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## RESEARCH ARTICLE

# Temporal order of RNase IIIb and loss-of-function mutations during development determines phenotype in *DICER1* syndrome: a unique variant of the two-hit tumor suppression model [v1; ref status: approved with reservations 1, <http://f1000r.es/5I9>]

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## Abstract

Pleuropulmonary blastoma (PPB) is the most frequent pediatric lung tumor and often the first indication of a pleiotropic cancer predisposition, *DICER1* syndrome, comprising a range of other individually rare, benign and malignant tumors of childhood and early adulthood. The genetics of *DICER1*-associated tumorigenesis are unusual in that tumors typically bear neomorphic missense mutations at one of five specific "hotspot" codons within the RNase IIIb domain of *DICER1*, combined with complete loss of function (LOF) in the other allele. We analyzed a cohort of 124 PPB children for predisposing *DICER1* mutations and sought correlations with clinical phenotypes. Over 70% have inherited or

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*de novo* germline LOF mutations, most of which truncate the *DICER1* open reading frame. We identified a minority of patients who have no germline mutation, but are instead mosaic for predisposing *DICER1* mutations. Mosaicism for RNase IIIb domain hotspot mutations defines a special category of *DICER1* syndrome patients, clinically distinguished from those with germline or mosaic LOF mutations by earlier onsets and numerous discrete foci of neoplastic disease involving multiple syndromic organ sites. A final category of patients lack predisposing germline or mosaic mutations and have disease limited to a single PPB tumor bearing tumor-specific RNase IIIb and LOF mutations. We propose that acquisition of a neomorphic RNase IIIb domain mutation is the rate limiting event in *DICER1*-associated tumorigenesis, and that distinct clinical phenotypes associated with mutational categories reflect the temporal order in which LOF and RNase IIIb domain mutations are acquired during development.

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## Introduction

Pleuropulmonary blastoma (PPB) is the most common primary lung cancer of childhood (OMIM #601200)<sup>1,2</sup>. Early PPB (type I) presents as lung cysts that are at risk for transformation into high grade sarcomas, which may have both cystic and solid components (PPB type II) or be entirely solid (PPB type III)<sup>2,3</sup>. Not all PPB type I cysts progress to sarcoma; those that do not are designated type Ir (regressed)<sup>1,3</sup>. The genetic and epigenetic events responsible for initiation of cyst formation and subsequent progression to sarcoma are just beginning to be understood<sup>3-6</sup>. PPB is pathognomonic for a childhood cancer syndrome that features a range of other benign and malignant neoplasms including ovarian Sertoli-Leydig cell tumor (SLCT), cystic nephroma (CN) and renal sarcoma or Wilms tumor, nodular hyperplasia and carcinoma of the thyroid gland, nasal chondromesenchymal hamartoma (NCMH), embryonal rhabdomyosarcoma (ERMS), pituitary blastoma and pineoblastoma<sup>2,4,7-30</sup>. We previously identified inherited loss of function (LOF) mutations in *DICER1* (OMIM #606241) as the major genetic factor in this syndrome<sup>4</sup>. *DICER1* syndrome thus became the first cancer predisposition associated with a systemic defect in microRNA (miRNA) processing.

The *DICER1* gene encodes an RNase III-family endonuclease that cleaves precursor microRNAs (pre-miRNA) into active miRNA<sup>31,32</sup>. Sequencing studies of syndromic tumors have revealed biallelic, compound mutations of *DICER1*<sup>6,11,15,21,26,28-30,33-35</sup>. Generally, one allele (often germline) bears a nonsense or frame-shift mutation predicted to cause full loss of function (LOF), and one allele bears a missense mutation in the *DICER1* RNase IIIb domain. Biallelic LOF mutations have not been identified in PPB, suggesting that retention of some miRNA processing function is usually required for tumor survival<sup>6,35</sup>. RNase IIIb missense mutations in *DICER1* syndrome tumors affect five “hotspot” codons that encode key amino acids in the metal-binding catalytic cleft of the nuclease domain: E1705, D1709, G1809, D1810 and E1813<sup>6,26,29,30,33-35</sup>. Amino acid substitutions at these positions cause neomorphic *DICER1* function in miRNA processing, such that cleavage of mature 5p miRNAs from the 5' end of pre-miRNA hairpin structures fails, while mature 3p miRNAs continue to be cleaved from the 3' end normally<sup>6,26,33,35,36</sup>. The high overall ratio of 5p to 3p mature miRNAs seen in normal tissues is essentially inverted in *DICER1* tumors, suggesting that uncleaved 5p miRNAs are rapidly degraded<sup>6</sup>. Depletion of 5p miRNAs alters expression of numerous downstream target mRNAs across the exome, including some critical for embryogenesis or tumor suppression<sup>33,36</sup>. The pleiotropic nature of *DICER1* syndromic disease likely reflects the diverse array of genes regulated by miRNAs during organ development and in differentiated tissues.

Clinical features of *DICER1* syndrome are highly variable with regard to age at first occurrence of neoplastic disease, the number of discrete foci of disease that develop over time, and the specific organ sites involved. As a step toward understanding the basis of clinical variability, we explored the spectrum of predisposing *DICER1* mutations in a large cohort of PPB/*DICER1* syndrome patients. Correlation of genotypes with clinical features revealed a distinctive phenotype of early onsets and extensive, multifocal disease in patients who are mosaic for hotspot missense mutations in the RNase IIIb domain. We propose that the extreme phenotypes

of this patient group are attributable to the order in which allelic *DICER1* mutations were acquired during development, *i.e.*, an RNase IIIb hotspot missense mutation acquired early in embryogenesis and subsequently unmasked by LOF mutations or loss of the second allele. Understanding how the interplay of RNase IIIb missense and LOF mutations influences the expression of syndromic neoplasias can aid diagnosis at early stages, when they are most curable, and improve genetic evaluation and counseling for families with *DICER1* syndrome.

## Subjects and methods

### Patients and specimens

PPB patients (n = 124) and family members were ascertained through the International PPB Registry (IPPBR). Inclusion into this study required a pathologic diagnosis of PPB verified by central review (LPD, DAH). All subjects gave written consent for molecular and family history studies, as approved by the Human Research Protection Offices at Washington University in St. Louis (HSC#04-1154), Children's Hospitals and Clinics of Minnesota (IRB#98107), and Children's National Medical Center (IRB#4603; Pro0315). For families with more than one affected member, only data from the initial proband is included. Medical history and biological samples were collected and prepared for analysis as previously described<sup>4,30</sup>. Tumor tissue was available for sequencing from a subset of patients. For two of these cases, DNA was isolated from unstained tissue on glass slides using the Pinpoint Slide DNA Isolation System (Zymo, Irvine, CA).

### Definition of “disease foci”

Clinical data was abstracted from medical records and imaging studies. All children had pathologic confirmation of PPB. In addition, lung cysts, kidney cysts, CN, NCMH, SLCT, ERMS, thyroid cancer or nodules, pineoblastoma and/or pituitary blastoma were defined as evidence of syndromic disease. Lung cysts that were distinctly separate (anatomically separate in same lobe or in different lobes) were counted individually.

### Mutation testing

Initial sequencing of blood and saliva DNA samples was by standard Sanger methods described previously<sup>4</sup> or by a commercial laboratory (Ambry Genetics, Aliso Viejo, CA). Low-frequency variants were detected and quantified by targeted next-generation sequencing (NGS) using a custom multiplex PCR panel for *DICER1* coding regions (Ion Torrent Ampliseq, Life Technologies, Grand Island, NY, USA) (Table S1)<sup>30</sup>. NGS was performed on an Ion Torrent 318 v2 chip (ION PGM Sequencing 200 kit v2, Life Technologies) with an average of 6 samples per chip, to achieve an average depth of coverage of 3000 filtered reads. Signal processing, mapping and quality control were performed with Torrent Suite software v.4.0.2 (Life Technologies). Variant calls were made using the Torrent Variant Caller Plugin v.4.0, with somatic low stringency mutation workflow and default settings. BAM files of raw reads were reviewed using Integrative Genomics Viewer v2.3<sup>37,38</sup>.

### Annotation of sequence variants and the spectrum of possible mutations

*DICER1* sequence variants were annotated with Alamut Batch software (Interactive Biosoftware, Rouen, France), with reference

to *DICER1* transcript record NM\_177438.2. Nonsense, frameshift and canonical splice-site mutations were considered loss of function (LOF). Missense variants affecting codons 1705, 1709, 1809, 1810 and 1813 in the RNase IIIb domain were classified as “hotspot” mutations. For variants assayed by NGS, allele frequencies were calculated from filtered read counts. The SIFT algorithm was used to assess potential significance of novel missense mutations<sup>39–41</sup>. All variants identified were deposited into ClinVar (accession numbers SCV000195560-SCV000195643). The numbers of possible single-nucleotide changes that can produce amino acid substitutions at the five hotspot codons or nonsense mutations anywhere in the *DICER1* open reading frame, or disrupt canonical splice sites, were compiled from *DICER1* transcript record NM\_177438.2 and genomic record NG\_016311.1.

### NanoString genomic copy number assay

Molecular probes for NanoString Copy Number Assay at the *DICER1* locus were developed in collaboration with NanoString Technologies, Inc., Seattle, WA (Table S2). Genomic DNA was fragmented and hybridized using the nCounter Prep Station, and hybridization signals quantified using the nCounter Digital Analyzer, according to NanoString’s recommendations. Preliminary analysis and quality control of the data were performed using nSolver Analysis Software version 1.1 (NanoString) with default copy number variation (CNV) analysis settings. CNVs were confirmed with high-density CNV array hybridization in a commercial laboratory (Prevention Genetics, Marshfield, WI).

### Statistical analyses

The number of disease foci per patient and the age at *DICER1* syndrome diagnosis were compared between mutation categories using nonparametric tests, due to the skewness of both clinical features and to the unbalanced sample sizes. Kruskal-Wallis tests were used to compare medians among the four mutation categories. Where a significant overall association was found, pair-wise post-hoc Wilcoxon rank sum tests were used to compare medians, and resulting p-values adjusted for multiple comparisons using the Sidak method. A p-value of 0.05 was considered statically significant and all analyses were performed using Stata V13 (College Station, TX).

## Results

### Most predisposing *DICER1* mutations are inherited loss of function (LOF) mutations

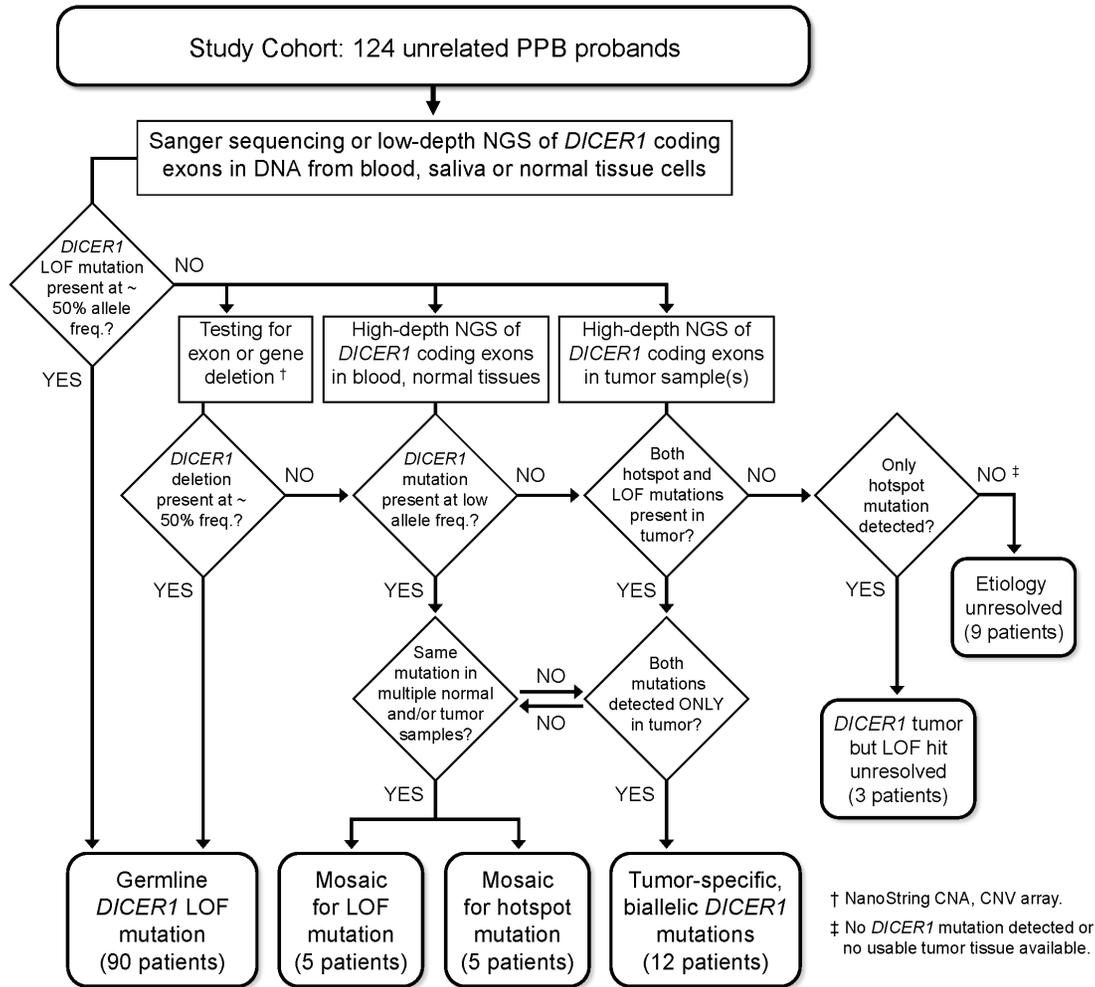
Our overall approach to detecting and categorizing predisposing *DICER1* mutations in PPB children is shown schematically in Figure 1. We identified germline, heterozygous *DICER1* mutations in 90 of the 124 probands in our cohort (72.6%; Table 1, Table S3). Nearly all (89) were detected by Sanger sequencing of exonic PCR amplicons. For one child in whom no mutation was detected by Sanger sequencing, blood DNA was probed by NanoString hybridization, which indicated deletion of one copy of exon 24. High-density CNV array hybridization was used to confirm a heterozygous deletion of ~ 1.1 kb, comprising all of exon 24 and parts of the flanking introns (c.5096-498\_5364+356del). Paternal DNA was positive for the deletion, which was anticipated as this child has an uncle with CN. Only one previous instance of a large, intragenic deletion as a germline *DICER1* mutation has

been reported, which suggests such mutations are very rare<sup>42</sup>. The actual prevalence of large deletions is difficult to estimate because they are not readily detected by the targeted sequencing strategies applied for mutation screening in this study and most others.

The spectrum of germline mutations is dominated by truncating, LOF mutations (Figure 2). These are mainly single-nucleotide substitutions that produce new stop codons (33 cases, 37%) and small insertions or deletions (indels) within exons that shift reading frame (44 cases, 49%). Seven mutations of consensus splice sites occur in our cohort; of which six are predicted to cause exon skipping during transcript splicing with resulting frameshift. The remaining splice site mutation, c.1752+1delG, is at the 5’ end of intron 10. Skipping of exon 10 would cause in-frame deletion of 81 amino acids near the end of the helicase domain. In all, 84 of 90 germline *DICER1* mutations discovered in patients (93%) truncate the open reading frame before the end of the critical RNase IIIb domain, and are thus predicted to result in complete loss of DICER1 protein function even if the message escapes nonsense-mediated decay. Six non-truncating germline mutations were identified, including the intron 10 splice site mutation described above and five non-hotspot missense changes: I582T, L1583R and G1708E (each seen once) and D1822V (identified in two patients) (Table S4). The I582T substitution is at the distal end of the helicase domain (Figure 2), the role of which is unclear. L1583R is within the RNase IIIa domain and segregates with disease in a family<sup>4</sup>. The G1708E and D1822V mutations both fall within the RNase IIIb domain, near the metal-binding catalytic site. These two missense mutations are predicted to compromise protein function by the SIFT algorithm but their precise functional significance in *DICER1* is unknown<sup>39–41</sup>.

DNA was available from both parents for 77 children with germline mutations, and Sanger sequencing of parental DNA was sufficient to confirm 67 of the mutations (87%) as inherited. Mutations in the ten patients whose parents had no *DICER1* mutation detected by Sanger sequencing were provisionally considered *de novo*. To confirm this, targeted next generation sequencing (NGS) was performed in eight of the ten trios, yielding mutant allele frequencies between 42.0% and 57.1% in the probands but no conclusive evidence of the variants in parental blood. There were no statistically significant differences between *de-novo* and inherited germline LOF patients with respect to age at onset, numbers of disease foci or survival.

Penetrance of familial *DICER1* LOF mutations was far from complete. Of the 67 families in this cohort with segregating LOF mutations, 29 include parents or siblings who are confirmed as mutation carriers but have no history of syndromic disease (Table S5). True penetrance is difficult to estimate because we have limited knowledge of how many germline *DICER1* mutation carriers are phenotypically normal, as only a subset with overtly affected family members have been ascertained. Moreover, subclinical disease is common. Preliminary data from an ongoing NCI-sponsored *DICER1* family history study indicate that ~ 87% of otherwise asymptomatic individuals with confirmed *DICER1* mutations have thyroid nodules detectable by ultrasound and ~ 43% have lung cysts detectable by CT scan (D.R. Stewart and L. Doros, unpublished).



**Figure 1. Study design – Detection and categorization of *DICER1* mutations in PPB probands.** A cohort of 124 children diagnosed with pleuropulmonary blastoma (PPB) was screened for predisposing *DICER1* mutations by targeted Sanger sequencing and/or low-depth, next-generation sequencing (NGS) of DNA amplified from peripheral blood cells, saliva (buccal cells) or non-neoplastic surgical specimens. Sequenced PCR amplicons covered the 26 coding exons of the *DICER1* open reading frame and flanking splice signals. *DICER1* coding sequence or splice site mutations detected at approximately heterozygous frequency in blood or normal tissue cells were categorized as germline mutations. For patients in whom screening revealed no germline mutation, blood and/or normal tissues were analyzed for the presence of intragenic deletions or larger genomic alterations using NanoString copy number assay and CNV array, and for coding or splice site mutations present at low allele frequencies using high-depth NGS on the Ion Torrent platform. Wherever possible, matched tumor specimens were also sequenced on the Ion Torrent platform. Low-frequency *DICER1* mutations detected in multiple normal and/or tumor samples, or in primary tumors of multiple organs, were categorized as mosaic mutations. Mosaic mutations were further categorized as loss-of-function (LOF) or RNase IIIb domain hotspot missense mutations. Patients for whom both LOF and hotspot mutations were identified in a single tumor, but not found in blood or normal tissue samples, were categorized as having tumor-specific, biallelic *DICER1* mutations.

Among children with germline LOF mutations, age at first diagnosis of PPB or other syndromic disease was typically one to five years (70 of 90 patients), but this ranged from diagnosis within days of birth to as late as eighteen years. The most frequent syndromic condition after PPB was cystic nephroma, followed by thyroid disease (nodular hyperplasia or carcinoma), nasal chondromesenchymal hamartomas and embryonal rhabdomyosarcomas (Table 1, Table S5). The number of discrete disease foci per patient ranged as high as five or six (in two patients), but the majority of children in this group had experienced no more than two at the time of their most recent exam, and nearly half had only a single PPB tumor. None of the six

patients with non-truncating germline mutations had unusual clinical features and as a group they were not distinguishable from patients with truncating mutations. Table S6 provides data on somatic hotspot mutations identified in all available tumors of PPB children.

#### Approximately 10% of predisposing *DICER1* mutations are mosaic rather than germline

We and others have previously described biallelic *DICER1* mutations in tumors of children who apparently have no germline mutation, inherited or *de novo*<sup>6,14,35</sup>. Because PPB children are typically so young when affected, we hypothesized that at least some cases of this

**Table 1. Clinical and Pathologic Features by Predisposing *DICER1* Mutation Category.**

	Germline LOF mutations	Mosaic mutations		Tumor-specific mutations
		Loss of function	RNase IIIb hotspot	
Number of patients	90	5	5	12
Sex distribution				
Male	44	3	2	10
Female	46	2	3	2
Age at first diagnosis, months <sup>a</sup>				
Median (range)	35 (0–227)	25 (12–46)	11 (0–15)	33 (24–139)
Mean (standard deviation)	36 (31)	27 (12)	9 (6)	42 (31)
P-value, vs. germline group <sup>d</sup>	–	0.97	0.048	0.99
Disease foci distribution				
Lung - cysts, PPB	90	5	5	12
Kidney - cysts, cystic nephroma	12	0	4	0
Kidney - Wilms tumor	1	0	0	0
Thyroid - nodular hyperplasia	4	0	2	0
Thyroid - cancer	4	0	1	0
Nasal cavity - NCMH	5	0	2	0
Ovary - Sertoli-Leydig cell tumor	3	0	2	0
Uterine cervix - ERMS	4	0	0	0
Urinary bladder - ERMS	2	0	0	0
Pineoblastoma	1	0	1	0
Ciliary body medulloepithelioma	1	0	1	0
Small intestine - juvenile polyps	0	0	4	0
PPB type distribution				
Type IR	9	0	5 <sup>c</sup>	0
Type I	25	2	0	1
Type II	31	1	1 <sup>c</sup>	6
Type III	25	2	0	5
Number of disease foci per patient <sup>b</sup>				
Median (range)	2 (1–6)	2 (1–2)	14 (10–24)	1 (1–1)
Mean (standard deviation)	1.8 (1.0)	1.6 (0.5)	16.8 (6.3)	1 (0.0)
P-value, vs. germline group <sup>d</sup>	–	0.99	0.0006	0.0072
Survival, number of patients (months)				
Alive (median age at present)	80 (100)	3 (46)	5 (96)	10 (85)
Deceased (median age at death)	10 (60.5)	2 (64.5)	0	2 (57)

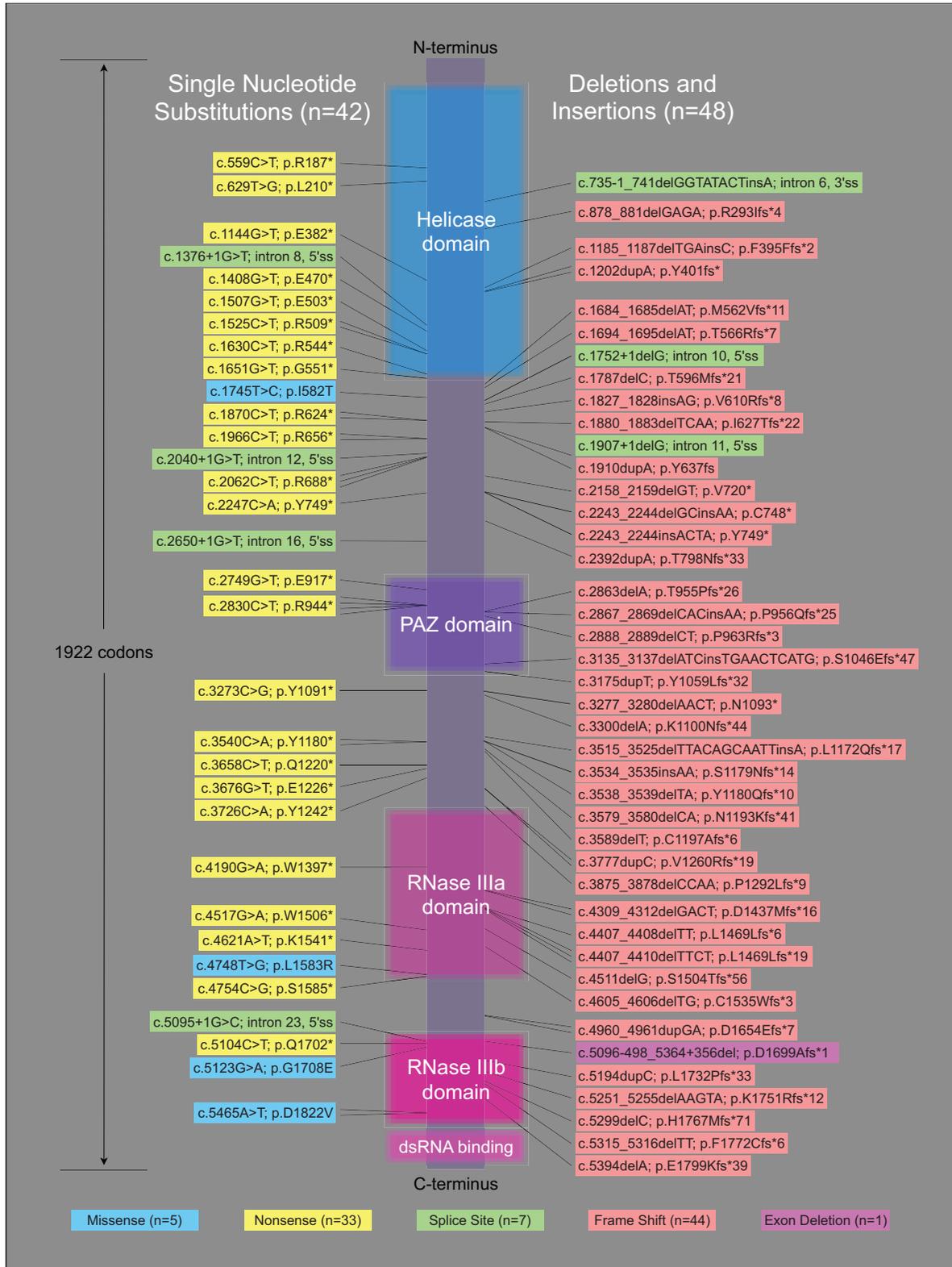
Abbreviations: PPB pleuropulmonary blastoma; NCMH nasal chondromesenchymal hamartoma; ERMS embryonal rhabdomyosarcoma.

a. Age at first clinical presentation with PPB or other *DICER1* syndrome pathology.

b. Total number of discrete disease foci, as defined in Subjects and Methods.

c. One patient with both type IR and type II PPB.

d. Medians compared using a Kruskal-Wallis test; *post-hoc* pair-wise tests adjusted for multiple comparisons.



**Figure 2. The spectrum of predisposing loss-of-function mutations in PPB/*DICER1* syndrome.** A linear schematic of the *DICER1* open reading frame is shown with annotated functional domains represented to scale. Sequence changes identified as inherited or *de novo* germline mutations in 90 PPB/*DICER1* syndrome patients are indicated by position along the coding sequence. Mutations linked to the schematic by two, three or four fine lines are those discovered in a corresponding number of individuals from unique families.

kind reflect mosaicism, *i.e.*, a mutation present in some but not all cells of the body, because it occurred during post-zygotic embryonic development rather than being present in the zygote (as a germline mutation would be). To explore this possibility, we performed targeted, high-depth NGS of *DICER1* coding exons in DNA from blood and/or other normal tissues of children who had tested negative for germline mutation by Sanger sequencing, and in matched samples of tumor tissue where available. We categorized a *DICER1* mutation detected by NGS as mosaic when the following criteria were met: *i.* The mutation was evidently not a constitutional, germline allele because it was present at sub-heterozygous frequency (arbitrarily taken as below 35% of reads) in peripheral blood and/or other normal tissue samples. *ii.* The mutation was evidently not specific to a tumor, because the same mutant allele was detected in one or more normal, non-neoplastic tissue samples, OR, the same mutant allele was detected in multiple primary tumors arising in different organs (Figure 1). We identified ten children with predisposing mosaicism for either LOF or RNase IIIb hotspot mutations (Table 1).

Mosaic LOF mutations were detected in five children, at frequencies that ranged from 1.1% to 17.2% of allelic reads in DNA from blood, saliva or normal fibroblasts (Table S7). For three of these children, archival PPB tumor tissue was available, and in each the LOF mutation was present, as was an RNase IIIb domain hotspot mutation. Two of the five children with mosaic LOF mutations had a single focus of disease in a lung. The other three children each had two foci of disease, also restricted to the lungs. It might be anticipated that children bearing mosaic LOF mutations tend to have fewer disease foci than those with germline LOF mutations because the number of cells at risk for second hits is generally lower. No statistically significant difference of this kind can be discerned from the five mosaic LOF children in our cohort, but notably, none have developed syndromic tumors other than PPB. As this was not a population study, we cannot estimate how many persons with mosaic LOF mutations are asymptomatic but, by analogy to the low penetrance of familial LOF mutations, it could be a large proportion.

Five children harbored mosaic RNase IIIb domain hotspot missense mutations, detected in multiple primary neoplasms and/or non-neoplastic tissues (Table 2). None had family members with features of *DICER1* syndrome, and the RNase IIIb hotspot mutations found in probands were not detected in parental blood, consistent with a postzygotic origin. NGS of tumor tissues from these children identified somatic LOF mutations or allele loss in some but not all specimens, with the caveat that allele loss can be difficult to detect in specimens with low tumor purity (*i.e.*, PPB Type I, CN and NCMH). For one mosaic hotspot patient, specimens of a thyroid carcinoma and two separate ovarian Sertoli-Leydig cell tumors (SLCT) were available for NGS. The thyroid carcinoma and one SLCT had lost the second *DICER1* allele, but the other SLCT had instead sustained a frameshift mutation (Table 2). This is consistent with underlying mosaicism for the RNase IIIb hotspot mutation and subsequent acquisition of independent LOF mutations in each tumor site.

#### Mosaic RNase IIIb hotspot mutations are associated with early-onset, multifocal disease

The five children with mosaic RNase IIIb domain hotspot mutations shared unusual clinical features. All were diagnosed with *DICER1*

syndrome early; within 15 months of birth. All presented with multiple cysts of the lungs and/or kidneys, which were accompanied or followed in all cases by multiple *DICER1* syndromic tumors (Figure 3). Four of the five had CN as well as PPB. Other tumors included SLCT, thyroid nodular hyperplasia or carcinoma, NCMH, ciliary body medulloepithelioma and pineoblastoma. In addition, four of the five children experienced episodes of intestinal intussusception, which in three cases were associated with polyps of the small intestine discovered upon surgical intervention. Total numbers of discrete disease foci per patient were extraordinarily high, ranging from a minimum of 10 to as many as 24. Despite the small number of patients in this group, statistical analysis confirms clinical impressions that they are distinct from those with predisposing LOF mutations. Mean age at first *DICER1* syndrome diagnosis was significantly earlier, and both mean and median numbers of disease foci are significantly greater in children with mosaic RNase IIIb mutations (Table 1). The association with juvenile-type intestinal polyps and intussusception is a novel feature of children with mosaic RNase IIIb hotspot mutations, not previously seen in children with other categories of *DICER1* mutation.

Four of the five children with mosaic *DICER1* hotspot mutations presented with cystic PPB (type I/IR) rather than sarcomatous disease (type II or type III) and all five have survived to date. However, this does not indicate a benign clinical course. Though all five hotspot mosaic children are alive, their clinical experiences have been complicated and arduous (Figure 3). Each has undergone multiple major surgeries and chemotherapies with concomitant morbidities. One child is alive with recurrent disease at last follow-up.

#### Tumor-specific, biallelic *DICER1* mutations account for about 10% of PPB cases

In twelve children, we identified biallelic *DICER1* mutations present at high allele frequencies in a PPB tumor, but not detectable in blood even with the benefit of high-depth NGS (Table S8). Tumors from these children had an RNase IIIb hotspot missense mutation and either a nonsense LOF mutation ( $n = 5$ ) or allele loss ( $n = 7$ ). All twelve children presented with a single PPB tumor and none developed additional foci of disease in the lungs or other organs over the course of subsequent follow-up. This is consistent with occurrence of both an RNase IIIb hotspot mutation and a LOF mutation or allele loss within a single, highly localized clone of somatic cells which then gave rise to the tumor. Absence of additional disease foci is a predictable outcome if both *DICER1* mutations are restricted to the initial site of tumorigenesis. However, the absence of additional disease foci among children in this category did not indicate less dangerous disease. Of the 12 patients, 11 had advanced PPB (type II or III), and two succumbed (Table 1).

#### Currently unresolved cases

Twelve PPB probands in our cohort are negative for predisposing *DICER1* mutations detectable in blood DNA by Sanger sequencing or NGS of coding exons. Ten of these children had a single focus of disease, and thus may be sporadic cases involving tumor-specific, biallelic *DICER1* mutations, but tumor tissue is either not available or not of sufficient quality for confirmation. Two had multifocal disease, possibly reflecting *DICER1* mosaicism that is not well represented in the blood lineage (below our limits of detection).

**Table 2. Sequence Results from Children with DICER1 Mosaic RNase IIIb Mutations.**

Study ID	Tissue source	Tumor purity <sup>a</sup>	RNase IIIb domain hotspot mutation	Hotspot allele freq. <sup>b</sup> (variant/total reads)	Loss of function mutation	LOF allele freq. <sup>b</sup> (variant/total reads)
101	Blood	-	c.5126A>G; p.D1709G	Sanger <sup>c</sup> (NR)	ND	-
	Normal lymph node	-	"	15.2% (10/66)	ND	-
102	Blood	-	c.5125G>A; p.D1709N	4.61% (22/477)	ND	-
	Brain, PPB metastasis	30%	"	51.0% (213/418)	Allele loss	-
103	Blood	-	c.5125G>A; p.D1709N	0.28% (18/6413)	ND	-
	Kidney, CN	40%	"	14.9% (174/1172)	c.1129G>A; p.V377I	3.1% (5/159)
	Lung, PPB Type IR	20%	"	16.2% (30/185)	c.1200G>A; p.W400*	4.1% (11/141)
	Small intestine, polyp	25%	"	17.5% (65/371)	c.96G>A; p.W32*	3% (13/431)
104	Blood	-	c.5428G>T; p.D1810Y	0.21% (13/6217)	ND	-
	Normal fallopian tube	-	"	7.19% (141/1961)	ND	-
	Lung, PPB Type IR	20%	"	27.8% (193/694)	ND	-
	Kidney, CN	20%	"	29.2% (64/219)	c.1711delT; p.S571Vfs*16	21.8% (73/192)
	Ovary, SLCT	95%	"	92.4% (1837/1988)	Allele loss	-
105	Blood	-	c.5437G>C; p.E1813Q	0.04% (1/2450) <sup>d</sup>	ND	-
	Nasal cavity, NCMH	20%	"	29.3% (579/1977)	ND	-
	Thyroid, follicular Ca	60%	"	66.6% (289/434)	Allele loss	-
	Ovary (right), SLCT	75%	"	76.8% (750/976)	Allele loss	-
	Ovary (left), SLCT	25%	"	31.8% (624/1962)	c.4626delC; p.Q1542Hfs*18	21.7% (430/1984)
	Blood	-	c.5138A>T; p.D1713V	21% (NR)	ND	-
Klein <sup>44</sup> case 1	Normal kidney	-	"	35% (NR)	ND	-
	Wilms tumor	NR	"	37% (NR)	c.1304C>T; p.P453L	variable
Klein <sup>44</sup> case 2	Blood	-	c.5125G>T; p.D1709Y	28% (NR)	ND	-
	Normal kidney	-	"	35% (NR)	ND	-
De Kock <sup>11</sup> case 12	Wilms tumor	NR	"	47% (NR)	ND	-
	Blood	-	c.5125G>C; p.D1709H	Sanger <sup>c</sup> (NR)	ND	-
	Pituitary blastoma	NR	"	Sanger <sup>c</sup> (NR)	Allele loss	-

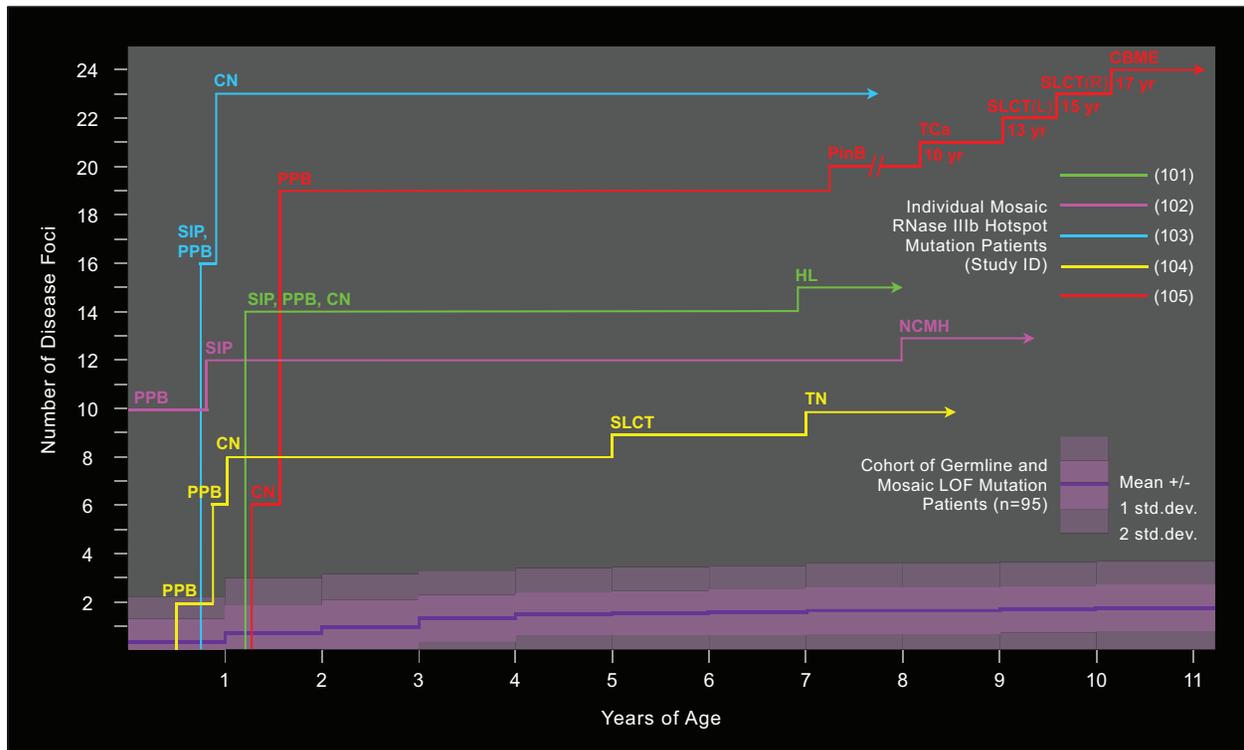
Abbreviations: Ca carcinoma; CN cystic nephroma; LOF loss of function; NCMH nasal chondromesenchymal hamartoma; ND none detected; NR not reported; PPB pleuropulmonary blastoma; SLCT Seroli-Leydig cell tumor.

a. Percent tumor cells in specimen, estimated visually by microscopy in tumor sections.

b. Allele frequency estimates were derived from NGS read counts in this study. In the two cases reported by Klein *et al*, allele frequencies were determined by pyrosequencing assays.

c. Hotspot allele detected by Sanger sequencing only; no NGS performed.

d. Variant allele frequency below estimated error rate for base substitutions (0.07%) with Ion Torrent using 200 bp sequencing kit<sup>50</sup>.



**Figure 3. Numbers and types of disease foci in *DICER1* syndrome patients with mosaic RNase IIIb domain hotspot mutations.** For each of the five mosaic hotspot children identified in this study, an individual timeline indicates numbers of discrete foci of neoplastic disease and their histopathological types, graphed with respect to patient age at diagnosis. Across the lower portion of the chart, a single aggregate timeline (dark violet) represents the mean number of disease foci for all PPB/*DICER1* syndrome patients with predisposing loss of function (LOF) mutations identified in this study, graphed with respect to patient age at diagnosis. The shaded areas (in lighter violet) surrounding the timeline for LOF mutation patients indicates one and two standard deviations above and below the mean. The range of foci number among all LOF mutation patients was 0 to 6 in all years of age represented (not shown). Abbreviations: CN cystic nephroma; CBME ciliary body medulloepithelioma (eye); NCMH nasal chondromesenchymal hamartoma; PPB pleuropulmonary blastoma; PinB pineoblastoma; SIP small intestinal polyp(s); SLCT Sertoli-Leydig cell tumor (ovary); TCa thyroid carcinoma; TN thyroid nodule(s).

Clinical features of the twelve unresolved cases and the status of further analyses pending or completed, including tumor sequencing, NanoString copy number assay and germline sequencing for additional candidate loci, are summarized in Table S9.

#### Dataset 1. Patient information dataset

<http://dx.doi.org/10.5256/f1000research.6746.d80768>

Excel file with deidentified raw data for patient ages at diagnosis and numbers of disease foci, and statistical analyses<sup>51</sup>.

## Discussion

### Genotype-phenotype correlation of predisposing mutations in PPB-*DICER1* syndrome

All germline *DICER1* truncating mutations are predicted to be essentially equivalent in their effect: complete loss of function in miRNA processing. This prediction is based partly on nonsense-mediated decay, but also reflects the functional domain structure

of the *DICER1* protein. All truncating mutations so far identified in PPB/*DICER1* syndrome patients interrupt the open reading frame before the end of the critical RNase IIIb domain (Figure 1, Table S3). Neomorphic RNase IIIb domain function (skewed 5p/3p miRNA production) is a recurring feature of *DICER1* tumors, and it is plausible that loss of all wildtype RNase IIIb function is required for it to become tumorigenic. Presumed equivalence of all truncating mutations is consistent with clinical findings: no correlations are apparent between locations of germline truncating mutations within the *DICER1* gene and clinical features such as age of onset, number of disease foci, specific tissue sites involved or survival. Non-truncating germline mutations are too rare for correlations with clinical presentations or outcomes to be ascertained.

The natural history of PPB indicates a multistep genetic pathogenesis, and so it is not surprising that in some cases where no germline *DICER1* mutation can be detected, one of the two “hits” required for tumorigenesis was acquired during embryogenesis in the form of somatic mosaicism. Mosaic mutations may ultimately prove

important in the pathogenesis of many other sporadic childhood neoplasias, as demonstrated recently for retinoblastoma (*RBI*)<sup>43</sup>.

Mosaicism for RNase IIIb domain hotspot missense mutations defines a special category of *DICER1* syndrome patients that are phenotypically distinct from those who bear germline or mosaic LOF mutations. RNase IIIb hotspot mutations have not been encountered as inherited alleles in this study or others, which suggests they are inviable<sup>4,8,10,11,14–16,21,26–30,34,44</sup>. In addition to the five mosaic RNase IIIb hotspot patients in our cohort, three apparently similar cases have been reported (Table 2). Klein *et al.* described two infants with bilateral Wilms tumor and multiple cysts of the kidneys and lungs<sup>44</sup>. Each child was found to be mosaic for a *DICER1* RNase IIIb domain missense mutation, although in one case the mutation was at D1713; also an acidic residue within the RNase IIIb catalytic cleft, but not an established hotspot. De Kock *et al.* described an infant with pituitary blastoma and bilateral cysts of the kidneys and lungs in whom a *de-novo* hotspot mutation was detected at high allele frequency in blood as well as tumor<sup>41</sup>.

Clinically, mosaic hotspot patients are distinguished by two features: *i.*) consistently early presentations of neoplastic disease, often by one year of age, and *ii.*) numerous discrete foci of disease developed concurrently or successively, usually involving more than one syndromic tissue/organ site (Figure 3, Table 2). The two features are related and can be interpreted within the conceptual framework of the emerging model for *DICER1* syndrome pathogenesis, which provides important insight as to how tumor suppression by *DICER1* fails<sup>6,26,33,35,36</sup>. *DICER1* is not a classical tumor suppressor gene for which “two hits” – loss of function in both alleles – are required to allow tumorigenesis. Neither is it haploinsufficient in the usual sense, *i.e.*, that cells with only one expressed allele make wild-type protein, but not in sufficient quantity to fulfill its function. Rather, it is neomorphic function by mutant *DICER1* protein, with substitutions of key amino acids in the RNase IIIb domain that causes tumor suppression to falter when it is not masked by expression of wild-type *DICER1* protein. Unmasking of an RNase IIIb hotspot mutation may arise through any form of LOF mutation in the wild type allele, including allele loss. The two mutational events, RNase IIIb missense and LOF, may occur in either order and both are generally required to foment the initiation of tumorigenesis. However, as outlined below, RNase IIIb hotspot mutation is a low-probability event and LOF mutation is, relatively, a very high-probability event. The projected consequence of these lopsided probabilities is that occurrence of an RNase IIIb hotspot mutation becomes the rate-limiting step in onset of pathogenesis.

#### Rationale for the distinctive phenotype of mosaicism for RNase IIIb hotspot mutations

The RNase IIIb domain hotspots in *DICER1* are a diminutive mutational target; five codons within an open reading frame of 1922 codons (0.26%). Moreover, molecular mechanisms by which RNase IIIb hotspot missense mutations can arise are restricted to errors of DNA replication and/or DNA repair that produce nucleotide substitution without disturbing the open reading frame. There are 36 possible single-nucleotide changes that can produce amino acid substitutions at these five codons, and only a subset of them has

ever been identified in *DICER1* syndrome tumors. The spectrum of pathogenic RNase IIIb hotspot mutations is thus very narrow. In contrast, the spectrum of possible LOF mutations is broad and mechanistically diverse. Of the 1922 codons in the *DICER1* open reading frame, 675 can be converted to a stop codon by a single nucleotide change. A subset can be converted in more than one way, giving a total of 736 possible single nucleotide changes that result in a nonsense mutation. Among the other 16,562 possible single nucleotide changes in the *DICER1* open reading frame, presumably some would be missense mutations that disrupt *DICER1* protein function. The five non-hotspot missense mutations we detected as predisposing alleles in PPB probands are likely examples (Figure 2). The individual nucleotides of the *DICER1* open reading frame present 5766 point locations at which insertion or deletion of one or a few nucleotides can shift reading frame. An additional 104 bases comprise canonical splice sites of the 26 *DICER1* introns, where small sequence changes may result in exon skipping, with or without frameshift. The possibilities for LOF mutations also include larger intra-locus deletions and allele loss through copy-neutral loss of heterozygosity, segmental deletions or complete loss of chromosome 14. Absolute frequencies of these diverse *DICER1* mutational mechanisms in a particular cell lineage cannot be modeled precisely, but it becomes clear that the aggregate likelihood of all possible LOF mutations is vastly greater than the likelihood of a neomorphic mutation in one of the five hotspot codons.

It follows that in a developing embryo or child with a germline (or mosaic) *DICER1* LOF mutation, “second hits” occurring in a somatic cell will almost always be another LOF mutation, usually resulting in cell death or limited proliferation at most. Rarely, a second hit will be an RNase IIIb hotspot missense mutation, which allows for continuing cell viability and growth, though at the cost of skewed miRNA processing that may ultimately promote tumorigenesis in the surviving clones of cells. However, the low likelihood of incurring an RNase IIIb hotspot missense mutation in somatic cells means that months, years or a lifetime may elapse before one occurs. Further, the developmental context in which a second, hotspot mutation occurs may be important. There are apparently windows of risk for transformation, perhaps coinciding with certain periods of organ/tissue development when an “onco-fetal” gene program is normally active and subject to miRNA modulation, *i.e.*, lung, kidney and brain in the embryo; uterine cervix and ovaries in pubertal girls<sup>1–3,8,9,45</sup>. A low probability of RNase IIIb hotspot mutations as second hits during windows of risk may underlie the low penetrance and variable expression of familial LOF mutations in *DICER1* syndrome.

For a developing child with a mosaic RNase IIIb hotspot mutation, the prospects are radically different. Somatic cells that bear the RNase IIIb hotspot mutation, masked by a wild type allele, will be viable and non-tumorigenic unless and until they sustain a second hit. However, cells with a preexisting RNase IIIb hotspot mutation are at high aggregate risk of acquiring a subsequent LOF mutation, because it can take any of the myriad forms outlined above. The probability of a secondary LOF mutation occurring during expansion of any given cell lineage over the course of prenatal and postnatal development is relatively high, and independent LOF

mutations in multiple lineages may occur. If sufficient fractions of cells in critical lineages are affected, disturbed regulation of developmental gene expression programs arising from defective miRNA processing may be lethal *in utero*. For surviving embryos, onsets of tumorigenesis will tend to be early and, depending on embryonic distribution of the RNase IIIb hotspot mutation, foci of tumorigenesis may arise in one or more organ sites characteristic of *DICER1* syndrome. Additionally, we hypothesize that in mosaic hotspot children, wider tissue/organ distribution of aberrant miRNA processing during development may produce syndromic features not seen in children with predisposing LOF mutations, such as juvenile-type small intestinal polyps, or the generalized somatic overgrowth noted by Klein *et al.*<sup>44</sup>.

### Implications for mutation testing, clinical evaluation, and genetic counseling

Recent publications have outlined general recommendations for mutation detection and clinical evaluation for syndromic disease in patients with suspected *DICER1* syndrome and family members<sup>9,46–48</sup>. Here we add considerations of risk for multifocal disease and reproductive transmission of *DICER1* mutations based on mutation category.

Most predisposing *DICER1* mutations are germline and detectable by targeted Sanger sequencing from blood. Initial testing should include parents, to distinguish inherited from *de novo* mutations. Sanger sequencing will usually suffice to detect a parental mutation that is also constitutional, but may fail to detect mosaicism. There is growing appreciation that apparently *de novo* mutations in children with genetic disease sometimes stem from mosaicism in a parent, which can often be detected by more sensitive methods<sup>49</sup>. For eight patients with apparently *de novo* mutations in this cohort, we found no evidence of mosaicism in parents by resequencing with high-depth NGS, but this limited finding does not exclude the possibility of parental mosaicism for families evaluated in the future.

For those patients who have a tumor with confirmed *DICER1* mutation(s), but test negative for germline mutation by Sanger sequencing from blood, it will be important to distinguish as rigorously as possible between tumor-specific, biallelic mutations and the presence of underlying mosaicism. Mutations confined to the tumor will confer no risk for new foci of primary disease in the proband, and family members including potential offspring will be unaffected. Mosaicism, whether for an LOF mutation or an RNase IIIb hotspot mutation, will confer some degree of risk for additional syndromic neoplasias. It may be impossible to unequivocally rule out mosaicism, but techniques such as targeted resequencing by high depth NGS in multiple tissues can greatly improve diagnostic confidence, particularly with respect to RNase IIIb hotspot mutations. For patients who have more than one focus of disease but no germline or mosaic LOF mutation identifiable by targeted NGS of exons, testing for intragenic deletions or larger genomic alterations is recommended.

Patients carrying mosaic RNase IIIb hotspot mutations are predicted, on the basis of both clinical observations and mechanistic rationale, to have extraordinarily high risk as a group for developing

multiple disease foci; approaching 100%. It will not be possible to predict individual risk for multifocal disease by allele frequency in blood, as this will not reveal the extent to which other somatic lineages harbor the mutation. Mosaic RNase IIIb hotspot patients will benefit from the most proactive program of family education and surveillance. The International PPB Registry recommends that potential benefits of renal ultrasound and surveillance chest CT be discussed with the family<sup>48</sup>. The frequency of follow-up chest CTs and chest radiographs should be determined individually, based on patient age, medical history and previous imaging results. Continuing evaluations should include a yearly complete review of systems by a clinician familiar with *DICER1* syndrome; yearly screening for ovarian SLCT with review of systems for endocrine dysfunction and pelvic ultrasound for females from early childhood through adulthood; yearly ophthalmologic examination and yearly thyroid examination by palpation and/or ultrasound. Pituitary blastoma and pineoblastoma are rare even in *DICER1* syndrome and typically limited to the infant and young child. There is no consensus at this time on screening for intracranial neoplasms.

As prospective parents, patients who are mosaic for a *DICER1* mutation face a theoretical risk for transmitting the mutation of up to 50%, depending upon whether and at what frequency it is present in germ cells. For carriers of a mosaic LOF mutation, the consequences of transmission will be similar to those of a germline LOF mutation carrier. For carriers of a mosaic RNase IIIb mutation, it is uncertain whether transmission could result in a live birth. The absence of RNase IIIb hotspot mutations as inherited alleles in all published studies implies they preclude development to term, but this remains speculative. The mosaic hotspot mutation identified in patient 101 of this cohort was discernable in blood by Sanger sequencing and present at 15% of NGS read counts in normal lymph node tissue (Table 2). Similarly in the two Wilms tumor patients reported by Klein *et al.* and one pituitary blastoma patient described by De Kock *et al.*, *de-novo* hotspot mutations were readily detected in blood by Sanger sequencing<sup>44,11</sup>. Whether the latter case is truly germline or mosaic with high representation in the blood lineage was unclear. Nonetheless, it is clear from these examples that human embryogenesis can tolerate a *DICER1* hotspot mutation at high allele frequency in at least some cell lineages. It thus seems possible, though unlikely, that an inherited RNase IIIb hotspot mutation could be viable.

### Data availability

The ClinVar accession number(s) for the variant sequences reported in this paper are SCV000195560-SCV000195643.

*F1000Research*: Dataset 1. Patient information dataset, [10.5256/f1000research.6746.d80768](https://doi.org/10.5256/f1000research.6746.d80768)<sup>51</sup>

### Web resources

ClinVar database, <http://www.ncbi.nlm.nih.gov/clinvar/>

Online Mendelian Inheritance in Man (OMIM), <http://www.omim.org/>

PPB Genetic Study In: Clinical Trials.Gov available from, <http://clinicaltrials.gov/show/NCT00565903>

International PPB Registry, <http://www.ppbregistry.org>

NCI *DICER1* Phenotype Study, <http://dceg.cancer.gov/research/clinical-studies/DICER1-ppb-study>

### Author contributions

DAH, YM, GW, LPD, JI and PG conceived the study. DAH, AF, JY, WY, AR designed the experiments. DAH, AF, JY, AR, PS, LD, GW carried out the research. CR, GW, AH, KPS contributed database support and analysis. DRS, MAB and JT provided expertise in genetics. HG performed statistical analysis. DAH and MAB interpreted the results and prepared the first draft of the manuscript. All authors were involved in the revision of the draft manuscript and have agreed to the final content.

### Competing interests

No competing interests were disclosed.

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## Supplemental data

**Supplemental data for 'Temporal order of RNase IIIb and loss-of-function mutations during development determines phenotype in *DICER1* syndrome: a unique variant of the two-hit tumor suppression model'.**

Supplemental data file comprises nine tables:

**Table S1.** *DICER1* coding region amplicons for Ion Torrent sequencing

**Table S2.** *DICER1* probes for NanoString copy number assays

**Table S3.** Summary of germline *DICER1* loss-of-function mutations identified in PPB children

**Table S4.** Germline missense *DICER1* mutations (non-hotspot) – additional details

**Table S5.** Clinical features of children with germline *DICER1* mutations

**Table S6.** Summary of somatic *DICER1* RNase IIIb domain "hotspot" mutations identified

**Table S7.** Sequence results from children with mosaic *DICER1* loss of function mutation

**Table S8.** Sequence results from children with tumor specific, biallelic *DICER1* mutations

**Table S9.** Clinical features of 12 unresolved cases; PPB children who tested negative for germline *DICER1* mutation

[Click here to access the data.](#)

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# Open Peer Review

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Brenneman *et al.* present an observational study on a cohort of patients with DICER1 Syndrome. The analysis of 124 probands in combination with available familial data increases the understanding of penetrance and variability of mutations in DICER1 and the effect of these mutations on phenotype. Their identification of additional mosaic, germline and sporadic mutations helps to further elucidate phenotypic differences between these groups and provides insight into disease pathogenesis. Their proposed temporal model of mutation acquisition correlating to disease presentation is supported by the data. However, the following points need clarification:

## Abstract:

On page two the authors state: "A final category of patients lack predisposing germline or mosaic mutations and have disease limited to a single PPB tumor bearing tumor-specific RNase IIIb and LOF mutations."

Cases with a single PPB tumor should not be included under the umbrella of the DICER1 syndrome. A single tumor bearing a causative mutation does not merit inclusion as syndromic, which requires wide spread distribution of mutations in that same gene. This classification is not accurate as these patients simply represent a sporadic neoplasm.

## Subjects and Methods:

### *Mutation Testing*

On page three the authors state: "Initial sequencing of blood and saliva DNA samples was by standard Sanger methods described previously or by a commercial laboratory (Ambry Genetics, Aliso Viejo, CA). Low-frequency variants were detected and quantified by targeted next-generation sequencing (NGS) using a custom multiplex PCR panel for *DICER1* coding regions (Ion Torrent Ampliseq, Life Technologies, Grand Island, NY, USA). NGS was performed on an Ion Torrent 318 v2 chip (ION PGM Sequencing 200 kit v2, Life Technologies) with an average of 6 samples per chip, to achieve an average depth of coverage of 3000 filtered reads."

Did the authors use the Ion Torrent PCR panel for the analysis of the isolated tumors? This is not mentioned in the methods section. The data presented in Table 2 displays a large distribution in read numbers, which may have implications for data analysis. Can the authors provide an explanation for the

wide distribution of read coverage in this table, particularly in the disparity of reads between blood and tumor samples? This should be included in the methods section.

*Annotation of sequence variants and the spectrum of possible mutations*

On page three the authors state their methods for annotating variants identified: “For variants assayed by NGS, allele frequencies were calculated from filtered read counts. The SIFT algorithm was used to assess potential significance of novel missense mutations.”

It would be helpful, and strengthen the author’s argument that these mutations are pathogenic, to include an analysis of the frequency of loss of function (LOF) and hotspot mutations in the population by determining their minor allele frequency (i.e. using ExAC or 1000 genomes).

*NanoString genomic copy number assay*

On page four the authors state: “Genomic DNA was fragmented and hybridized using the nCounter Prep Station, and hybridization signals quantified using the nCounter Digital Analyzer, according to NanoString’s recommendations.”

It is stated in this section that hybridization signals were quantified. However, in table two many of the second hit LOF mutations are simple listed as “allele loss” and not quantified. Can the specific allele region and its quantification be provided as a percentage of allele loss abundance (as was done in table S8 for “Informative SNP” in cases 91,111,112)? Without this data it cannot be determined why the hot spot variant and the allele loss are unequally distributed (cases 104 and 105). Furthermore this would confirm the tumor purity estimates provided as normal cells should not have loss of the second allele. As tumor purity increases so should percentage of allele loss if these mutations are in fact required for tumor formation. As the table reads now it is implied that the second allele loss is complete (50%) in the tumors where it was observed. If this is not the case we ask that the loss be quantified and included, otherwise the “-” should be replaced with “NM” (not measured).

Were any positive controls run to confirm the ability to specifically detect copy number events using the Nanostring assay? For example, isolating DNA from preserved tumor samples often yields sheared fragments varying in size, which may hinder probe hybridization across fragments. This may yield false positive allele loss results.

**Results:**

*Most predisposing DICER1 mutations are inherited loss of function (LOF) mutations*

On page four the authors state: “Our overall approach to detecting and categorizing predisposing DICER1 mutations in PPB children is shown schematically in Figure 1. We identified germline, heterozygous DICER1 mutations in 90 of the 124 probands in our cohort (72.6%; Table 1, Table S3).”

Are the identified LOF variants observed in the general population (ExAC, 1000 Genomes)?

On page four the authors state: “In all, 84 of 90 germline *DICER1* mutations discovered in patients (93%) truncate the open reading frame before the end of the critical RNase IIIb domain, and are thus predicted to result in complete loss of DICER1 protein function even if the message escapes nonsense-mediated decay.”

There is no mention of potential alternative splice isoforms of DICER1, which may be translated despite the presence of early stop and/or frameshift mutations. This is an oversight especially as there is an emerging role for a specific splice variant DICER1e (a splice variant composed of only the RNase IIIa, IIIb and dsRBD domains) in neoplasms. This isoform may utilize a distinct promoter as has been observed for

the glucocorticoid receptor gene (Russcher *et al.*, 2007) and not rely on faithful sequence integrity of upstream exons. Two independent reports (Cantini *et al.*, 2014 and Hinkal *et al.*, 2011) have shown increased DICER1e isoforms in oral cancer cells and breast cancer cells respectively. This may be an important factor in discerning potential sub categories of LOF mutations. If in fact DICER1e plays a pathogenic role it is possible that alleles bearing early stop and/or frameshift mutations upstream of the RNaseIIIb domain, which in this study account for 93% of the mutations, are still able to code for this isoform and contribute to disease. The mechanism for oncogenesis may not require a true loss of function first hit but the presence of a modified isoform which in combination with a hot spot mutation would lead to a neomorphic phenotype associated with tumor formation. The authors should acknowledge this possibility in the manuscript.

*Approximately 10% of predisposing DICER1 mutations are mosaic rather than germline*

On page six the authors present table 1 “Clinical and Pathologic Features by Predisposing DICER1 Mutation Category.”

In table 1 there is a single case reported of a germline LOF mutation and a Wilms tumor (WT). Could the authors speculate on the rarity of WT in their large cohort given the described association of both single hot spot and biallelic mutations in DICER1 with this tumor type (Klein *et al.*, 2014; Wu *et al.*, 2013)?

On page eight the authors state: “NGS of tumor tissues from these children identified somatic LOF mutations or allele loss in some but not all specimens, with the caveat that allele loss can be difficult to detect in specimens with low tumor purity (*i.e.*, PPB Type I<sub>r</sub>, CN and NCMH).”

If the second hit LOF mutation is indeed a “driver” mutation of neoplasm one would expect those mutations to occur early in tumor formation and then clonally expand and be present in a majority of tumor cells. The very difficulty to detect these second hit LOF mutations argues that these tumors are in fact genetically heterogeneous; suggesting that these second hit mutations may represent passenger or modifying mutations but not drivers. The caveat mentioned supports both the authors’ and the alternative hypotheses and this should be included as a possible mechanism of pathogenesis.

On page eight the authors state: “Four of the five children with mosaic *DICER1* hotspot mutations presented with cystic PPB (type I/IR) rather than sarcomatous disease (type II or type III) and all five have survived to date.”

It is surprising that none of the mosaic hot spot cases present with PPB type II/III compared to two thirds of the germline LOF cases. This is not consistent with the more complicated clinical course and numerous neoplasms observed in the mosaic cases. Is it possible that the distribution in the tissue ultimately dictates the severity of the PPB? The authors should discuss as part of their disease model why the mosaic cases have a more complex clinical course while having more benign lung pathology.

On page eight the authors state: “Though all five hotspot mosaic children are alive, their clinical experiences have been complicated and arduous (Figure 3).”

As the authors state, cases with mosaic *DICER1* hot spot mutations present with a complex clinical course. Therefore, including a more detailed clinical description of the five mosaic cases, specifically paying attention to their phenotype, would strengthen this statement. For example, including growth parameters as well as developmental and physical exam findings may help to define this subgroup of the *DICER1* syndrome. On page 14 of the supplement a footnote to table S9 mentions “This data set is limited, pursuant to concerns for potential identification of study participants based on particular combinations of clinical and pathologic features. Qualified investigators with specific questions about the study not answered by the data in these tables are invited to contact the International PPB Registry. The

Registry will try to accommodate requests for additional data while preserving protected health information.” This concern may be the very reason why more detailed phenotypic data is lacking. However this limits the ability for the reader to draw genotype-phenotype correlations and to compare these cases to those already reported in the literature. We request that at least common clinical findings shared by mosaic cases (if any) are presented in a table without reference to individual cases. This should limit the risk of potential identification of study participants.

#### *Table 2*

On page eight the authors state: “We categorized a *DICER1* mutation detected by NGS as mosaic when the following criteria were met: *i.* The mutation was evidently not a constitutional, germline allele because it was present at sub-heterozygous frequency (arbitrarily taken as below 35% of reads) in peripheral blood and/or other normal tissue samples. *ii.* The mutation was evidently not specific to a tumor, because the same mutant allele was detected in one or more normal, non-neoplastic tissue samples, OR, the same mutant allele was detected in multiple primary tumors arising in different organs.”

It is possible that low abundance mutations detected in blood are not present in a blood cell lineage but may represent metastatic disease (for example in case 102 where the brain tumor is a metastatic event that originated in the lung PPB)? Furthermore cases 102, 103, 104, and 105 have very low mutation abundance in blood with cases 103-105 carrying less than 0.3%. These low numbers are perhaps evidence of unrecognized metastatic disease in these patients, the detection of which has been previously described (Haber and Velculescu, 2014). Testing of more “normal” tissues is needed for fulfilling the mosaicism criteria as proposed by the authors. It is more likely that the cases in table 2 have lower levels of mosaicism limited to a small number of tissues in contrast to other cases with more widely distributed hot spot mutations (Klein *et al.*, 2014). Additional phenotypic clinical data for these cases (as requested above) is needed to properly make a comparison.

Tumor purity is very low in mosaic cases samples (Table 2), and this is used as an argument for why second hit mutations are lower in abundance. However, it is also possible that the second hit mutation is unequally distributed throughout the tumor and in fact absent from some regions of the tumor. This possibility should be mentioned in the manuscript.

#### *Tumor-specific, biallelic DICER1 mutations account for about 10% of PPB cases*

On page eight the authors state: “In twelve children, we identified biallelic *DICER1* mutations present at high allele frequencies in a PPB tumor, but not detectable in blood even with the benefit of high-depth NGS (Table S8).”

These cases likely represent sporadic neoplasm mutations. We question if these cases should be classified as having the “*DICER1* syndrome.” A more clear distinction between isolated PPB and the “*DICER1* Syndrome” should be included.

#### *Currently unresolved cases*

On page eight the authors state: “Twelve PPB probands in our cohort are negative for predisposing *DICER1* mutations detectable in blood DNA by Sanger sequencing or NGS of coding exons.” The authors include an additional 12 unresolved cases (Table S9). We suggest that a potential etiology for cases in which *DICER1* mutations are absent would be caused by mutations in *DROSHA* or other genes involved in the microRNA processing pathway. It has been established that at least in the pathogenesis of sporadic Wilms tumor RNaseIIIb mutations in *DROSHA* phenocopy those in *DICER1*, although possibly by distinct mechanisms (Rakheja, 2014). While the authors pursued *DROSHA* testing via Sanger sequencing exclusively in blood (Table S9 Footnote: “Sanger sequencing in blood DNA for

DROSHA, XPO5, and the DICER1 promoter region”) this approach might have missed low abundance mosaic mutations. Furthermore, some of these “unresolved” cases (121, 123 and 124) have been identified as carrying hot spot mutations in their tumors without the presence of a second hit. This may illustrate that the hotspot mutations alone may be sufficient for tumorigenesis.

On page 5 the authors present Figure 1 “Study Design”

After reviewing this cohort we call into question the designation of “unresolved” in one case. We would like to propose that case 123 is actually mosaic due to the presence of the same hotspot mutation in two distinct disease foci. This case cannot be excluded as mosaic based on the absence of the mutation in blood as it is similar to case 105 where the sequencing from blood is not above the error threshold. Case 123 should therefore be moved to table two, and the study design/mosaic criteria amended to classify case 105 and 123 as mosaic even in the absence of the hotspot mutation in normal tissue. The absence of a LOF second hit in the presence of a mosaic hot spot mutation should represent a distinct subset of mosaic cases and not classified as “unresolved.”

### **Discussion:**

#### *Genotype-phenotype correlation of predisposing mutations in PPB-DICER1 syndrome*

On page ten the authors state: “All germline *DICER1* truncating mutations are predicted to be essentially equivalent in their effect: complete loss of function in miRNA processing.”

As mentioned above this conclusion must be tempered by the possibility of an alternatively spliced variant of *DICER1* that could be expressed despite a truncating mutation upstream of the RNase IIIb domain.

On page ten the authors state: “Neomorphic RNase IIIb domain function (skewed 5p/3p miRNA production) is a recurring feature of *DICER1* tumors, and it is plausible that loss of all wildtype RNase IIIb function is required for it to become tumorigenic.”

The statement “loss of all wildtype RNase IIIb function is required for it to become tumorigenic” does not apply to all categories of the *DICER1* syndrome. From the data as it is presented in this study the only category where this can be concluded is from the sporadic tumors, which are distinct from the *DICER1* syndrome. In these tumors it is clear from the data in table S8 that all hot spot mutations are accompanied by LOF mutations with corresponding abundances, which are certainly a characteristic of these aggressive lung neoplasms. However, we cannot conclude causality for the second hit mutations in all *DICER1* tumors since (1) in the mosaic hot spot cases (Table 2) the observed abundance for the second LOF hit mutations is always less than the hot spot mutation and (2) there are cases of tumors in this report that lack a second hit (Case 105: NCMH, Case 123 CN and PPB). A main objection to the analysis and interpretation of these results is the lack of an explanation for the differences in mutation abundance between LOF and hot spot mutations within a tumor and the presence of tumors without a second hit. This raises questions as to whether the LOF mutations are in fact drivers of tumorigenesis or passenger mutations. Although tumor purity could be partially responsible for these inconsistencies, the data on its own does not sufficiently establish that these LOF mutations are required for tumor formation in non-sporadic tumor cases when they are not present in all neoplastic cells.

In the model as it is proposed by the authors, cases that are mosaic for RNase IIIb mutations display no clinical findings until a second LOF mutation occurs which drives and is essential for tumor formation (Page 11, last paragraph). We believe there is the possibility for another explanation. A single RNase IIIb mutation alone could have a pro-oncogenic effect on distinct cell types at specific developmental stages. As tumorigenesis proceeds, a LOF mutation in the other allele may arise as the tumor drifts, further aggravating the 5p/3p imbalance in a sub population of tumor cells. Supporting this alternative model,

cases 105, 121, 123 and 124 are reported to have neoplasms with no second hit detected. If this second hit is essential why do these tumors lack the LOF second hits? In aggregate there are 4 mosaic cases (2 in this report and 2 in the literature), which, in combination with the absence of germline true heterozygote hot spot mutations, support the alternate model that mosaic hot spot mutations are likely pathogenic on their own and the authors should expand their model to include this alternate explanation.

### Supplemental Data

#### *Table S5 Clinical features of children with germline DICER1 mutations*

Can the authors comment on why mortality is higher in germline LOF mutation carriers than it is in the mosaic “hot spot” mutation carriers even though the latter have a more complicated clinical course? Could this be due to the association of PPB type I/IR with mosaic cases and PPB type II and III with germline LOF cases?

### Minor Changes

1. The authors should include appropriate references to any manuscripts in which any of these PPB registry cases have been previously reported.
2. While not essential, it would be informative to include any affected siblings for the 10 identified *de novo* LOF cases to support or refute potential germline mosaicism in the parents (Page 4)?
3. Table 1 includes a single case of a germline LOF mutation and a Wilms tumor (WT). However, little else is described about this case. Furthermore there is no mention of this case in the supplementary materials. Please include mutation analysis and additional phenotypic information for this case.

**We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.**

**Competing Interests:** No competing interests were disclosed.

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