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Pineoblastoma segregates into molecular sub-groups with distinct clinicopathologic features: A Rare Brain Tumor Consortium registry study

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Abstract

Pineoblastoma (PB) are rare, aggressive pediatric brain tumors of the pineal gland with modest overall survival despite intensive therapy. We sought to define the clinical and molecular spectra of PB to inform new treatment approaches for this orphan cancer. Tumor, blood, and clinical data from 91 patients with PB or supratentorial primitive neuroectodermal tumor (sPNETs/CNS-PNETs), and 2 pineal parenchymal tumors of intermediate differentiation (PPTIDs) were collected from 29 centres in the Rare Brain Tumor Consortium. We used global DNA methylation profiling to define a core group of PB from 72/93 cases, which were delineated into five molecular subgroups. Copy number, whole exome and targeted sequencing, and miRNA expression analyses were used to evaluate the clinico-pathologic significance of each subgroup. Tumors designated as group 1 and 2 almost exclusively exhibited deleterious homozygous loss of function alterations in miRNA biogenesis genes (*DICER1*, *DROSHA*, and *DGCR8*) in 62 and 100% of group 1 and 2 tumors respectively. Recurrent alterations of the oncogenic *MYC-miR-17/92-RB1* pathway were observed in the RB and MYC subgroup, respectively characterized by *RB1* loss with gain of *miR-17/92*, and recurrent gain or amplification of *MYC*. PB sub-groups exhibited distinct clinical features: group 1-3 arose in older children (median ages 5.2-14.0 years) and had intermediate to excellent survival (5-year OS of 68.0-100%), while Group RB and MYC PB patients were much younger (median age 1.3-1.4 years) with dismal survival (5-year OS 37.5% and 28.6%, respectively). We identified age <3 years at diagnosis, metastatic disease, omission of upfront radiation, and chr 16q loss as significant negative prognostic factors across all PBs. Our findings demonstrate that PB exhibit substantial molecular heterogeneity with sub-group associated clinical phenotypes and survival. In addition to revealing novel biology and therapeutics, molecular sub-grouping of PB can be exploited to reduce treatment intensity for patients with favorable biology tumors.

Keywords

pineoblastoma; PNET; PPTID; miRNA; RB; MYC

Introduction

Malignant brain tumors are the leading cause of pediatric cancer-related death and disability. Embryonal brain tumors (EBTs) are the largest group of brain tumors diagnosed in children 0-14 years old and comprise 20% of all pediatric brain neoplasms [47]. Although historically classified based on tumor location and similar primitive neuroectodermal tumor (PNET) histology [24], EBTs are known to comprise a spectrum of molecular diseases with distinct clinico-pathologic features [8]. Medulloblastoma (MB) which represents 60% of childhood EBTs has been most studied, while rare EBTs, which comprise ~40% of EBTs are understudied and poorly understood. These include atypical rhabdoid/teratoid tumors (ATRTs), embryonal tumors with multilayered rosettes (ETMRs), as well as pineoblastoma (PB) - all historically treated as high-risk brain tumors with intensified regimens [30,29].

PBs comprise 30% of all pineal region tumors and may be difficult to distinguish from other tumors including germ cell tumors, high-grade gliomas, ATRTs, ETMRs and lower-grade pineal parenchymal tumors of intermediate differentiation (PPTIDs) [32]. PB have been grouped in clinical and biological studies with other EBTs arising in cerebral locations, called supratentorial primitive neuro-ectodermal tumors (sPNETs or CNS-PNETs) [42]. As there are few dedicated PB studies, the clinical and molecular spectra, and best treatment approach for these highly malignant tumors remains to be established. A recent large clinical retrospective study indicated radiotherapy (RT) but not high-dose chemotherapy (HDC) improved survival of PB patients ≥4 years old [45], although prospective consortia studies show improved survival for older children with intensified multi-modal approaches [29,27,11]. Historical sPNET studies also reported 5-yr OS of 50-65% for older children with pineal region EBTs, while patients < 3-5 years old had poorer 5-yr OS of 15-40% [30,20]. Whether these observations reflect age-related treatment biases or biological differences remain unknown.

Limited animal modeling data [57] and clinical studies of heritable “tri-lateral” retinoblastoma [14,6] suggest a role for *RB1* and related tumor suppressor pathways in PB. In addition, miRNA biogenesis gene defects have also been recently implicated in PBs [15,58]. MiRNAs which are critical post-transcriptional regulators, undergo complex processing by endonucleases (DROSHA, DGCR8, and DICER1) into mature miRNAs that function in RNA-induced silencing complexes (RISC) [37]. Although, several small studies have reported *DICER1* and *DROSHA* alterations in PB, the spectrum of RB and miRNA biogenesis alterations and their clinical significance in PBs remains to be fully evaluated. In this study, we integrated global DNA methylation profiling, copy number, and whole exome (WES) and targeted sequencing analyses on a large cohort of PB patients enrolled in a rare brain tumor registry to investigate the molecular and clinic-pathologic spectrum of PB.

Materials and Methods

Tumor, blood, and clinical data

Tumor tissue, blood, and clinical data from 93 patients diagnosed with PB, related sPNETs/CNS-PNETs, or PPTID were collected from 29 centres as part of the global Rare Brain Tumor Consortium biorepository and clinical registry (rarebraintumorconsortium.ca) using

procedure approved by Research Ethic Board at the Hospital for Sick Children and participating institutions (Supplementary Table 1, online resource) . All cases were diagnosed at their referring institutions. Available pathology reports and prepared slides were all reviewed by an experienced pediatric neuropathologist. Six of these cases have previously been analyzed by Affymetrix 100K single-nucleotide polymorphism (SNP) array and reported by Miller et al.[42]. DNA from frozen tissue or formalin-fixed, paraffin-embedded materials, and blood were extracted using the Qiagen AllPrep DNA/RNA Mini kit (Qiagen, Germany), and total RNA from 6 tumors was prepared with nCounter miRNA prep kit according to standard protocol.

Molecular and bioinformatic analyses

Tumor DNA was analysed on the Illumina HumanMethylation450 or MethylationEPIC methylation arrays (Illumina, San Diego, CA) as described previously (www.tcag.ca) [64,59] and 5000-15,000 most variable probes (standard deviation >0.3) were used for all downstream analyses (R v3.3.1). Tumor types were determined using unsupervised cluster analyses of methylation data against 1200 reference tumor profiles [59].

For t-distributed stochastic neighbor embedding, default parameters were used, except for perplexity = 10 (Rtsne v0.15, R v3.5.3). For hierarchal clustering, 1-Pearson correlation was used for distance measuring, with average linkage (pheatmap R package, R v3.61). k-means clustering was performed with Euclidean for distance measuring, and average linkage (ConsensusClusterPlus R package). Non-matrix factorization (NMF) analysis was performed with ranks (k) 2-10 at 100 runs (NMF v0.20.6). Tumor copy number profiles were determined using Conumee (version 1.8.0) and GISTIC2 (v2.0.23) [41] analyses on methylation and Illumina Omni SNP array.

WES analysis was performed on the Illumina HiSeq 4000 platform (Genome Quebec, TCAG), with variant calling using the Mutek2 pipeline (Ontario Institute for Cancer Research). Targeted sequencing was performed on the Ion Torrent platform using custom primers (Thermo Fisher Scientific) and the Ion Reporter variant calling pipeline (Genome Quebec, ResourcePath) [41]. Mutations were called deleterious or potentially deleterious based respectively on calls by both or one of the Sorting Intolerant From Tolerant (SIFT) (<0.05) or Polyphen-2 (>0.909) tool scores [66,1]. MiRNA expression was determined based on the NanoString miRNA panel (NanoString Technologies Inc.) [59] for available tumor-derived miRNA.

Statistical analyses

Event-free survival (EFS) was defined as interval between time of diagnosis to first event: tumor recurrence or progression, death from any cause, or last follow-up for those without events. Overall survival (OS) was defined as interval between time of diagnosis and death from any cause or last follow-up. Survival and prognostic factor analyses were performed on cases treated with curative intent and for which complete treatment and outcome information were available. Survival estimates were performed using Kaplan-Meier method with 95% CI, with log-rank testing used for comparisons. Fisher exact and Kruskal-Wallis analyses were used to evaluate association of specific clinical features (age, tumor location, stage)

with PB sub-groups, while univariate Cox proportional hazards regression modelling was used to identify clinical and treatment prognostic factors. All statistical analyses were performed in R v3.6.1.

Results

PB segregates into five molecular subgroups with distinct copy number profiles

Global methylation data from 93 tumors diagnosed as PB, sPNETs/CNS-PNETs, or PPTID were analysed against a reference cohort of 1200 pediatric brain tumors [59] using unsupervised orthogonal clustering (t-distributed stochastic neighbour embedding, NMF, K-means and hierarchal clustering) analyses (Fig. 1a, b). Attributable to the difficulty in diagnosing PB, 21/93 cases clustered with other tumor entities (11 germ cell tumor, 5 ATRTs, 2 MB, 2 high-grade glioma, 1 ETMR), and were excluded from further analysis. The remaining 72 tumors which segregated in one distinct cluster were further characterized using NMF, hierarchal clustering, and K-means clustering which revealed 5 robust sub-groups with highest co-phenetic co-efficient score at k=5 (Supplementary Fig. 1, online resource). We designated these as group 1, 2, 3, RB, and MYC PB sub-groups, respectively consisting of 21, 11, 13, 9, and 18 tumors, based on specific copy number and mutational features described below.

To further investigate PBs sub-groups, we performed copy number analyses using Conumee and GISTIC2 analyses on methylation and SNP array data (Fig. 2a), which revealed few significant overlapping copy number alterations except for chr 16 loss seen in all but group 3 PBs. Group 1 tumors most frequently exhibited broad gains of chr 7 (5/21; 24%) and chr 12 (6/21; 29%) and losses of chr 16 (5/21; 24%) and 22q (6/21; 29%). More detailed analysis revealed 14% (3/21) of group 1 tumors exhibited recurrent loss of a minimal 1.4Mb region on chr 5p13.3 encompassing *DROSHA,* which mediates primary-miRNA processing (Fig. 2b). In group 2 tumors, DNA methylation (Fig. 2c) and SNP array (Supplementary Fig. 2a, online resource) data showed broad chr 14q (9/11; 82%) losses where miRNA endonuclease gene *DICER1* maps, and focal homozygous *DROSHA* loss in one sample. Additionally, group 2 tumors exhibited loss of chr 8 (5/11; 45%), 16 (3/11; 27%), and 20 (3/11; 27%). In contrast, group 3 PB had no significant recurrent copy number alterations except for chr13q loss in 3/13 (23%) samples (Fig. 2d).

In the fourth designated RB sub-group, methylation and SNP arrays showed recurrent losses of a focal 0.6Mb chr 13q14.2 region spanning *RB1* in 56% (5/9) of samples (Fig. 3a; Supplementary Fig. 2b, online resource); 80% (4/5) of these also harbored focal gains of a 1.9Mb chr13q13.3 region spanning the *miR-17/92* oncogene previously implicated in retinoblastoma [12]. Nanostring expression profiling on a cohort of 6 primary PBs indicated copy number driven *miR-17/92* expression in a group RB PB (RBTC746), without significant changes in expression of paralogous loci, *miR-106b/25* and *miR-106a/363*, or the unrelated *let-7* locus (Fig. 3b). The RB sub-group also exhibited broad chr 1q (3/9; 33%) and 6p (5/9; 55%) gains and chr 16 losses (7/9; 78%) (Fig. 3a). The fifth sub-group, designated as the MYC PB, exhibited recurrent focal gains (7/18; 39%) or amplification (2/18; 11%) of a 1.2 Mb chr 8q24.21 segment encompassing *MYC* and chr 16q losses (8/18; 44%) (Fig. 3c).

miRNA biogenesis defects, *RB1* loss, and *MYC* activation characterize PB sub-groups

To extend our copy number analyses we performed WES for 11 samples and targeted sequencing of *DICER1*, *DROSHA*, *DGCR8*, *XPO5*, *TARBP2*, *RB1,* and *TP53* for 48 tumor and 21 matched blood samples with limited materials; 2 additional tumors and 2 blood samples only had materials sufficient for targeted *DICER1* and *TP53* sequencing only.

Sequencing analyses revealed mutually exclusive recurrent, deleterious, loss of function mutations of *DICER1*, *DROSHA*, or *DGCR8* almost exclusively in group 1 and 2 PBs (Fig. 4a). Significantly 11/15 *DICER1*, 6/8 *DROSHA* and 3/3 *DGCR8* alterations were novel cancer mutations not reported in COSMIC (https://cancer.sanger.ac.uk/cosmic) (Table 1). Of 15 unique *DICER1* mutations in 16 PBs, 11 were nonsense/frameshift and 4 were missense mutations. Truncating *DICER1* mutations were located within or prior to the RNase IIIb domain, while missense mutations mapped to the RNase IIIb and Helicase domains. *DROSHA* mutations, which were distributed throughout the gene, were also predominantly truncating (5/8), while only 1/3 *DGCR8* mutations was predicted to be truncating. Less common alterations included two novel, potentially deleterious missense mutations of *XPO5*, which functions in pre-miRNA export. No alterations in *TARBP2*, a DICER miRNA loading complex gene, mutated in a spectrum of cancers [21,16], were seen in our PB cohort. Significantly, we also identified germline *DICER1* mutation in 5 patients and a potential deleterious missense germline *DROSHA* mutation (RBTC717, c.199C>A; p.P67T) in one patient. Of note, all *DICER1* mutations in group 1 (6/6) and 2 (9/9) PBs were accompanied by deleterious somatic *DICER1* mutations or heterozygous chr 14q loss, variant allele frequency >96%, or complete chr14q loss. Similarly, three tumors with *DGCR8* mutations, both in groups 1 and 2 PBs, also exhibited chr 22q loss. Collectively our data shows critical miRNA biogenesis genes are targeted by copy number alterations and/or mutations in 62 (13/21) and 100% (11/11) respectively of group 1 and 2 PBs (Fig. 4).

Targeted sequencing of ten group 3 PB did not reveal any miRNA biogenesis genes, *RB1* or *TP53* alterations. Interestingly, additional WES analyses of group 3 PB samples revealed 2/8 (RBTC786 and −793) harbored similar in-frame insertions (c.935_936insCGTGGG and c.937_938insGCCGTG, respectively) in *KBTBD4*, which encodes a Cul3 E3 ubiquitin ligase adaptor, resulting in a p.P311_R312dup affecting the Kel substrate binding domain (Supplementary Fig. 3, online resource) [7]. While the c.937_938insGCCGTG mutation has recently been proposed to be a marker for PPTIDs [35], both cases of PPTID in our cohort, which were group 3 tumors, did not have this alteration on WES [18]. While both our tumors with this mutation were institutionally diagnosed as PB, they had lower Ki67 labeling indices (Supplementary Table 2, online resource) more consistent with PPTID based on values reported by Fèvre-Montange et al. [18]. Examining all group 3 tumors diagnosed as PB, Ki67 scores were at the threshold between PPTID and PB (mean 19.2%, range 10-40%). In total, group 3 tumors (mean 16.6%, range 3-40%) had significantly lower Ki67 scores than tumors belonging to other groups (mean 39.6%, range 10-75%) (p=0.003 by Kruskal-Wallis test).

In contrast to group 1 and 2 tumors, sequencing of 22 RB and MYC sub-group PBs revealed only two potentially deleterious *DICER1* mutations without evident LOH, one each in a MYC (RBTC779, c.1468N>T; p.R490C) and a RB subgroup (RBTC758, c.5240C>T;

p.S1747L) tumor. Consistent with copy number analyses, sequencing revealed 3/8 (38%) RB sub-group tumors had recurrent stop-gain *RB1* (p.R320* and p.Q121*) mutations previously reported in other cancers including retinoblastoma [39,22]. Amongst the RB subgroup patients, one (RBTC1231) presented in the context of tri-lateral retinoblastoma for which confirmatory germline testing could not be performed. Targeted sequencing of 21 blood samples, including 5 from RB subgroup patients, did not reveal any additional RB1 germline mutations. Notably, we did not identify somatic or germline *TP53* mutations in 48 PBs and 10 matched blood DNA samples sequenced.

PB subgroups have distinct clinico-pathologic features

Although PB predominantly arises in children, we observed a wide range of ages from six months to 60 years among 61/72 patients with available data, with 87% of patients ≤18 yrs of age, and children <3yrs comprising 28% of all patients (Supplementary Table 3, online resource). Comparison of clinical features showed no gender bias in the entire cohort (p=0.127) although, there was a predominance of females and males respectively in the RB (1 male:3.5 female) and MYC (2.6 male: 1 female) group of patients (Table 2). While children with group 1-3 PBs had respective median ages of 5.2, 12.5, and 14.0 years at diagnosis, the RB and MYC group patients were much younger with median ages of 1.3 and 1.4 years respectively (p<0.0001) (Fig. 5a). Staging data available for 54 patients indicated 39% (21/54) of primary PB were metastatic; 20/21 patients presented with M3/M4 disease, and only one with M1 disease. Incidence of metastases at diagnosis differed significantly across PB groups (p= 0.028) with group 2 and RB group patients respectively exhibiting the lowest (13% M2; 1/8 patients) and highest incidence (100% M3; 5/5 patients) of metastases (Fig. 5b).

46/72 PB patients were treated with curative intent and had complete treatment and outcome information available (Table 2). Univariate analyses revealed age <3yrs as a significant negative prognostic factor for EFS (HR 3.1, CI 1.3-7.4, p=0.008) and OS (HR 3.8, CI 1.4-10.1, p=0.008). EFS (HR 2.7, CI 1.2-6.3; p=0.017) and OS (HR 3.6, CI 1.3-9.7, p=0.012) were also significantly inferior in patients with metastatic disease at diagnosis. Patients who did not receive upfront RT also had inferior EFS (HR 6.5, CI 2.7-15.6, p<0.001) but not OS (HR 2.3, CI 0.8-6.7, p=0.115), while receipt of conventional chemotherapy only vs. HDC, and extent of surgery were not significantly associated with EFS or OS. As PB patients <3yrs are often treated without RT or with delayed RT regimens, we also examined prognostic factors stratified by age <3 and ≥3yrs at diagnosis. These analyses showed no significant prognostic factors except a trend toward poorer EFS with conventional dose chemotherapy compared to HDC among 11 children <3yrs, while metastatic disease remained a significant negative prognostic factor for OS (HR 4.3; 95% CI 1.1-16.7; p=0.035) but not for EFS in children ≥3yrs of age at diagnosis.

Kaplan-Meier survival analyses for all PB patients treated with curative intent revealed 5-yr EFS and OS respectively of 48.1 and 65.0%. Consistent with our Cox proportional hazards regression model, patients < 3 yrs, metastatic disease at diagnosis, and who were not treated with upfront RT had significantly poorer survival. The 5-yr EFS and OS for patients stratified by ≥ 3 vs. < 3 yrs of age were 58.2% vs. 18.2% (p=0.005) and 77.0% vs. 24.2%,

(p=0.005) respectively (Supplementary Fig. 4a, online resource), while patients with localized and metastatic disease had 5-yr EFS and OS of 60.5% vs. 29.4% (p=0.012) and 78.7% vs. 44.8% (p=0.008) respectively. Patients treated with and without upfront RT had respective 5yr EFS of 58.8% vs 10% (p<0.0001), while upfront RT was also associated with a trend towards improved survival: 5-yr OS for patients who received upfront RT was 71% vs. 40% for patients who did not receive upfront RT (p=0.106) (Supplementary Fig. 4b-c, online resource).

In addition to clinical risk factors, our analyses indicated PB survival also correlated with molecular features of tumors. Notably, EFS differed significantly across the five molecular subgroups of PB (p=0.009) while OS trended toward significance (p=0.096), with group 2 PB patients exhibiting a striking 100% 5-yr EFS/OS (Fig. 5d). In contrast, the RB and MYC sub-groups of PB, which correlated with youngest age at diagnosis and highest frequency of chr 16q loss, had the lowest 5-yr EFS/OS of only 25%/37.5% and 14.3%/28.6%, respectively (Table 2). Because chr 16q loss was associated with these high-risk groups but also seen recurrently in groups 1 and 2, we analyzed whether it was independently associated with poorer outcomes. Indeed, across all cases, those with chr 16q loss compared to unaltered chr 16q were associated respectively with 16.1% vs. 63.0% 5yr-EFS (p=0.015) while OS were respectively 47.0% vs. 72.4% (p=0.139) (Supplementary Fig. 5, online resource). Collectively our data suggest distinct tumor biology are associated with different clinical risk features and may contribute significantly to disparate treatment-related outcomes in PBs.

Discussion

PBs are high-risk brain tumors with only modest long-term survival despite multi-modal intensive regimens and for which there remains limited data to inform novel therapeutic approaches [20,29,45,27,11]. Here we performed an integrated molecular and clinic-pathologic analyses of a large cohort and demonstrate PBs comprise 5 molecular sub-groups with distinct clinico-pathologic and survival features. Group 1 and 2 PB which arise in older children exhibit recurrent miRNA biogenesis gene defects; group 3 PB which affects adolescents and adults exhibit few alterations, while the RB and MYC sub-groups affecting children age <18 months harbor *RB1* and *MYC* alterations. Our data indicate age <3yrs, metastases at diagnosis and tumor molecular features as important determinants of survival in PB patients (summarized in Fig. 6) and provide an important framework for prospective studies.

Strikingly, we identified deleterious mutations in multiple components of the miRNA processing machinery almost exclusively in group 1 and 2 PBs. Consistent with association of PBs with DICER1 predisposition syndrome [15,58], we identified germline and somatic *DICER1* mutations in addition to somatic *DROSHA* and *DGCR8* mutations, which have not been reported in PBs to date. With the exception of reported nonsense mutations [15] in RBTC717 and −745, all of the *DICER1* mutations identified in our study were novel and those in group 1 PB most commonly affected the RNase IIIb domain which selectively processes 5p miRNA [26]. Interestingly, imbalanced abundance of 5p versus 3p miRNAs due to RNase IIIb domain mutations have been implicated as important oncogenic

mechanisms [28,3,53]. In contrast, mutations in group 2 tumors affecting both RNase domains were predicted to completely impair miRNA maturation, as reported in Wilm's tumors [54]. Group 1 and 2 PBs with *DICER1* mutations exhibited LOH as reported in smaller PB studies [15,58]. This is in stark contrast to *DICER1* mutations in other tumors, where LOH is rare and truncating germline mutations are associated with hotspot missense mutation of the RNase IIIb domain [19]. In PBs, the second hit appears to be either chr 14q loss or a second truncating mutation of both RNase domains. Interestingly, murine tumors with bi-allelic *Dicer1* knockout appear to be selected against [33] suggesting PB tumors likely retain some residual DICER1 activity, either through conserved RNase IIIa domain (in group 1) or other aberrant functions not involving the RNase domains (in group 2). The unique pattern of *DICER1* somatic and germline mutations observed in our cohort suggest specificity of the second hit in the formation of this tumor.

We identified truncating and damaging missense mutations of both *DROSHA* and *DGCR8*, but at much lower frequency than *DICER1*. As these mutations were seen only in group 1 and 2 PBs, and in only 10% (6/59) of tumors in our study, it is perhaps not surprising that *DROSHA* and *DGCR8* mutations were not reported in recent WES or whole genome sequencing studies of 19 PBs [60,35]. *DROSHA* mutations are frequent in Wilm's tumors, where > 70% are missense mutations at E1147 in the RNase IIIb domain [68,67]. Although the IIIa and b domains respectively processes the 3p and 5p arms of pri-miRNA, the reported missense mutations do not appear to cause an imbalance in 5p and 3p mature miRNAs, but may act via dominant-negative mechanisms to globally downregulate miRNA production [54,65]. Our findings suggest miRNA maturation may also be globally downregulated in a subset of group 1 and 2 PBs via homozygous loss or biallelic truncating mutations of several critical miRNA endonucleases. All *DGCR8* mutations in our PB samples were accompanied by chr 22 loss or LOH, similar to LOH in Wilms tumors with hotspot *DGCR8* dsRBD mutations that impair mature miRNA expression [65,67,68]. Interestingly, one group 1 PB (RBTC757) exhibited loss of chr 22 copy in the context of the chr 22q11.2 deletion syndrome (22q11.2DS, DiGeorge syndrome). The minimal chr 22q11.2DS region which encompasses *DGCR8* has also been linked to two prior cases of PB [46,61,34], and suggest *DGCR8* loss may predispose to PB.

Although heritable retinoblastoma is associated with increased risk for PB [43], *RB1* alterations have not been described in sporadic PB. In the RB subgroup, we observed recurrent *RB1* homozygous loss or inactivating stop-gain mutation with LOH consistent with a classic two-hit mechanism. Associated with *RB1* loss, we observed recurrent copy number gains of chr 13q31.3 which encompasses the oncogenic *miR-17/92* cluster. In Rb/p107-deficient mice, *miR-17/92* overexpression drives retinoblastoma formation by targeting *Cdkn1a* (p21/Cip1) to increase retinal cell proliferation [12], an oncogenic process that requires intact Dicer1 function [48] and may explain the paucity of miRNA biogenesis gene mutations in the RB sub-group of PBs. Of note we observed that *MYC*, which is also known to drive neoplastic growth by upregulating *miR-17/92* [36], was recurrently gained/amplified in the MYC PB sub-group. These observations suggest common oncogenic mechanisms driven by a *MYC-miR-17/92-RB1* axis [55,56] may underlie the aggressive biological features seen in these PB subgroups

KBTBD4 is a member of a large family of Bric-a-brac/Tramtrack/Broad (BTB) complex-containing adaptor proteins that complex with CUL3 E3 ubiquitin ligase and serve as a bridge between CUL3 and its substrate via a kelch interaction domain [10,7]. Substrates are then ubiquitinated and marked for degradation in the ubiquitin proteasome pathway. Hotspot mutations affecting the kelch domain have been reported in group 3 and 4 MB [49], and three cases of PPTID [35], and have been proposed as an oncogenic driver. While the targeted substrate of KBTBD4 has not been demonstrated, similar mutations in other BTB proteins that affect the substrate-binding domain or cause loss-of-function have been reported in a variety of cancers [10]. For example, in prostate cancer, androgen receptor signaling is implicated in tumor initiation and progression, as well as development of resistance to anti-androgen therapy [9]. Mutations affecting the androgen receptor-binding domain of BTB protein SPOP [5] leads to the failure of ubiquitination by CUL3 and thus, enhanced androgen receptor signaling [2]. Our WES analyses identified hotspot kelch domain mutations in 2/8 sequenced group 3 tumors. Although both these cases, diagnosed as PB, had lower Ki67/MIB-1 proliferation indices more consistent with PPTIDs [18], we did not observe this alteration in our two cases of PPTID or other group 3 PBs. While the hotspot *KBTBD4* mutation have been proposed to be a marker for PPTID [35], our data suggests this mutation is characteristic for at least some group 3 tumors rather than exclusively all PPTIDs. With the caveat that Ki67/MIB-1 scores can be subjective and variable depending on tumor sample size, our review of scores in our cohort suggest group 3 is mainly composed of PBs with lower Ki67 indices in the range of PPTIDs, and tumors diagnosed as PPTIDs. Thus, tumors diagnosed as PPTID may be biologically similar to a proportion of PBs based on their shared global DNA methylation profile and silent chromosomal copy number landscape. Alternatively, some PPTIDs may be mis-diagnosed as PBs.

We observed on univariate analysis that loss of chr 16q was a significant negative prognostic marker for EFS and trending toward significance for OS. Interestingly, in another childhood embryonal cancer, Wilm's tumor, chr 16q loss is also an established independent negative prognostic marker for relapse and death, and is being used to risk stratify patients with favorable histology tumors for intensified therapy [25]. Whether the loss-of-heterozygosity (LOH) of chr 16q disrupts a putative tumor suppressor or is a result of greater genomic instability remains to be elucidated for Wilm's tumor. Some groups have proposed that LOH 16q may involve the effects of tumor-associated genes E2F4, COX4 [50], and CTCF [44], which all reside on chr 16q. We did not see mutations affecting these genes in our limited WES.

However, RB family tumor suppressor *RBL2* (p130) also resides on 16q and is inactivated or lost in multiple cancers, including retinoblastoma [4,62,13,69,52]. We found that group RB and MYC tumors are characterized by chr 16q loss and an oncogenic MYC-miR-17/92-RB1 axis. Interestingly, in pancreatic adenocarcinoma, high expression of one member of the *miR-17/92* cluster, *miR-17-5p*, directly targets *RBL2* to inhibit RBL2-mediated repression of E2F4 target genes (*MYC*, *CCND1*, *JUN*), thereby enhancing proliferation [69]. *RBL2* targeting is also seen in ovarian carcinoma via overexpression of *miR-17/92* paralog *miR-106a* [38]. *RBL2* could be similarly targeted by loss of chr 16q in PB. However, we did not observe *RBL2* mutations in our limited WES, nor that chr 16q loss and *miR-17/92* gain/

amplification were mutually exclusive. Further studies will have to be completed to fully characterize the MYC-miR-17/92-RB axis and the role of *RBL2* in PB.

Clinical studies of PBs to date have been limited by its rarity and lack of large, disease specific prospective cohorts. The recently completed Children's Oncology Group high-risk EBT trial ACNS0332, enrolled 34 patients >3yrs, however separate clinical and molecular analyses of the PB cohort has not been reported [29]. Our clinical findings are limited by the retrospective nature of our registry-based cohort, and relatively smaller numbers compared to other studies of far more common childhood EBTs. Indeed, only recently have two clinical analyses with larger numbers, both retrospective, been published: a single institution study of 41 patients from St. Jude Research Hospital [51] and a pooled analysis of 135 patients from SIOP-E and US Head Start trial groups [45]. No previous published study has yet performed a combined molecular and clinical analysis as we have sought to do here. The clinical applicability of our findings will likely require further validation through continued collaboration with other research groups to pool enough data to power subgroup-specific risk stratification and inform therapy.

Nonetheless, consistent with prior studies [31,23,40,63,51,45], we identified young age at diagnosis (<3 yrs), metastatic disease, and omission of upfront RT as negative prognostic factors for PB survival. Also in agreement with published observations [51,45,17] our analysis did not reveal prognostic correlations with HDC or extent of surgery across all PB patients, although there was a trend toward improved EFS in children <3yrs who received HDC.

In contrast to the excellent outcome in group 2 PB (5-yr OS 100%), groups 1 and 3 patients had intermediate outcomes (68.0 and 80%), while groups RB and MYC patients had poorest outcomes (37.5 and 28%). Metastatic disease and chr 16 loss, which correlate with poorer survival across the entire cohort, was also enriched in group 1, RB, and MYC PBs, thus suggesting adverse molecular and clinical risk features may account partly for the poorer outcomes of these patients.

While the difference in EFS and OS between group 2 and 3 is due to just one group 3 patient who recurred then died from disease, another group 3 patient was only treated with palliative chemotherapy and thus not included in our intent-to-treat analysis. Both patients had extra-CNS (M4) metastasis at diagnosis. In contrast, of nine patients with group 2 tumors and clinical data, two were excluded from our intent-to-treat analysis: one who refused treatment, and another who died from intraoperative complications. No treated patients recurred or died. These differences in clinical features between the two groups not captured by intent-to-cure only EFS/OS estimates have led us to assign group 2 a superior prognosis to group 3.

The impact of different age-related therapeutic approaches likely contributes to differences in outcomes across PB patients. Indeed, we observed a significant difference in proportion of patients <3yrs (4/11; 36.4%) vs. those ≥3yrs of age at diagnosis (33/36; 91.7%; $p<0.001$) who received upfront RT, suggesting RT avoidance may play a role in adverse outcomes seen in younger patients who primarily had group 1, RB and MYC PB. Of note, group 2 and

3 PB had the highest median age at diagnosis, including three patients >20yrs of age who were alive at last follow-up after therapy that included only up-front RT. In contrast, two adult patients >20yrs at diagnosis who had group 1 and MYC tumors, both died despite receiving multimodal therapy including CSI, suggesting intensive therapy may not completely negate adverse tumor biology. Despite the prognostic impact of RT demonstrated by our study and that of others, it is also interesting to note that 5/29 long term survivors in our cohort who never received radiation therapy were young patients with group 1 (2 patients), MYC (2 patients), and RB (1 patient) PBs.

Our integrative molecular and clinico-pathologic analyses in this study which has identified five distinct molecular sub-groups of PB has provided important new insights into the pathogenesis of PB and confirm the importance of cancer predisposition related to miRNA biogenesis and RB1 gene defects in PB patients. Our study indicates groups 1-3 PBs patients treated with contemporary multi-modality regimens have intermediate to excellent outcomes but also highlight critical treatment gaps for younger PB patients most susceptible to radiation-related toxicities. Although our retrospective study has limitations, it represents one of the largest integrated clinical and molecular analyses of PB to date and provides new and critical information to inform therapy reduction in prospective clinical trials for favorable risk patients and development of novel therapies for high risk patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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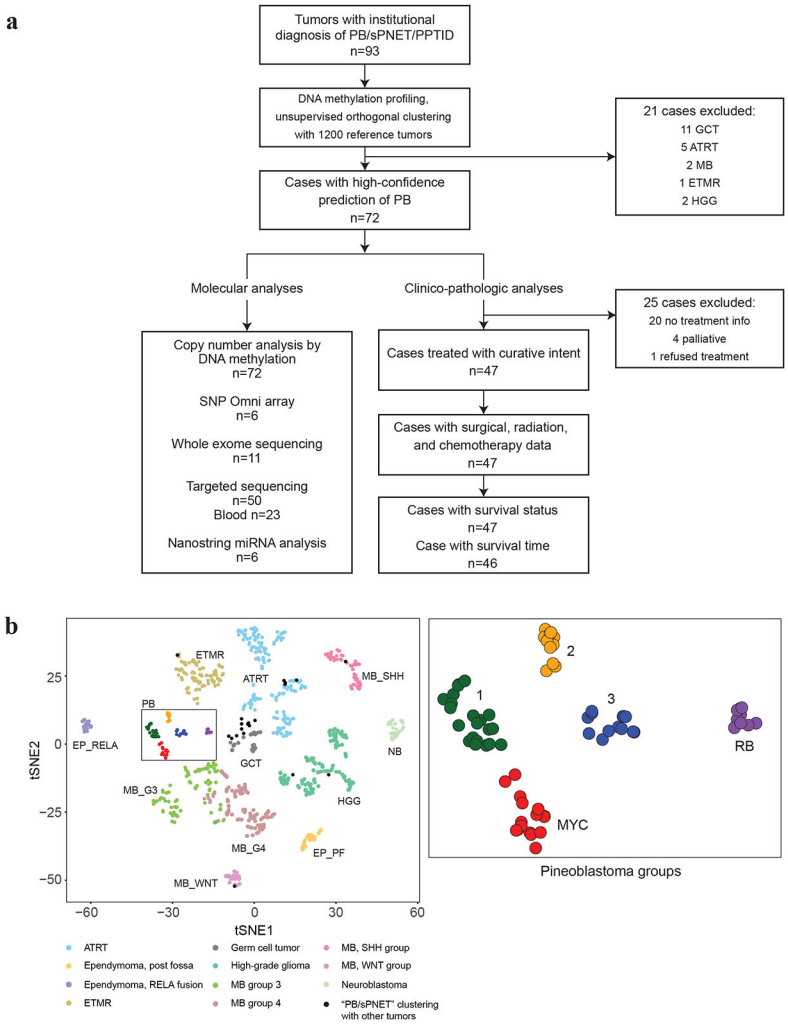

Figure 1. PB comprise five molecular sub-groups

a. Flow diagram of analyses performed: 93 primary tumors with institutional diagnosis of pineoblastoma (PB) or supratentorial PNET (sPNET) were analysed using global methylation profiling and compared against a reference cohort of 1200 pediatric brain tumors to identify and exclude samples that segregated with other brain tumors. A cluster of robust, molecularly confirmed 72 PBs were further characterized using methylation and SNP arrays for copy number alterations, mutational analyses using WES and targeted sequencing, and Nanostring analyses for miRNA expression. Clinical, treatment, and molecular sub-group data available for 46 PB patients treated with curative intent were integrated for clinic-pathologic analyses.

b. t-Distributed stochastic neighbour embedding (tSNE) plots of DNA methylation clustering patterns of 93 presumed PB samples relative to 951/1200 representative pediatric brain tumor entities demonstrate PB clusters separately from other tumor entities. Plots using the top 12,500 most varying methylation probes by standard deviation (SD) are shown. Tumors are shown as colored spheres which include atypical teratoid rhabdoid tumor (ATRT), ependymoma posterior-fossa (EP_PF) or supratentorial, RELA-fusion (EP_RELA), embryonal tumor multiple rosettes (ETMR), germ cell tumor (GCT), high-grade glioma

(HGG), neuroblastoma (NB), medulloblastoma WNT (MB_WNT), SHH (MB_SHH), group 3 (MB_G3), and group 4 (MB_G4). Black spheres indicate tumors with an institutional diagnosis of PB that segregated with other known brain tumor entities are (n=21). A robust cluster of 72 PBs is boxed; blow-up image of PB cluster on right shows five molecular PB sub-groups designated as 1, 2, 3, RB, and MYC.

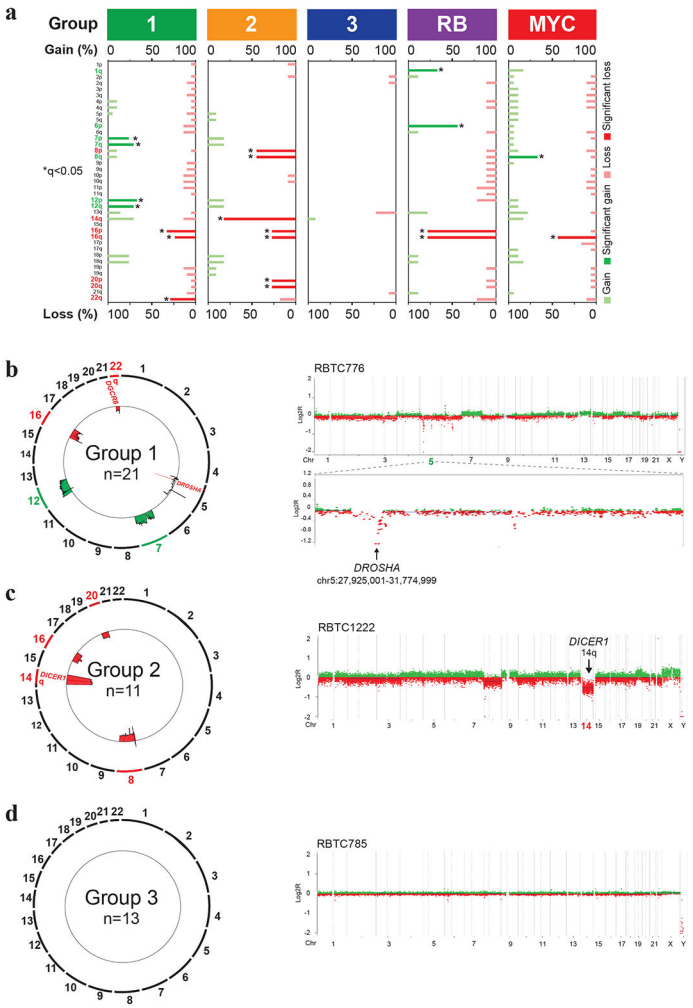

Figure 2. PB molecular subgroups have distinct copy number landscapes

a. Pattern of copy number alterations across PB molecular sub-groups as determined using GISTIC analyses of global methylation data. Chromosomal regions with recurrent copy number gains (green) or losses (red) significantly enriched within each PB sub-group are highlighted; asterisk indicates false discovery rate of q<0.05.

b, c. Composite circos plots of global methylation profiles showing recurrent copy number gains (green) and losses (red) in 21 group 1 and 11 group 2 PBs. Focal or broad alterations associated with miRNA biogenesis loci *DICER1*, *DROSHA* and *DGRC8* are highlighted. Higher resolution copy number profiles generated using Conumee, of representative group 1 and group 2 samples with respective focal chr 5p13.3 targeting *DROSHA* and chr 14q loss associated with *DICER1*, are shown on the right.

d. Composite circos plot of global methylation profiles in 13 group 3 PBs. Higher resolution copy number profile generated using Conumee of a representative group 3 sample is shown on right.

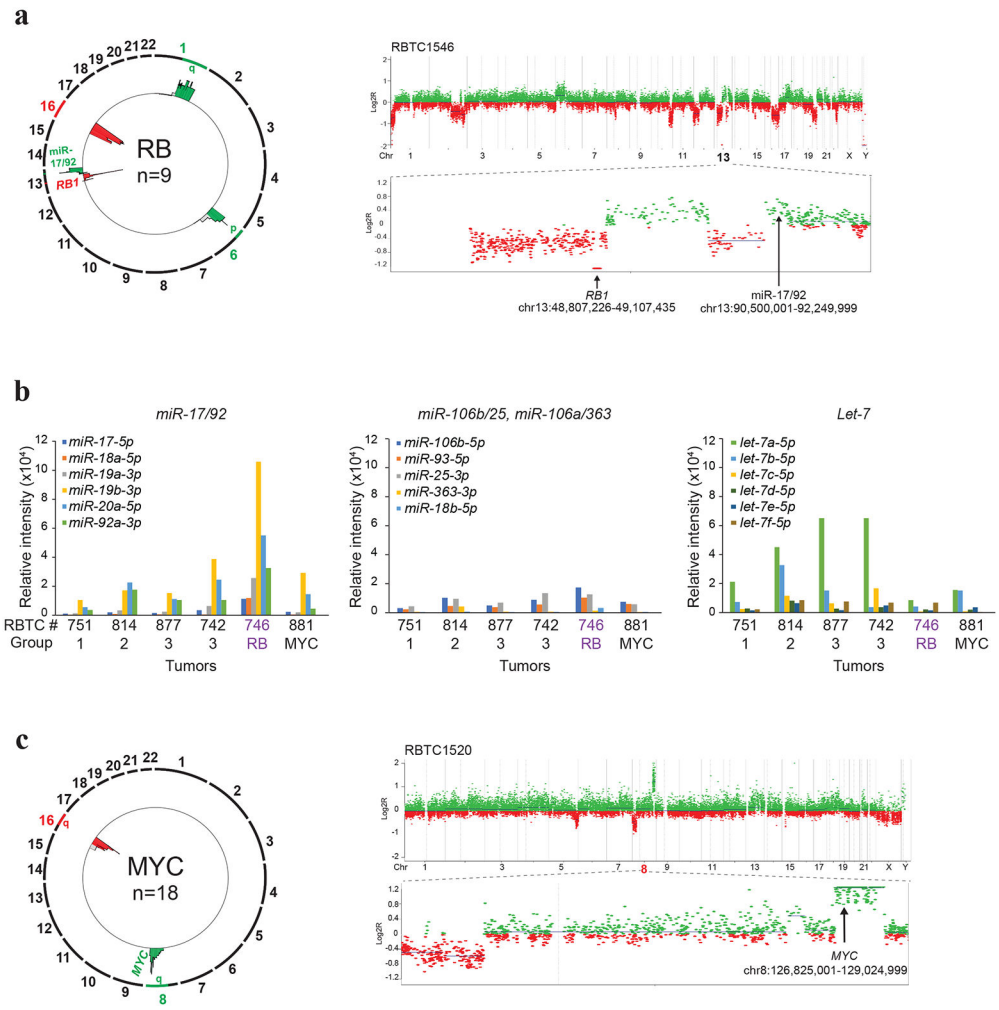

Figure 3. Recurrent copy number alterations in RB and MYC sub-group PBs

a. Composite circos plot of global methylation profiles from 9 RB subgroup PBs depicting recurrent copy number gains (green) and losses (red); recurrent copy number alterations associated with *miR-17/92* and *RB1* are highlighted. Higher resolution copy number profile of a representative tumor RBTC 1546 with homozygous loss of *RB1* at chr13q14.2 and copy number gain encompassing *miR-17/92* at chr 13q31.3 is shown on right.

b. Copy number driven expression of *miR-17/92* in RB sub-group PB. MiRNA expression levels for the *miR-17-92*, paralogous *miR106a-363*, *miR-106b-25* and unrelated *let-7* loci was determined from NanoString(v.3) miRNA expression data from 6 PBs. Plots show relative, normalized probe intensities of miRNAs in PB sub-groups; miRNA expression levels of RBTC746 with focal chr13q13.3 copy number gains targeting *miR-17-92* shown in Figure A, is highlighted.

c. Composite circos plot of global methylation profiles from 18 MYC subgroup PBs. Recurrent focal chr 8q amplification/gains (green) and chr 16q losses (red) are highlighted. Higher resolution copy number profile of a representative tumor, RBTC 1520, with focal *MYC* amplification is shown on right.

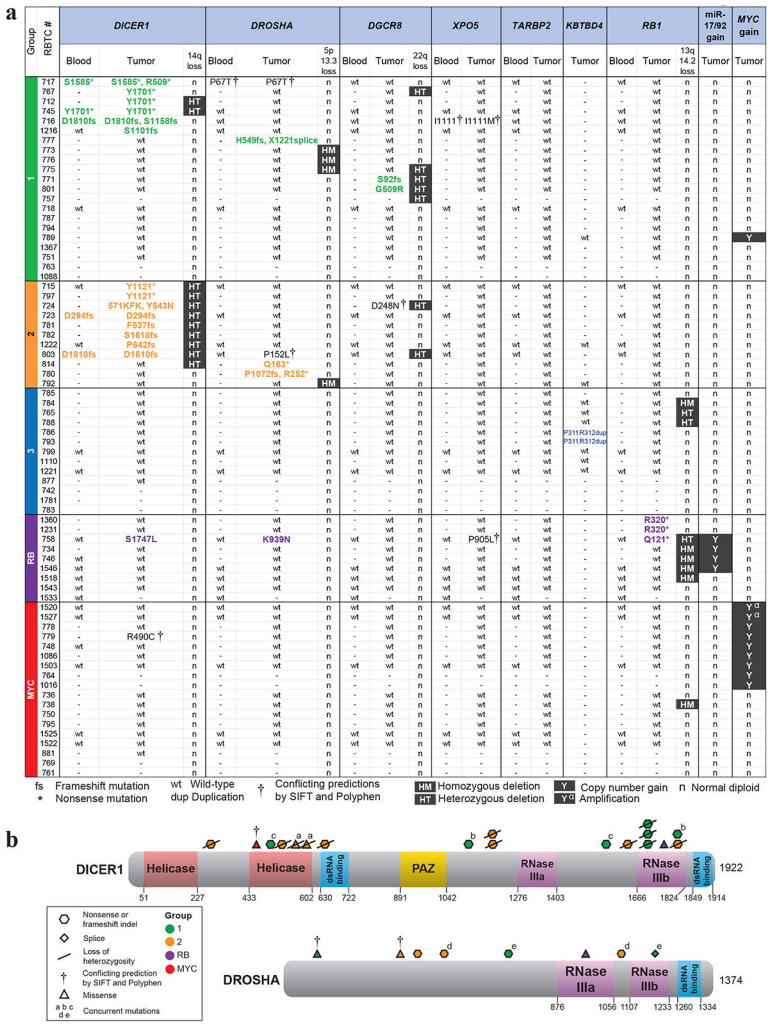

Figure 4. Recurrent mutations/alterations of miRNA biogenesis genes, *RB1* and *MYC* characterize PB sub-groups.

a. Summary of mutations and copy number alterations associated with miRNA biogenesis gene (*DICER1*, *DROSHA*, *DGCR8, XPO5, TARBP2*), *KBTBD2*, *RB1*, *miR-17/92*, and *MYC* determined using a combination of targeted sequencing, WES, methylation and SNP array based copy number analyses in individual PBs of different sub-groups with tumor and matched blood DNA available for study. Samples lacking materials for specific assay are indicated by (-); broad copy number alterations determined by methylation or SNP-based copy number analyses are indicated by HT (heterozygous), HM (homozygous), n (normal diploid) or presence (Y) of MYC focal gains or amplification (α) is indicated. Status or specific gene alterations determined by targeted sequencing or WES is indicated as wt (wild-type); * (stop-gain mutation), fs (frameshift insertion or deletion), † (deleterious missense mutation predicted by SIFT and Polyphen2). All predicted truncating gene mutations are highlighted.

b. Schema of *DICER1* and *DROSHA* mutations relative to maps of corresponding proteins. Type and location of mutations are shown as colored symbols relative to amino acid

sequence numbers and known or predicted functional domains; colors of mutation symbols correspond to tumor sub-group.

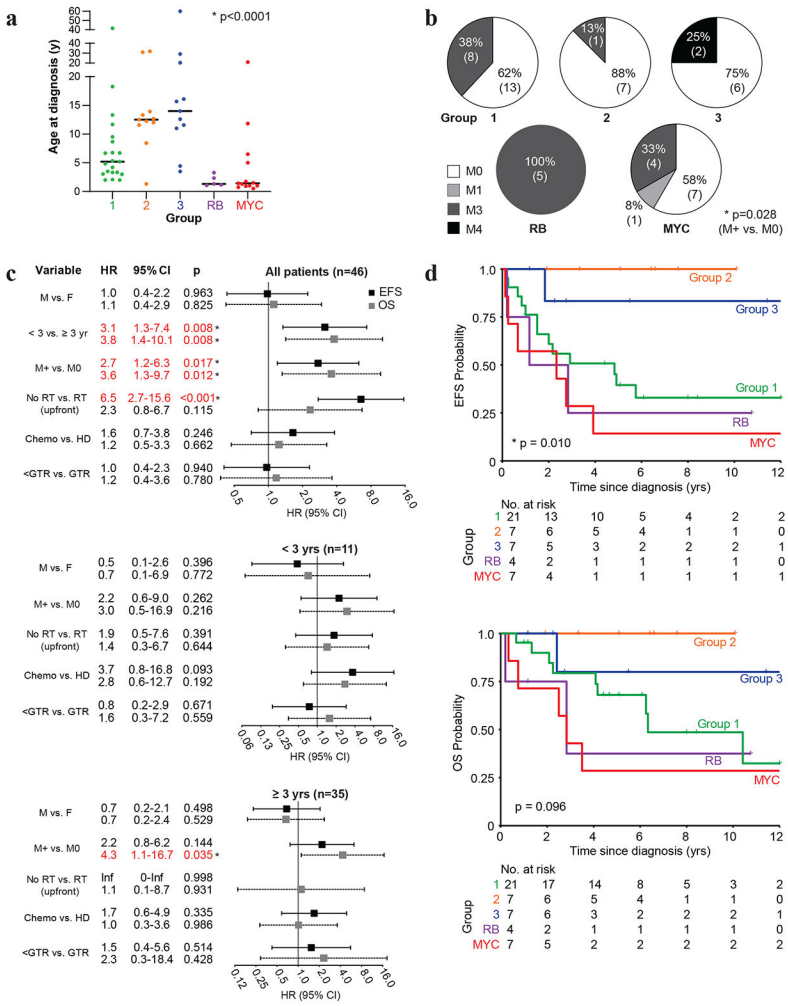

Figure 5. Molecular sub-groups of PB have distinct clinicopathologic features

a. Scatterplot of age at diagnosis for PB patients relative to tumor molecular sub-group. Bar indicates median age as determined using Kruskal-Wallis test.

b. Frequency of metastatic (M+; M1, −3, −4) and non-metastatic (M0) disease determined as per the Chang staging system is shown relative to PB sub-groups; significance in distribution of M+ versus M0 patients across all PB sub-groups was determined using Fisher exact test.

c. Forest plot of Hazard ratio (HR) from univariate Cox proportional hazards regression model of gender (male/M vs. female/F), age, metastatic status (M+ vs M0), radiotherapy (no upfront RT vs. upfront RT), conventional chemotherapy only (chemo) vs. high-dose chemotherapy (HD), and extent of tumor removal (less than gross total resection/GTR vs GTR) on EFS (black) and OS (gray) was performed on data from 46 patients treated with curative intent. Whiskers denote 95% confidence interval.

d. Kaplan-Meier survival analyses of event free (EFS) and overall survival (OS) for 46 patients treated with curative intent stratified by PB sub-groups. Plots abbreviated to maximum of 12 years from diagnosis. For patients with group 1-3, RB, and MYC PBs EFS were respectively, 39.5%, 100%, 83.3%, 25.0%, and 14.3%; 5-year OS were 68.0%, 100%, 80%, 37.5%, and 28.6%.

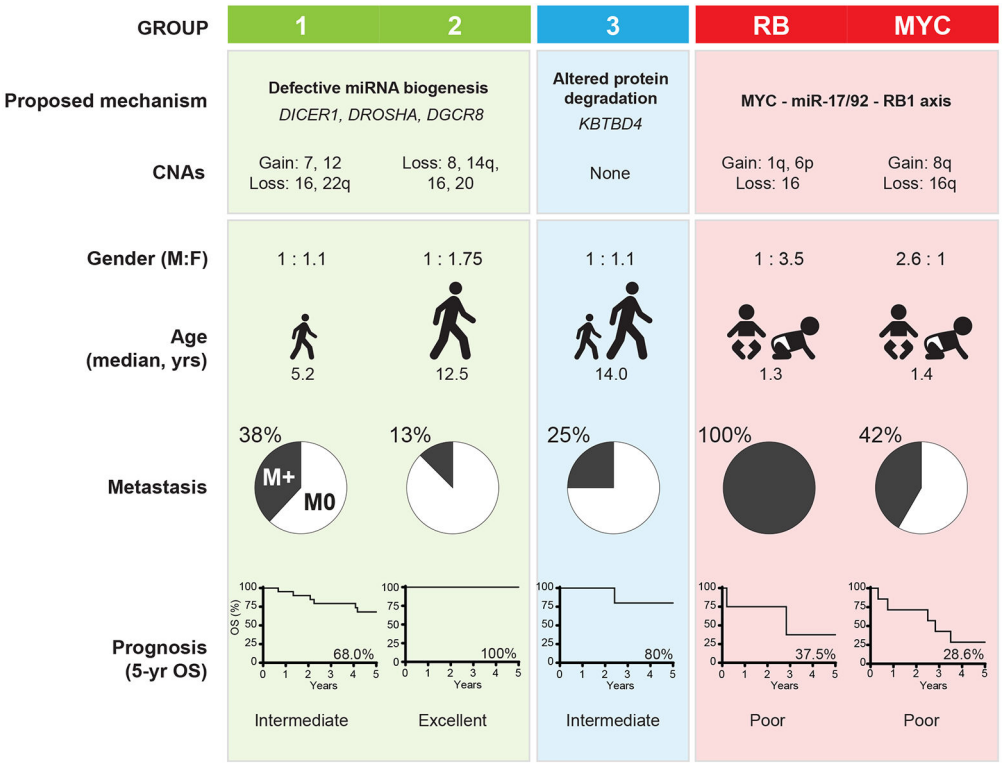

Figure 6.
Schematic summary of molecular and clinical features across PB sub-groups

Table 1

Summary of PB mutations identified in this study

Gene	Mutation	Type	Predicted deleterious effect of mutation	Observed in PB cohort	Observations in COSMIC	Cancer types in COSMIC
DICER1	Y1701*	Nonsense	Truncating	3	1	Liver
	S1585*	Nonsense	Truncating	1	Novel	
	R509*	Nonsense	Truncating	1	3	Melanoma
	Y1121*	Nonsense	Truncating	2	Novel	
	D1810fs	Frameshift indel	Truncating	2	Novel	
	S1158fs	Frameshift indel	Truncating	1	Novel	
	S1101fs	Frameshift indel	Truncating	1	Novel	
	D294fs	Frameshift indel	Truncating	1	Novel	
	F537fs	Frameshift indel	Truncating	1	Novel	
	S1618fs	Frameshift indel	Truncating	1	Novel	
	P642fs	Frameshift indel	Truncating	1	Novel	
	Y543N	Missense	Altered helicase domain	1	Novel	
	S1747L	Missense	Altered RNase IIIb domain	1	2	Breast
	R490C	Missense	Helicase Domain †	1	1	Bladder
	571_573delinsKFK	Missense	Helicase Domain - unknown	1	Novel	
DROSHA	Q163*	Nonsense	Truncating	1	Novel	
	R252*	Nonsense	Truncating	1	Novel	
	H549fs	Frameshift indel	Truncating	1	Novel	
	P1072fs	Frameshift indel	Truncating	1	Novel	
	X1221_splice	Splice site	Truncating	1	Novel	
	P152L	Missense	Not in functional domain †	1	Novel	
	P67T	Missense	Not in functional domain †	1	1	Large intestine
	K939N	Missense	Altered RNase IIIa domain	1	1	Breast
DGCR8	S92fs	Frameshift indel	Truncating	1	Novel	
	G509R	Missense	Immediately adjacent to DRBM1 domain	1	Novel	
	D248N	Missense	Not in functional domain †	1	Novel	
XPO5	I1111M	Missense	Unknown †	1	Novel	
	P905L	Missense	Unknown †	1	Novel	
RB1	R320*	Nonsense	Truncating	2	21	Retinoblastoma, endometrial, breast
	Q121*	Nonsense	Truncating	1	2	Lung, thyroid
KBTBD4	p.P311_R312dup	In-frame insertion	Altered Kelch binding domain	2	0	PPTID [a], MB [b]

Abbreviations:

† conflicting prediction by SIFT and Polyphen2

[a] reported by Lee et al. 2019

[b] Northcott et al. 2017

Table 2

Summary of patient features and treatment across PB sub-groups

		Total		Group 2						RB		MYC		
		n	%	n	%	n	%	n	%	n	%	n	%	
	Number of patients	72		21	29	11	15	13	18	9	13	18	25	**p-value**
Clincial features	**Gender**	72		21		11		13		9		18		0.127
	Male	36	50	10	48	4	36	7	54	2	22	13	72	
	Female	36	50	11	52	7	64	6	46	7	78	5	28	
	Age	61		21		11		11		5		13		<0.0001 *
	Median (yrs) (range)	6.5	(0.5-60)	5.2	(2.0-41.5)	12.5	(1.3-31.9)	14.0	(3.5-60)	1.3	(1.1-3.3)	1.4	(0.5-21.0)	
	<1	3	5	0	0	0	0	0	0	0	0	3	23	
	1 to <3	14	23	3	14	1	9	0	0	4	80	6	46	
	3 to <10	20	33	14	67	1	9	2	18	1	20	2	15	
	10 to <18	16	26	2	10	7	64	6	55	0	0	1	8	
	≥18	8	13	2	10	2	18	3	27	0	0	1	8	
	Stage	54		21		8		8		5		12		0.028 *
	M0	33	61	13	62	7	88	6	75	0	0	7	58	
	M+	21	39	8	38	1	12	2	25	5	100	5	42	
Treatment features: intent to tre at group	**Surgery**	47		21		7		7		4		8		0.431
	GTR	17	36	5	24	3	43	4	57	1	25	4	50	
	<GTR	30	64	16	76	4	57	3	43	3	75	4	50	
	Radiotherapy	47		21		7		7		4		8		0.041 *
	Yes	37	79	17	81	7	100	7	100	2	50	4	50	
	No	10	21	4	19	0	0	0	0	2	50	4	50	
	Chemo	46		21		7		6		4		8		0.749
	HDC	29	63	15	71	4	57	3	50	3	75	4	50	
	Conventional	17	37	6	29	3	43	3	50	1	25	4	50	
Survival analysis	**Status**	47		21		7		7		4		8		0.019 *
	Dead	18	38	9	43	0	0	1	14	2	50	6	75	
	Alive	29	62	12	57	7	100	6	86	2	50	2	25	
	Recurrence	46		21		7		7		4		7		0.003 *
	Yes	21	46	12	57	0	0	1	14	2	50	6	86	
	No	25	54	9	43	7	100	6	86	2	50	1	14	
	Median follow-up time (yrs) (range)	4.2 (0.2-20.3)		4.7 (0.7-20.3)		6.4 (1.9-10.1)		2.8 (1.2-13.9)		2.0 (0.2-10.8)		3.2 (0.3-17)		
	5-yr survival (%)													
	EFS (95% CI)	48.1 (32.2-62.3)		39.5 (18.5-60.0)		100 (n/a)		83.3 (27.3-97.5)		25 (0.9-66.5)		14.3 (0.7-46.5)		0.009 *

	Total		Group 2				RB		MYC		
	n	%	n	%	n	%	n	%	n	%	
OS (95% CI)	65.0 (47.8-77.7)		68.0 (42.0-84.2)		100 (n/a)		80 (20.4-96.9)		37.5 (1.1-80.8)	28.6 (4.11-61.2)	0.096