Introduction

Pleuropulmonary blastoma (PPB) is an extremely rare and highly aggressive pulmonary malignancy occurring in early childhood. It is characterized histologically by a primitive blastoma and a malignant mesenchymal stroma in the lung that often shows multidirectional differentiation (1). PPB may be sporadic or hereditary and may also present as a part of a familial tumor syndrome (2) consisting of cystic nephroma and other tumors, such as ovarian tumor, embryonal rhabdomyosarcoma, and malignant germ cell tumors (2). Recently, germline DICER1 mutations have been demonstrated in majority of patients with PPB and DICER1 syndrome (2, 3). DICER1 is a member of the ribonuclease III (RNase III) protein family that is involved in the generation of microRNAs (miRNA), modulating gene expression at the posttranscriptional level (4). The DICER1 protein contains RNase IIIa and RNase IIIb domain, which are considered to dimerize intramolecularly with Mg^{2+}/Mn^{2+} to form the active site of the enzyme (5). In PPB, almost all mutations are reported to be heterozygous frameshift or nonsense mutations of germline origin, suggesting an important role of DICER1 haploinsufficiency in PPB pathogenesis (2, 3). However, most obligate carriers of DICER1 mutations and heterogeneous DICER1-deficient mice did not develop PPB or other types of tumors, suggesting that DICER1 haploinsufficiency alone is insufficient for tumor development but requires additional genetic alterations (3, 6). To identify a complete set of genetic alterations underlying PPB pathogenesis, we performed whole-exome sequencing of paired tumor and normal DNA from seven cases with sporadic PPB, of which two cases were analyzed for samples obtained at the initial presentation and relapse. Mutations in DICER1 and other genes were examined by targeted deep sequencing in 16 samples from 12 sporadic PPB cases, including three analyzed by whole-exome sequencing.

Materials and Methods

Specimens

Genomic DNA for 11 cases was extracted from fresh-frozen samples stored at −80 °C and obtained approximately 2 to 15 years previously. Paraffin-embedded samples were used as tumor samples for cases 10 (at relapse) and 11 (at diagnosis). These samples were stored for approximately 1 year. For
germline control, DNA was obtained from bone marrow blood, peripheral blood, or bone marrow smears in which absence of tumor cells was pathologically confirmed. Bone marrow smears were used as normal samples for cases 05, 07, 08, and 12. This study was approved by The University of Tokyo Ethics Committee (Tokyo, Japan; approval number 1598), and informed consent was obtained from the parents of all participants.

Whole-exome sequencing
Whole-exome sequencing of primary tumor and matched normal specimens of cases 01, 02, 04, 07, 09, 10, and 12 was performed as previously described (7, 8). Relapsed tumor specimens of cases 01 and 02 were also analyzed. Whole-exome capture was accomplished using liquid-phase hybridization of sonicated genomic DNA having a 150 to 200-bp mean length to a bait cRNA library synthesized on magnetic beads (SureSelect Human All Exon Kit V3 or V5, Agilent Technology) according to the manufacturer’s protocol. The captured targets were subjected to sequencing using HiSeq 2000 (Illumina) according to the manufacturer’s instructions. Raw sequence data were processed using Genomon-exome (http://genomon.hgc.jp/exome/en/index.html) for detection of cancer exome sequencing data through the in-house pipeline constructed at the Human Genome Center, the Institute of Medical Science, The University of Tokyo. Analyses using Genomon are summarized in Supplementary Fig. S1. Sequence data have been deposited at the European Genome-phenome Archive (EGA, http://www.ebi.ac.uk/ega/), which is hosted by the European Bioinformatics Institute, under accession number EGAS00001000662.

Deep sequencing for validation of variants detected by whole-exome sequencing
To validate the mutations detected by whole-exome sequencing, deep sequencing was performed using pair or trio DNA specimens (primary/relapse tumor and normal) using HiSeq 2000 or MiSeq (Illumina). Primers used for this validation are listed in Supplementary Table S1. Mutations were amplified using PCR with a Nol linker individually attached to each primer and pooled together on a per-sample basis after successful amplification was confirmed by gel electrophoresis. Pooling was followed by purification of DNA using the FastGene Gel/PCR Extraction Kit (Nippon Genetics) and digestion with Nol. The digested DNA was purified again, and an aliquot of purified DNA was ligated with T4 DNA ligase for 5 hours, sonicated into approximately 200 bp fragments on an average using Covaris (Covaris), and used for generation of sequencing libraries with the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs) according to the manufacturer’s protocol. Data processing was performed according to previously described methods (7, 8). Each single-nucleotide variant and each insertion/deletion (indel) whose variant allele frequency (VAF) in the tumor sample was equal to or more than 2.0% and in the germline sample less than 2.0% were assigned as a somatic mutation. If the mutant allele frequency in the matched nontumor sample was more than 2.0%, the mutation was discarded (8). The mutation was evaluated for pathogenicity using online mutation predicting tool, Mutation Taster (http://www.mutationtaster.org).

Small RNA sequencing
RNA was extracted using the miRNeasy Kit (Qiagen). Total RNA was quantified and evaluated for quality using a bioanalyzer (Agilent Technology). Libraries for small RNA sequencing were generated using the TruSeq small RNA Sample Preparation Kit (Illumina) and analyzed using the Illumina MiSeq according to the manufacturer’s protocol. Small RNA sequencing was performed for four cases (cases 01, 07, 08, and 09). Read sequences were aligned against miRBase (release 16) using MiSeq Reporter v2.3 (Illumina). After alignment, the number of read sequences aligned to each miRNA or pre-miRNA was calculated. Gurtan and colleagues demonstrated that the RNase IIIA and IIIB domains of DICER1 process the 3′ (3p) and 5′ (5p) arms of miRNAs, respectively, in vivo (9). We defined the pre-miRNA cleavage ratio as the read counts of pre-miRNA/ (read counts of pre-miRNA + miRNA). This ratio was calculated for 5p or 3p miRNA, and then compared tumor specimens with fetal lung as normal control. Statistical differences were calculated by Wilcoxon rank-sum test.

Single-nucleotide polymorphism genotyping microarray
DNA of 11 cases (excluding case 11) as well as that of three relapse cases was hybridized to Affymetrix GeneChip 250K Nsp arrays (Affymetrix). DNA of cases 10 (at relapse) and 11 was not hybridized because of the poor quality of DNA from the paraffin-embedded samples. After appropriate normalization of mean array intensities, signal ratios between tumors and anonymous normal references were calculated in an allele-specific manner, and allele-specific copy numbers were inferred from the observed signal ratios based on a hidden Markov model using CNAG software (http://www.genome.umin.jp).

Sanger sequencing and targeted deep amplicon sequencing
Sanger sequencing of DICER1 and TP53 was performed for samples from all cases and relapsed tumor samples from four cases. Germline DNA was sequenced for nine cases (including case 02 without DICER1 mutation). Sanger sequencing of PDCD2L and UBAC2 was performed for 11 cases. Deep amplicon sequencing of target exons of TP53, GPR182, and CTNNB1 was performed for 14 samples from 11 cases. Exons harboring mutations in DICER1 were sequenced for 11 cases, and all coding exons of DICER1 were sequenced for case 02. Details of deep sequencing have been provided above. All primer sequences for these genes are listed in Supplementary Table S2–S4.

Results
The mean coverage in the whole-exome sequencing of tumor and germline samples was 126× and 128× for the 50 Mb target regions, respectively. More than 93% of the coding sequences were represented by more than 20 independent reads on an average (Supplementary Fig. S2). GC content and mean coverage are shown in Supplementary Fig. S3. Mean coverage of high-GC (≥60%) exons was lower than that of low GC (<60%). In total, 217 nonsilent substitutions and 12 indels
were detected across nine tumor specimens, of which 191 (88%) and 12 (100%), respectively, were successfully confirmed by deep sequencing (Supplementary Table S5). The number of nonsilent mutations per sample at presentation (13–35 mutations) was lower than that reported in most solid tumors in adults (10–12), but comparable with the number reported for other pediatric tumors such as neuroblastoma and medulloblastoma (18 and 16, respectively; Fig. 1A; refs. 13, 14). In two cases for which serial samples could be analyzed, relapsed samples had higher mutation number than corresponding samples at initial presentation (Fig. 1A and B). In both cases, intratumoral subpopulations were evident at the time of initial presentation (Fig. 1C). As previously reported for other cancers (15, 16), the clonal architecture of tumor subpopulations underwent dynamic evolutionary alterations during tumor progression. Serial samples in each case had several clonal mutations in common as well as harbored private subclonal mutations of their own (Fig. 1B and C). In case 01, some of the subclonal mutations (purple) found in the initial sample disappeared at relapse and were replaced by new mutations carried by new subpopulations (red), whereas most of the mutations found in the subclones (green) were retained at similar relative allele frequencies in the relapse sample in case 02. In both cases, relapsed tumors were accompanied by newly acquired gene mutations in each subpopulation and/or by appearance of new subclones that were totally absent from the original initial samples (Fig. 1C).

DICER1 mutations were detected for six cases (cases 01, 04, 07, 09, 10, and 12) but not for case 02; targeted deep sequencing was unable to detect any DICER1 mutations. DICER1 mutations were found in the major tumor populations in these six cases (Fig. 1C and D). In contrast with previous reports where all DICER1 mutations were heterozygous and had germline origin, we identified two homozygous somatic DICER1 mutations in cases 09 and 10, prompting us to investigate the status of DICER1 mutations in five additional cases. DICER1 mutations were found in 11 of 12 (92%) cases (Table 1; Fig. 2A and Supplementary Fig. S4), in which six of the 11 cases with DICER1 mutations carried compound heterozygous mutations. Two cases carried homozygous DICER1 mutations (Fig. 2B), presumably caused by copy number-neutral LOH (or uniparental disomy; UPD) involving the 14q arm harboring the DICER1 locus. In total, biallelic DICER1 mutations were found in eight of the 11 (73%) cases with DICER1 mutations. We failed to demonstrate biallelic alterations in three cases (case 01, 05, and 11; Table 1 and Supplementary Fig. S4). We confirmed the same DICER1 mutation status in initial and relapse samples in all four cases, for which both serial samples were available, indicating that DICER1 mutations are involved in tumor development rather than progression.

Germline DNA was available in eight cases to confirm germline/somatic origins of DICER1 mutations, of which four (cases 04, 07, 08, and 12) were compound heterozygous for a germline nonsense/frameshift and a somatic missense mutation, two (cases 09 and 10) were homozygous for somatic, missense mutations caused by an acquired UPD, and the remaining cases were heterozygous for a somatic missense mutation (case 01) or a germline frameshift mutation (case 05; Table 1). Among the three cases without normal samples, the combination of a nonsense and missense mutation was also found in the two cases with compound heterozygous mutations. In these cases, a somatic origin was suspected for a

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Mutations and mutant allele frequencies detected by whole-exome sequencing in 7 PPB cases. A, type and number of somatic mutations in each tumor. Each mutation type is distinguished using the indicated color. Primary (pr) and relapsed (re) tumors of cases 01 and 02 were examined independently by whole-exome sequencing. B, Venn diagram of somatic mutations found in cases 01 and 02. Both relapsed (re) tumors had increased number of somatic mutations compared with primary (pr) tumors. C, VAF distribution of validated mutations in relapsed cases. VAF was obtained from deep sequencing. Allele frequencies were corrected for copy numbers determined by SNP array analysis. DICER1 mutation is discriminated by the indicated color in case 01. Case 02 harbored no DICER1 mutation. Subclonal mutations in case 01 at primary (pr) and relapse (re) are distinguished by purple and red, respectively. Subclonal mutations in case 02 are distinguished by green. D, VAF distribution of validated mutations in nonrelapsed cases. DICER1 mutations were included in the major tumor population.
missense mutation (D1810Y) in case 06, in that the VAF of that mutant deviated significantly from the expected value (0.5) for germline variants (Supplementary Table S6). Conspicuously, all nine missense DICER1 mutations found in our cohort were located within the RNase IIIb domain with a mutational hotspot at G1809 (Fig. 2C), for which a somatic origin was confirmed or highly suspected in eight mutations. Combined with previous reports for PPB (2, 3), this high frequency of germline mutations supported the incomplete penetrance of DICER1 mutations in both familial and sporadic PPB. To assess the effect of DICER1 mutation in RNase IIIb domain on RNA cleavage, we performed small RNA sequencing in tumors with mutational hotspots at G1809R and D1810Y. Total RNA including miRNA extracted from fetal lung was used as a normal control. Given that the RNase IIIA and IIIB domains of DICER1 process the 3p and 5p arms of miRNAs, respectively (9), DICER1 mutations in RNase IIIb domain are expected to affect 5p rather than 3p miRNA expression. Comparing the pre-miRNA cleavage ratio of tumor samples to that of the fetal lung control, we confirmed dramatically reduced 5p miRNA expression in the tumors with G1809R and D1810Y mutations (P < 7.1 × 10−7; Fig. 3A and B). In contrast, 3p miRNA expression was significantly higher in the tumor samples than in fetal lung control (P < 1.4 × 10−3), suggesting that G1809R and D1810Y mutants have opposite effects on 3p miRNA cleavage. Taken together, our results suggest that a mutational hotspot at G1809R has a pathogenic effect.

Except for DICER1, several genes were found to be recurrently mutated in whole-exome sequencing, including TP53, CTNNB1, GPR182, MYH8, PDE2A, and TMX3 (Supplementary Table S7). TP53, CTNNB1, and GPR182 were investigated by targeted deep sequencing in an additional five cases, although these genes were not mutated in CTNNB1 and GPR182. The result of targeted deep sequencing in TP53 is described below. To identify additional genetic alterations, we next performed single-nucleotide polymorphism (SNP) array-based genome-wide copy number analysis in 14 samples of 11 cases for which high-quality genomic DNA was available (including three cases with both primary and relapsed tumors). Chromosome 8q gain was the most common copy number change and was found in 10 of the 11 cases in varying combinations with other genetic changes, including loss of chromosomes 10 and 17p and high-grade amplification of 19q (Fig. 4A and Supplementary Figs. S4 and S5). Chromosome 17p LOH was found in 10 samples and was caused by UPD (N = 1) or deletions (N = 9), and commonly involved an 8.5-Mb region that contained TP53. To investigate a possible role of TP53 mutations in PPB, we analyzed the TP53 mutation status in 14 tumor samples from all 12 cases by Sanger and deep sequencing. We detected recurrent missense or frame shift mutations in five of the 12 cases (42%; Fig. 4B; Table 1), in which all five cases were accompanied by 17p LOH and led to biallelic TP53 inactivation. Intriguingly, in case 04, the relapsed tumor had a different TP53 mutation (G105S) from that found at the time of initial presentation (C176F), suggesting that the relapse originated from a different subclone in which the two DICER1 mutations predated TP53 mutations. We also found several focal amplifications involving 5q23, 6q16-21, 15q23-24, and 19q13.11. However, none of

<table>
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<th>AA change</th>
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Abbreviations: ND, not determined; AA, amino acid; Pr, primary; Re, relapse.

<sup>a</sup>Primary tumor.

<sup>b</sup>Relapse tumor.
these amplifications were recurrent, except for those involving 19q13.11, which were found in three (25%) of the 12 cases (Supplementary Fig. S5). The amplified region contains five genes, including LSM14A, KIAA0355, GPI, UBA2, and PDCD2L, but mutations were detected in none of these genes.

Discussion

The most striking discovery in the present study is the frequent biallelic involvement of DICER1 mutations in majority of PPB cases with an obligatory missense mutation involving the RNase IIIb domain. In our cohort, biallelic DICER1 mutations were documented in eight of the 11 DICER1-mutated cases with sporadic PPB, with RNase IIIb domain-involving mutations found in all cases and somatic origins demonstrated in all evaluable cases. This result was in stark contrast with previous reports, where all DICER1 mutations in PPB or DICER1 syndrome cases were heterozygous and inherited from parents; all mutations were either nonsense or frameshift changes except for two cases, of which one had a missense mutation in the RNase III domain (2, 3). Interestingly, a recent study reported frequent recurrent DICER1 mutations affecting the RNase IIIb domain in nonepithelial ovarian cancers, especially Sertoli–Leydig cell tumor, in which 26 of 43 tumors carried exclusively RNase IIIb domain mutations with only four tumors being compound heterozygotes of a germline nonsense/frameshift mutation and an RNase IIIb domain mutation (5). Conspicuously, no germline mutations involving the RNase IIIb domain and no biallelic nonsense or frameshift mutations have been reported in any human cancers, possibly accounting for the different spectrum of DICER1 mutations between PPB and ovarian cancers. These unique features of DICER1 mutations suggest distinct oncogenic roles of both nonsense/frameshift and RNase IIIb domain mutations. It could be hypothesized that complete loss of DICER1 function, beyond haploinsufficiency through targeted mutations within the RNase IIIb domain, could be required or effective for the tumor cells to be clonally selected.

The RNase IIIb domain in DICER1 and other RNase III protein family members is involved in excision of double-stranded miRNA stems, which are then cleaved to single-stranded miRNA through the activity of the RNase IIIa domain (5). A mutation of the conserved amino acids in the RNase IIIb domain could thus lead to compromised miRNA processing, especially in excision of miRNAs. In fact, four mutational hotspots at metal-binding sites (E1705, D1709, D1810, and E1813) found in nonepithelial ovarian cancer were shown to have decreased RNase IIIa activity (5). In the current study, we found an additional mutational hotspot within the RNase IIIb domain affecting a highly conserved amino acid position

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Figure 2. DICER1 abnormalities detected in 12 PPB cases. A, frequency of identified DICER1 mutations in 12 cases. B, homozygous DICER1 mutation with 14q LOH without copy number loss. Right panels show a sequence chromatogram of a G1809R homozygous mutation. Left panels show 14q LOH obtained from SNP array analysis. tCN, total copy number; AsCN, allele-specific copy number. C, a schematic of DICER1 protein structure with the positions of alterations. Top and bottom portions indicate mutations detected in our study and previously reported mutations in references 2 and 3, respectively. All the nine missense DICER1 mutations found in our cohort were located within the RNase IIIb domain with a mutational hotspot at G1809. fs, frameshift.
(G1809) in the vicinity of the two known hotspot codons (D1810 and E1813). Our small RNA sequencing revealed that mutational hotspots at G1809 and a D1810 mutation showed a dramatically reduced cleavage ratio of 5p miRNA, and D1810 mutation also showed the same results in PPB. D1810 mutation is one of the hotspot mutations in nonepithelial ovarian cancer (5), of which reduced 5p miRNA expression has been already confirmed (17). This finding suggests that a specific mutational hotspot of PPB, G1809, is functionally equivalent to hotspot mutations in nonepithelial ovarian cancer. Anglesio and colleagues showed no significant change in 3p miRNA expression (17); however, its cleavage ratio was increased in our analysis. This result may be due to the existence of some mechanism that activates DICER1 to compensate the loss of 5p miRNA production (Fig. 3C). Gurtan and colleagues also mentioned an increased ratio of miRNA star to mature strands relative to cells expressing native hsDicer (9). MiRNA star means less abundant mature miRNA, which usually consists of 3p miRNA, so that this result is compatible with our observation. Thus, it seems that mutations at G1809 could lead to a biologic consequence similar to that of known hotspot mutations (5), although the oncogenic mechanism of the defective cleavage but not excision of miRNAs in the pathogenesis of PPB and other cancers awaits elucidation.

Besides DICER1 mutations, TP53 mutations with or without 17p loss as well as trisomy 8 and other chromosomal abnormalities were among the common genetic lesions in PPB. With respect to DICER1 mutations, it is of note that TP53 also plays a critical role in the regulation of miRNA processing (18). Indeed, tumor-derived transcriptionally inactive TP53 mutants suppress precursor and mature miRNA levels, whereas native TP53 increases them (18), indicating that TP53 plays an important role in cancer biology via regulation of miRNA processing. A recent study showed that TP53 regulates DICER1 expression via transcriptional miRNAs such as let-7 (19). In contrast, Wang and colleagues showed that knockdown of DICER1

![Figure 3. Significant reduction of pre-miRNA cleavage of 5p strand in four tumor specimens by small RNA sequencing. A, 5p miRNA biogenesis was significantly reduced in tumor samples. P values were calculated by Wilcoxon rank-sum test. B, 3p miRNA biogenesis was retained in tumor samples. In contrast with 5p miRNA expression, 3p miRNA expression in tumor samples exceeds normal control. C, schematic model of aberrant pre-miRNA cleavage by hotspot mutant DICER1. The miRNA biogenesis pathway by normal DICER1 is indicated in the top panel. A proposed model of hotspot DICER1 mutant is presented in the lower panel. Hotspot DICER1 mutant could not cleave the 5p strand of pre-miRNA. Loss of 5p miRNA may prompt DICER1 to cleave pre-miRNA so that 3p miRNA may be overprocessed.](https://www.aacrjournals.org/cancerres/article-pdf/74/10/2747/3081881/cancerres-2013-2470-a.pdf)
expression in BxPC-3 and Panc-1 pancreatic cancer cells resulted in significant increases in TP53 protein levels (20), suggesting the existence of a regulatory loop between TP53, DICER1, and let-7, deregulation of which may play a role in PPB development.

In conclusion, biallelic DICER1 mutations were common in PPB, invariably accompanied by a somatic RNase IIIb domain mutation. Majority of cases had mutated DICER1 alleles in germline with or without an additional RNase IIIb domain mutation in the remaining allele. Recurrent mutations were rare in PPB, except for frequent TP53 deletions/mutations. Our results provide novel insight into the critical role of DICER1 mutations and importance of TP53 inactivation in the pathogenesis of PPB.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M. Seki, S. Ogawa, J. Takita
Development of methodology: Y. Shiraishi, Y. Okuno
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Seki, K. Yoshida, Y. Sato, R. Nishimura, Y. Okuno, K. Kato, R. Hanada, Y. Nomura, M.-J. Park, T. Ishida

Figure 4. Overview of DICER1 and TP53 mutations with copy number alterations. A, copy number alterations by SNP array analysis in 14 PPB samples from 11 cases. The regions of DICER1 and TP53 are indicated by arrows. Amplification, gain, loss, and UPD are distinguished by the indicated colors. Copy number (CN) gain was defined as copy number between 3 and 5. Amplification was defined as an inferred copy number of more than 5. Copy number loss was defined as copy number less than one copy and LOH was assigned when one allele was retained. B, distribution of DICER1 and TP53 mutations with frequently detected copy number alterations. pr, primary; re, relapse; NA, not available.
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Seki, A. Oka, S. Ogawa, J. Takita
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Seki, A. K. Okubo, and Drs. Y. Tanaka, K. Ida, A. Motomura, S. Shinogawa, K. Watanabe, and A. Kozaki for assisting with clinical care for patients and collecting samples.

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