Anaplastic ganglioglioma—A diagnosis comprising several distinct tumour types

Annekathrin Reinhardt¹,² | Kristin Pfister¹,²,³ | Daniel Schrimpf¹,² | Damian Stichel¹,² | Felix Sahm¹,² | David E. Reuss¹,² | David Capper⁴,⁵ | Annika K. Wefers¹,²,⁶ | Azadeh Ebrahimi¹,²,⁷ | Martin Sill⁸,⁹ | Joerg Felsberg¹⁰ | Guido Reifenberger¹⁰,¹¹ | Albert Becker⁷ | Marco Prinz¹²,¹³,¹⁴ | Ori Staszewski¹² | Christian Hartmann¹⁵ | Jens Schittenhelm¹⁶ | Dorothee Gramatzki¹⁷ | Michael Weller¹⁷ | Adriana Olar¹⁸ | Elisabeth Jane Rushing¹⁹ | Markus Bergmann²⁰ | Michael A. Farrell²¹ | Ingmar Blümcke²² | Roland Coras²² | Jan Beckervordersandforth²³ | Se Hoon Kim²⁴ | Fabio Rogerio²⁵ | Petia S. Dimova²⁶ | Pitt Niehusmann²⁷ | Andreas Unterberg²⁸ | Michael Platten²⁹,³⁰ | Stefan M. Pfister⁸,⁹,³¹,³² | Wolfgang Wick⁸,³³ | Christel Herold-Mende³⁴ | Andreas von Deimling¹,²,⁸

Correspondence Andreas von Deimling, Department of Neuropathology, University Hospital Heidelberg, Im Neuenheimer Feld 224, 69120 Heidelberg, Germany. Email: andreas.vondeimling@med.uni-heidelberg.de

Present address Annekathrin Reinhardt, Centre for Human Genetics Tübingen, Tübingen, Germany.

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Abstract

Aims: Anaplastic ganglioglioma is a rare tumour, and diagnosis has been based on histological criteria. The 5th edition of the World Health Organization Classification of Tumours of the Central Nervous System (CNS WHO) does not list anaplastic ganglioglioma as a distinct diagnosis due to lack of molecular data in previous publications. We retrospectively compiled a cohort of 54 histologically diagnosed anaplastic gangliogliomas to explore whether the molecular profiles of these tumours represent a separate type or resolve into other entities.

Methods: Samples were subjected to histological review, deoxyribonucleic acid (DNA) methylation profiling and next-generation sequencing. Morphological and molecular data were summarised to an integrated diagnosis.

Results: The majority of tumours designated as anaplastic gangliogliomas resolved into other CNS WHO diagnoses, most commonly pleomorphic xanthoastrocytoma (16/54), glioblastoma, isocitrate dehydrogenase protein (IDH) wild type and diffuse paediatric-type high-grade glioma, H3 wild type and IDH wild type (11 and 2/54), followed by low-grade glial or glioneuronal tumours including pilocytic astrocytoma, dysembryoplastic neuroepithelial tumour and diffuse leptomeningeal glioneuronal tumour (5/54), IDH mutant astrocytoma (4/54) and others (6/54). A subset of tumours (10/54) was not...
INTRODUCTION

Patients diagnosed with ganglioglioma (GG) World Health Organization (WHO) Grade 1 usually have a favourable clinical outcome with a recurrence rate of 1% and a 10 year overall survival rate of 84–86% [1–3]. Histological features of anaplasia have been observed in about 1–8% of GGs prompting the designation of anaplastic GG (aGG) [1, 4–6]. The 5th edition of the World Health Organization Classification of Tumours of the Central Nervous System (CNS WHO) states that anaplasia has been observed in the glial component including conspicuous mitotic activity, high Ki67 proliferation index, necrosis and microvascular proliferation [7]. Based on the data available, patients with aGG fare worse than patients with GG CNS WHO Grade 1. Recurrence was observed in 50% of the aGG patients, and median overall survival ranged from 25 to 44 months [2, 5, 6, 8–12].

Similar to GGs, v-Raf murine sarcoma viral oncogene homologue B (BRAF) V600E mutations also have been described in aGGs. Other alterations observed in individual cases of aGGs include cyclin-dependent kinase inhibitor 2A (CDKN2A)/p16 loss, cyclin-dependent kinase 4 (CDK4) gain/amplification, p53 accumulation, telomerase reverse transcriptase (TERT) promoter mutations, alpha thalassemia/mental retardation syndrome X-linked (ATRX) loss and H3 histone family protein (H3)-3A K27M mutations [6, 13–17]. However, no characteristic molecular profile has been established so far, and the histological features described for this entity allow for an ample range of interpretation. According to the 5th edition of the CNS WHO classification, further studies are required to confirm aGG as a distinct tumour type [7]. In the present study, we systematically analysed the histological findings and molecular profiles of a cohort of 54 tumours originally diagnosed by histology as aGG in order to explore whether these tumours constitute a distinct group.

MATERIALS AND METHODS

Tissue samples and clinical data

Tissue samples from 54 patients histologically diagnosed as aGG between the years 2000 and 2018 were collected for this study. Cases were included based on the original diagnosis of the supplier. Formalin-fixed, paraffin-embedded (FFPE) tissue samples of these tumours were retrieved from the archives of the Institutes of Neuropathology in Heidelberg, Erlangen, Bonn, Düsseldorf, Freiburg, Hanover, Tübingen, Bremen Mitte, Zürich, Basel, the Beaumont Hospital Dublin and the University of Texas, MD Anderson Cancer Center. The following clinical data were acquired when possible: histological diagnosis, patient sex, age at diagnosis of aGG, tumour localisation and resection state (primary surgery or re-resection).

Histological examination and immunohistochemistry

In order to identify potential associations between histological and molecular profiles, haematoxylin and eosin (HE)-stained slides were systematically reviewed by A. R. and K. P. with the following histological criteria evaluated for each tumour: general morphological growth pattern, infiltration pattern, cellularity, nuclear pleomorphism, presence of xanthomatous, multinucleated or giant cells, mitoses, necrosis, vascular changes, presence of eosinophilic granular bodies or Rosenthal fibres, lymphocytic infiltration, calcification and the presence of reticulin fibres in the tumour. Reticulin stains were performed in 31 of 54 cases. For tumours with sufficient tissue available, immunohistochemistry with antibodies specific for glial fibrillary acidic protein (GFAP, n = 43), synaptophysin (n = 44), cluster of differentiation 34 (CD34) (n = 32), marker of proliferation (Ki67/MIB-1) (n = 42), ATRX (n = 15), K27M mutation in a histone H3 family protein
(H3 K27M) (n = 2), isocitrate dehydrogenase protein 1 (IDH1) R132H (n = 18) or BRAF V600E (n = 36) was performed on a Ventana BenchMark XT Immunostainer (Ventana Medical Systems, Tucson, Arizona, USA) using established protocols. For dilutions and antibody details, see Table S1.

BRAF V600E, IDH1 R132H and H3 K27M immunohistochemistry was scored as either positive or negative in the tumour cells. Loss of nuclear ATRX expression was considered, when more than 80% of tumour cell nuclei showed loss of expression, whereas nuclei of intermingled non-neoplastic cells serving as internal control were positive [18]. Slides were scanned on either a NanoZoomer Digital Slide Scanner (Hamamatsu, Hamamatsu, Japan) or an AT2 Aperio Digital Pathology Slide Scanner (Leica Biosystems, Wetzlar, Germany) and photographed using Aperio ImageScope software (v11.0.2.725, Aperio Technologies, Vista, California, USA).

Desoxyribonucleic acid (DNA) extraction and methylation data generation

DNA was extracted from FFPE or fresh frozen 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. From each tissue sample, 200 to 500 ng of DNA were processed for DNA methylation analysis. Either the Infinium HumanMethylation450 BeadChip (450K) array or the Infinium MethylationEPIC BeadChip (850K) array (Illumina, Carlsbad, California, USA) was used to determine the DNA methylation status of CpG sites according to the manufacturer’s instructions. From Cases 6 and 43, DNA was extracted from two different areas (glial and neuronal fraction). Therefore, 56 DNA samples were gathered from 54 patients.

Methylation-based classification: Unsupervised t-distributed stochastic neighbour embedding (t-SNE)/ uniform manifold approximation and projection (UMAP) analyses, classifier predictions and copy number profile calculation

Initially, a t-SNE plot was computed via R package Rtsne (https://github.com/jkrjith/htsne) using the 20,000 most variable CpG sites according to standard deviation, 3000 iterations, a perplexity value of 10 and selected reference classes. In addition, the methylation profiles of all tumours were matched with unselected reference classes in a more comprehensive t-SNE plot comprising 90,000 tumours so far analysed via the Department of Neuropathology Heidelberg, the Deutsches Krebsforschungszentrum (DKFZ [German Cancer Research Center]) and the website https://www.molecularneuropathology.org [19]. In addition, a UMAP analysis was performed with the reference classes of the brain tumour classifier V11b4 using the 20,000 most variable CpG sites according to standard deviation and a minimum distance of 0.1.

In a second step, DNA methylation profiles were classified using the brain tumour classifier versions V11b4, V12.3 and V12.5 (https://www.molecularneuropathology.org/mnp/classifiers) [19].

In addition, copy number profiles were calculated from the methylation array data as previously described using the ‘conumee’ package in R (http://bioconductor.org/packages/release/bioc/html/conumee.html).

Sanger sequencing, DNA panel sequencing and ribonucleic acid (RNA) sequencing

Sequencing of the hotspot mutations in H3 histone gene family member 3A (H3-3A), histone cluster 1 H3 family member B (H3C2) (HIST1H3B), BRAF, isocitrate dehydrogenase gene 1 (IDH1), isocitrate dehydrogenase gene 2 (IDH2) and the TERT promoter was performed according to standard protocols. Primer sequences and Refseq NM accession numbers for the respective genes are listed in Table S2. For the amplification reaction, the following reagents were used: 12.5 μl GoTaq G2 DNA polymerase (Promega, Madison, Wisconsin, USA), 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 (~25 ng/μl). PCR conditions are listed in Table S3. For subsets of tumours, gene panel sequencing (n = 12) or RNA sequencing (n = 3) was performed as previously described [20, 21]. Gene panel sequencing data were filtered as follows: Exonic and splicing single-nucleotide variants were selected. Synonymous and stop-loss variants were not considered. Thereafter, variants with a frequency not exceeding 0.1% 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&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 dbSNP database. The remaining items (non-synonymous, stop-gain or splice-site variants and frameshift insertions/deletions) were evaluated for their potential clinicopathological relevance using the databases http://cancer.sanger.ac.uk/cosmic, http://www.ncbi.nlm.nih.gov/clinvar/ and https://varsome.com/. According to these databases, variants were categorised as outlined in Table S4. A full list of the genes represented in the applied gene panel is provided in Table S5. The deFuse and Arriba software tools [22] were used to identify gene fusions (https://sourceforge.net/projects/defuse/ and https://github.com/suhrig/arriba/).

Statistical analysis and reference data set

The DNA methylation array data were processed with the R/Bioconductor package minfi (Version 1.20) [1]. The reference classes of the t-SNE analysis were as follows: GG (14 cases); IDH glioma, subclass astrocytoma (A IDH; 14 cases); IDH glioma, subclass high-
grade astrocytoma (A IDH HG; 15 cases); diffuse midline glioma, H3 K27 altered (DMG K27; 13 cases); glioblastoma (GBM) of the midline (GBM MID; 15 cases); GBM, v-my c avian myelocytom atosis viral oncogene neuroblastoma derived homologue (MYCN) subtype (GBM MYCN; 12 cases); GBM, mesenchymal subtype (GBM MES; 15 cases); GBM, receptor tyrosine kinase protein (RTK) I subtype (GBM RTK I; 15 cases); GBM, RTK II subtype (GBM RTK II; 14 cases); GBM, RTK III subtype (GBM RTK III; 15 cases); pleomorphic xanthoastrocytoma (PXA; 27 cases); high-grade astrocytoma with piloid features (HGAP; 15 cases); pilocytic astrocytoma (PA) of the supratentorial hemispheres and GG (PA CORT/GG; 14 cases); PA of the posterior fossa (PA PF; 15 cases); PA of the midline (PA MID; 13 cases); dysembryoplastic neuroepithelial tumour (DNET; 15 cases); diffuse leptomeningeal glioneuronal tumour (DLGNT; 9 cases); ependymoma, NF-kappa-B transcription factor p65 (RELA) fusion-positive (EPN RELA; 15 cases); atypical teratoid/rhabdoid tumour, subclass MYC (ATRT MYC; 15 cases); desmoplastic myxoid tumour, SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily B, member 1 (SMARCB1) mutant (DMT SMARCB1; 9 cases); infantile hemispheric glioma (IHG; 10 cases); subependymal giant cell astrocytoma (SEGA; 11 cases); desmoplastic infantile astrocytoma/GG (DIG/DIA; 8 cases); supratentorial subependymoma (SEPN SUP; 10 cases); neuroepithelial tumour, pleomorphic adenoma gene 1-like zinc finger 1 (PLAGL1) fusion-positive (EPN ST 1; 15 cases); and control tissue, hemispheric cortex (HEMI CORT; 10 cases). The characteristics of the reference methylation classes (MCs) are described under https://www.molecularneuropathology.org/mnp/classifiers [4].

RESULTS

aGG was primarily diagnosed in young adults with a predilection for the temporal region

Median age of the 54 patients was 25 years ranging from 1 to 81 years. The male/female ratio of 1.2 demonstrates a slight male predominance consistent with previous reports [5, 10]. The tumour localisation was known for 45 patients, with 87% in supratentorial and 18% in infratentorial compartments. Half of the tumours were located in the temporal lobe, 20% in the frontal lobe and the remaining fraction in other areas.

aGG presented with heterogeneous histological features

The aGGs in our cohort exhibited highly variable histological patterns precluding the definition of general histological criteria. Table S6 provides an overview of the histological features. Most aGGs showed a predominantly glial and some a mixed glioneuronal morphology. Most of the tumours exhibited increased cellularity, moderate to high nuclear pleomorphism and diffuse infiltration into the adjacent brain tissue. In only two tumours, neuronal and glial compartments appeared spatially separated. Figure 1 shows as an exemplar the histological and immunohistochemical features of one aGG (Case 50 in Figure S1) presenting with two distinct morphologies: an oligodendroglial and a mixed glioneuronal component. In Table S7, the results of immunohistochemical stains are summarised with most of the

![FIGURE 1](https://www.molecularneuropathology.org/mnp/classifiers)
tumours expressing GFAP in the majority and synaptophysin in a lower fraction of the tumour cells. In approximately half of the aGGs, CD34 expression was observed [1]. The Ki67 index was highly variable with a median of 10% and a range from 1% up to 70%. Figure S1 provides morphological and immunohistochemical findings of each tumour.

Methylation profiles of aGGs were assigned to various methylation clusters in unsupervised analyses and to various MCs of the brain tumour classifier.

Initially, the methylation profiles of the aGGs were clustered in unsupervised analyses comprising a t-SNE plot (Figure 2) and a UMAP plot.

**FIGURE 2** t-SNE analysis of 54 tumours* with the histological diagnosis of anaplastic ganglioglioma, indicated in black circles, and 342 reference cases of established glioma methylation classes, indicated in different colours. Tumours of the same class are depicted in one colour. For this analysis, the 20,000 most variably methylated CpG islands were used. Reference methylation classes: A IDH, IDH glioma, subclass astrocytoma; A IDH HG, IDH glioma, subclass high-grade astrocytoma; HGAP, high-grade astrocytoma with piloid features; DMG K27, diffuse midline glioma, H3 K27 altered; GBM MID, glioblastoma, IDH wild type, subclass midline; GBM MES, glioblastoma, IDH wild type, subclass mesenchymal; GBM RTK I, glioblastoma, IDH wild type, subclass RTK I; GBM RTK II, glioblastoma, IDH wild type, subclass RTK II; GBM RTK III, glioblastoma, IDH wild type, subclass RTK III; GBM MYCN, glioblastoma, IDH wild type, subclass MYCN; (a)PXA, (anaplastic) pleomorphic xanthoastrocytoma; DIGNET, diffuse leptomeningeal glioneuronal tumour; ATRT MYC, atypical teratoid/rhabdoid tumour, subclass MYC; PA CORT/GG, low-grade glioma, subclass hemispheric pilocytic astrocytoma and ganglioglioma; PA MID, low-grade glioma, subclass midline pilocytic astrocytoma; PA PF, low-grade glioma, subclass posterior fossa pilocytic astrocytoma; DIGNET, low-grade glioma, dysembryoplastic neuroepithelial tumour; GG, low-grade glioma, ganglioglioma; IHG, infantile hemispheric glioma; SEGAL, low-grade glioma, subependymal giant cell astrocytoma; DIG/DIA, low-grade glioma, desmoplastic infantile astrocytoma/ganglioglioma; SEPN SUP, subependymoma, supratentorial; EPN RELA, ependymoma, RELA fusion-positive; EPN ST 1, neuroepithelial tumour, PLAGL1 fusion-positive; DMT SMARCB1, desmoplastic myxoid tumour, SMARCB1 mutant; HEMI CORT, control tissue, hemispheric cortex; int. dia., integrated diagnosis; meth. cluster, methylation cluster. *Two patients are represented twice.
In most aGGs, single-gene alterations were in line with the methylation-based classification

Previous series of aGGs reported alterations in BRAF, cyclin-dependent kinase inhibitor 2A/B (CDKN2A/B), the TERT promoter, CDK4 and H3-3A [6, 13–17]. In our series, BRAF mutations were found in 30% of the tumours. The main fraction of these reached the highest classifier scores for the MC PXA or the MCF family (MCF) PA, whereas a subset was unclassifiable. Homozygous CDKN2A/B deletions were observed in 50% of cases, mainly in tumours with the highest classifier scores for the MC PXA and the MCF GBM IDH wild type. TERT promoter mutations were detected in 20% of the tumours. These tumours were predominantly assigned to the MCF GBM IDH wild type, whereas one tumour was classified as MCF diffuse paediatric-type high-grade glioma, H3 wild type and IDH wild type (Case 20), and two tumours were allotted to the MC PXA (Cases 1 and 7). TERT promoter mutations have been described in single cases of (anaplastic) PXAs [23, 24] and have been suggested to be associated with malignant transformation of these tumours [25, 26]. Focal amplifications occurred in 13% of tumours affecting the genes epidermal growth factor receptor (EGFR), cyclin D2 (CCND2), MYCN, CDK4, mouse double minute 2 homologue (MDM2), platelet-derived growth factor receptor alpha (PDGFRα) and hepatocyte growth factor receptor (MET) and were predominantly found in tumours assigned to the MCF GBM IDH wild type, in one tumour classified as diffuse paediatric-type high-grade glioma, H3 wild type and IDH wild type [7] and in one with an elevated classifier score for the MC HGAP. Presence of the combination of gain of chromosome 7 and loss of chromosome 10 (7/10 signature) (15%) was restricted to tumours assigned to the MCF GBM IDH wild type. Three tumours with the highest classifier score for the MC DMG K27 harboured an H3-3A K27M mutation, with one additionally carrying a BRAF V600E mutation. RNA sequencing revealed a protein phosphatase 1, catalytic subunit, beta isoform (PPP1CB)/anaplastic lymphoma tyrosine kinase (ALK) fusion in a tumour with an elevated classifier score for the MC IHG and an Ewing sarcoma breakpoint region 1 (EWSR1)/PLAG1 fusion in a tumour classified as MC neuroepithelial tumour, PLAG1 fusion-positive (V12.5) (MC EPN ST 1) [27]. DNA panel sequencing indicated a KIAA1549/BRAF fusion in a tumour assigned to the reference cluster of DLGNTs. One case classified as MC ATRT showed immunohistochemical loss of integrase Inteactor 1 (INI1) expression in the tumour cells (see Figure S2) and a homozygous SMARCB1 deletion in the copy number profile.

The most common integrated diagnoses were PXA and GBM, IDH wild type

Histological and immunohistochemical findings, methylation profile, copy number variations and sequencing data were summarised to an integrated diagnosis (Figure S1). Figure 3 shows how the integrated diagnoses were distributed among the 54 patients. The diagnosis (anaplastic) PXA was assigned to 30% of the tumours, whereas ~20%
were categorised as GBM IDH wild type. In four instances, the diagnosis A IDH was allotted. One or two tumours each were assigned to DMG K27, diffuse midline glioma H3 K27 altered, CNS WHO Grade 4; DNET, dysembryoplastic neuroepithelial tumour, CNS WHO Grade 1; GBM (IDH wt), glioblastoma IDH wild type, CNS WHO Grade 4; HGGped H3 wt IDH wt, diffuse paediatric-type high-grade glioma, H3 wild type and IDH wild type (CNS WHO Grade 4); IHG, infant-type hemispheric glioma; NET PLAGL1, neuroepithelial tumour PLAGL1 fusion-positive; PA, pilocytic astrocytoma, CNS WHO Grade 1; PXA, pleomorphic xanthoastrocytoma, CNS WHO Grades 2–3; ST EPN ZFTA, supratentorial ependymoma ZFTA fusion-positive.

Two tumours (Cases 17 and 20), which would have been designated as GBM IDH wt according to the 4th edition of the CNS WHO classification, turned out as diffuse paediatric-type high-grade glioma, H3 wild type and IDH wild type according to the 5th edition. The tumour of Patient 17, who was 35 years old, was classified as MC diffuse midline glioma, not otherwise specified (NOS). The HE-stained sections of two of these tumours (Cases 36 and 54) are shown in Figure S3 and additional molecular details in Figure S1. We found that copy number variations were predominantly observed in the glial component. Upon molecular analysis, the glial fraction of Case 6 was diagnosed as PXA, whereas the neuronal fraction finally turned out as infiltration zone. In contrast, both the glial and neuronal fraction of Case 43 were considered as tumour subclones that both carried a BRAF V600E mutation. The glial fraction was assigned to the MC PA CORT/GG. A quite interesting finding in this fraction was a heterozygous deletion of chromosome arm 22q, which is unusual for PA. The neuronal fraction was assigned to SEGAs in the t-SNE in Figure 2, but probably due to a limited set of reference classes. In the updated classifier versions V12.3 and V12.5, it was classified as MC GLOIOMA NORM HI (glioma with high proportion of normal cells) and clustered accordingly in a more comprehensive t-SNE. This MC comprises gliomas with a high fraction of immune or stromal cells. However, the histological slides did not reveal high infiltration or normal brain tissue. Instead, enlarged and very pleomorphic tumour cells with prominent nucleoli were observed. Due to the two histologically and molecularly different compartments with unusual profiles that do not fit to a known entity, the tumour was finally considered as unclassifiable. A summary of the molecular findings and integrated diagnoses for the neuronal and glial component of Cases 6 and 43 is given in Table S8.

Three aGGs transpired to be infiltration zone of glial tumours

After molecular analyses and histological review, Cases 28 and 29 were finally diagnosed as infiltration zone of GBM, whereas the neuronal component of Case 6 was diagnosed as infiltration zone of PXA. The glial component of the latter presented with high cell density, heterozygous deletion of chromosome 9, homozygous deletion of CDKN2A/B and clustered to PXAs in the t-SNE. The histological and molecular profiles were therefore indicative of PXA. In contrast, the neuronal component histologically presented with low cell density and only single, enlarged neuronal cells of unusual morphology. The methylation profile was assigned to GG; copy number alterations were not obvious (see also Table S8 and Figure S1). Hence,
the neuronal component was retrospectively designated as infiltration zone, and the single neurons of unusual morphology were interpreted as non-neoplastic. Notably, the finding that infiltration zones reach high classifier scores for GG is not uncommon due to their oftentimes high percentage of neuronal cells. In Cases 28 and 29, our initial histological impression was inconclusive. However, both molecular profiles presented with a 7/10 signature and a homozygous CDKN2A/B deletion and were therefore highly suggestive of GBM. Re-evaluation after molecular analyses led to the consensus that the histology was at least compatible with an infiltration zone of GBM. In Case 28, the low tumour cell content was also reflected by the methylation profile, which was classified as MC GLIOMA NORM HI. In contrast, Case 29 was classified as MC GBM MES (see also Figure S1).

**FIGURE 5** HE-stained sections of two tumours with the histological diagnosis of anaplastic ganglioglioma and unclear integrated diagnosis, even after molecular analyses. (A) Unclassifiable tumour (low-grade) (Case 54): pleomorphic neuroectodermal tumour with microcystic architecture, vesicular appearing nuclei and prominent nucleoli; the copy number profile (not shown) revealed no indications for chromosomal aberrations. (B) Unclassifiable tumour (high grade) (Case 36): In the HE-stained section, multilayered vessels and pleomorphic tumour cells are visible, among these a binucleated tumour cell with prominent nucleoli; the copy number profile (not shown) revealed a homozygous CDKN2A/B deletion.
Unclassifiable tumours had heterogeneous profiles that were not assignable to an established entity

The methylation profiles of five tumours (Cases 36, 37, 48, 49 and 50 in Figure S1) were found in vicinity to the HGAP cluster in the t-SNE. However, none of them entirely fulfilled the required molecular criteria.

In Case 36, a homozygous CDKN2A/B deletion and an NF1 frameshift deletion were compatible with the diagnosis of HGAP. However, additional mutations in phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) and SET domain containing 2, histone lysine methyltransferase (SETD2) were more in favour of GBM IDH wild type. The histological (Figure 5) and molecular results suggested a high-grade glioma. However, the criteria for HGAP were not entirely fulfilled, and distinction from GBM IDH wild type was not possible.

Case 37 reached a sub-threshold score for the MC HGAP. However, the copy number profile with numerous chromosomal gains, losses and focal amplifications was unusual for this entity. Although ATRX loss and the assignment in the t-SNE were compatible with the diagnosis of HGAP, panel sequencing analysis did reveal neither a MAPK pathway alteration nor an ATRX mutation. In summary, not all criteria for HGAP were met.

Case 50 clustered between HGAP, GBM MID and DLGNT upon t-SNE/UMAP analyses. Panel sequencing revealed a Kirsten rat sarcoma viral oncogene homologue (KRAS) G12R hotspot mutation described in various cancers and tectal gliomas [29]. In a study on HGAPs, two of 83 tumours also harboured a KRAS mutation, but additionally had CDKN2A/B and ATRX alterations [30]. As these were not evident in the present case, it was supposed to belong to an entity not yet characterised.

The methylation profile of Case 49 was inconsistently assigned to HGAP or PXA. Besides a CDKN2A/B homozygous deletion, a BRAF D594N mutation and a PIK3CA E600K mutation were detected. PIK3CA mutations have been shown to occur in 6–15% of GBMs and have been linked to adverse outcome [31]. However, alterations of the PIK3 pathway were also found in other glioma entities [32, 33]. BRAF D594N is known as a kinase inactivating mutation leading to a reactive increase of RAS activity and extracellular signal-regulated kinase 2 (ERK) signalling [34]. Whether this tumour represents a rare variant of GBM, HGAP or PXA or belongs to an as yet unknown entity remained unclear.

In Case 48, an H3-3A K27M and a BRAF V600E mutation were found. Single patients with glial/glioneuronal tumours and this constellation of mutations have been described. As these patients appeared to have a more favourable clinical outcome than patients with DMG K27, a separate categorisation has been suggested [35]. However, as the exact nature of these tumours is still unclear, they are not yet represented as a separate entity in the CNS WHO classification [7].

The methylation profile of Case 51 was assigned to DMG K27, but no K27M mutation in H3-3A or HIST1H3B was detectable. Immuno-histochemistry for loss of tri-methylation of lysine 27 of histone H3 (H3 K27me3) or RNA sequencing for EZH inhibitory protein (EZHIP) expression [36] could not be performed due to insufficient material. The tumour was therefore designated as diffuse midline glioma, NOS.

DISCUSSION

The 5th edition of the WHO Classification of Tumours of the Central Nervous System states that previous studies on aGGs lacked comprehensive molecular testing and suggests further analyses to validate the existence of this entity [7]. In this study, we assessed the morphological and molecular profiles of 54 aGGs from multiple institutions. All cases were included based on the original histological diagnosis of the submitting centre. We explored whether these tumours could be allotted to established tumour types or whether they represent a separate entity. The majority of aGGs turned out as belonging to established glial tumour types [4], most commonly PXA and GBM IDH wild type. Only three tumours of the aGG cohort were finally categorised as glioneuronal tumours (two as DNET and one as DLGNT).

However, many of the aGGs contained neuronal cells of partly dysplastic appearance. Although the morphology of dysmorphic ganglion cells is described in the CNS WHO classification, it is also discussed that no objective identification marker exists and that distinction from an infiltration zone of other glial tumours may be challenging [7]. Likewise, further literature in the field of glioneuronal tumours stresses diagnostic issues like the variable microscopic appearance of glial and neuronal components and the poor inter-observer agreement in the designation of GG [1, 37]. Particularly regarding aGG, the WHO book does not indicate how many mitoses should be considered as conspicuous and how many of the described anaplastic features have to be present to justify this designation. Another complicating aspect is that ganglion cell differentiation may also be observed in other entities, for instance, PXA or DLGNT [4, 7].
Hence, the constellation of features applied to assign the diagnosis of aGG may substantially depend on the observer’s interpretation. Accordingly, the results of this study indicated that a definition based on dysplastic neurons and signs of anaplasia allows for a wide spectrum of tumour types. This was supported by molecular analyses suggesting different entities. As these comprised predominantly glial tumours, our findings also raise the question whether the dysplastic neurons observed in aGGs are more likely entrapped cells of compromised appearance due to adjacent or infiltrative tumour. The fact that three tumours of our series finally turned out as infiltration zone also underlines this issue. In summary, the wide spectrum of integrated diagnoses among aGGs indicates substantial subjectivity of this designation and in the declaration of dysplastic neurons.

Interestingly, cells with neuronal appearance have been observed in a small subset of PXAs. These tumours have been designated as composite PXA and GG [37–39]. The morphology of such tumours may come close to the morphology described for aGG. However, as the results of our study suggest, it has to be considered that aGG may be a misdiagnosis in case of a PXA with unusual morphology. In a recent study, we similarly found that GG and also GBM IDH wild type are common misdiagnoses among tumours of the MC PXA. These findings underline the importance of molecular analysis, particularly methylation-based classification, to differentiate between entities that may show histological overlap on the one hand and also considerable histological variability on the other hand [26].

The 5th edition of the CNS WHO classification lists a new tumour type designated as diffuse high-grade glioma, paediatric-type, H3 wild type and IDH wild type [7]. The methylation profiles of two aGGs were allotted to the correspondent MCF in the classifier version V12.5, whereas in previous classifier versions reached elevated scores for the MCF GBM IDH wild type (MCF GBM IDH wt). Indeed, their copy number profiles did not show typical features of classical adult GBM (see Figure S1). Previous studies and empirical data also suggest that copy number aberrations of paediatric high-grade gliomas are distinct from classic GBM in adults [40–42]. Analysis of methylation profiles of diffuse high-grade gliomas, paediatric-type, H3 wild type and IDH wild type showed that this tumour type is a heterogeneous group comprising various MCs [42, 43]. As first results on the clinical course show that these tumours behave aggressively, CNS WHO grade 4 is considered, albeit differences in survival time between the MCs subsumed under this tumour type are observed [7, 42].

One tumour with aGG morphology (Case 45, Figure 4) turned out to be ATRT. Single cases of gliial/glioneuronal tumours transforming to ATRT have been described [44]. Recently, a study about tumours with epigenetic similarity to ATRT designated low-grade diffusely infiltrative tumours, SMARCB1 mutant has been published. Two of these tumours showed high-grade areas and were initially considered as ATRT with a GG component [45]. These data suggest that in unclear cases of aGG, INI1/SMARCB1 testing should be considered.

Case 52 was designated as NET PLAGL1, histologically presented as clustered small, round tumour cells with intermingled ganglion cells (Figure 4) and had an EWSR1/PLAGL1 fusion. In a case series of gliial/glioneuronal tumours with unusual EWSR1 fusions, a tumour with similar histology and this particular alteration has been reported [46]. A recent study about the histological and molecular characteristics of PLAGL1 fusion-positive neuroepithelial tumours described a heterogeneous morphology with a high fraction of cases showing ependymoma-like features [27].

Our study further showed that 10 tumours with heterogeneous histological and molecular profiles could not be allotted to an established WHO diagnosis and also did not build a separate methylation cluster. For these tumours, discrepancies between the morphology, the positioning in comprehensive t-SNE analyses, the classifier predictions and single molecular parameters were evident. Moreover, molecular alterations with unclear diagnostic significance were frequently detected, for instance, combined H3-3A K27M and BRAF V600E mutation (Case 48) [35, 47], a KRAS G12R mutation (Case 50), combined BRAF D594N and PIK3CA E600K mutation (Case 49) and a BRAF V600E mutation in combination with chromosome 22q loss (Case 43). Taken together, the heterogeneous histology as well as mutational and methylation profiles provided evidence neither for a known entity nor for a group suggestive of a new tumour type beyond the 5th edition of the CNS WHO classification.

The methylation profiles of seven aGGs were assigned to the reference cluster of HGAPs (see Figure 2). Although HGAPs may comprise a wide spectrum of morphological features, a neuronal or glioneuronal appearance has not been observed [30]. Hence, it is not surprising that after histological re-evaluation and molecular testing, the integrated diagnosis of HGAP was not assigned to any of these cases. After taking the molecular results into account, five of these tumours were finally designated as unclassifiable, mostly because of inconclusive molecular constellations, whereas two cases were diagnosed as GBM IDH wild type and DMG K27 (Figure S1). Further investigations are necessary to clarify whether the five tumours with unclear molecular findings may belong to an unusual subgroup of HGAP or to other types of high-grade glioma, which have not yet been described.

As a limitation of this work, we considered that single types or subtypes defined in the CNS WHO classification may possibly not be assignable or even distinguishable from other types by methylation profile. One example to mention is multinodular and vacuolating neuronal tumour. Another aspect to keep in mind is that, in single instances, the classifier is not able to distinguish neuronal and glial subtypes of related entities. This is the case in desmoplastic infantile GG vs desmoplastic infantile astrocytoma and also in some examples of hemispheric PA vs GG. For these entities, common MCs, namely, MC PA CORT/GG and MC DIG/DIA, exist despite of histological differences with regard to the ratio of glial and neuronal components. The fact that a relevant fraction of aGGs molecularly turned out to be PXAs therefore raises the question whether some of these tumours may similarly represent neuronal and glial predominant variants of a molecular entity with a common methylation profile. Recent analyses of the molecular landscape of composite PXAs and GGs further underline this consideration. Notably, both components of these tumours were positive for BRAF V600E, whereas selectively in the...
PXA component, a homozygous \textit{CDKN2A/B} deletion/p16 loss and a higher number of chromosomal aberrations were obvious. Hence, these composite tumours were suggested to represent a stage of progression from GG to PXA \cite{48,49}. Further, more comprehensive analyses like whole-genome or proteomic approaches may serve to further address this issue and to clarify whether this also applies for tumours designated as aGG.

**CONCLUSION**

Our results suggest that most tumours with the histological diagnosis of aGG belong to established CNS WHO tumour types. A small fraction was not assignable but showed heterogeneous histological and molecular profiles. As common features pointing to a separate tumour type or subtype were not evident in this subset of cases,
suggests an algorithm for a reasonable and systematic molecular workup, which might help with further classification of difficult cases.

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CONFLICTS OF INTEREST
A. v. D., D. C., M. S. and D. Schrimpf have pending patents on DNA methylation-based methods for classifying tumour species/tumour species of the brain. F. S. declares an honorary from Illumina. W. W. reports to be an inventor and patent holder on ‘Peptides for use in treating or diagnosing IDH1R132H positive cancers’ (EP2800580B1) and ‘Cancer therapy with an oncolytic virus combined with a checkpoint inhibitor’ (US11027013B2). He consulted for Apogenix, AstraZeneca, Bayer, Enterome, Medac, MSD and Roche/Genentech with honoraria paid to the Medical Faculty at the University of Heidelberg. S. M. P. declares a grant from ITCC-P4 companies: Lilly, Roche, Pfizer, Charles River, Bayer HealthCare, PharmaMar, Amgen, Sanofi, AstraZeneca and Servier. The other authors do not declare any competing interests.

AUTHOR CONTRIBUTIONS
A. v. D. contributed in the conceptualisation of the project, supervision of the project, data inquiry, data analysis, manuscript writing, contribution of tissue or patient information, suggestions about manuscript design or content, and critical review and approval of the manuscript; A. R. in the conceptualisation of the project, data inquiry, data analysis, manuscript writing, contribution of tissue or patient information, suggestions about manuscript design or content, and critical review and approval of the manuscript; K. P. and D. C. in the conceptualisation of the project, data inquiry, data analysis, contribution of tissue or patient information, suggestions about manuscript design or content, and critical review and approval of the manuscript; and other authors in the contribution of tissue or patient information, suggestions about manuscript design or content, and critical review and approval of the manuscript.

AFFILIATIONS
1Department of Neuropathology, University Hospital Heidelberg, Heidelberg, Germany
2Clinical Cooperation Unit Neuropathology, German Cancer Research Center (DKFZ), Heidelberg, Germany
3Institute of Pathology, Kantonsspital Winterthur, Winterthur, Switzerland
4Department of Neuropathology, Charité—Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, Berlin, Germany
5German Cancer Consortium (DKTK), Partner Site Berlin, German Cancer Research Center (DKFZ), Heidelberg, Germany
6Institute of Neuropathology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
7Department of Neuropathology, University of Bonn, Bonn, Germany
8German Cancer Consortium (DKTK), Core Center Heidelberg, Heidelberg, Germany
9Hopp Children’s Cancer Center Heidelberg (KITZ), Heidelberg, Germany
10Institute of Neuropathology, Heinrich Heine University, Medical Faculty, University Hospital Düsseldorf, Düsseldorf, Germany
11German Cancer Consortium (DKTK), Partner Site Essen/Düsseldorf, German Cancer Research Center (DKFZ), Heidelberg, Germany
12Institute of Neuropathology, Medical Faculty, University of Freiburg, Freiburg, Germany
13Center for Basics in NeuroModulation (NeuroModulBasics), Faculty of Medicine, University of Freiburg, Freiburg, Germany
14Signalling Research Centres BIOS and CIBSS, University of Freiburg, Freiburg, Germany
15Department of Neuropathology, Hannover Medical School, Hanover, Germany
16Institute of Pathology and Neuropathology, Comprehensive Cancer Center Tübingen, University Tübingen, Tübingen, Germany
17Department of Neurology, University Hospital and University Zürich, Zürich, Switzerland
18NOMIX Laboratories, Denver, Colorado, USA
19Institute of Neuropathology, University Hospital Zürich (USZ), Zürich, Switzerland
20Institute of Neuropathology, Center for Pathology, Klinikum Bremen Mitte, Bremen, Germany
21Department of Neuropathology, Beaumont Hospital, Dublin, Ireland
22Department of Neuropathology, University Hospital Erlangen, Member of EpiCARE ERN, Erlangen, Germany
23Department of Pathology, GROW School for Oncology and Developmental Biology, Maastricht University Medical Centre, Maastricht, The Netherlands
24Department of Pathology, Yonsei University College of Medicine, Seoul, South Korea
25Department of Pathology, University of Campinas (UNICAMP), Campinas, Brazil
ANAPLASTIC GANGLIOGLIOMA—A DIAGNOSIS COMPRISING SEVERAL DISTINCT TUMOUR TYPES

Epilepsy Surgery Center, Department of Neurosurgery, St. Ivan Rilski University Hospital, Sofia, Bulgaria
Section of Neuropathology, Department of Pathology, Oslo University Hospital, Oslo, Norway
Clinic for Neurosurgery, University Hospital Heidelberg, Heidelberg, Germany
Department of Neurology, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany
Clinical Cooperation Unit Neuroimmunology and Brain Tumour Immunology, German Cancer Research Center (DKFZ), Heidelberg, Germany
Division of Paediatric Neurooncology, German Cancer Research Center (DKFZ), Heidelberg, Germany
Department of Paediatric Oncology and Hematology, University Hospital Heidelberg, Heidelberg, Germany
Neurology Clinic, University Hospital Heidelberg, Heidelberg, Germany
Division of Experimental Neurosurgery, Department of Neurosurgery, University Hospital Heidelberg, Heidelberg, Germany

ETHICS STATEMENT
Tissue collection and processing as well as data collection were performed in compliance with local ethics regulations and approval.

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DATA AVAILABILITY STATEMENT
All data collected, generated and evaluated for this manuscript are available in Figure S1. Digitalised slides and raw data of the DNA methylation/sequencing analyses can be provided on reasonable request for non-commercial use.

ORCID
Annekathrin Reinhardt † https://orcid.org/0000-0002-0295-0312
Felix Sahm † https://orcid.org/0000-0001-5441-1962
Annika K. Wefers † https://orcid.org/0000-0001-9394-8519
Albert Becker † https://orcid.org/0000-0003-2661-3705
Jens Schittenhelm † https://orcid.org/0000-0002-9168-6209
Pitt Niehusmann † https://orcid.org/0000-0002-3247-5241

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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