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***DICER1* Mutations in Embryonal Rhabdomyosarcomas from Children With and Without Familial PPB-Tumor Predisposition Syndrome**

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Abstract

Embryonal rhabdomyosarcoma (ERMS) is the most common sarcoma of childhood and is a component of the familial Pleuropulmonary Blastoma (PPB)-predisposition syndrome. Using the PPB model, we hypothesized that *DICER1* mutations would be found in familial and sporadic forms of ERMS. Blood samples from four children with familial PPB and ERMS, and an additional 52 sporadic ERMS tumors were tested for *DICER1* mutations. *DICER1* mutations were found in all 4 patients with familial PPB and in 2 of 52 (3.8%) patients with sporadic ERMS. Our findings confirm the pathogenetic relationship between ERMS and PPB and suggest that ERMS may result from abnormal miRNA regulation.

Keywords

pleuropulmonary blastoma; *DICER1*; *DICER*; rhabdomyosarcoma; children; miRNA

Introduction

Embryonal rhabdomyosarcoma (ERMS) is the most common soft tissue sarcoma of early childhood. The majority of ERMS arise in luminal organs or structures such as the bladder; uterus or vagina of the genitourinary tract; the nasal cavity, paranasal sinuses or nasopharynx in the head and neck; or within the common bile duct. Thus, ERMS can be categorized as an organ-based embryonal neoplasm similar to pleuropulmonary blastoma (PPB) of the lung, nephroblastoma of the kidney, and neuroblastoma of the adrenal gland. Each of these embryonal neoplasms appears to arise during organ development. Morphologically these tumors resemble the embryonic form of the organ.

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Conflict of interest: nothing to declare

We have recently uncovered heterozygous germline loss of function mutations in the major miRNA processing gene *DICER1* in familial PPB.[1] Familial PPB - tumor predisposition syndrome is a unique syndrome in which individuals are predisposed to PPB and other embryonal tumors such as ERMS, cystic nephroma, neuroblastoma, nasal chondromesenchymal hamartoma, ocular medulloepithelioma and ovarian sex cord-stromal tumors. [2-5] We hypothesize that in PPB, decreased *DICER1* expression affects the concentration of key miRNAs responsible for the appropriate downregulation of growth signals expressed during development (presumably via growth factors regulated by miRNAs or perhaps miRNAs themselves). [1] Given that ERMS is seen in PPB families and is the major sarcomatous element of PPB, we hypothesized that *DICER1* mutations would be found in sporadic ERMS.

Methods

All participants or their guardians from familial PPB cases gave written informed consent to genetic testing and medical history collection. The presumed sporadic samples of ERMS were obtained from the Cooperative Human Tissue Network (CHTN) without identifying information except age in years, site and a pathologic description. Medical and/or family histories were not available. Tumor samples from the CHTN were from individuals who were enrolled on protocols of the Children's Oncology Group (COG). This study was approved by the Institutional Review Board at Children's National Medical Center.

Individuals from 4 different PPB families with pathologically confirmed ERMS were studied. For the set of ERMS, we obtained tumor samples from 52 children enrolled on protocols from the COG between 1986 and 2004. Frozen sections were reviewed for quality and then tumor DNA was extracted using QIAampDNA blood mini kit (Qiagen). Germline DNA for 1 of the 52 children was available and was requested following the initial experiment. For PPB family members, blood or saliva was collected through family visits made by the research team, or mailed directly by the research subject or his/her health care professional. Germline DNA was extracted from peripheral blood lymphocytes using the PAXgene blood DNA kit (Qiagen, Germantown, MD) or from saliva using the Oragene kit (DNA Genotek, Ontario, CA).

Primers were designed to amplify all of the coding exons including intron-exon boundaries of *DICER1* (NM_177438) as described previously. [1] The resultant products were purified and directly sequenced. Sequence traces were assembled and scanned for variations using Sequencher version 4.8 (Gene Codes, Ann Arbor, MI) and visual inspection of chromatograms.

Results

We sought to determine the incidence of *DICER1* mutations in an unbiased cohort of ERMSs obtained from the CHTN as well as cases of ERMS occurring in PPB families. In our PPB cohort, 3 of 99 probands and one paternal uncle of a child with PPB were diagnosed with ERMS (Table 1). There were five ERMSs total, three located in the urinary bladder and two in the cervix. Two of the five tumors were from a child who had Type II PPB at 4 years of age and then primary ERMS of the cervix at 8 years, and a second ERMS of the bladder at 12 years. These tumors were each diagnosed 4 years apart without interval recurrent tumor. [6] One child presented with signs and symptoms related to the PPB with the bladder ERMS diagnosed during staging evaluation of the intrathoracic tumor. One additional child was first diagnosed with cervical ERMS and then a large multiloculated cyst was discovered in the right upper lobe of the lung during the staging evaluation. Resection of the lung cyst showed a multicystic mass without residual primitive mesenchyme, which

we have termed as Type I PPB “regressed.” Finally, one child with PPB had an uncle with a history of bladder ERMS diagnosed at 6 weeks of age. Sequence analysis identified germline *DICER1* loss-of-function mutations in all 4 of these affected individuals (Table 1).

A total of 52 presumed sporadic cases of ERMS were available from the CHTN for study. Ages at diagnosis ranged from 1 to 22 years with a median age of 6.7 years at diagnosis. There were 31 males (70.5%) and 13 females (29.5%). Sites of primary tumors included 25 tumors in organ-based sites including naso- or oropharynx (n=6), bladder (n=5), uterus/female pelvis (n=4), vagina (n=3), male pelvis/perineum (n=2), kidney (n=1) and abdominal organ sites (n=4). Twenty-four tumors were soft tissue based including paratesticular/scrotal region (n=18) and one each in the calf, orbit, neck, flank, diaphragm and chest wall. Three tumors did not have a designated site. Two of 52 tumors were found to have *DICER1* mutations. One of the cases, a 12-year-old female with uterine ERMS harbored a base substitution in the last base of the intron before exon 25 (c.5365-1G>T) which is predicted to disrupt the splice site. The second mutation was a three base pair deletion resulting in deletion of a glutamic acid residue (p.1418_1420delE). This glutamic acid residue is one of three consecutive glutamic acid residues in an acidic portion of the *DICER1* protein in between the two RNase III domains. These glutamic acid residues are conserved in mammals and although we have no functional data specifically for this allele, based on conservation, location in the protein, and other examples of deleterious loss of a single glutamic acid residue in other proteins, [7-8] we predict this deletion to have a deleterious effect on this *DICER1* allele. Germline DNA was available from the child with uterine ERMS and was negative indicating a tumor-specific, somatic *DICER1* mutation.

Discussion

Prior to its introduction as a distinct entity in 1988 [9], PPB had often been diagnosed as variations of ERMS. The cystic type I PPBs account for the multiple reports of sarcomas arising in congenital cystic adenomatoid malformations (CCAM) [10]. It is likely that most of the published solid RMSs of the lung would be categorized today as the solid variant of PPB.[11] The basis for this literature confusion is that the ERMS pattern is a prominent feature of solid types II and III PPB and is the exclusive malignant element in the purely cystic type I lesions. [12] PPB, like the botryoid variant of ERMS, is characterized by a population of primitive and differentiated rhabdomyoblasts which reside as a condensed population of cells beneath epithelia appropriate for the organ whether it is alveolar-type epithelium in the lung, urothelium in the case of the bladder or squamous mucosa in the vagina. (Figure 1) Both of these tumors originate in organogenesis with early PPB comprised of immature, benign-appearing epithelium and mesenchymal elements resembling the fetal lung at 10-16 weeks gestation and ERMS with morphology similar to developing muscle at 5-6 weeks gestation.

A genetic link between PPB and ERMS was first postulated in 1993 by Sciort et al, who uncovered cytogenetic abnormalities of 2q in a case of PPB. [13] Similar 2q abnormalities had previously been described in ERMS cases. Although the majority of cases of RMS occur sporadically, there are known associations with other syndromes such as Neurofibromatosis Type 1 (NF-1) [14] noted in 0.5% of patients evaluated by the International Rhabdomyosarcoma group [15], Li-Fraumeni syndrome, [14] and most recently familial PPB predisposition syndrome. Similarly, two children with PPB have NF-1 and one child with PPB has a Germline p53 mutation, suggesting Li-Fraumeni syndrome. [16] Within the PPB syndrome, we and others have found heterozygous germline, loss-of-function *DICER1* mutations in cystic nephroma, ocular medulloepithelioma, Sertoli-Leydig tumors and nodular thyroid hyperplasia. [17] Conversely in patients without a family history

of PPB, *DICER1* mutations have also been found in cystic nephroma, [18] Sertoli-Leydig tumors [5] and familial multinodular goiter. [19]

This sequencing study confirms that like the other tumors described above, germline *DICER1* mutations do appear to have a role in the pathogenesis of ERMS in the setting of the familial PPB predisposition syndrome. Slade et al did not find *DICER1* mutations in 19 RMS cases [20]. In contrast, we found *DICER1* mutations in 2 of 52 (3.8%) in our larger cohort. Our findings further suggest that a small, but not inconsequential proportion of sporadic ERMS tumors do harbor *DICER1* mutations. The identification of mutations in *DICER1* in a broader range of pediatric embryonal tumors suggests that in some of these tumor types the miRNA pathway is important in the regulation of growth and differentiation during organ development.

The current study is limited by the relatively small number of cases of sporadic ERMS cases, especially of the genitourinary subtype, obtained from the CHTN and the fact that germline DNA was not readily available for these cases. In addition, we have only analyzed one of the many genes involved in the ever-expanding miRNA biogenesis pathway. It is likely that ERMS is not a genetically homogeneous group, and that *DICER1* and miRNAs may be more important in a subset of these tumors characterized by site (e.g. uterus and bladder) and type (especially the botryoid variant given its histologic similarity). Physicians treating patients with organ-based ERMS should be aware of the association between ERMS and PPB, and obtain detailed family history that may require genetic testing for patients and their family members. The discovery of more examples of ERMS and PPB may help strengthen the pathogenetic link between these two childhood neoplasms.

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Table 1
Clinicopathologic features of the children with embryonal rhabdomyosarcoma and *DICER1* mutations

Patient ID	Age PPB	Sex	PPB type	Age at ERMS	Site	Sample tested	Mutation	Amino acid change
fERMS1	4 yr	F	II	8 yr 12 yr	Cervix Bladder	Blood	NM_177328.2: c.4309_4312delGACT	NP_803187.1: p.D1437fs
fERMS2	9 yr	F	I-R	9 yr	Cervix	Blood	NM_177328.2: c.5104C>T	NP_803187.1: p.Q1726X
fERMS3	NA	M	NA	6 wks	Bladder	Blood	NM_177328.2: c.2247C>A	NP_803187.1: p.Y749X
fERMS4	5 mo	M	I	5mo	Bladder	Blood	NM_177328.2: c.1910dupA	NP_803187.1: p.Y637fs
CHTN 1	NA	F	NA	12 yr	Uterus	Tumor and Blood	Tumor NM_177328.2: c.5365-1G>T Germline DNA normal	N/A
CHTN2	NA	Unk	NA	Unk	Abdomen	Tumor	Tumor NM_177328.2: c.4259_4261delGAG	NP_803187.1: p.1418_1420delE

fERMS-familial cases; Unk - unknown