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ORIGINAL PAPER

Papillary glioneuronal tumor (PGNT) exhibits a characteristic methylation profile and fusions involving *PRKCA*

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Abstract

Papillary glioneuronal tumor (PGNT) is a WHO-defined brain tumor entity that poses a major diagnostic challenge. Recently, *SLC44A1–PRKCA* fusions have been described in PGNT. We subjected 28 brain tumors from different institutions histologically diagnosed as PGNT to molecular and morphological analysis. Array-based methylation analysis revealed that 17/28 tumors exhibited methylation profiles typical for other tumor entities, mostly dysembryoplastic neuroepithelial tumor and hemispheric pilocytic astrocytoma. Conversely, 11/28 tumors exhibited a unique profile, thus constituting a distinct methylation class PGNT. By screening the extended Heidelberg cohort containing over 25,000 CNS tumors, we identified three additional tumors belonging to this methylation cluster but originally histologically diagnosed otherwise. RNA sequencing for the detection of *SLC44A1–PRKCA* fusions could be performed on 19 of the tumors, 10 of them belonging to the methylation class PGNT. In two additional cases, *SLC44A1–PRKCA* fusions were confirmed by FISH. We detected fusions involving *PRKCA* in all cases of this methylation class with material available for analyses: the canonical *SLC44A1–PRKCA* fusion was observed in 11/12 tumors, while the remaining case exhibited a *NOTCH1-PRKCA* fusion. Neither of the fusions was found in the tumors belonging to other methylation classes. Our results point towards a high misclassification rate of the morphological diagnosis PGNT and clearly demonstrate the necessity of molecular analyses. *PRKCA* fusions are highly diagnostic for PGNT, and detection by RNA sequencing enables the identification of rare fusion partners. Methylation analysis recognizes a unique methylation class PGNT irrespective of the nature of the *PRKCA* fusion.

Keywords Papillary glioneuronal tumor · *SLC44A1* · *PRKCA* · *NOTCH1* · DNA methylation · RNA sequencing

Introduction

Papillary glioneuronal tumor (PGNT) is a rare neoplasm that was first reported by Komori et al. [10] in 1998, and introduced as a separate entity in the 2007 World Health Organization (WHO) Classification of Tumors of the Central Nervous System (CNS) and assigned to WHO grade I [12]. Histologically, it is characterized by a biphasic differentiation pattern comprising pseudopapillary structures composed of flat-to-cuboidal glial fibrillary acidic protein (GFAP)-positive astrocytes lining hyalinized vessels, and interpapillary collections of synaptophysin-positive

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neurocytes with occasional ganglion cells [11, 13]. Outcome is usually favorable, with a reported median 5-year progression-free survival higher than 80% [1].

A t(9;17)(q31;q24) translocation was initially identified as the sole karyotypic anomaly in two PGNTs, and the respective *SLC44A1* (solute carrier family 44, member 1)–*PRKCA* (protein kinase C alpha) fusion was detected by reverse transcription-polymerase chain reaction (RT-PCR) and fluorescence in situ hybridization (FISH) analysis [5]. This fusion was further confirmed by FISH analysis in four PGNT cases, while it was not found in 15 cases of potential histological PGNT mimics [17]. More recently, the fusion was also identified by dual-color interphase FISH analysis in two out of three PGNT [16], and a PGNT case report found an *FGFR1* N546K mutation [8].

DNA methylation profiling has proven to be a powerful tool for tumor classification and identification of molecular subclasses. Moreover, this approach allows refining and improving the accuracy of current histology-based diagnosis of CNS tumors [7, 18–20]. So far, genome-wide DNA methylation profiles of PGNT have not been established. Therefore, PGNT was not recognized by recently published DNA methylation-based brain tumor classifier [6].

In this study, we set out to subject tumors histologically diagnosed as PGNT to DNA methylation analysis, DNA copy-number analysis, and RNA sequencing, with the aim of exploring the molecular landscape of this tumor entity to improve diagnostic accuracy.

Materials and methods

Tissue samples

We analyzed 31 tumor samples from the following pathology institutions: Berlin, Münster, Erlangen, Freiburg, Hannover, Coimbra, Moscow, Essen, Basel, Frankfurt, Dusseldorf, Milan, Copenhagen, Hamburg, London, Paris, and Heidelberg. Twenty-eight of these 31 tumors had been histologically diagnosed as PGNT. The remaining three tumors initially received a different diagnosis, but turned out to exhibit features of a core group defined as PGNT by molecular findings. Two of the tumors with FISH data have been published previously [17].

A reference group with diagnoses based on DNA methylation analysis was selected from the files in the Department of Neuropathology, Heidelberg. The reference group included the potential morphological mimics of PGNT represented by the methylation classes (MC) dysembryoplastic neuroepithelial tumor (DNT); MC rosette-forming glioneuronal tumor (RGNT); MC ganglioglioma (GG); MC central neurocytoma (CN); MC diffuse leptomeningeal glioneuronal tumor (DLGNT); MC chordoid glioma of the third ventricle (CG); MC low-grade glioma (LGG), MYB/MYBL1; MC pleomorphic xanthoastrocytoma (PXA); MC hemispheric pilocytic astrocytoma (PA HEMI); MC midline pilocytic astrocytoma (PA MID); MC posterior fossa pilocytic astrocytoma (PA PF); MC ependymoma RELA fused (EP RELA); MC normal hemispheric cortex (NORM HEMI) [6]. Each of the reference methylation groups contained ten samples.

The sampling of tumors and clinical data collection were performed in accordance with standards approved by the local ethical committees.

Morphological and immunohistochemical examination

We evaluated hematoxylin–eosin (H&E)-stained slides, applying the diagnostic criteria provided by the 2016 WHO Classification of Tumors of the Central Nervous System [13]. The following features characteristic of PGNT were reviewed and documented: biphasic differentiation pattern, pseudopapillary component, vessel hyalinization, interpapillary neurocytes, ganglion cells, and microcalcifications. Immunohistochemistry was provided by the contributing centers if available.

Extraction of DNA and RNA

A suitable area with tumor cell content exceeding ≥ 70% was identified on H&E slides and macrodissection performed by punch biopsy (pfm medical, Köln, Germany). DNA and RNA were extracted from formalin-fixed paraffin-embedded (FFPE) tissue using the Maxwell 16 FFPE Plus LEV DNA Kit and Maxwell 16 LEV RNA FFPE Purification Kit (Promega, Madison, WI, USA), according to the protocols supplied with the kits.

Genome-wide DNA methylation profiling

All tumor samples were submitted to DNA methylation analysis. The Illumina Infinium Human Methylation EPIC (850 K) BeadChip array (Illumina, San Diego, CA, USA) was employed, following the manufacturer's instructions. Copy-number profile (CNP) analysis was assessed using R package "conumee" [21] after an additional baseline correction (https://github.com/dstichel/coumee).

RNA sequencing and FISH

RNA was able to be obtained from 19 samples and, upon reverse transcription, was subjected to next-generation sequencing on a NextSeq 500 (Illumina, San Diego, CA, USA), as described previously [19]. We used deFuse [15] and Arriba (https://github.com/suhrig/arriba/) methods for

the detection of gene fusions. FISH analysis procedures for two cases have been described previously [17].

Statistical analysis

DNA methylation data were processed with the R/Bioconductor package "minfi" (version 1.20) [3]. For unsupervised hierarchical clustering, we selected the 15,000 methylated CpG sites with the highest median absolute deviation. Clustering was performed using Ward's linkage method and Euclidean distance as described previously [18]. The t-SNE plot was computed using the R package "Rtsne" from the 15,000 most variable cpg sites across the data set, 2000 iterations, and a perplexity value of 10. Comparison of nominal variables was performed using Fisher's exact test.

Results

Distinct methylation profile of PGNT entity

Using the methylation data for 28 tumors having received a diagnosis of PGNT, we performed t-SNE (Fig. 1) and cluster analysis [Supplementary Figure 1 (Online Resource 1)] together with 130 reference cases from 13 distinct methylation classes. Seventeen of 28 tumors diagnosed as PGNT

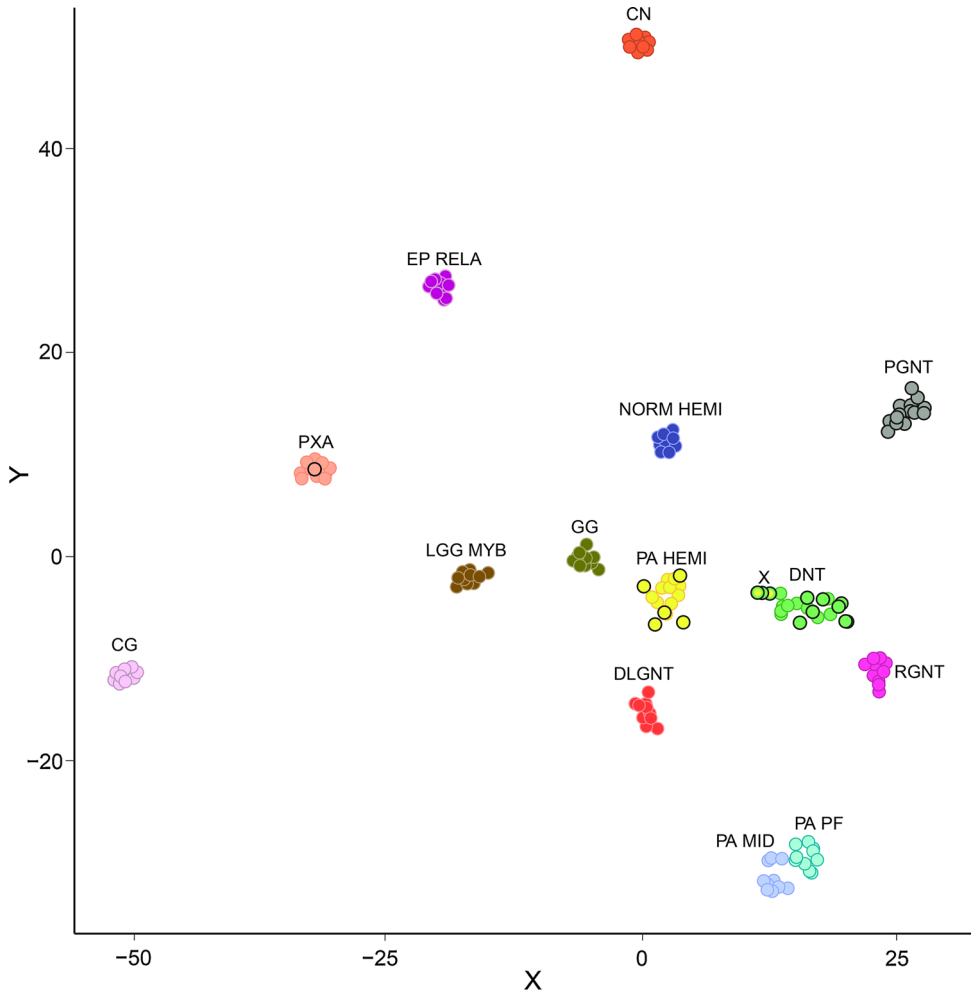

Fig. 1 t-SNE analysis of PGNT and reference tumors. Twenty-eight cases with histological diagnosis of PGNT and the three cases retrospectively identified as belonging to MC PGNT are each indicated by a black circle. Fourteen cases formed a novel distinct methylation group (depicted in gray). Fourteen cases matched with the other reference groups. Three cases showed no clear distinction between MC DNT and MC PA HEMI in repeated t-SNE analysis, indicated by dual color. *PGNT* MC papillary glioneuronal tumor, *DNT* MC dysembryoplastic neuroepithelial tumor, *RGNT* MC rosette-forming glioneuronal tumor, *CG* MC chordoid glioma of the third ventricle, *GG* MC ganglioglioma, *CN* MC central neurocytoma, *DLGNT* MC diffuse leptomeningeal glioneuronal tumor, *LGG MYB* MC low-grade glioma, MYB/MYBL1, *PXA* MC pleomorphic xanthoastrocytoma, *PA HEMI* MC hemispheric pilocytic astrocytoma, *PA MID* MC midline pilocytic astrocytoma, *PA PF* MC posterior fossa pilocytic astrocytoma, *EP RELA* MC ependymoma RELA fused, *NORM HEMI* normal hemispheric cortex. X: three cases showed no clear distinction between DNT and PA HEMI in repeated t-SNE analysis

clearly clustered to methylation classes of other tumor entities: eight tumors clustered to reference cases for MC DNT, five with MC PA HEMI, and a single case with MC PXA in the t-SNE analysis (Fig. 1). For three tumors, analysis of methylation data did not clearly distinguish between MC PA HEMI and MC DNT in repeated t-SNE analysis. However, 11/28 tumors diagnosed as PGNT formed a novel separate cluster, which we termed MC PGNT. The MC PGNT has not been identified in previous analyses of brain tumors.

Subsequently, in a large t-SNE plot containing over 25,000 methylation profiles of CNS tumors from the extended Heidelberg cohort, we screened and identified three additional cases [cases 10, 11, and 12 in Tables 1 and 2 and Supplementary Table 1 (Online Resource 2)] that clustered to our MC PGNT.

Copy-number variations in the PGNT methylation group

Copy-number variations of the 14 cases in the MC PGNT are described in Fig. 2 and Table 1. Six cases showed a flat copy-number profile and five cases showed a focal gain in a region on 17q including *PRKCA* (case 3 is shown as an example in Fig. 2a). Case 7 (Fig. 2b) and case 13 exhibited a narrow focal loss at the *PRKCA* locus on chromosome 17q. Case 4 (Fig. 2c) harbored a partial 9q loss and partial 17q gain including the *PRKCA* locus. Among the five cases harboring the 17q focal gain, recurrent case 8 (Fig. 2d) exhibited additional gains and losses.

RNA sequencing and FISH confirm *PRKCA* as a defining feature of MC PGNT

Nineteen tumors were suitable for RNA sequencing, including 10 out of 14 cases from MC PGNT. Fusions involving *PRKCA* were detected in all ten tumors belonging to MC PGNT. Nine tumors of MC PGNT carried the canonical *SLC44A1–PRKCA* gene fusion and one tumor a novel *NOTCH1–PRKCA* gene fusion. *NOTCH1-PRKCA* fusion has not been reported previously. None of the nine tumors of our series belonging to other methylation classes contained either of the fusions. The results of RNA sequencing of tumors in MC PGNT are described in Table 1. FISH

Table 1 Clinical and molecular features of the 14 cases belonging to methylation class PGNT

MC PGNT	Initial diagnosis	Age (years)	Gender	Location	CNV by 850 K	Fusion
Case 1	PGNT	39	F	Left temporal	Flat	*SLC44A1* (exon 15)-*PRKCA* (exon 9)*
Case 2	PGNT	17	F	Left lateral ventricle	Flat	*SLC44A1* (exon 15)-*PRKCA* (exon 9)*
Case 3	PGNT	13	M	Left mesial parietal	Gain at 17q *PRKCA* locus	*NOTCH1* (exon 29)-*PRKCA* (exon 9)*
Case 4	PGNT	32	M	Right parieto-occipital	Partial loss at 9q; partial gain at 17q involving *PRKCA* locus	N/A
Case 5	PGNT	27	M	Right temporal	Gain at 17q *PRKCA* locus	N/A
Case 6	PGNT	15	F	Right frontal	Gain at 17q *PRKCA* locus	*SLC44A1* (exon 15)-*PRKCA* (exon 9)*
Case 7	PGNT	14	F	Supratentorial	Loss at 17q *PRKCA* locus	*SLC44A1* (exon 15)-*PRKCA* (exon 9)*
Case 8	Recurrent PGNT	54	M	N/A	Gain 7, 18, 20, 21 and 17q *PRKCA* locus	*SLC44A1* (exon 15)-*PRKCA* (exon 9)*
Case 9	PGNT	16	M	Right lateral ventricle	Flat	*SLC44A1* (exon 15)-*PRKCA* (exon 9)*
Case 10	Ependymoma	14	F	Intraventricular	Flat	*SLC44A1* (exon 15)-*PRKCA* (exon 9)*
Case 11	Central neurocytoma	16	F	Left intraventricular	Gain at 17q *PRKCA* locus	*SLC44A1* (exon 15)-*PRKCA* (exon 9)*
Case 12	Glioneuronal tumor	N/A	F	Frontal cortex	Flat	*SLC44A1* (exon 15)-*PRKCA* (exon 9)*
Case 13	PGNT	6	F	Left parietal	Loss at 17q *PRKCA* locus	*SLC44A1-PRKCA* (FISH)**
Case 14	PGNT	14	M	Right temporal	Flat	*SLC44A1-PRKCA* (FISH)**

N/A not available

*Fusion detected by RNA sequencing, **fusion detected by FISH

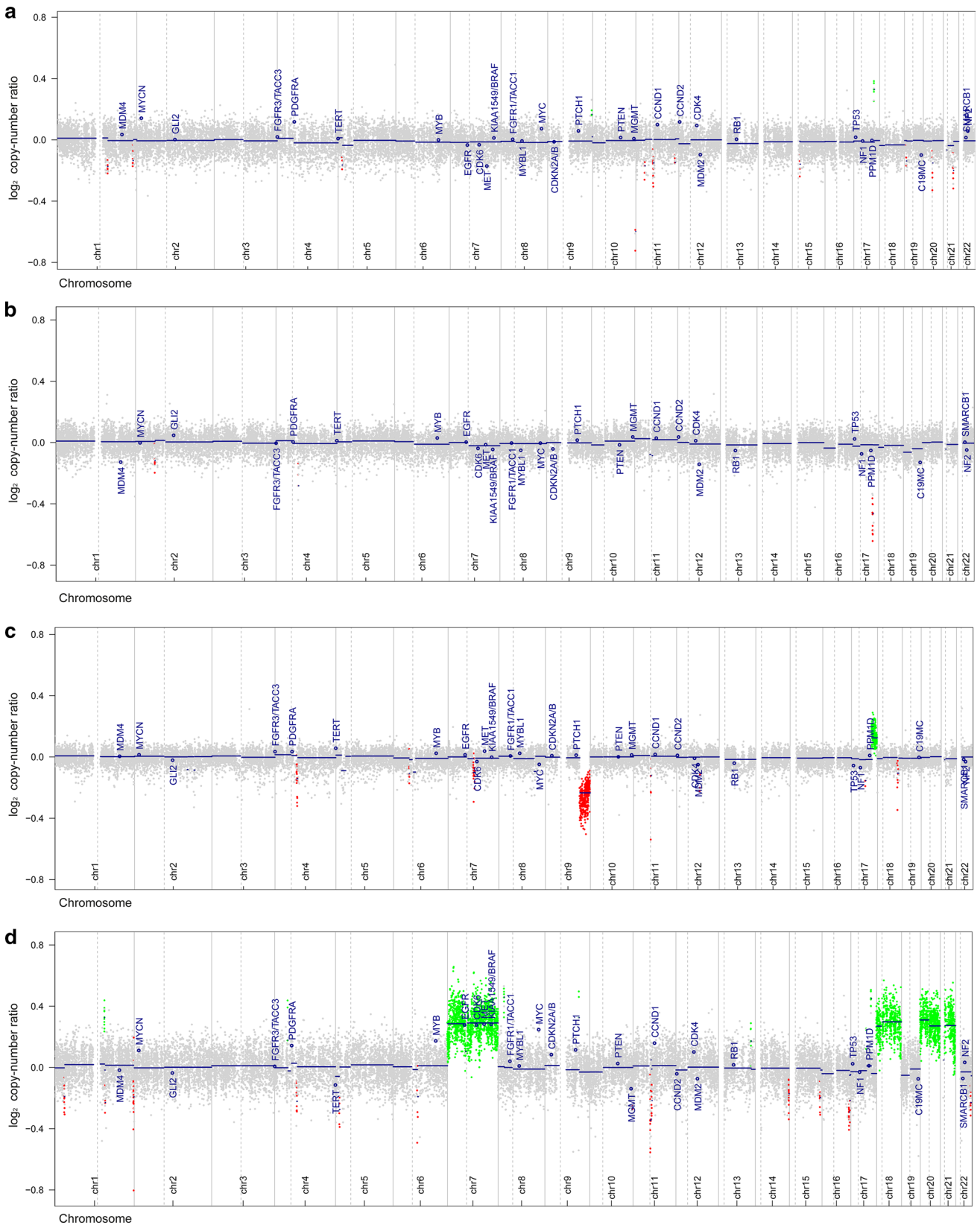

Fig. 2 CNP in MC PGNT. Gains (**a**, **c** and **d**) and a loss (**b**) were detected at the *PRKCA* locus on 17q in case 3 (**a**), case 4 (**c**), case 8 (**d**), and case 7 (**b**). A partial loss at 9q and gain at 17q was revealed in case 4 (**c**). The recurrent tumor in case 8 (**d**) exhibited multiple copy-number alterations

Table 2 Morphological features of cases belonging to methylation class PGNT

MC PGNT group	Biphasic differentiation pattern	Pseudopapillary structures	Hyalinized vessels	Interpapillary neurocytes	Ganglion cells	Microcalcifications
Case 1	−	±	+	+	±	+
Case 2	±	+	+	+	−	−
Case 3	+	+	+	+	±	−
Case 4	+	+	+	+	−	−
Case 5	−	+	±	−	−	−
Case 6	±	±	±	+	−	+
Case 7	+	+	+	+	±	−
Case 8	+	+	+	+	−	−
Case 9	+	+	+	+	−	+
Case 10	+	+	+	+	−	−
Case 11	±	−	−	+	−	+
Case 12	±	+	±	+	−	+
Case 13	+	+	+	+	−	−
Case 14	+	+	+	+	−	−

Diffuse presence is documented as "+", focal as "±", and absent as "−"

analysis from 2/14 tumors from MC PGNT has been published previously [17].

Furthermore, in the analysis of RNA sequencing performed in 273 brain tumors, using the Arriba method, we revealed the *SLC44A1–PRKCA* gene fusion in only one central neurocytoma. In this series, we also detected a *PRKCA–FAT1* fusion and a *PRKCA–FAM91A1* fusion in two tumors belonging to methylation class low-grade glioma, MYB/MYBL1 [6], and a *PRKCA–PTPRS* fusion in one peripheral nerve sheath tumor.

Clinical data and morphological features of MC PGNT cases

The clinical data for the cases are documented in Table 1 and Supplementary Table 1 (Online Resource 2). No clear sex predilection was found, and the median age at diagnosis was 16 ($n = 13$, range: 6–54). All cases with the available tumor location were supratentorial, with no clear predilection of lobes. Of these, three cases were located in close proximity to the lateral ventricles. Characteristic morphological features of PGNT are documented in Table 2. All of these cases exhibited a low-grade neuroepithelial tumor appearance, with cellularity varying from case to case. Vessel hyalinization was prominent, as well as the presence of interpapillary neurocytes. The characteristic biphasic differentiation pattern of PGNT was diffusely present in 8/14 cases. Ganglion cells were identified focally only in three cases on H&E-stained sections. Microcalcifications were present in 5 of 14 cases. Occasional mitotic figures could be found, but no necrosis or microvascular proliferation occurred in any of these cases. Histologically, we present one typical PGNT (Fig. 3a–c), three PGNTs with atypical morphology (Fig. 3d–f), and three PGNT mimics (Fig. 3g–i).

Discussion

From a series of 28 tumors diagnosed as PGNT on morphological grounds, we extracted a core set of 11 tumors exhibiting a unique molecular fingerprint, thereby defining a distinct tumor entity. This molecular fingerprint relied on both a highly characteristic methylation profile and invariable detection of a gene fusion involving *PRKCA*. Based on the methylation profile, this group was termed MC PGNT. The remaining 17 tumors belonged to other well-established entities. Comparing further tumors in our research database with this highly characteristic methylation profile, we detected three other cases exhibiting features of the MC PGNT. RNA sequencing revealed an *SLC44A1–PRKCA* fusion in all three cases.

Frequency of *PRKCA* fusions in PGNT and other brain tumors

PRKCA encodes protein kinase C alpha (PKC α), which is a member of the family of calcium and phospholipid-dependent serine/threonine kinases involved in tumor formation and progression [14]. PGNT has been recognized as a distinct tumor entity and subsequently included into the WHO Classification of Tumors of the Central Nervous System [10, 11]. However, the diagnosis on morphological grounds is difficult, with a major problem being the separation from dysembryoplastic neuroepithelial tumors [4]. More

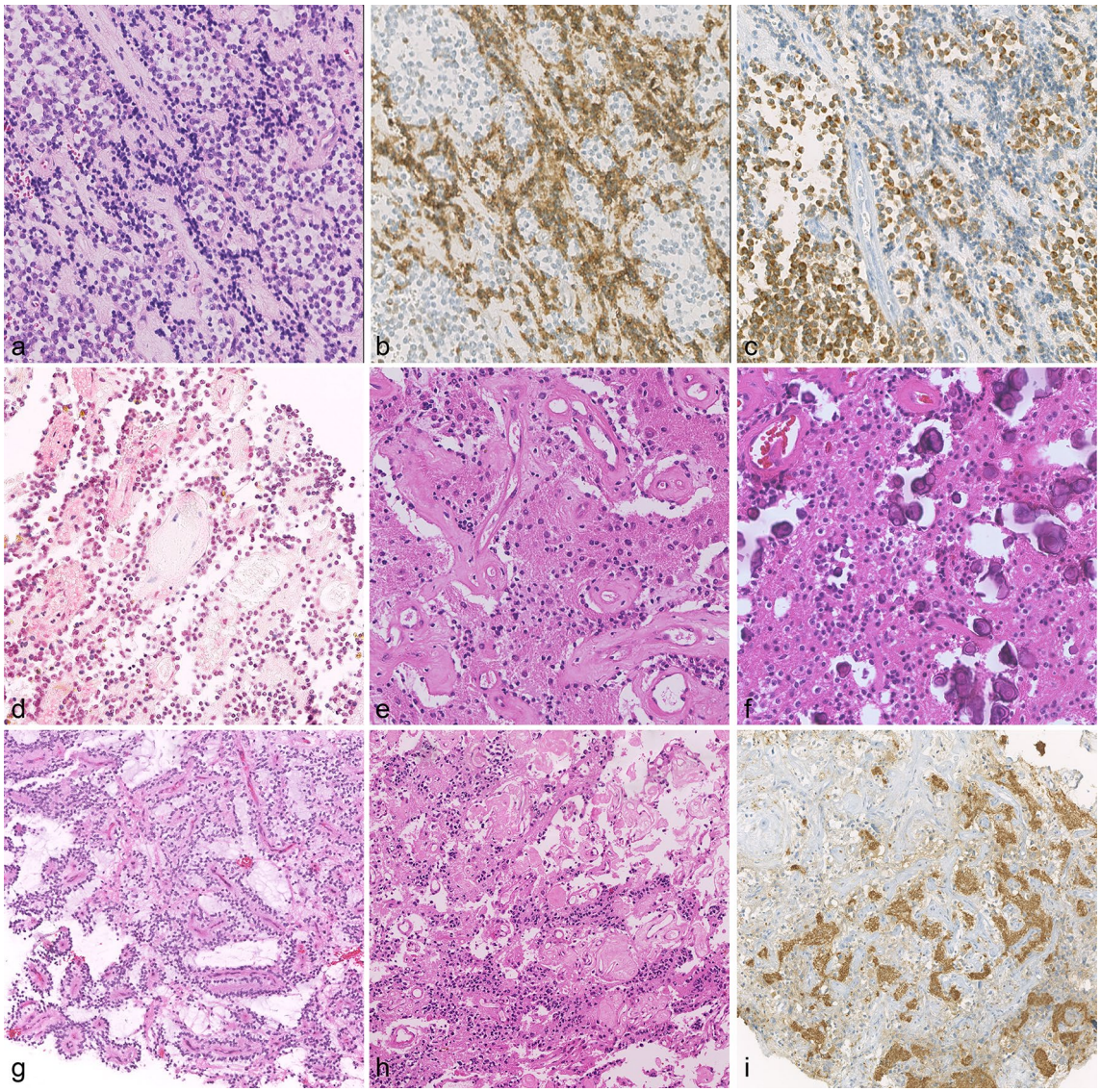

Fig. 3 Histology and immunohistochemistry of MC PGNT cases (**a**–**f**) and mimics (**g**–**i**). In case 2, H&E (**a** ×200), GFAP (**b** ×200), and synaptophysin staining (**c** ×200) demonstrate the typical morphology. The biphasic differentiation pattern is present, with pseudopapillary darker and denser GFAP-positive astrocytic cells with scant cytoplasm in contrast to synaptophysin-positive oligodendroglia-like neurocytes in the interpapillary areas. In case 5, H&E (**d** ×200) depicts pseudopapillary structures, but no biphasic differentiation pattern is found due to the lack of interpapillary neurocytes. In case 6, H&E-staining (**e** ×200) shows no clearly evident pseudopapillary structures and no biphasic differentiation pattern. In case 1, H&E (**f** ×100) reveals prominent microcalcifications, while the typical biphasic differentiation pattern of PGNT is missing. H&E (**g** ×100) of case 24 exhibits PGNT-like morphology, but methylation analysis yielded MC DNT. Perivascular pseudorosettes and a somewhat biphasic differentiation pattern with oligodendroglia-like neurocytes mimic PGNT. H&E (**h** ×200) of case 25 also suggests PGNT but methylation analysis yielded between MC DNT and MC PA HEMI. Note the similarity to case 6 (**e**). In case 23 (**i** ×100), synaptophysin positivity in interpapillary areas with low tumor cell content may be misinterpreted as PGNT, but this case belonged to MC DNT

recently, the *SLC44A1–PRKCA* fusion has been detected in PGNT [5, 16, 17]. The frequency of this fusion in PGNT varies among studies, mainly due to the low number of tumors examined. In the first study, two of three PGNT carried an *SLC44A1–PRKCA* fusion [5]. Subsequent analyses found this alteration in four of four [17] and in two of three PGNT [16]. In our series, 9 of 18 morphologically defined PGNT which could be examined by RNA sequencing or FISH exhibited a fusion involving *PRKCA*, approximating a frequency of 50%. However, employing methylation-based diagnoses, this association changed dramatically to 12 *PRKCA* fusions in 12 tumors allotted to MC PGNT. Our observation of *PRKCA* fusions in 100% of MC PGNT raises the question of whether the lower frequency of this alteration

in the previous series was caused by morphology-based misinterpretation of some other tumor entities as PGNT.

In our series of 273 brain tumors submitted to RNA sequencing, we detected fusions involving PRKCA in only four cases, none of which was recurrent. However, PRKCA mutations have been described to consistently occur in chordoid glioma [9]. These tumors characteristically carry a PRKCA D463H point mutation. Analysis of chordoid glioma by methylation analysis demonstrates a profile distinct from that of MC PGNT.

Re-definition of PGNT

PRKCA fusions invariably occur in tumors of MC PGNT but very rarely in other entities. Tumors diagnosed on morphological grounds as PGNT but not harboring a fusion involving *PRKCA* can be readily allotted to other entities by methylation analysis. This constellation clearly demands a re-definition of PGNT. In a lesion exhibiting features of glioneuronal tumors, PGNT should be diagnosed either if tumors based on methylation analysis are sorted to MC PGNT or if a fusion involving the *PRKCA* gene is detected. The invariable association of the fusion event with the distinct methylation profile is underlined by a *p* value smaller than 0.0001 [Fisher's exact test, Supplementary Figure 2 (Online Resource 3)]. DNA methylation analysis appears to identify the same set of tumors, independent of the gene partnering with *PRKCA* in the fusions, and is more adaptable to diagnostic workflows, requiring DNA extracts only. We suggest the future designation "papillary glioneuronal tumor, *PRKCA*-fused". Without appropriate testing, the designation "papillary glioneuronal tumor, NOS" may be used.

Copy-number alterations in MC PGNT

Overall, the number of copy-number alterations in tumors of MC PGNT is low comparable to many benign tumors of the central nervous system [7]. The canonical *SLC44A1–PRKCA* fusion results from a translocation t(9;17)(q31;q24). Analysis of CNV of tumors belonging to MC PGNT reveals focal abnormalities on the chromosomal arm 17q involving the *PRKCA* locus in 7 of 14 cases, possibly as a genomic "scar" of the translocation formation. Typical examples of focal gains are depicted in Fig. 2a, d. In contrast, a focal CNV of the 17q *PRKCA* locus together with chromosome 6, 11, and 12 gains was found in only one of 17 tumors not belonging to MC PGNT. Therefore, such alterations cannot be taken as definite proof of an *SLC44A1–PRKCA* fusion; however, they can be considered as an indirect evidence for such a fusion gene. Comparable copy-number alterations are seen in pediatric ependymomas carrying the *YAP–MAMLD1* fusion and exhibiting focal alterations of the *YAP1* locus at 11q22.1–11q21.2 and *MAMLD1* locus Xp28 [2].

Conclusion

We describe the invariable coincidence of a specific methylation profile with the presence of a fusion gene involving *PRKCA* in PGNT. All tumors histologically diagnosed as PGNT exhibiting neither of these traits could be identified as belonging to other tumor entities by DNA methylation-based classification. We propose re-defining PGNT, with the presence of either the methylation profile of MC PGNT or the presence of a fusion involving *PRKCA* of a glioneuronal tumor as stringent criteria for the PGNT diagnosis.

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