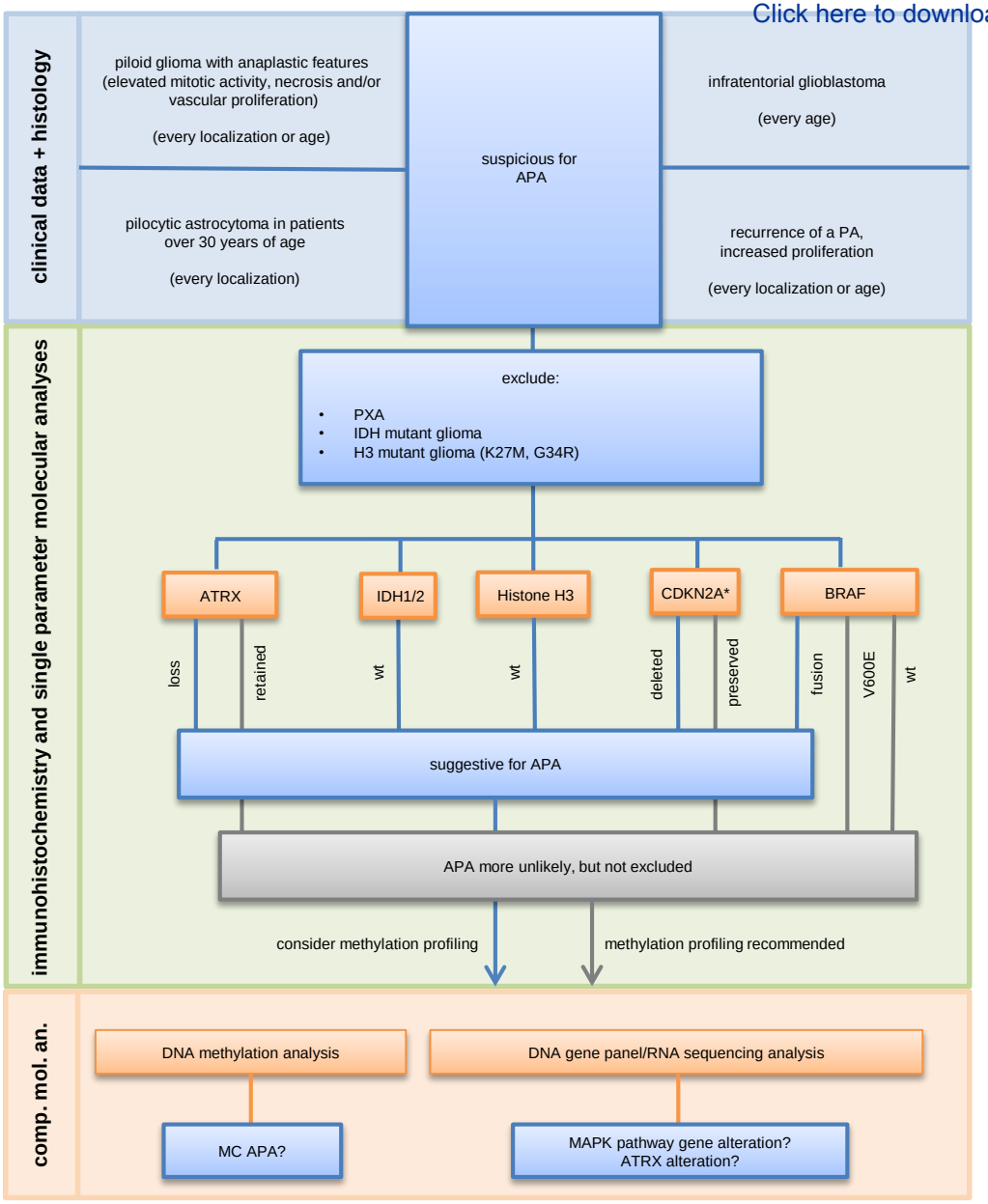
Legend:

diagnostic considerations

investigation

comp. mol. an. … comprehensive molecular analyses

*… In the present study, p16 immunohistochemistry showed a poor correlation with the CDKN2A/B status. Hence, we do not recommend p16 immunohisto-chemistry for diagnostic purposes.



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