SF 424 R&R and PHS-398 Specific

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PI: HILL, DANA ASHLEY	Title: DICER1 and the Pleuropulmonary Blastoma Family Cancer Syndrome	
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	Organization: CHILDREN'S RESEARCH INSTITUTE	
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.

RESEARCH & RELATED OTHER PROJECT INFORMATION

1. * Are Human Subjects Involved? ☒ Yes ☐ No

1.a If YES to Human Subjects

Is the Project Exempt from Federal regulations? ☐ Yes ☒ No

If no, is the IRB review Pending? ☒ Yes ☐ No

2. * Are Vertebrate Animals Used? ☐ Yes ☒ No

3. * Is proprietary/privileged information included in the application? ☐ Yes ☒ No

4a. * Does this project have an actual or potential impact on the environment? ☐ Yes ☒ No

5a. * Does this project involve activities outside of the United States or partnerships with International Collaborators? ☐ Yes ☒ No

PROJECT SUMMARY/ABSTRACT

Studies of inherited cancer syndromes have provided unique opportunities to uncover and explain important cellular pathways. Pleuropulmonary blastoma (PPB) is a rare lung sarcoma that arises during fetal lung development and affects children under 6 years of age. In approximately 20% of cases, PPB appears to develop as the result of inherited cancer susceptibility. We have recently demonstrated germline loss of function *DICER1* mutations in familial PPB. We hypothesize that inactivation of the wild-type *DICER1* allele leads to tumor formation through dysregulation of genes controlled by miRNAs. Three highly integrated aims are proposed to further define the role *DICER1* plays in PPB. In Aim 1, we will define the heritability of PPB and further characterize the PPB tumor predisposition syndrome by systematically collecting comprehensive personal medical histories from extended family members from 100 PPB families. In Aim 2 we will determine *DICER1's* role in PPB predisposition performing *DICER1* mutation analysis in 100 PPB children. For each proband with a *DICER1* mutation, we will test parents, siblings, grandparents and more distantly related members for the presence of family-specific mutations. We will explore candidate genes in cases lacking *DICER1* mutations. These experiments will shed light on the genetic heterogeneity of PPB and will allow us to measure the penetrance and expressivity of *DICER1* mutations. In Aim 3 we will determine *DICER1's* role in PPB initiation and evaluate potential mechanisms of oncogenesis. We will use immunohistochemistry to evaluate DICER1 expression in both tumor-associated epithelial and mesenchymal components and to guide subsequent somatic mutation studies of *DICER1*. Conceptually, this tumor may represent a naturally-occurring model of mesenchymal cell dysregulation. Preliminary data suggests that loss of *DICER1* (and mature miRNAs) may occur primarily in the non-neoplastic *epithelial* component of the tumor leading to dysregulation of "tumor promoting factor(s)" that stimulate proliferation of the mesenchyme and predisposes the mesenchyme to sarcomatous transformation. The investigation of dysregulation in the context of malignancy may add to our knowledge base of epithelial-mesenchymal interactions in normal and abnormal lung development. The work proposed in this application will help test and refine this hypothesis and build the foundation for future mechanistic studies. A better understanding of this syndrome is essential to the development of clinical criteria for identifying these families and for guiding their medical care. Intensified radiologic screening examinations could be directed toward at-risk family members, leading to earlier detection of PPB in its purely cystic stage when the disease is more readily cured. In addition, study of families that show predisposition to PPB represent a unique opportunity to learn about the cellular processes in the borderland between development and neoplasia and to study how tissue-specific loss of *DICER1* (and the miRNAs it regulates) manifests in human disease.

PROJECT NARRATIVE

This work will shed light on the role genetics plays in a new pediatric tumor syndrome and could ultimately lead to new strategies to the detection and treatment of PPB and related tumors such as rhabdomyosarcoma and Wilms tumor.

FACILITES AND OTHER RESOURCES

Laboratory

Dr. Hill's laboratory includes approximately 250 square feet, and is located in the Research Center for Cancer and Immunology Research, Children's Research Institute (CRI). It is a fully equipped molecular biology laboratory and shares a BL-2 facility for cell/virus culture including six carbon dioxide incubators, a roller bottle apparatus, two BL-2 safety cabinets, two inverted microscopes (one uv), a centrifuge, and a water bath laboratory shared by all investigators.

Clinical

In mid-2009, the anatomic pathology laboratory at CNMC will have a fully automated immunohistochemistry service using a Bond immunohistochemistry system (Leica microsystems). This machine also performs heat induced epitope retrieval, deparaffinization and in situ hybridizations. This immunohistochemistry service is a shared service between clinical laboratory and research. Dr. Hill serves as the pathology laboratory Chief.

Animal

Not applicable.

Computer

CRI has a network, employs full time systems engineers, Oracle and SQL database designers, PhD level bio-informatics and bio-statistics personnel. The PI has a network linked computer in her office and there is an additional computer in the laboratory.

Office

The PI has approximately 150 square feet of office space in a building connected to the laboratory building. In addition, there is desk space in the laboratory for the Research Assistant.

Major Equipment

CRI genomics resources include 4 Affymetrix GeneChip stations (4 GeneChip® Fluidics stations, 4 GeneArray® scanners, 2 GeneChip® hybridization ovens). Affymetrix data resides in an Oracle LIMS system, served by dedicated personnel. There are 2 ABI 3700 96 capillary sequencers, 2 ABI 3100 16 capillary sequencers, and 2 Transgenomic WAVE DHPLC machines operating in a 96 well plate format, a quantitative PCR machine, TaqMan genotyping, MWG PCR robot, and Affymetrix 100k SNP Chips and 300kb re-sequencing microarrays.

CRI core laboratory research equipment (ultra-low freezers, centrifuges, liquid nitrogen tanks), beta counters, gamma counters, lyophilizers, Beckman DU70, Shimadzu CS9000 densitometer, bacterial incubators, PCR thermocyclers, laminar flow hoods, ELISA readers) is housed in 5,000 sq. feet of space shared by all investigators.

The Research Center for Genetic Medicine houses two Affymetrix GeneChips stations, including GeneChip Fluidics Station, GeneArray scanner, and GeneChip hybridization ovens. Affymetrix data resides in an Oracle LIMS system. In addition we have an Axon GenePix4000 cDNA array dual channel scanner and a Gene Machine robotic arrayer with 16 printing pins and a 36 plate robotic plate feeder for the arrayer. The Research Center houses a Biomek FX robot, two ABI3700 96 capillary sequencers, two ABI3100 16 capillary sequencers, one ABI377 slab sequencer, five 96-well MJ tetrad PCR machines (20 blocks total), and eight Perkin-Elmer 9600 PCR machines. In addition, the Center has two 8-capillary automated sequencers (CEQ2000) and a LiCor infrared gel-based automated sequencer and four single 96-well block MJ PCR machines. Further equipment includes a Beckman XL-90UC ultracentrifuge, Savant speedvac plus, Brinkman polytron, Hitachi GeneSpec III spectrophotometer, Nikon Microphot FXT microscope with fluorescent filter blocks and Optonics PE750 digital camera, cryostat, and Licor infrared imager for western blots. An Arcturus PicCell II Laser Capture Microdissection System was recently purchased.

RNA expression profiling: The Research Center for Genetic Medicine is the single largest contributor of microarray data in the public domain (15% of NCBI GEO), and is well equipped to assist with microarray work proposed here.

PHS 398 Cover Page Supplement

OMB Number: 0925-0001

1. Project Director / Principal Investigator (PD/PI)

Prefix: Dr.

* First Name: Dana

Middle Name: Ashley

* Last Name: Hill

Suffix: M.D.

* New Investigator? ☐ No ☒ Yes

Degrees: M.D.

2. Human Subjects

Clinical Trial? ☒ No ☐ Yes

* Agency-Defined Phase III Clinical Trial? ☐ No ☐ Yes

3. Applicant Organization Contact

Person to be contacted on matters involving this application

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4. Human Embryonic Stem Cells

* Does the proposed project involve human embryonic stem cells? [X] No [ ] Yes

PHS 398 RESEARCH PLAN

1. Application Type:

From SF 424 (R&R) Cover Page and PHS398 Checklist. The responses provided on these pages, regarding the type of application being submitted, are repeated for your reference, as you attach the appropriate sections of the Research Plan.

*Type of Application:

☒ New ☐ Resubmission ☐ Renewal ☐ Continuation ☐ Revision

SPECIFIC AIMS

(Please note: the bibliography with the references listed throughout this section in the original grant application was omitted in order to decrease the file size.)

Pleuropulmonary blastoma (PPB) is a rare lung sarcoma that arises during fetal lung development and affects children under 6 years of age.[1] The early stage of PPB (Type I PPB) is characterized by epithelial cysts and small numbers of uncommitted mesenchymal cells which in later stages overgrow the cysts (Type II PPB) and become purely solid high grade, multipatterned sarcomas (Type III PPB).[2] PPB is a component of a recently described tumor predisposition syndrome in which family members are also at increased risk for developing Wilms tumor-related neoplasms, rhabdomyosarcoma and perhaps other conditions.[3] Using linkage analysis we recently mapped a PPB locus to chromosome 14q31-32. Subsequent candidate gene sequencing identified heterozygous germline *DICER1* mutations in all 10 PPB families investigated. These mutations appear to confer a loss-of-function. *DICER1* encodes an RNase III protein that is required to cleave precursor microRNAs (pre-miRNA) into active miRNAs (and siRNAs). We hypothesize that inactivation of the wild-type *DICER1* allele results in tumor initiation through dysregulation of genes controlled by miRNAs and that this could represent a novel pathway for neoplastic transformation.

Aim 1: Define the heritability of PPB and further characterize the PPB tumor predisposition syndrome
Our recent work describing inherited *DICER1* mutations associated with predisposition to PPB was based on a small subset of families. This highly focused study resulted in the identification of an etiologic factor, but many important questions about the clinical features of the syndrome remain. What fraction of PPB patients has inherited disease? What tumors or other medical conditions are directly attributable to *DICER1* mutation? How penetrant are *DICER1* mutations? We will address these questions by systematically collecting comprehensive personal medical histories from extended family members from an estimated 100 families (62 families already consented). We will use a questionnaire designed by the research team to obtain past or present history of developmental and anatomic anomalies, cysts, tumors, autoimmune conditions and pregnancy history and review medical records. A better understanding of this syndrome is essential to the development of clinical criteria for identifying these families and for guiding their medical care. In addition, the study of families that show predisposition to PPB represent a unique opportunity to gain a better understanding of cellular processes in the borderland between development and neoplasia and to study how tissue-specific loss of *DICER1* (and the miRNAs it regulates) manifests in human disease.

Aim 2: Determine *DICER1's role in PPB predisposition: DICER1* mutation analysis for approximately 100 PPB cases and evaluation of other candidate genes.
Our preliminary studies have demonstrated germline mutations in *DICER1* in family members from 10 different families showing susceptibility to PPB providing a convincing argument that DICER1 has an important role in the pathogenesis of this disease. Our analyses have, however, been focused on families with multiple affected individuals. The frequency of *DICER1* mutations in PPB cases without familial disease in unknown. Do isolated cases of PPB (sporadic) with multiple lung cysts carry germline mutations in *DICER1* and if so, how often are these molecular defects *de novo* mutations? The penetrance and expressivity of *DICER1* mutations is also yet to be determined. In this aim we will test 100 PPB children with PPB for germline *DICER1* alterations. For each proband with a *DICER1* mutation, we will test parents, siblings, grandparents and more distantly related members for the presence of family-specific mutations. The mutational status will be used to measure the rate of new mutations, estimate the penetrance of mutations (relying on the family and medical history developed in Aim 1) and to better define the expressivity. This information will facilitate implementation of gene-based testing and will help further refine the information used to counsel these families. Individuals and families for whom we exclude a role for *DICER1* aberrations then become an additional cohort for sequencing of other miRNA processing genes such as *DROSHA*, *DGCR8, Exportin-5* or for new linkage/association studies.

Aim 3: Determine *DICER1's* role in PPB initiation and evaluate potential mechanisms of oncogenesis
It is not known how haploinsufficiency for DICER1 contributes to PPB susceptibility. Animal models haploinsufficient for DICER1 and the human PPB families provide compelling evidence that a single copy of *DICER1* is sufficient for normal development. Conditional knock-out of Dicer1 in embryonic mouse lung epithelium produces a PPB-like cystic phenotype.(4) We hypothesize that **1) Development of PPB, and related tumors seen in these families requires a second genetic "hit" namely, somatic inactivation of the wild-type *DICER1* allele** and **2) Loss of *DICER1* (and mature miRNAs) occurs in the *epithelial***

component of the tumor leading to dysregulation of "tumor promoting factor(s)" that stimulate mesenchymal proliferation and predispose to neoplastic transformation. We will use immunohistochemistry to evaluate DICER1 expression in both tumor-associated epithelial and mesenchymal components. These results will guide subsequent somatic mutation studies of *DICER1*. Finally we will assess the gene expression profiles in Type III PPB to identify signatures associated with this aggressive form of PPB.

BACKGROUND AND SIGNIFICANCE

Distinct pathologic types of PPB indicate disease progression and predict clinical outcome

Pleuropulmonary blastoma is a rare pediatric lung cancer that was first recognized as a unique pathologic entity in 1988.[1] PPB almost exclusively affects children less than 6 years of age. PPB is analogous to other organ-based embryonal tumors such as rhabdomyosarcoma of skeletal muscle, Wilms tumor of the kidney and neuroblastoma of the adrenal gland in which the neoplastic process appears to arise from a particular stage of the developing organ. Approximately 15 to 25 children in the United States and 30 to 40 children worldwide are diagnosed with PPB annually. Despite its rarity, the tumor has been well characterized from a clinical and pathologic perspective. PPB is one of the few pediatric cancers whose distinctive natural history can be discerned by gross and microscopic examination. Much of what we have learned about the biology of PPB has been from morphologic observations of tumors at different time points.[5] Based on our study of the earliest PPBs (from 31 weeks gestation), we believe the tumor arises from an embryonic uncommitted lung mesenchymal cell. The embryonic lung mesenchyme originates from mesoderm and is characterized by loosely packed primitive cells that are the source of progenitors for endothelial cells, fibroblasts, smooth muscle and cartilage.[6] Lung mesenchyme plays an important role in the growth of the developing lung.[7] One of the key functions of the mesenchyme is signaling the endodermally-derived epithelium to proliferate, branch and differentiate into the various types of lining cells.[8] Similarly, one of the key roles of the epithelium (and mesothelium) is to drive proliferation and regulate differentiation of the mesenchyme.[9] Over the past two decades it has become evident that many human malignancies arise and progress as a consequence of mutations in master regulators of normal developmental processes. Study of PPB holds promise for providing novel insights into how dysregulation of normal developmental processes leads to tumor formation.

The architecture of PPB early in development is characterized by multilocular cyst within the lung parenchyma (Type I PPB).[5] The cyst walls are lined by benign-appearing alveolar-type epithelium. The cyst walls contain variable number of primitive mesenchymal cells, some of which show differentiation along either skeletal muscle or cartilaginous lines. Typically these early cases present in infants and young children (median 9 months of age) who come to medical attention for difficulty breathing due to a large space-occupying cyst in the lung or due to a rupture of the air-filled cyst in the chest (pneumothorax). Very often these benign-appearing cysts are mistaken for unrelated entities congenital cystic adenomatoid malformations or lobar emphysema. In later stages of tumorigenesis, the mesenchymal cells expand and overgrow the epithelial cysts forming an overtly malignant cystic and solid (Type II) or purely solid sarcoma (Type III).[1;2] These children are older than those with Type I PPB; the median ages at diagnosis for Type II and Type III PPB are 36 and 42 months respectively. A child with Type II or III PPB typically presents with weight loss, fever, shortness of breath and a large lung mass. These pathologic types of PPB reflect the observed biological progression within individual patients and in age-segregated subgroups.[2;10] Children presenting with Type III PPB represent approximately one-third of all PPBs. It is unclear if all the type III PPBs go through a cystic stage; most have not had radiographic studies prior to presentation. On the other end of the spectrum, it recently has become clear that not all Type I PPB are destined to progress to Type II and then Type III PPB.[5] Through our pilot family study, we identified multiple older children and adults with benign lung cysts. Histologic review of these cysts demonstrated the unique Type I PPB architecture but without the mesenchymal tumor cells. Similar cysts can be seen in PPB patients themselves where one or more of the cysts show signs of progression and others appear “dormant”. In our recent review of 51 early (Type I) PPBs we have observed that *de novo* regressive changes such as cyst wall necrosis are common and these cyst walls appear to heal over with fibrosis.[5] Whether benign cysts in these patients represent regressed Type I PPB, incompletely developed Type I PPB or have a different underlying pathogenesis entirely remains an open question but the implication is that sarcomatous transformation of a Type I PPB requires additional genetic alterations.

Pathologic type is the only known statistically significant predictor of outcome.[11] Type is used to stratify patients into different therapeutic groups. Type I PPB is treated with complete surgical removal and adjuvant chemotherapy. With chemotherapy, the overall survival for this subgroup is over 90%.12 Children with Types II and III PPB are subject to an aggressive chemotherapy regimen, however, the survival rates are only 60% and 45%, respectively. **These survival statistics suggest that screening methods which improve our ability to identify PPB in its early stages may dramatically improve outcome.**

PPB is a component of a hereditary tumor predisposition syndrome
One of the early observations in children with PPB was the recognition that PPB was commonly multifocal and could run in families.[1;3;13] Approximately 20% of children with PPB have a personal or family history of other childhood cancers (unpublished PPB Registry data). Cancers and other conditions described in these families include other PPBs, lung cysts, embryonal rhabdomyosarcoma, Wilms tumor, cystic nephroma, medulloblastoma, glioblastoma, gonadal germ cell and stromal tumors, primitive retinal tumors, thyroid cancers and hyperplasias, melanoma, autoimmune thyroiditis, Crohn's disease, intestinal polyps, nasal chondromesenchymal hamartomas, sarcomas and hematopoietic malignancies. This observation is remarkable for the collective association of rare childhood neoplasms associated with organ development. While this information was initially sufficient to provide a working definition of the syndrome for successful linkage analysis (see preliminary data section C.1.2.), it is insufficient for understanding the full spectrum of disease and relative risks for the various conditions. The collection of family history, including medical recordpathologic confirmation of tumors, has varied from family to family with little uniformity in pedigree development and ascertainment of information in family members. The family data collected are thus 1) incomplete, 2) biased towards the "most" familial cases, and 3) focused largely on pediatric tumors. With a systematic approach and unbiased ascertainment proposed in Aim 1 (see experimental design section D.1.1.), we will produce a more accurate and inclusive data set of medical histories to facilitate the complete clinical characterization of the PPB Family Tumor Predisposition Syndrome.

The International Pleuropulmonary Blastoma Registry and its role in the study of PPB
The International PPB Registry (IPPBR) (Research Director, Jack Priest) was formed concurrent with the initial description of PPB in 1988 and is responsible for many of the clinical and pathologic observations described above http://www.ppbregistry.org. The IPPBR's clinical operations at Children's Hospitals and Clinics of Minnesota (HSC#98107) and Pathology Operations at Washington University Medical Center (HSC#04-1154) and now Children's National Medical Center are funded by Children's Hospitals and Clinics of Minnesota Foundation and its associated charitable event, The Pine Tree Apple Tennis Classic. We follow approximately 300 children and in the last 4 years have been referred 30 to 35 new cases per year from five continents. The P.I. of the proposed research serves as the Pathology Research Director for the Registry and plays a leadership role in clinical investigation, strategic planning and family advocacy. One of the most important initiatives by the P.I. in this role was to lay the foundation for scientific investigation by establishing a tumor specimen, DNA and cell line biorepository (W.U. HSC#05-0192) (see Preliminary Studies C.1.1). This biorepository combined with the Registry's infrastructure for identifying and collecting new cases and existing comprehensive clinical and pathologic database is a unique resource and facilitates the type of detailed clinical and translational research proposed in this study. Importantly, the Registry is preparing to initiate the first International Treatment Protocol and Biology Study of PPB (PI: Yoav Messinger, see letter of support). Engaged with collaborators across the U.S., Canada, South American, Europe and Australia we seek to define the best care plan upon which future treatment protocols can be built. We estimate that patient accrual will begin in late 2009. With relevance to this proposal, DNA and tissue banking collection requirements are embedded within the treatment protocol to ensure that precious tumor samples will be available for biological studies for years in the future. Given their critical role in patient accrual, Drs. Priest and Messinger will be collaborators in this proposal.

Mutations in *DICER1* are a cause of familial PPB and *DICER1* is essential in lung morphogenesis
We have recently shown inherited PPB is associated with *DICER1* mutations. Ten of 10 families studied had mutations (see Preliminary Studies, Section C below). This is the first demonstration of inherited mutation in *DICER1* associated with cancer.

Only one *DICER1* gene exists in humans. It encodes a ~ 218 kDa protein RNase III protein that plays a key role in microRNA biogenesis. miRNAs are short, single stranded RNAs that regulating protein expression by binding specific sequences in the 5' or 3' untranslated regions of mRNA molecules and either targeting them for destruction or inhibiting their translation.[14] miRNAs are often expressed in temporal and organ-specific patterns. miRNA regulatory processes appear to be very important in 1) human developmental timing events, 2) stem cell proliferation,[15;16] 3) cell cycle control,[17] 4) regulatory control of some lymphocyte subsets,[18;19] and 5) oncogenesis.[20] It appears that there is intrinsic redundancy in miRNA regulation; multiple miRNAs can suppress a single target mRNA and single miRNAs can suppress multiple target mRNAs.[15] The function of DICER1 is to perform the final cleavage of pre-processed small RNAs to produce the ~22 nucleotide "mature"

effector molecules that can then go on to bind mRNAs.[14] Other important proteins in this process include Drosha which processes small RNAs in the nucleus,[21] DGCR8 which recognizes the RNA substrate, exportin-5 which transports the small RNAs into the cytoplasm for DICER1 and then proteins involved in the RNA-induced silencing complex (RISC) that assist mature miRNAs in target repression.[14] Without a functional DICER1 protein, the miRNA regulatory control pathway would not be operational.

Abnormalities in miRNA processing appear to be feature of a variety of cancers.[22] Evidence is accumulating that most miRNAs appear to be downregulated as tumors become less differentiated.[23] Some miRNAs function as tumor suppressors,[20;24;25] whereas others, such as the *mir-17-92*, can have both suppressor and oncogenic effects.[26] It has been postulated that miRNAs are responsible for "fine-tuning" growth regulatory networks.[26] Recently, reduced *DICER1* levels have been associated with aggressive tumor phenotypes in a range of different malignancies.[27-29] Mouse models of globally impaired miRNA processing show an enhancement of cellular transformation and tumorigenesis through dysregulation of oncogenes but this is insufficient to initiate tumors.[20] How loss of *DICER1* might collaborate with other genetic events in what appears to be a multi-step tumor progression is currently unknown. The importance of DICER1 protein in development has been shown in genetic models. Murine Dicer-/- embryonic stem cells proliferate slowly and are defective in differentiation suggesting some aspects of differentiation (and stem cell proliferation) depend on Dicer.[16] Dicer-/- mouse embryos survive until embryonic day 7.5 but then die before axis formation.[30] Dicer-/- zebrafish have miRNA processing ability early on, perhaps from maternal Dicer activity, but experience slow growth and developmental delay before dying at 14-21 days post fertilization.[31;32] Mutant embryos have abnormal morphogenesis of heart, brain and other organs.

To study later effects of Dicer loss in development, a conditional Dicer allele was engineered with loxP sites inserted around the exon that encodes most of the second RNase III domain and upon recombination excises this domain which renders the allele non-functional.[33] Using this construct, Harris and colleagues generated a mouse model in which Dicer is lost in lung epithelium at ~ d.10.5 in embryogenesis.[4] The resultant phenotype shows marked cystic dilation of the airspaces and mesenchymal expansion which resembles the early stage of PPB.[4] The *Dicer*-null lung tissues showed increased expression of FGF10 protein with an expanded distribution in lung mesenchyme. Harris and colleagues speculated that lack of miRNA control might account for the observed dysregulation of FGF10.[4]

SIGNIFICANCE

Historically, studies of inherited cancer syndromes have provided unique opportunities to uncover and explain important cellular pathways (e.g. *Rb* in retinoblastoma leading to Knudson's "two-hit" hypothesis of tumor suppressor genes).[34;35] Based on our preliminary results below, we anticipate that study of PPB families may shed light on a novel pathway of oncogenesis.

As we have identified the first described human germline mutations in *DICER1*, PPB is an important human model for studying how tissue-specific loss of DICER1 (and the miRNAs it regulates) may manifest clinically and biologically in humans. We know that these families are at risk for rare pediatric cancers but we know nothing about the relative risks for more common adult cancers such as breast cancer, prostate cancer, melanoma, leukemia and others. In addition, animal model data suggests that *Dicer1* has an important role in regulating T lymphocytes; mouse models of loss of *Dicer1* in T regulatory cells results in fatal autoimmunity.[18;19] Might we expect to see an excess of autoimmune conditions such as Hashimoto's thyroiditis, Crohn's disease, celiac disease, diabetes, lupus and/or other related conditions in these families? And if the mechanisms for predisposition to these conditions could be resolved, might they be relevant in sporadic cases of the same diseases? And could identifying the key miRNAs or target proteins form the basis for the development of novel, rational (pathway-specific) therapeutic options?

It is clear from survival statistics that early detection of PPB is critically important to achieving a high cure rate. Mutation testing in PPB probands and their families may eventually prove useful in the clinical management of the disease. Knowing which children have inherited a predisposition to the disease would help direct intensified screening computed tomography scans to only those children who are at risk. We anticipate that the work proposed in Aim 1 and 2 will lead to a better understanding of the genetic heterogeneity of PPB with respect to *DICER1* mutations and will lead to more accurate estimations of penetrance, expressivity and the frequency of de novo mutations. Each of these will be important in developing comprehensive screening

guidelines and educating families. This study parallels that of another rare family cancer syndrome, multiple endocrine neoplasia 2a (MEN2a), and related syndromes where the discovery of mutations in the RET protooncogene that cause medullary carcinoma of the thyroid led to the development and application of presymptomatic gene-based testing and surgical prophylaxis in childhood which is curative.[36-40]

Finally, based on the current evidence that PPB arises from a lung mesenchymal stem cell and the central role of the latter in lung growth and differentiation, we anticipate the genes and pathways important in PPB may be relevant to key cellular processes in the developing lung. Conceptually, this tumor may represent a naturally-occurring model of mesenchymal cell dysregulation. Although not a focus of this application, the investigation of this dysregulation in the context of malignancy may nevertheless add to our knowledge base of epithelialmesenchymal interactions in normal and abnormal lung development.

PRELIMINARY STUDIES

Recruitment of families and procurement of samples

As detailed in the background, the PPB Registry provides an efficient and effective mechanism for identifying patients with PPB. In preparation for this research proposal the P.I. received a seed grant from the Siteman Cancer Center at Washington University (Hill NIH 2P30CA9184204, 7/1/2005-6/30/2007) and formed a team to begin the systematic collection of family history data with medical record confirmation and biologic specimen accrual. Since Human Studies Approval in March 2005 (WU IRB#05-0192) we have now enrolled over 60 families from 22 different US states and 7 foreign countries and continue to identify new families at a rate of approximately 2 per month. To date, 460 research subjects have been enrolled (median 3 subjects per family). All research subjects provide written consent for molecular and family history studies as approved by the Human Research Protection Office at Washington University in St. Louis (WU05-0192). Blood and/or saliva specimens are collected as a source of genomic DNA. Detailed family histories are obtained by Jennifer Ivanovich, a genetic counselor experienced in family studies and a co-investigator for this proposal.[41-45] We have banked approximately 430 DNA samples from probands and family members. We have formalin-fixed paraffin-embedded (FFPE) material from approximately 75 PPB tumors. We have snap frozen PPB tumor tissue from 36 patients. We have lymphoblastoid cell lines on 25 PPB patients. Each DNA and tumor sample is linked to extensive clinical, pathological, treatment and outcome data. Family recruitment, collection of history and medical records and biological specimens and refinement of the clinical aspects of the PPB syndrome will continue through the terms of this proposed research.

The obvious challenge that accompanies any study of a rare disease is ensuring collection of an adequate number of specimens to perform the proposed research. We have used multiple approaches to identify and enroll families and procure precious tissue resources.

a. International PPB Registry referral The International PPB Registry is an unparalleled resource and is a full participant in this proposed research. To our knowledge, there are no other efforts like this in the world for this tumor. The PPB Registry enrolled 39 cases in 2005 and 37 cases in first 8 months of 2006. The PPB Registry asks each newly diagnosed patient to enroll in the genetic study. The overwhelming majority of these referred patients and families have expressed their willingness to participate in the study. Dr. Jack Priest, the Director of the Registry is a collaborator in this application.

b. Raising awareness among physicians Recent educational efforts by PPB Registry oncologists and pathologists and the development of websites http://ppbstudy.wustl.edu and http://www.ppbregistry.org have led to increased recognition of this condition and increased referral of patients to the PPB Registry.

c. PPB Family Conferences The research team in collaboration with the PPB Registry hosts biannual informational conferences for children and families affected by PPB. There have been two meetings in St. Louis (2006) and Minneapolis (2008) with 24 and 35 families attending respectively. The next meeting will be held in Washington, DC in 2010. These meetings are supported by charitable contributions from our foundations and individual donors. The attendees have become both participants in the study and a vocal group of patient advocates.

d. PPB International Consortium As described in the background section the PPB Registry and collaborators throughout the U.S., Europe, Canada, Australia and South America will initiate the first prospective treatment protocol for PPB in the upcoming months. The protocol originates from a PPB International Consortium composed of surgeons, oncologists and pathologists from Europe, United Kingdom, Brazil, and Australia (see letters of support) that first met in the fall of 2006 in Geneva Switzerland. The goal of this International Consortium was to collaborate on establishing a standard of care for treatment of PPB and develop standards for acquiring DNA and tissue resources for biology studies. Dr. Yoav Messinger is the P.I. of the treatment protocol for PPB and is a collaborator in this application. This protocol will ensure that samples from around the world are referred for the work proposed in this application.

Initial characterization of the PPB syndrome

The clinical data collected by the Registry in the past had insufficient detail to fully characterize the PPB Family Cancer Syndrome. A thorough evaluation of the family histories of all PPB patients, including those presumed sporadic, has not been performed. The data is lacking in such areas as: 1) confirming number of relatives and

status of each; 2) reporting of adult tumors and preneoplastic conditions; 3) confirming pathologic diagnoses in the reported cancers and precancerous lesions in the family; and 4) additional clinical, developmental and pregnancy history. In preparation for this proposal, the available data from 198 PPB Registry families was descriptively analyzed to 1) generate hypotheses about the syndrome, 2) inform the design of a questionnaire to collect health information from each research subject, and 3) create a working definition of "disease affected" status in families for the pilot linkage study described in section C.1.3.

Working definition of PPB Syndrome

a. Multifocal Disease: Forty of 166 (24%) PPB children with radiographic material available for review had multifocal lung tumors/lung cysts. Accounting for PPB type, 40% of the early PPBs (Type I PPB) are multifocal.

b. Defining the phenotype in the PPB Family Cancer Syndrome: We took a conservative approach in generating the phenotypic definition of "affected" for the linkage study. We based our definition on evidence of repeated occurrence of these tumors in multiple families, the frequency of these tumors in family members compared with the general population, as well as likely biological relationships between PPB and these tumors. The following four clinically recognizable features are pathognomonic:

PPBs: There are 9 families with > 1 PPB (2 sibling pairs, 1 parent/child, 1 child/uncle and 4 sets of cousins).

Cystic nephroma (CN): Cystic nephromas and related renal tumors including WT occur in 9.2% of all PPB Registry patients,[13] whereas CN is exceedingly rare in the general population. All CN in affected patients/relatives have come to clinical attention by age 4 years,[13] compared to the primarily adult occurrence of non-PPB cystic nephromas.

Table 1: Descriptive Analysis of tumors and tumorous conditions in 198 PPB Families (data collected by PPB Registry)

Tumor type	Probands	Family Members
PPB/lung cysts	198	19
Cystic Nephroma	16	6
Embryonal RMS	3	1
Gonadal Germ cell tumor	0	7
Ovarian stromal tumor	2	5
Leukemia	0	6
Thyroid nodular hyperplasia	4	26
Thryoid carcinoma	2	6
Melanoma	0	3
Small intestinal polyps	4	2
Nasal chondromesenchymal hamartoma	3	0

Non-epithelial tumors or conditions with less than 3 examples in probands/family members: neuroblastoma (1), Wilms tumor (1), Hodgkin disease *(1), non-Hodgkin lymphoma (2), retinal tumors (2), non-RMS sarcoma (2), NF-1 (2)*

Embryonal rhabdomyosarcoma (ERMS): Rhabdomyosarcomatous differentiation is a common morphologic pattern in PPB lung tumors. According to the National Cancer Institute's Surveillance Epidemiology and End Results (SEER) database, the population incidence of rhabdomyosarcoma is 5.1 per million children aged 0-14 years (95% CI=4.6-5.8/10^6). We have already seen four examples of ERMS associated with PPB, three as synchronous primaries in PPB probands and one in an uncle of a proband. Although the strength of association between ERMS and PPB has yet to be estimated, as does the heritability of the PPB syndrome, a preliminary estimate suggests that it is extremely unlikely to see 4 cases in 198 families if the cases are independent.

Lung cysts: Benign-appearing lung cysts are part of the multifocal disease in children with PPB and have also been described in 10 relatives of children with PPB. PPB-associated cysts are distinct from (but often mistaken for) other developmental cysts such as cystic adenomatoid malformation.[5] Pathologic review confirmation of all lung cysts by the P.I. will be critical in efforts to define the syndrome. We believe that PPB-associated cysts seen in family members represent Type I PPBs that have undergone spontaneous regression, lacked the additional genetic insults to progress or alternatively have a different (but related) pathogenesis. Cysts may not be symptomatic but can reliably be detected by CT scans. Routine chest xrays are not sensitive enough to detect small cysts.

Family members with no observable phenotype or those with conditions that did not meet our initial criteria are currently designated as "*unaffected*."

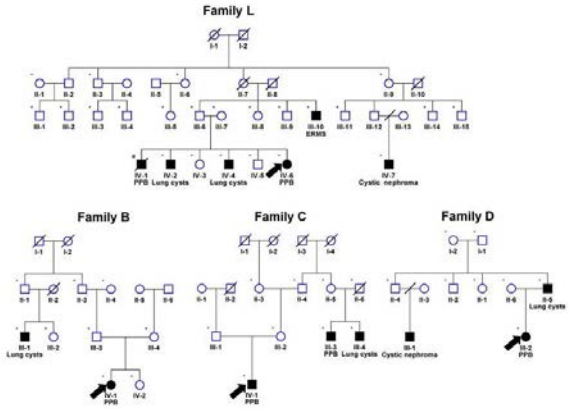
The proposed research in Aim 1 will address these families in a more comprehensive, systematic manner. We anticipate that with this clinical data from approximately 100 families the spectrum of disease will expand to involve additional tumor types (see Table 1) and perhaps additional non-neoplastic medical conditions. This information can then be incorporated into counseling guidelines regarding relative risks for disease and potential preventative care.

Mapping a PPB susceptibility locus: linkage studies

From a screen of the first 62 families enrolled, we identified ten families with apparent inherited predisposition to PPB as evidenced by two or more relatives with PPB, lung cysts, cystic nephroma and/or embryonal rhabdomyosarcoma. We selected four of these families for linkage studies based on the availability of DNA specimens from affected members of the kindreds and family structure (Fig.1). Genotyping was performed using Affymetrix Human SNP arrays version 6.0. A subset of the over 900,000 polymorphic markers represented on the SNP array was selected for linkage analysis based on pairwise measurements of linkage disequilibrium (LD) and estimates of heterozygosity.4[6]

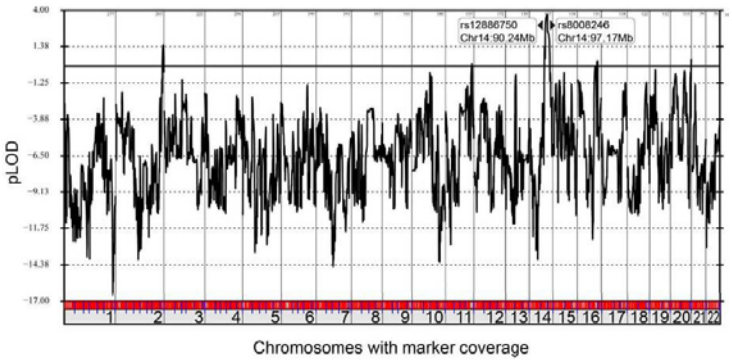
We used Affymetrix 6.0 data from 30 CEPH (Caucasian) families as a reference data set.[46] In short, r^2 was calculated for each pair of adjacent markers. Because marker selection was intended to minimize the use of markers in high LD which may contribute to Type I error, we were conservative with our approach. For marker pairs showing an $r^2>0.1$, the marker with the least heterozygosity was discarded. The method was reiterated sequentially for all markers on each chromosome using a one Mb sliding window. Genotypes for 4117 SNPs selected for linkage analysis were then imported into the easyLINKAGE Plus program v5.08.[47] Markers with call rates <95% (n=281) and any creating Mendelian errors (n=110) were removed from the data set.[48] Multipoint non-parametric and parametric linkage analyses on all four families combined was then performed using the Genehunter v.2.1r5 algorithm.[49;50] The parametric analysis assumed autosomal dominant inheritance and obligate heterozygotes were modeled as unaffected, unknown, and affected. All other individuals were classified as unaffected. We chose to model obligate heterozygotes in this manner to take into account the fact that many lung and kidney cysts in older children and adults may be asymptomatic; the lung cysts in individuals IV-2 and IV-4 from family L were picked up on CT scans done for unrelated pathology. All three of these parametric models yielded similar results; logarithm of odds ratios (LOD score) did not vary by more than 0.3. Penetrance was set at 0, 0.25 and 0.25 for +/+, +/- and -/- genotypes respectively with + indicating wild-type. The disease allele frequency was set at 0.001. Results demonstrated evidence for linkage in a segment of distal chromosome 14q (Fig. 2). RFLP analysis of the markers rs10873449 and rs11160307 using formalinfixed paraffin-embedded (FFPE) tissue from a deceased affected member of family L (Figure 1, individual IV-1) revealed transmission of the allele segregating with disease, further supporting linkage to the 14q region.

Figure 1: PPB Families used for linkage analysis



Probands are indicated by arrows. Individuals with PPB, lung cysts, cystic nephroma or embryonal rhabdomyosarcoma (ERMS) are shown as filled in symbols. Circles represent females. Squares represent males. Symbols with a slash through them indicate deceased individuals. Generations are listed I to IV and individual family members are identified by number. Individuals genotyped for linkage analysis are indicated with an asterisk. Individual IV-1 (#) from Family L, genotypes were determined by RFLP analysis using DNA prepared from FFPE tissue.

Figure 2: Results of genome-wide parametric linkage analysis



Evidence for linkage in a segment of distal chromosome 14q; the maximum multipoint lod score in the parametric analysis when modeling obligate heterozygotes with normal phenotypes as "unaffected" was 3.71. No other interval showed a lod score greater than 1.40. Haplotype analysis for markers spanning the sited of the peak parametric LOD score of 3.71 in 14q31.1-32 region pointed to a 7 Mb interval flankedby rs12886750 and rs8008246.

Identification of mutations in *DICER1* associated with PPB

The candidate region of linkage on distal 14q was further evaluated by creating individual haplotypes using an expanded set of ~ 7000 Affy 6.0 markers from region surrounding the linkage peak. The minimum overlap for the PPB susceptibility locus was then inferred based on recombination events visualized in affected individuals from each of the four families.[51] The approximately 7 Mb candidate region (flanked by rs12886750 and rs8008246) included 72 annotated genes.[52] One gene, *DICER1*, was a particularly compelling candidate because of its known role in organ development, including lung branching morphogenesis, cell cycle control and oncogenesis.[4]

We developed primers to amplify all coding exons and intron-exon boundaries of *DICER1* using Primer3 and the UCSC genome browser PCR module (see appendix).[53;54] We first sequenced germline DNA from the proband from Family L. We identified a single variant, a heterozygous mutation that generates a stop codon Y749X (Fig.3). This stop codon is predicted to result in a protein that is truncated before DICER1's critical functional RNase III domains. The *DICER1*$_{Y749X}$ showed complete linkage to the other affected individuals in Family L. Next the three remaining probands from the linkage families and six other multiplex families were sequenced. The sequence traces were assembled and scanned for variationsusing Sequencher version 4.8 (Gene Codes, Ann Arbor, MI). All variants were confirmed via bidirectional sequencing, confirmed in additional affected individuals within the respective families and queried against the NCBI dbSNP Build 128 database. All 10 study families revealed unique heterozygous *DICER1* germline mutations (Table 2). Five families (including the proband from Family L) had single base substitutions resulting in stop codons. Three families had insertion or deletion mutations resulting in frameshifts. One family had a single base insertion resulting in a stop codon. For each of these nine families, **the predicted mutant protein would be truncated proximal to DICER1's two important carboxy-terminal RNase III functional domains.** Since these domains are responsible for DICER1 cleavage of miRNAs, the loss of these domains would certainly render this truncated protein nonfunctional. The tenth family investigated had a single base substitution in exon 23, resulting in a leucine to arginine change (L1583R) in the region between the two carboxy-terminal RNase III domains (Fig 4). We analyzed this amino acid substitution with the SIFT algorithm which suggested this non-polar to charged amino acid change would not be tolerated.[55] According to the NCBI SNP database Build 128, this sequence variant has not been previously reported. In addition, this missense alteration was absent in 360 cancer-free controls assessed by Pyrosequencing.™

Figure 3: Unique DICER1 sequence alterations present in the probands of each of four families in the linkage study

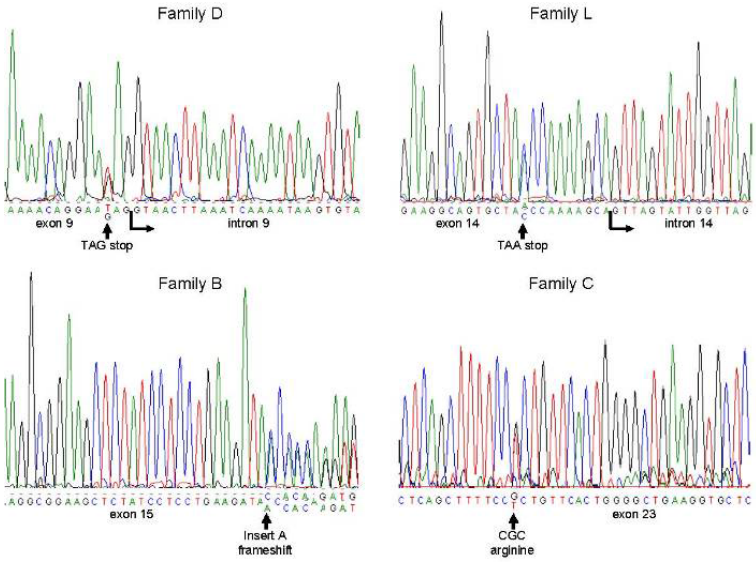
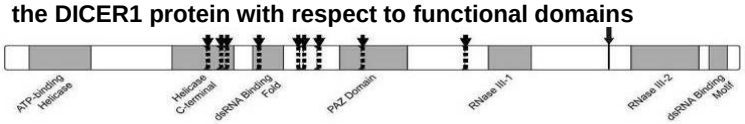


Table 2: Summary of *DICER1* mutations (variants) identified in 10 PPB Families

Family ID	Mutation	Exon	Amino acid change	Mutant RNA	Mutant protein
A	3012C→T	18	R944X	NA	NA
B	2574insA	15	T798Nfs	Reduced	Not detectable
C	4930T→G	23	L1583R	NA	Normal
D	1689G→T	9	E503X	Reduced	ND
E	2092insA	12	Y637X	NA	NA
F	1866-1867delAT	10	M562Vfs	NA	NA
G	2430insTACC	14	P750Lfs	Reduced	ND
H	3722C→A	21	Y1180X	NA	NA
I	1812C→T	10	R544X	NA	NA
L	2429C→A	14	Y749X	Reduced	ND

Figure 4: Schematic drawing of locations of mutations in the DICER1 protein with respect to functional domains

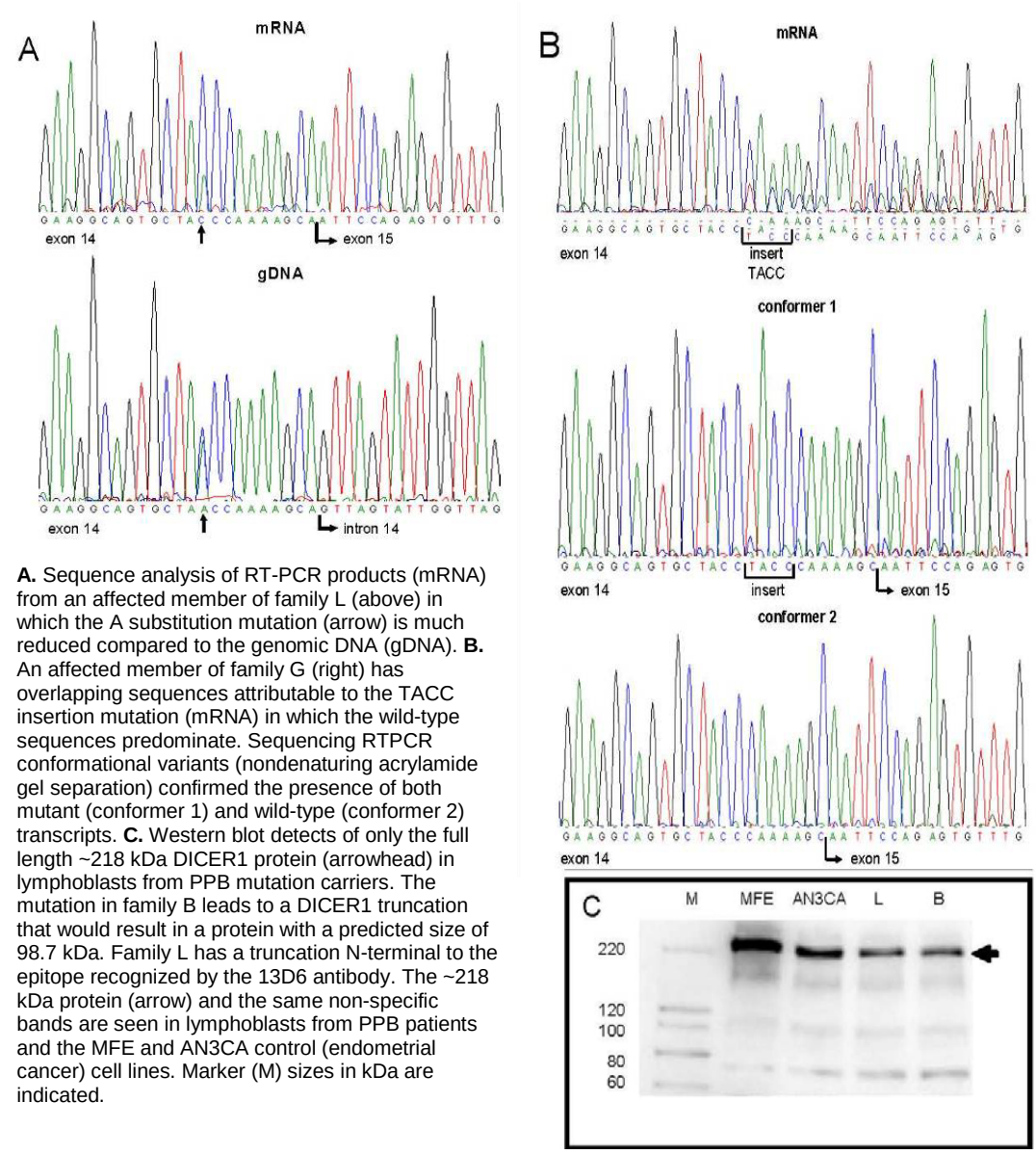


Vertical dotted lines indicate the location of insertion/deletions or truncating mutations in nine families. Each of these occurs before the key RNase III functional domains. The solid line marks the location of the missense mutation predicted to be deleterious in one family.

Levels of mutant mRNA are decreased consistent with nonsense-mediated decay

Lymphoblastoid cell lines were available from affected members from four families expected to produce prematurely truncated proteins. RNA was extracted from these cell lines and RT-PCR was performed to assess regions of family-specific mutations. The resultant products were directly sequenced. The heights of chromatogram peaks of wild-type and mutant RNA were compared to assess relative amounts of each product. RT-PCR of lymphoblastoid cell line mRNA and direct sequencing of PCR products revealed marked reductions in levels of the mutant messages compared to wild-type mRNA, suggestive of nonsense mediated decay.[56;57] The single base substitution (2429C→A) in exon 14 in family B was detectable, but at a low level (Figure 5A). The four base insertion (2430insTACC) mutation seen in exon 14 in family G, represented approximately one-quarter of the *DICER1* transcripts based on relative peak heights (Figure 5B). The significant reduction in mutant mRNA in lymphoblastoid cell lines from the four mutation carriers investigated suggests they may have reduced transcripts in a range of somatic tissues and potentially reduced DICER1 protein levels.

Figure 5: Reduction in mutant mRNA in lymphoblasts from mutation carriers.



A. Sequence analysis of RT-PCR products (mRNA) from an affected member of family L (above) in which the A substitution mutation (arrow) is much reduced compared to the genomic DNA (gDNA). **B.** An affected member of family G (right) has overlapping sequences attributable to the TACC insertion mutation (mRNA) in which the wild-type sequences predominate. Sequencing RTPCR conformational variants (nondenaturing acrylamide gel separation) confirmed the presence of both mutant (conformer 1) and wild-type (conformer 2) transcripts. **C.** Western blot detects of only the full length ~218 kDa DICER1 protein (arrowhead) in lymphoblasts from PPB mutation carriers. The mutation in family B leads to a DICER1 truncation that would result in a protein with a predicted size of 98.7 kDa. Family L has a truncation N-terminal to the epitope recognized by the 13D6 antibody. The ~218 kDa protein (arrow) and the same non-specific bands are seen in lymphoblasts from PPB patients and the MFE and AN3CA control (endometrial cancer) cell lines. Marker (M) sizes in kDa are indicated.

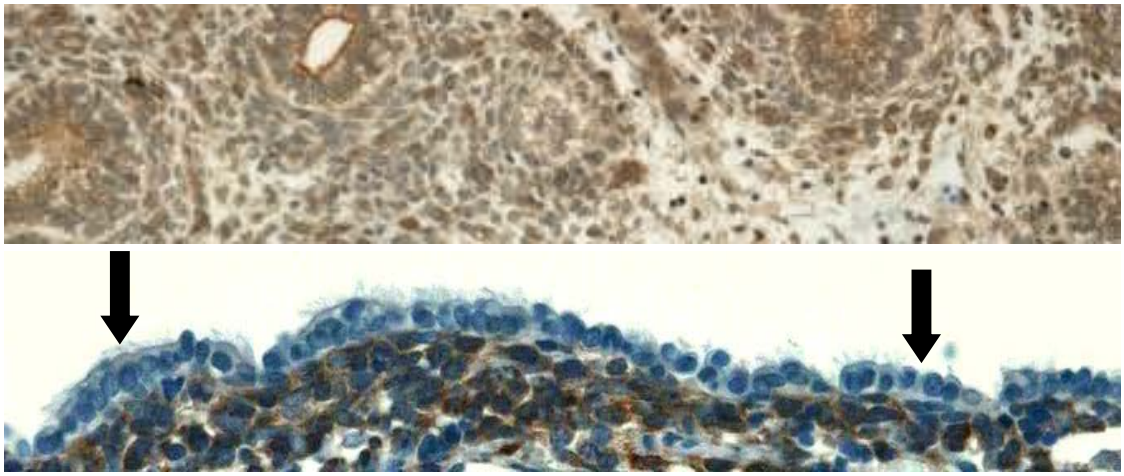
Western blot analysis was performed using an anti-DICER1 N-terminal antibody raised to a peptide from amino acid 749 to amino acid 798 (13D6, Abcam, Cambrige, MA) to determine if the truncated protein was present. Only family B was informative (the other three with cell lines had protein truncations that were more Nterminal than the epitope detected by the 13D6 antibody. As predicted by the RT-PCR analysis, the truncated mutant ~99 KDa protein from proband B was not detectable(Fig.5C).

Preliminary immunohistochemical analysis of DICER1 in PPB tumor tissue

A DICER1 immunohistochemistry assay was developed for use with FFPE samples using a rabbit polyclonal antibody (HPA000694, rabbit anti-human, Sigma-Aldrich, St. Louis, MO). Tissue sections were pretreated using citrate buffer (pH 6.0) and steam heat. Antibody concentrations were titrated using prostate carcinoma and normal lung as controls. We selected a final antibody concentration of 1:75. Sections were stained using a

modified avidin-biotin-peroxidase technique on an automatic stainer.[58] We examined a series of fetal tissues obtained from postmortem examinations. DICER1 appears to be expressed in essentially all organ tissues during early embryogenesis. In lung, DICER1 protein staining is seen in bronchial-alveolar epithelium, mesenchyme, cartilage and vasculature from fetal pseudoglandular (Fig. 6 upper panel), canalicular and saccular stages and in lung epithelium, inflammatory cells and endothelial cells in newborn and adult lung. Tumors from two children with known DICER1 mutations were stained with this antibody (family A, R944X, Type II PPB; family L, Y749X, Type III PPB). Bronchial and alveolar epithelium and endothelium served as positive internal tissue controls. In both cases the mesenchymal tumor cells showed positive staining for DICER1 in the cytoplasm consistent with presence of protein. The Type III PPB from the child in family L did not have a tumor- associated epithelial component in the tumor. The tumor-associated epithelial component from the child in family A showed absence of DICER1 staining consistent with loss of the protein (Fig. 6 lower panel).

Figure 6: DICER1 Immunohistochemistry in fetal lung and DICER1-mutant PPB



Upper Panel. DICER1 staining in normal fetal lung showing cytoplasmic positivity in epithelial and mesenchymal components indicated by brown staining in cytoplasm. **Lower Panel.** DICER1 staining in child with PPB from Family A shows strong cytoplasmic staining of the mesenchymal tumor cells but the ciliated lining epithelium (arrows) is negative. This child has a truncating mutation in one *DICER1* allele. This pattern of staining suggests that the wildtype allele has been lost in the tumor-associated epithelium and is retained in the mesenchyme tumor cells. (Rabbit polyclonal anti-DICER1 with hematoxylin counterstain. Original magnifications x20 (upper); x 40 (lower))

The observed staining pattern, if confirmed in additional patients, suggests that the wild-type allele of DICER1 has been lost in the tumor-associated epithelium but not in the mesenchymal tumor cells and forms the basis of our mechanistic hypothesis on the role of DICER1 in oncogenesis.

HYPOTHESIS:

Loss of *DICER1* (and mature miRNAs) occurs in the *epithelial* component of the tumor leading to dysregulation of one or more "tumor promoting factor(s)" that stimulates mesenchymal proliferation and predisposes the mesenchyme to neoplastic transformation.

The implication inherent in this hypothesis is that **THE GENETIC ABNORMALITY DRIVING TUMORIGENESIS DOES NOT OCCUR IN THE CELLS THAT ULTIMATELY TRANSFORM.** In other words, a genetic event occurring in one cell type induces, promotes or predisposes another cell type to malignant transformation. The work proposed in this application will help test and refine this hypothesis and build the foundation for future mechanistic studies.

RESEARCH DESIGN AND EXPERIMENTAL METHODS

Aim 1: Define the heritability of PPB and further characterize the PPB tumor predisposition syndrome

Overall approach: Our recent work describing inherited *DICER1* mutations associated with predisposition to PPB was based on a small subset of families. This highly focused study resulted in the identification of an important etiologic factor, but many important questions about the clinical features of the syndrome remain. What fraction of PPB patients have inherited disease? What tumors or other medical conditions are directly attributable to *DICER1* mutation? How penetrant are *DICER1* mutations? We will address these questions by systematically and uniformly collecting comprehensive personal medical histories from extended family members from an estimated 100 families (62 families already consented). We will use a questionnaire designed by the research team to obtain past or present history of developmental and anatomic anomalies, cysts, tumors, autoimmune conditions and pregnancy history and review medical records. A better understanding of this syndrome is essential to the development of clinical criteria for identifying these families and for guiding their medical care. In addition, the study of families that show predisposition to PPB represent a unique opportunity to gain a better understanding of cellular processes in the borderland between development and neoplasia and to study how tissue-specific loss of *DICER1* (and the miRNAs it regulates) manifests in human disease.

Overview
We will develop detailed family histories for 100 PPB patients. Three generation pedigrees will be created with medical record verification of reported malignancies (when possible). Biologic specimens (primarily peripheral blood or saliva) will be collected as a source of normal DNA from family members. Archived surgical pathology specimens will be requested for use of a source of genomic DNA for deceased family members. The medical histories generated in this aim will be combined with data from Aim 2 in estimates of penetrance and to better define the expressivity of *DICER1* mutations.

PPB study population and recruitment

Our study population will include 100 children with PPB (probands) and their family members. The study will include a retrospective cohort (children who were diagnosed with PPB before the initiation of the study with potential ascertainment bias) and a prospective cohort (every child diagnosed with PPB since study initiation is approached for study enrollment). The study population will be derived from three sources: 1) The International PPB Registry (IPPBR) at the Children's Hospitals and Clinics of Minnesota (www.ppbregistry.org), 2) treating physicians of children with PPB, and 3) families of children with PPB. The vast majority of cases will be referred by Drs. Jack Priest and Yoav Messinger of the IPPBR, with whom we have long standing scientific collaboration and who are collaborators on this proposal (see letters of support). There are nearly 120 families currently enrolled in the IPPBR and 52 new families have enrolled since 2006. Prior to registry enrollment, confirmation of a PPB diagnosis is made through the registry's centralized pathology review. We recognize treating physicians and families may self-refer to study. Information about our study is available to families via our website, http://ppbstudy.wustl.edu.

Family Recruitment
Dr. Jack Priest (collaborator) will refer all patients currently enrolled in the IPPBR. Dr. Priest has served as the Research Director for the IPPBR since its inception more than 20 years ago and has had direct contact with all patients currently enrolled. Dr. Priest will contact families by telephone or email to inform them of the opportunity to participate. The majority of PPB cases are children and therefore contact is most often with a parent or guardian. For PPB patients or families who are not enrolled with the Registry, Dr. Priest will contact the patients' physicians, by telephone, email or letter seeking his/her assistance in notifying the families about the study and specifically asking the physician to refer the patients to the Registry for more information. In some instances, physicians caring for PPB patients may refer them directly to the family studies proposed in this application and would be encouraged to do so by Dr. Priest. Histologic confirmation of the PPB diagnosis will be performed through the IPPBR for these cases. Once a family has agreed to be contacted about the study, Dr. Priest or the referring physician will notify Jennifer Ivanovich (Co-Investigator) at Washington University School of Medicine with the family contact information.
Ms. Ivanovich will contact the proband and his/her parents to explain the study in detail and obtain verbal

consent for the research. Detailed family medical histories will be obtained for each PPB proband. For probands with a personal history (e.g. second primary) or a family history suggestive of hereditary disease (e.g. three-generation history of cancer, multiple affected relatives, etc) we will invite extended family members to participate in the study. Based on recruitment of the four families used for our linkage analysis (see C.1.3.), we anticipate at least 24 additional family members on average will participate.

Supplemental recruitment
Our collaborating institutions, Washington University School of Medicine and the International PPB Registry at the Children's Hospitals and Clinics of Minnesota conducted two education symposia for PPB families in 2006 and 2008. Over 25 families attended each conference including families who live outside of the United States. These conferences provide an opportunity to inform families of ongoing clinical investigations focused on PPB and to facilitate their participation in these studies. We plan to host our third PPB education symposium in the Summer 2010 in Washington, DC and with immediate recruitment of families into the study.

Development of extended pedigrees

Jennifer Ivanovich (Co-Investigator) will be responsible for the development of detailed three-generation pedigrees for all 100 probands. Most often the initial family history information will be provided by one or both of the probands' parents/guardians. However, grandparents or other family representatives may be asked to provide or clarify additional family history information. Pedigrees will be developed, essentially as was performed for the four families included in the linkage study (see C1.3 figure 1) using information obtained from the written health/family history questionnaires, face-to-face interactions and telephone interviews. Ms. Ivanovich will consult with Alison Whelan (Co-Investigator) to interpret data and set priorities for extended family member enrollment, data and specimen collection. Dr. Paul Goodfellow (Co-Investigator) will meet with Ms. Ivanovich and Dr. Whelan on a monthly basis to review and discuss progress with family history and specimen collection. Dr. Hill (PI) will participate in the monthly meeting by teleconference. Ms. Ivanovich and Dr. Whelan have expertise in both clinical cancer genetics and cancer genetics research and have worked together for more than 15 years (see Ivanovich and Whelan biosketches for examples of clinical and translational joint publications). Dr. Goodfellow has worked with Ms. Ivanovich and Dr. Whelan for more than 15 years (see Goodfellow biosketch) and all three have worked closely with Dr. Hill since 2004 on the genetics of PPB.

All participants (probands and family members) will ask to complete a written health and family history questionnaire, devised as part of our ongoing studies. In brief, the questionnaire addresses each research subject's 1) demographics (including race and ethnicity per NIH guidelines); 2) personal history of cancer including cancer screening, surgical history specifically involving the lungs thyroid, gonads or kidneys (note the focus on organ sites for which are known sites of involvement in the PPB syndrome), 3) past radiographic studies of chest and kidney (to identify presence or absence of conditions that may or may not be clinically apparent, e.g. small lung or kidney cysts); 4) personal history of autoimmune diseases (e.g. Hashimoto's thyroiditis, Crohn's disease, etc…); and 5) location of medical records pertaining to the above. Following receipt of the written questionnaire, Ms. Ivanovich, with the assistance from the Hereditary Cancer Core at the Siteman Cancer Center, Washington University/Barnes-Jewish Hospital in St. Louis, will contact each participant by telephone to verify the reported history. With 15 years experience of family based studies and clinical genetics practice, we have found individuals report more accurate and extensive family and medical history if given the opportunity to complete the questions on their own, before direct questioning by a healthcare professional. Many research families live outside the US, however our experience to date with international study participants makes us confident follow-up phone interviews can be used to reliably verify reported medical and family history information. For participants who do not speak English as their primary language, we will utilize the translator service at Barnes-Jewish Hospital, Washington University School of Medicine. This team can translate 29 languages (www.barnesjewish.org/groups/defaul.asp?NavID=3749). We will request medical records (or death certificates if applicable) for all of the following: lung cysts or disease, any form of cancer, cystic nephroma, benign tumors, polyps, autoimmune diseases, congenital abnormalities, pregnancy losses, developmental or intellectual delay, or for previous relevant surgeries or radiographic studies. Representative histologic slides will be requested from the outside hospitals when applicable, and will be reviewed by Dr. D. Ashley Hill (Principal Investigator) for diagnosis confirmation. Collected pedigree and medical record data will be reviewed on an on-going basis. When appropriate the questionnaire will be revised to capture additional information. The entire research team will meet via

teleconference on a quarterly basis to specifically address what changes in the data collection process should be brought into place. The retrieval of medical records and data entry will be supported by the Hereditary Cancer Core at Siteman Cancer Center. Ms. Ivanovich, in consultation with Dr. Alison Whelan (Co-Investigator), will oversee all requests for medical records to confirm reported disease among participants. The Hereditary Cancer Core has developed an efficient protocol for procuring medical records (see Ivanovich biosketch). To ensure timeliness and uniformity in data collection, research subjects will contacted a second time approximately 18 months after the initial interview for medical follow-up. As we learn more about these families, the second contact will also provide an opportunity to ask questions that are newly relevant to our understanding of the disease.

Medical follow-up
The median age at PPB diagnosis is 38 months. Young children often reflect young families. We recognize the full phenotype of families with an inherited cancer predisposition syndrome may not have expressed itself yet in these young families. We will take advantage of the five year grant period and follow-up with participants 18 months after enrollment. Participants will be contacted by telephone or by mail and asked to complete a one-page medical and family health update. Ms. Ivanovich will oversee the medical follow-up of all participants and the Hereditary Cancer Core at the Siteman Cancer Center will assist with re-contacting participants. Reconsent will not be required. We believe that two separate contacts of research subjects are essential to ensure the most accurate and timely data for subsequent analyses.

Biological specimen collection and banking
For each *proband* enrolled we will request 1) whole blood for DNA extraction and lymphoblastoid cell line generation (Coriell laboratories, Camden, NJ), 2) snap frozen tumor tissue and normal lung tissue, and 3) paraffin blocks. In some cases, saliva samples or buccal swab samples will be obtained for DNA extraction. These samples are obtained using the Oragene collection kits (http://www.oragene.com). For each *family member* enrolled we will request 1) whole blood or saliva or buccal smear for DNA extraction, and for family members with a PPB-related tumor we will also request 2) histology slides, and 3) paraffin blocks. The collection of biological specimens is arranged at the time of history ascertainment. Kits containing consent and medical release forms, medical/family health questionnaires, as well as UN3373 approved specimen shipping containers with two ACD tubes per family member (three for the proband), labeled Federal Express UN3373 Pak and instructions for collection, billing and mailing are prepared and sent to families. Participants arrange for phlebotomy services with their physicians. Biologic and pathology specimens will be banked and stored at Children's National Medical Center. *DICER 1* mutation (Aim 2) and tumor analyses (Aim 3) will be performed at Children's National Medical Center.

Data Management
We utilize a relational database to store all clinical and molecular data (ProgenyLab2000, Progeny Software, South Bend, IN). Each participant is assigned a code number. Code numbers will be used to link the medical and family history data collected as part of the extended pedigree development, and molecular studies conducted in Aim 2. A master list linking the code numbers and each participant's identity will be kept separate from the research data. The master list will be kept in a locked file and only the P.I. and designated members of the research team will have access. Only names of family members consented to the study will beenteredinto the master list. Other family members will only be identified by their study number, relationship to the proband, age, and gender.

Defining the heritability of PPB
These studies will be entirely descriptive, documenting disease in probands and their relatives in a cohort unselected for family history or multifocal disease in the proband. We will determine if PPB-related tumors (PPB, lung cysts, cystic nephroma and embryonal rhabdomyosarcoma) AND other neoplasms are significantly over-represented in first- , second-, and third-degree relatives, noting the relationship to the proband.

Potential problems and alternate strategies
Ensuring adequate family accrual
Although this is a rare tumor, we believe we have the infrastructure to accrue the necessary patients and specimens to accomplish the aims described in this proposal. Our efforts at education and outreach to families

and treating physicians as well as a new treatment protocol are described in preliminary data (C.1.1). Five years should be sufficient time to enroll approximately 100 families (62 already enrolled) and analyze the data.

Development of extended pedigrees
We do not anticipate insurmountable problems with the collection of clinical and family history information from individuals. Ms. Ivanovich, Drs. Goodfellow, and Whelan have extensive experience in data collection for similar family-based studies. Based on previous work by our group and others, we recognize retrieval of all medical records or death certificates when applicable will not be feasible.[41-45] Pedigree family history information will be noted as "confirmed" or "not confirmed", thus allowing for separate analysis in the future. Utilization of a written medical and family history questionnaire followed by direct telephone contact from the research team will assist in the procurement of relevant information and ensure efficient and consistent data acquisition.

Aim 2: Determine *DICER1's role in PPB predisposition: DICER1* mutation analysis for approximately 100 PPB cases and evaluation of other candidate genes.

Overall Approach: Our preliminary studies have demonstrated germline mutations in *DICER1* in family members from 10 different families showing susceptibility to PPB providing a convincing argument that DICER1 has an important role in the pathogenesis of this disease. Our analyses have, however, been focused on families with multiple affected individuals. The frequency of *DICER1* mutations in PPB cases without familial disease in unknown. Do isolated cases of PPB (sporadic) with multiple lung cysts carry germline mutations in *DICER1* and if so, how often are these molecular defects *de novo* mutations? The penetrance and expressivity of *DICER1* mutations is also yet to be determined.

In this aim we will test 100 PPB children with PPB for germline *DICER1* alterations. For each proband with a *DICER1* mutation, we will test parents, siblings, grandparents and more distantly related members for the presence of family-specific mutations. The mutational status will be used to measure the rate of new mutations, estimate the penetrance of mutations (relying on the family and medical history developed in Aim 1) and to better define the expressivity. This information will facilitate implementation of gene-based testing and will help further refine the information used to counsel these families. Individuals and families for whom we exclude a role for *DICER1* aberrations then become an additional cohort for sequencing of other miRNA processing genes such as *DROSHA*, *DGCR8, Exportin-5* or for new linkage/association studies.

***DICER1* mutation analysis**

Direct sequencing
We will use direct sequencing to identify *DICER1* mutations, essentially as described in C.1.4. Genomic DNA will be prepared from peripheral blood leukocytes or buccal mucosal cells from PPB probands. PCR amplicons for all 27 exons including intron-exon boundaries (see Table 3 below for primer sequences) will be sequenced using BigDye Terminator chemistry (v3.1 Applied Biosystems, Valencia CA) and an ABI3730 sequencer (Applied Biosystems). Sequence traces will be assembled and scanned for variation against the reference sequence using Sequencher version 4.8 (Gene Codes, Ann Arbor, MI). We have tested all these primer sets and they produce robust and complete bi-directional sequencing of the exons and intron-exon junction sequences. Three amplicons (exons 7, 15 and 20) have polymorphic mononucleotide repeats in intronic sequences which results in overlapping sequence peaks in heterozygotes. Additional assays for these three exons that will afford complete double pass (forward and reverse) sequencing are being devised. For these exons, sequence variants not resolved with the alternative amplicon will be evaluated by TA cloning and sequencing single molecules. All variants are queried against the NCBI dbSNP Build 128 database. For all missense changes, PyrosequencingTM assays will be designed for any non-synonomous variants identified. These assays will be used to interrogate ~550 cancer-free controls (http://www.siteman.wustl.edu/internal.aspx?id=2570). The potential signficiance of missense variants will also be evaluated using the SIFT algorithm.[55]

Table 3. Gene-specific primers for *DICER1* Sequencing of all coding exons and intron-exon boundaries.

NAME	LEFT_PRIMER	RIGHT_PRIMER	PRODUCT_SIZE
Exon1*	AATCACAGGCTCGCTCTCAT	GTCTCCACCTCCGCTGCT	762
Exon2	TCAAATCCAATTACCCAGCAG	GCAATGAAAGAAACACTGGATG	358
Exon3	TCTGCCAGAAGAGATTAAATGAG	TTTTGTAAATTTATTGGAGGACG	429
Exon4	AAATCAGACAACCAAGGCTACAG	TTTTGGAGGATAACCTTGGAAC	390
Exon5	TTTAATATTCATTCATTCATACACTG	TTGTCGTCAAGACATGCTTTC	518
Exon6	GAATTCTTACTCTTGCCCATTCC	TAGTGGCATTTCCACCAAAC	437
Exon7	GAGCCGCATTAAGCATATTTTC	CCCACTGCTAACATTCTGGC	395
Exon8	TCACATCACAACACAGGACG	AAATCCCAGTTAAACCCCAC	614
Exon9	AAATCACTCTACAGCTACCTCATGG	TAAATCACCGTCGCCAAATC	820
Exon10	TTCCTATGGATACAAAGAATAACAAAG	CATGTGTGTCAGAAATGACAGTTG	431
Exon11	AACTTTTATTGCTGCACGATACTG	AGCAGGTTACTTTGGAGTACTGAAG	760
Exon12	TGAACATGTAGATGACTACAAAAGC	TCACATTTCAAGTGCTCACC	777
Exon13	AAGTGTTCATGGTGCATGATTC	TTTTACTAGGCAGGACTTTTAAAGATG	585
Exon14	AAGCTGTGAATCGGAGAAAG	TTTGCAGTCCAGCTCATATTG	760
Exon15	TCTAGTGGAGAAATAGAAGAGGCAC	TAAGAAGTGTCATGCCTCGG	468
Exon16-17	TTTTAGTAGAGACGAGGTTTCACC	GAAAGCATCATTTCTGTTCTGAAG	754
Exon18	TTTGTGTGCAAAGCATCTCC	TGTAAAGGTGCCATTTAGCTTC	589
Exon19	TTTGTGATATATTAATGGGCCAAG	ATTGCACTTGAGGGATTCTTACC	582
Exon20	TCTCACTCCAACTGTTATGGCTTA	TTGGCCCATTAATATATCACA	776
Exon21_1	GAGTACATTCATCGCTGGGC	AATTGCTGTTGCTCTCAGCC	508
Exon21_2	ACTGCAAACCACTTTCAGGC	ACAAGCAGGAAATACCCGTG	501
Exon22	AGAAATTTGCCTCCATCAAA	AAAGCATAGAATATGTGGGAATT	725
Exon23_1	CAGGGCTTCCACACAGTCC	AACCCTTGCTTTTATTGAGTTTC	574
Exon23_2	TACAAGGCCAACACGATGAG	AAACTGTGGTGTTGACACGG	571
Exon24	TGCCGTCAGAACTCTGAAAC	TGTGGGGATAGTGTAAATGCTTC	403
Exon25-26	TGAACTTTTCCCCTTTGATG	TGGACTGCCTGTAAAAGTGG	450
Exon27	TCTGCCTTCAATTCATTCCA	CCTGTCTGTCGGGGGTATG	448

*plus 1.3M Betaine
All primer sequences are listed 5' to 3'.
M13 primers were appended to the 5' ends of the DICER1 specific sequences above to facilitate sequencing.
We used Taq polymerase and 1.5 ul of each primer (10 mmol dilution) in total reaction volume of 50 ul.

The following cycling conditions were used: 95° 5 min then 14 cycles {94°. 30 sec, 63°. 45 sec, 70° 45 sec} then 20 cycles {94°: 30 sec. 56° 45 sec. 70° 45 sec} then 70° 10 min. hold at 4°

All sequence variants will be classified based on predicted effects on the DICER1 protein. Stop and frameshifts resulting in loss of one or more functional domains will be considered deleterious mutations. Missense changes will be categorized as *probable deleterious* mutations when several of the following are seen: absence of sequence variant in normal controls; co-segregation with disease in families; SIFT intolerant change in a conserved residue.

Deletion/insertion analysis

Multiplex Ligation-Dependent Probe Amplification

Deletions undetectable by conventional PCR sequencing approaches are seen in a variety of cancer susceptibility syndromes including hereditary nonpolyposis colorectal cancer, familial adenomatous polyposis, hereditary diffuse gastric cancer and familial breast cancer. For PPB probands in which direct sequencing fails to identify mutations, we will test for *DICER1* deletions or insertions using multiplex ligation-dependent probe amplification (MLPA) methods.59-61 MLPA is a PCR based technique for identifying gene dosage alterations. We will use MLPA and ABI GeneMapper 4.0 software, essentially as has been developed for clinical use for BRCA1, BRCA2, MMR gene testing.60 Given *DICER1* is a large gene (27 exons), we expect we will need to devise 3-4 independent MLPA sets, each including representative extragenic regions used for ligation and PCR monitoring controls. The FAM-labeled primers will be designed to amplify fragments that can be distinguished by size on a polyacrylamide sequencing gel and so that all PCR reactions have a similar optimal amplification condition. Serial DNA dilutions will be used to optimize amplification conditions for multiplex PCR so that the amplification product is linear to the template concentration and thus will reflect a 2-fold difference

in template concentration. Any exon with a change (decrease or increase) in peak height of 70% will be classified as a deletion or duplication, respectively. Each multiplex PCR indicating a *DICER1* deletion will be confirmed by Southern blotting, if sufficient DNA is available, or in a second independent multiplex PCR reaction as well as by comparative genomic hybridization (CGH) see below.

Deletion of one or more exons and/or insertions that disrupt the *DICER1* reading frame will be classified as mutations.

Comparative genomic hybridization (CGH) microarrays
A CGH array strategy will be used to confirm deletions/insertions identified by MLPA, to identify the location of deletions not revealed by MLPA, and to map the breakpoints. We will design a custom CGH array for the region including *DICER1* on chromosome 14q using Agilent's eArray software (https://earray.chem.agilent.com/earray/index.jsp). Probes for the array are selected from the Agilent HD CGH Database Catalog. In brief, CGH will be performed according to the recommended guidelines. High molecular weight genomic DNA (1.5 µg) will be labeled using Agilent Genomic DNA Labeling Kit PLUS (Agilent Technologies, Santa Clara, CA). DNA samples will be labeled with Cy3 and co-hybridized with Cy5 labeled gender-matched normal reference gDNA to the *DICER1* CGH microarrays. Washing will be performed using Agilent CGH wash buffers and the arrays scanned on an Agilent DNA Microarray Scanner. Image analysis will rely on Agilent Feature Extraction (version 8.5) and data will be analyzed using Agilent CGH software. To take advantage of the economy of scale, this assay will be performed at the end of year 2 for all *DICER1* mutationnegative samples.

The CGH analyses will serve to confirm the results of MLPA and may identify alterations not revealed using the MLPA method. Furthermore, the breakpoints for insertions/deletions will be defined by the CGH studies which could then be used to devise mutation-specific PCR assays (long-range PCR). Such PCR-based tests for deletions or insertions would then be used to test family members for mutations as we begin to determine the penetrance and expressivity of inherited *DICER1* mutations.

Defining the penetrance and expressivity of *DICER1* mutations in PPB families

Our efforts to assess penetrance and expressivity of *DICER1* mutations bring together the genotypic characterization of relatives (*DICER1* mutation status) and the comprehensive family and medical histories developed in Aim 1. For each proband for whom a *DICER1* variant with a likely functional consequence (presumed mutation) is identified, we will test relatives for the presence of the same defect. We expect that for most families, direct sequencing of the exon of interest will be the assay of choice. First, the biologic parents will be tested for the presence of the mutations. If neither parent carries the variant, the proband will be classified as having a new (*de novo*) mutation. The biologic relationship of the proband and parents will be confirmed using highly polymorphic microsatellite markers (to exclude non-paternity or sample mixups). The new mutation rate can be calculated once all probands have been evaluated fully.

When one parent carries the variant, then we will proceed with typing all blood relatives of that parent enrolled in the study. Sequence data will then be correlated with medical history data obtained in Aim 1 to determine penetrance and expressivity.

Penetrance will be estimated by assessing the fraction of individuals who have a *DICER1* mutation and manifest conditions clinically associated with the syndrome. The most stringent measure of penetrance will be the fraction of individuals who have a *DICER1* mutation and manifest PPB, lung cysts, cystic nephroma or rhabdomyosarcoma. We realize this clinical definition of syndromic disease may be too stringent, and for that reason we will have a second measure of penetrance that includes the conditions above as well as gonadal stromal tumors and nasal chondromesenchymal hamartoma which based on data obtained since the linkage study we believe are also part of the syndrome.

Tumor type
PPB
Lung cysts Cystic nephroma
Rhabdomyosarcoma
Gonadal stromal tumor
Nasal chondromesenchymal hamartoma
Others

Expressivity will be defined by assessing the phenotypic manifestation of *DICER1* mutations within the kindreds. We will determine what manifestations of disease (medical record confirmed and considered as distinct entities) develop in family members carrying the mutations or in high risk family members (first and second-degree relatives with unknown mutation status). The most stringent measure of expressivity is the demonstration that clinical abnormalities develop in carriers of mutations and manifest as a phenotype consistent with the PPB susceptibility syndrome (PPB, lung cysts, cystic nephroma, rhabdomyosarcoma). As with penetrance we will also have a second measure of expressivity which includes the above conditions as well as gonadal stromal tumors, juvenile polyps and nasal chondromesenchymal hamartoma. As the family studies progress in Aim 1 we may learn of other tumors or medical conditions that appear to be overrepresented in PPB families and these would then be considered in studies of expressivity.

Sequence analysis of candidate genes in miRNA biogenesis pathway

For PPB probands for which *DICER1* mutations are not identified (D.2.1 above), we will investigate other candidates for germline mutations, focusing on genes known to be important in miRNA processing . The genes that will be investigated include, but are not limited to, *DROSHA*, *DGCR8 and EXPORTIN-5.* As our understanding of PPB, lung tumorigenesis and miRNA biology evolves, additional candidate genes and pathways are likely to emerge. The scope of the work in this subaim depends entirely on what fraction of PPB cases *do not* harbor *DICER1* mutations (genetic heterogeneity) and what fraction is attributable to somatic (acquired) *DICER1* or other gene defects. Similar to our approach with *DICER1*, we can perform standard sequencing and genomic analysis for these genes.

Potential problems and alternate strategies

Mutation analysis: Next generation sequencing and oligo-arrayed based methods for deletions of all classes of mutations are evolving rapidly. We will consider new methods for mutation detection as they are developed and validated. Nonetheless, we believe the tried and true approaches proposed above will allow us to effectively assess the role *DICER1* plays in PPB susceptibility and be more readily converted into tests for clinical use. We cannot exclude the possibility that inherited or acquired epimutations might explain some PPB (family and sporadic, respectively). Assessing epigenetic etiology is beyond the scope of this proposal.
Ascertainment bias: We realize selection bias toward more severely affected individuals in a study population derived primarily from a clinical registry is possible because these individuals (or families) might be more likely to seek out research studies. These factors should be considered in the broad application of heritability, penetrance and expressivity calculations. With the inherent nature of PPB as a rare and deadly pediatric cancer, we believe that a bias toward enrollment of *familial* cases in the International PPB Registry may be more theoretical than real. In the initial report of 11 cases, two were siblings and the percent of obviously familial cases enrolled in the Registry has not significantly varied from around 20% in the period since. Families with PPB and physicians caring for families of PPB approach the Registry physicians at the time of PPB diagnosis for information regarding treatment and prognosis (www.ppbregistry.org). In our experience it is rare for a family to decline enrollment. Another reasonable concern is that the Registry is biased toward cases in advanced stages as those would be most in need of treatment recommendations. This was almost certainly true in the first 15 years of the Registry when only 16% of cases enrolled were Type I PPB. With focused education of pediatric pathologists by Registry pathologists Hill (P.I.) and Dehner (refs) on the pathologic diagnosis of PPB in the early stages as well as the controversy over management of these early cases, Type I PPBs now represent the most common type at enrollment pathologic review (42% in 2008). If or how the distribution of cases in the various stages would affect calculations of penetrance and expressivity cannot be estimated. Regardless, this is a rare tumor and a population-based series, which would address the question of a bias, due to the rarity of *DICER1* gene mutations, would be logistically challenging and costly.
Expressivity analysis: We realize that assessing the expressivity of *DICER1* mutations might be problematic because the population of *DICER1* gene mutation carriers might not be large enough to consider standard incidence ratio (SIR) calculations. We will therefore simply list and count the phenotypic features present in gene mutation carriers. Data will provide important descriptive characterization of the clinicopathologic manifestations in *DICER1* mutation carriers and provide a foundation for future biology studies.

Aim 3: Determine *DICER1's* role in PPB initiation and evaluate potential mechanisms of Oncogenesis

Overall approach: It is not known how haploinsufficiency for DICER1 contributes to PPB susceptibility. Animal models haploinsufficient for DICER1 and the human PPB families provide compelling evidence that a single copy of *DICER1* is sufficient for normal development.[4;62] Conditional knock-out of Dicer1 in embryonic mouse lung epithelium produces a PPB-like cystic phenotype.[4] We hypothesize that **1) Development of PPB, and related tumors seen in these families requires a second genetic "hit" namely, somatic inactivation of the wild-type *DICER1* allele** and **2) Loss of *DICER1* (and mature miRNAs) occurs in the *epithelial* component of the tumor leading to dysregulation of "tumor promoting factor(s)" that stimulate mesenchymal proliferation and predispose to neoplastic transformation.** We will use immunohistochemistry to evaluate DICER1 expression in both tumor-associated epithelial and mesenchymal components. These results will guide subsequent somatic mutation studies of *DICER1*. Finally we will assess the gene expression profiles in Type III PPB to identify signatures associated with this aggressive form of PPB.

Immunohistochemical evaluation of DICER1 protein expression in PPB

DICER1 immunohistochemistry (IHC)
We will first assess DICER1 expression in PPB tumors using immunohistochemistry (IHC). To properly evaluate our hypothesis by immunohistochemistry we must evaluate DICER1 protein staining in tumor-associated epithelium as well as the mesenchymal tumor cells. Thus our efforts will begin with early stage tumors (Type I and II PPB) that typically include residual tumor-associated epithelial elements. Patterns of DICER1 staining in the epithelial and mesenchymal components of Type I and II tumors should shed light on whether DICER1 is lost in either or both cell types and will help guide the molecular studies testing for somatic loss of DICER1 as outlined in "Test for somatic *DICER1* mutations" below.

Methods for DICER1 immunohistochemistry for formalin-fixed, paraffin embedded (FFPE) PPB samples tumors have already been established (Prelim Studies C.1.6., fig. 6). In brief, we will use the rabbit polyclonal DICER1 antibody (HPA000694, Sigma-Aldrich, St. Louis, MO) and polymer-based detection of signal. All IHC will be performed using a Bond Immunohistochemistry system (Leica). Prostate carcinoma and normal lung will be used as positive controls for each experiment. Negative (no antibody) tumor tissue controls will be run in parallel (when sufficient tumor material is available).

Scoring and interpretation of DICER1 antibody staining
DICER1 antibody staining will be reviewed using standard light microscopy. Stained sections will be scored using a semi-quantitative scoring system from 0 to 3+ with 0 = no staining; 1+ = weak; 2+ = moderate; 3+ = strong. Scoring will be done on tumor-associated epithelium and mesenchymal tumor cells separately. The presence of nuclear staining will be recorded when noted.

If our hypothesis is correct, in children who have germline *DICER1* mutations (identified in Aim 2) we expect to see loss of DICER1 staining in cystic non-malignant epithelium and retention in the mesenchymal tumor cells as seen in our pilot case (Fig. 6, prelim. data).

Test for somatic *DICER1* mutations in PPB tumor cells and tumor-associated epithelium

Type I and II PPB tumors from patients with germ-line DICER1 mutations
Tumors from patients with known germ-line *DICER1* mutations will be evaluated for 'second hit' (somatic) *DICER1* mutations in tumor tissue. Because we propose that loss of the wild-type allele in epithelial cells is responsible for the early pathogenesis of PPB, we will first focus on Type I and II PPB tumors as these types will have tumor-associated epithelium for evaluation. We anticipate that we will demonstrate DICER1 somatic mutations in tumor epithelium but not in mesenchyme from the same tumor.

Type I and II PPB tumors from patients without demonstrable germ-line DICER1 mutations
For tumors from patients without demonstrable germ-line DICER1 mutations, but IHC studies suggest a role for *DICER1* (absence of immunodectable protein in either epithelium or mesenchyme or both), we will also evaluate these tumors for somatic *DICER1* mutations.

Type III PPB tumors with or without demonstrable germ-line DICER1 mutations
We will also test for *DICER1* somatic aberrations in tumor cells from approximately 20 Type III PPBs (composed solely of mesenchymally-derived cells) using the sequencing and deletion assays described in Aim 2. We will use a combination of tumors from known *DICER1* mutation carriers and from patients for whom no *DICER1* germline mutations were identified. If our hypothesis is correct, we expect that we will not find somatic *DICER1* mutations in mesenchymal tumor tissue. If our hypothesis is incorrect, and *DICER1* loss in mesenchyme is the key initiating factor, then we would identify somatic mutations in these cases with this approach.

Laser capture microdissection

Tumor DNAs will be prepared from laser-dissected (LCM) formalin-fixed tissues. Both the mesenchymal and epithelial compoments of tumors will be isolated by LCM and analyzed separately (Fig. 7). Tissues will be assessed both for deletion (loss of heterozygosity) at the *DICER1* locus and/or for point mutations or small insertions/deletions. Sequential FFPE sections will be cut at 5 microns thickness. Sections will be lightly stained with hematoxylin so as to visualize epithelial and mesenchymal components. Using an Arcturus Laser Capture system pure epithelial and pure mesenchymal tumor cells will be dissected and transferred into sterile Eppendorf tubes separately. DNA will be extracted using Recoverall™ (Applied Biosystems, Foster City, CA).

Fig 7. Example of DICER1 IHC-guided mutation analysis. The type I tumor arising in a child with an exon 18 mutation (R944X) shows absence of DICER1 in the epithelium (arrow head) but not in the neoplastic mesenchyme (arrow). IHC staining will be used to identify areas of negative staining for DICER1 protein. Parallel sections stained lightly with hematoxylin will direct LCM and the DICER1 IHC-positive and IHCnegative tissues will be analyzed separately.

LOH studies

Direct sequencing of exons carrying mutations

The first and the most straightforward analysis for loss of heterozygosity will be to analyze the tumor tissues in patients with known protein-truncating inherited *DICER1* mutations for loss of the wild-type *DICER1* allele by sequencing (cases described in Hill et al. submitted and identified in Aim 2). 125-200bp amplicons (appropriate for analysis of DNAs prepared from fixed tissues) spanning family specific mutations will be devised. DNA will be extracted from separate aliquots of epithelial cells and mesenchymal cells obtained by LCM. Following PCR, the products will be directly sequenced. Absent or reduced wild-type sequences will be taken as evidence of a deletion second hit. We expect the cell types that lack DICER1 staining will show loss of the wild-type allele whereas the tumor tissues showing positive staining for DICER1 will not. If we find two hits (germline and somatic mutation) that are identified in only in epithelium and never in the neoplastic stroma we will conclude the primary *DICER1* effect in PPB is epithelial.

If we encounter cases of sporadic PPB with absent DICER1 staining by IHC but with *no* germline mutation by mutation analysis in Aim 2) we will search for tumor cell somatic deletion mutations in *DICER1* using two different methods: Taqman SNP genotyping assays and fluorescence *in situ* hybridization (FISH). Both of these techniques are applicable to FFPE tissues and build on the PI's clinical and research expertise in diagnostic pathology. FISH has the advantage of visualization of both copies or loss of one copy directly in the cell type(s) of interest. If we see homozygosity of a series of SNPs across the DICER1 locus or loss of one or both copies of *DICER1* by FISH, we will conclude that *DICER1* loss may play a role is pathogenesis of that tumor.

Taqman SNP genotyping assays

24 pre-validated SNPs with a minor allele frequencies of > 35% were selected using SNPbrowser software (Applied Biosystems, Foster City, CA) to cover an approximately 1 Mb region surrounding the *DICER1* locus will be used to compare genotypes in microdissected epithelial tissue with microdissected mesenchymal tumor tissue from the same tumor specimen. First, the normal cellular DNA will from each proband will evaluated to determine which markers are informative (heterozygous). 1 to 5 ng of DNA will be mixed with Taqman universal master mix and differentially labeled forward and reverse primers for one or more informative prevalidated SNP alleles. PCR reactions are run on a Taqman system. Allelic discrimation reads are visually inspected. Loss of heterozygosity will be determined by reviewing homozygous allele type calls (genotypes) for the segment covered by the SNPs and comparing genotypes between epithelial and mesenchymal tissues.

Fluorescence in situ hybridization (FISH)

Tumor sections 5-6 µm thick will be cut onto poly L-lysine coated slides. Tissue sections will be deparaffinized and then pre-treated in a steamer with citrate buffer for 20 minutes followed by a 20 minute cool down period and a 5 minute distilled water wash. The sections will then be digested using Pepsin (4mg/ml) at 37° C for 15-30 minutes, washed in 2X SSC, and allowed to air dry. BAC fluorescent labeled, locus specific probes for the DICER1 region will be used for FISH (Empire genomics). Probes are prepared using DenHyb buffer (Insitus Laboratories, Albuquerque, NM, USA) at dilutions of 1:100. The hybridization mix (10-20 µl per slide) will then be applied to the sections, followed by simultaneous denaturing of probe and target at 90° C for 13 minutes. Hybridization is performed overnight at 50° C in a humidified chamber. After a series of post-hybridization washes in 50% formamide/2X SSC (3 minutes at room temperature) and 2X SSC (2 minutes at room temperature), the sections are allowed to air dry and then stained with DAPI nuclear stain (0.2 µl/ml) (Insitus Laboratories, Albuquerque, NM, USA). Slides are viewed with a Leica fluorescent microscope with appropriate filters. Each case will first be evaluated for hybridization efficiency and tissue quality. If both are adequate (>90% nuclei with signals), 100-300 non-overlapping, intact nuclei were scored for the number of fluorescent signals with data collected for the percentage of nuclei harboring 0 - 4 hybridization signals. Non-neoplastic tissue will be used as normal controls (2 signals per nucleus) with each assay. Cases will be considered deleted for DICER1 when > 30% (median +2 standard deviations) of nuclei harbor one signal.

Tumor sequencing

For cases with known germline DICER1 mutations but apparent retention of the wild-type allele by SNP allelic discrimination or FISH, we will perform sequencing of a limited number of exons. Templates prepared from microdissected formalin-fixed tissues are often less reliable due to DNA degradation. DNA quality is related to the length of time tissues were fixed, and the age of the blocks. The *DICER1* primers sequences used successfully to identify mutations in high molecular weight DNA yield amplimers of 350-820bp (submitted manuscript).

Because we will be analyzing DNAs from fixed tissues it will be necessary to design additional sets of primers to amplify smaller products for exons (<225bp). This will be a daunting task for a gene as large as *DICER1*. We will therefore prioritize the exons investigated based on the observed frequency of mutations (work in Aim 2). From our first 10 familial cases analyzed, 4 of the 10 mutations were seen in two exons (10 and 14, two mutations each). As analyses proceed we may find there are mutational hotspots on which we will focus our analyses.

We will also test for *DICER1* somatic aberrations in tumor cells from approximately 20 frozen Type III PPBs (composed solely of mesenchymally-derived cells) using the sequencing and deletion assays described in Aim 2. Will we use a combination of tumors from known DICER1 mutation carriers and from patients for whom no germline mutations were identified. We expect that we will not find somatic mutations in these purely mesenchymal tumors which would lend further credence to our model in which DICER1 defects occur exclusively in the epithelium

Investigating the PPB transcriptome for common pathways of PPB progression

We are proposing that dysregulation of a "tumor promoting factor(s)" from the epithelium stimulates mesenchymal proliferation and as a secondary effect, deregulated growth of the mesenchyme increases susceptibility to additional genetic insults and neoplastic transformation. Given that lung epithelium has an important role in stimulating rapid mesenchymal proliferation during development (to help grow the lung), it is reasonable to expect that the dysregulation will involve a known growth factor (Fig. 8). Although we could test for activation of these pathways individually, we believe the downstream effects of this growth factor may be more readily reflected in the gene expression profile of mesenchymal tumor cells and with an expression array we would also be able to evaluate how homogeneous or heterogenous these tumors are. It is beyond the scope of this proposal to evaluate if any of these growth factors demonstrate regulation by miRNAs but this would be an important next step (if the hypothesis holds to be true). Mouse models of conditional *DICER1* inactivation in lung epithelium, preferably with the ability to induce loss of *DICER1* at different timepoints, will likely be required to work out and confirm the specific targets involved (see future directions).

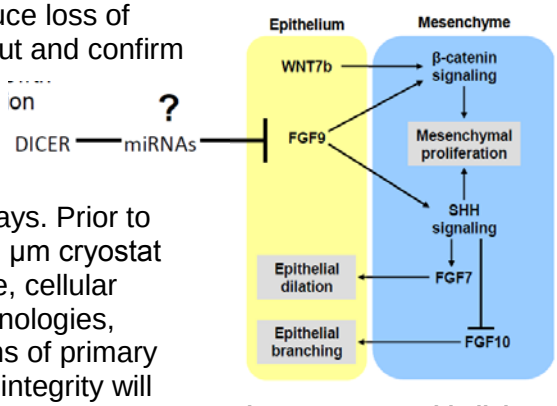


Figure 8: Lung epithelial-mesenchymal signaling. Epithelial expression of WNT7b and FGF9 induce β-catenin and SHH signaling that results in mesenchymal proliferation. Subsequent mesenchymal expression of FGFs in turn regulates epithelial airway branching and luminal dilation (adapted from [63-65])

Affy expression arrays and data analysis

Blocks of snap frozen tumor tissue are used for expression arrays. Prior to RNA extraction, tumor content and quality is assessed with a 5 µm cryostat section. All cases were required to contain >80% volume viable, cellular tumor. RNA will be extracted using Trizol (GibcoBRL Life Technologies, Gaithersburg, MD) from 5, 10 micrometer thick cryostat sections of primary tumors yielding 1 to 3 microgram total RNA. RNA quantity and integrity will be assessed by electrophoresis using the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). cDNA and cRNA will be prepared and analyzed using GeneChip Human Gene 1.0 ST arrays (Affymetrix Incorporated, Santa Clara, CA) according to manufacturer's recommendations. Bioinformatic support of the data analysis will be provided by the genomic core at Children's National Medical Center directed by Eric Hoffman (see biosketch and letter of support).

Potential problems and alternate strategies

Significant technical problems with IHC, PCR, FISH are not anticipated as these techniques are routinely performed in our laboratory. For immunohistochemistsry, there are reasons why certain types of cases would not be informative as to loss of *DICER1* gene and these are listed in the table below.

DICER1 function is lost in tumor-associated epithelium but staining for DICER1 protein is seen	DICER1 function is not lost and DICER1 protein is seen
The inherited germline mutation is missense rather than nonsense and the mutant protein is stable.	The wild-type copy of *DICER1* is *not* lost or mutated; phenotype results from haploinsufficiency.
The inherited germline mutation is nonsense but the truncated protein is made and is not truncated proximal to the antigen binding site.	The wild-type copy of *DICER1* is lost or mutated in the tumor cells but not the epithelium as predicted.
The wild-type copy of *DICER1* is rendered non-functional by a missense mutation ratherthan a deletion or nonsense mutation with resultant decay.	A gene other than *DICER1* is involved in a given child/family
The clone of epithelial cells that lose *DICER1* and initiate tumorigenesis during development are not present on the sections available for immunohistochemistry.*	

* Type I PPBs can have a large amount of epithelial surface area. Depending on the timing of the insult during development, the clone of cells that lose DICER1 may only be present focally (and not be represented on the tissue slides available). This is may limit the number of informative cases for the LCM-guided studies.

Expression analysis
Significant technical problems with expression analysis are not anticipated. We will use properly preserved, histologically characterized snap frozen tissue for these experiments. The core laboratory in Dr. Hoffman's genetic facility at Children's National Medical Center is very experienced in the technical and interpretative aspects of expression array (see Dr. Hoffman biosketch and resources pages). The concern with expression arrays relates to their informativity with respect to the pathways involved in tumor initiation. Although we think that *DICER1* inactivation is disrupting a key signaling pathway and the disruption of this pathway should lead to overt changes in gene expression, there are many reasons why analysis of late stage tumors would not reflect the initial insult.
1. The initiating "tumor promoting factor (TPF)" may be turned off through mechanisms of gene inactivation other than miRNA, thus the response to this TPF may subside.
2. We may not see the downstream effects of an initial "tumor promoting factor(s)" at the time the tumor comes to clinical attention presumably months to years after tumor initiation.
3. The TPF may not lead to activation of a common pathway and second, third, fourth hits may be random in different cells throughout the tumors. This may explain in part the heterogeneity of tumors. Thus there may or may not be genetically homogenous path of tumor progression.

Expected Outcomes

The first two aims described in this proposal will work synergistically to improve our understanding of this unique family cancer syndrome. A better understanding of the clinical features of the PPB syndrome achieved through Aim 1 is essential to the development of clinical criteria for identifying these families and for guiding their medical care. In Aim 2, the mutational status of 100 probands will be used to evaluate the incidence of *DICER1* mutations in familial and sporadic PPB, measure the rate of new mutations, estimate the penetrance of mutations and to better define the expressivity. This information will facilitate implementation of gene-based testing and will help further refine the information used to counsel these families. In addition, the study of families that show predisposition to PPB represent a unique opportunity to gain a better understanding of cellular processes in the borderland between development and neoplasia and to study how tissue-specific loss of *DICER1* (and the miRNAs it regulates) manifests in human disease. We anticipate that the clinical observations related to the DICER1 expressivity may provide a foundation for future mechanistic studies in a number of disease entities. Using a combination of pathologic and molecular characterizations in Aim 3, we will test our hypothesis that *DICER1* loss in epithelium is responsible for initiation of PPB, a tumor of lung mesenchyme. This model, if true, would represent a paradigm shift in human cancer biology – that a genetic aberration in epithelium can drive tumorigenesis in mesenchymal cells.

TIMELINE		**Year 1**	**Year 2**	**Year 3**	**Year 4**	**Year 5**
AIM 1	Patient/family enrollment; pedigree development; follow-up	X	X	X	X	
AIM 1	Biological specimen accrual including blood, cell lines, frozen tumor and normal tissue, paraffin blocks	X	X	X	X	X
AIM 1	Medical histories from extended PPB families		X	X	X	X
AIM 2	*DICER1* mutation analysis for PPB probands	X	X	X		
AIM 2	*DICER1* mutation analysis for family members		X	X	X	
AIM 2	Assess penetrance and expressivity of DICER1 mutations			X	X	X
AIM 2	Sequence *DROSHA*, *DGCR8* and other candidate genes in *DICER1* wild-type probands			X	X	X
AIM 3	Immunohistochemical studies	X	X	X		
AIM 3	Tumor *DICER1* mutation studies		X	X	X	
AIM 3	Profile gene expression in PPB tumors		X	X		

Future studies

The work in this proposal will lead directly to:

1. The development of clinical diagnostics for PPB focused on early detection of children at risk for the disease
2. Detailed molecular characterization of sporadic tumors seen in the syndrome (e.g. rhabdomyosarcoma).
3. miRNA profiling studies in cells affected by *DICER1* loss
4. Development of an appropriate mouse model that recapitulates the loss of *DICER1* in PPB. As indicated by our hypothesis, we expect that this will involve conditional inactivation of *DICER1* in lung epithelium. Although there is already a lung epithelium DICER-/- model (described in Harris[4]), we believe the developmental timing of *DICER1* loss will be critical to understanding the clinical heterogeneity of early Type I PPB. Studies with this model would include phenotypic and miRNA analysis of DICER1-deficient lung tissues, analysis of cellular proliferation and survival in lung mesenchyme, analysis of signaling molecules mediating epithelial-mesenchymal interactions and potentially testing if p53 and DICER1 loss cooperate in later PPB tumorigenesis.
5. Creation of a model to test for miRNA regulation of key epithelial-derived mesenchymal growth factors.

PROTECTION OF HUMAN SUBJECTS

This proposal involves direct interaction with human subjects. The initial full protocol for this research program was approved on March 9, 2005 (Washington University School of Medicine (WUSM) Human Research Protection Office HSC#05-0192) via full board review. Approval has been maintained without interruption since that date. The study is currently approved until 1/28/2010. The P.I. Dr. Hill recently relocated to Children's National Medical Center (CNMC) in Washington D.C. HRPO approval is pending in the new location.

RISKS TO SUBJECTS

A. Human Subjects Involvement and Characteristics

Study Population

All children with PPB and their family members are encouraged to enroll in the research study regardless of age, gender, ethnicity or race. Because this is a family study and families are ascertained most commonly through a child with a diagnosis of PPB, recruitment of women, men and children from all race/ethnic groups around the world is intrinsic to the study design. The inclusion enrollment report shows data from families enrolled in the study to date and is likely reflective of the general distribution of race/ethnicity among PPB families.

Collaborative organizations

This research study is done in collaboration with the International PPB Registry (IPPBR), WUSM and CNMC. The role of the IPPBR in this research is to support the patient accrual process (see below). The IPPBR has IRB approval for its patient enrollment activities both at Children's Hospitals and Clinics of Minnesota (IRB#98107), Washington University (HSC#04-1154) and approval is pending at CNMC.

B. Sources of Materials

In agreeing to participate in the study the patient/family member is asked to complete a health history questionnaire, release medical records and tissue specimens related to PPB, submit a blood or saliva sample and contact other family members about study participation. Phlebotomy services are typically performed by the research subject's own physician's office or laboratory.

Family structure and medical history information is collected through paper questionnaires and telephone interviews. Medical record information is collected using a signed release of information request to the respective hospital's medical records department. This information is collected specifically for this study. We record information regarding family structure (pedigrees), medical history, demographics and contact information for each research subject. Each family is assigned a unique family code and each individual within the family is assigned a subject code. Jennifer Ivanovich (co-investigator), the P.I. and appropriate investigators who collect the pedigree information and perform medical history interviews have access to data with identifiers. All of these research team members have completed the appropriate training in HIPAA and Protection of Research Subjects.

C. Potential Risks

The potential risks to the subject are outlined in the informed consent and include discomfort, bruising, and/or bleeding at the site of the needle insertion when giving a blood sample. For individuals for whom both parents also agree to participate, we acknowledge there is the possibility of identifying non-parental associations. That is, we may learn that one of the parents is not the biological parent. Since research subjects do not receive the results of any such analysis we view this as a minor risk. Blood or tissue samples are being collected to identify genes that may cause or contribute to the development of PPB and related tumors. Results of genetic testing are not released to participants or to research subject's physicians. Although the research record is not a component of the subject's medical chart, *participation* in this research study may become part of the subject's medical chart if the subject discusses his/her participation with his/her doctor.

ADEQUACY OF PROTECTION AGAINST RISKS

A. Patient accrual and informed consent

All children or parents/guardians referred for participation in this study have indicated a willingness to be informed about other studies as part of the informed consent process for enrolling in the PPB Registry. Jennifer Ivanovich (co-investigator) or the P.I. contacts the family of each patient to discuss the study and obtains

verbal informed consent. For cases in which the proband is a young child, consent is obtained from the parent and assent is obtained from the child (if appropriate).

The PPB proband's parents, grandparents or other family representative will be asked to contact extended family members about participating in the study. This approach is taken to maintain the confidentiality of family members who are unknown to the research team. Once a family member expresses interest in learning about the research (as communicated by the family representative), Ms. Ivanovich will contact him/her to obtain consent for participation.

B. Protection against risk

The primary risk in this study is that of a breach of confidentiality. We store all paper study materials with identifying information in locked file cabinets in the co-P.I.'s locked private office. Electronic records including scanned documents and databases used to track participant accrual and medical record requests are stored on servers housed in a secure environment in the Siteman Cancer Center. All servers are stored in a physically secure room requiring key-code entry. This room is protected by both a door with a physical lock as well as a door requiring a key-code. All servers housing electronic private health information (EPHI) will be located behind the network firewall on the WUCON network and meet all HIPAA security requirements. Only the PI, Jennifer Ivanovich, the study research assistant and IT staff have access to this data electronically.

POTENTIAL BENEFITS OF THE PROPOSED RESEARCH TO THE SUBJECTS AND OTHERS

There are no medical or financial benefits for taking part in this study. By agreeing to participate in the study the research subjects allow the use of his/her tissue and medical history information for research that may benefit others by helping us understand how this tumor begins and what makes it grow. One day this knowledge may result in new tests or treatments for other families who have children with PPB and related tumors. It may help to prevent or cure diseases. We believe these potential long-term benefits to society greatly outweigh the minimal risks to research subjects participating in the study.

IMPORTANCE OF THE KNOWLEDGE TO BE GAINED

Historically, studies of inherited cancer syndromes have provided unique opportunities to uncover and explain important cellular pathways (e.g. *Rb* in retinoblastoma leading to Knudson's "two-hit" hypothesis of tumor suppressor genes). Based on our preliminary results detailed below, we anticipate study of *DICER1* loss in PPB families may shed light on a novel pathway of oncogenesis.

This study parallels that of another rare family cancer syndrome, multiple endocrine neoplasia 2a (MEN2a), and related syndromes where the discovery of mutations in the RET proto-oncogene that cause medullary carcinoma of the thyroid led to the development and application of presymptomatic gene-based testing and surgical prophylaxis in childhood which is curative.

It is clear from survival statistics that early detection of PPB is critically important to achieving a high cure rate. Mutation testing in PPB probands and their families may eventually prove useful in the clinical management of the disease. Knowing which children have inherited a predisposition to the disease would help direct intensified screening computed tomography scans to only those children who are at risk. We anticipate that the work proposed in Aim 1 and 2 will lead to a better understanding of the genetic heterogeneity of PPB with respect to *DICER1* mutations and will lead to more accurate estimations of penetrance, expressivity and the frequency of de novo mutations. Each of these will be important in developing comprehensive screening guidelines and in education of families.

As we have identified the first described human germline mutations in *DICER1* these families showing predisposition to PPB represent an important model for studying how tissue-specific loss of DICER1 (and the miRNAs it regulates) may manifest clinically and biologically in humans.

Finally, based on the current evidence that PPB arises from a lung mesenchymal stem cell and the central role of the latter in lung growth and differentiation, we anticipate the genes and pathways important in PPB may relate to key cellular processes in the developing lung. Conceptually, this tumor may represent a naturally-occurring model of mesenchymal cell dysregulation. Although not the primary focus of this application, the

investigation of this dysregulation in the context of malignancy may nevertheless add to our knowledge base of epithelial-mesenchymal interactions in normal and abnormal lung development.

We believe that the importance of the knowledge to be gained greatly outweighs the minimal risks to research subjects participating in the study.

DATA SAFETY AND MONITORING PLAN

Not applicable. This research is not a clinical trial.

CLINICALTRIALS.GOV REQUIREMENTS

NCT00565903
Title: Elucidating the Genetic Basis of the Pleuropulmonary Blastoma (PPB) Familial Cancer Syndrome
Sponsor: Washington University
P.I. D. Ashley Hill, M.D.

INCLUSION OF WOMEN AND MINORITIES

Because this is a family study and families are ascertained through a child with a diagnosis of PPB, the recruitment of women, men and children from all race/ethnic groups around the world is intrinsic to the study design. The inclusion enrollment report shows data from families enrolled in the study to date and is generally reflective of the general distribution of race/ethnicity among PPB families within the International PPB Registry. A recent review of race and ethnicity report by the IPPBR showed that in 125 cases where race/ethnicity was known 7.2% were Hispanic, 84% were white, 6.4% were black/African-American, 4% were mixed race, 3.2% were and the remaining 2.4% were Pacific Islander and American Indian. These percentages are similar to what we have seen in our study to date.

TARGETED/PLANNED ENROLLMENT TABLE

Study Title: DICER1 and the Pleuropulmonary Blastoma Family Cancer Syndrome

Total Planned Enrollment: 1500

TARGETED/PLANNED ENROLLMENT: Number of Subjects			
Ethnic Category	**Females**	**Males**	**Total**
Hispanic or Latino	20	20	25
Not Hispanic or Latino	730	730	475
Ethnic Category: Total of All Subjects *	750	750	1,500

Racial Categories	**Females**	**Males**	**Total**
American Indian/Alaska Native	10	10	20
Asian	10	10	20
Native Hawaiian or Other Pacific Islander	0	0	0
Black or African American	70	70	140
White	660	660	1,320
Racial Categories: Total of All Subjects *	750	750	1,500

* The "Ethnic Category: Total of All Subjects" must be equal to the "Racial Categories: Total of All Subjects."

INCLUSION OF CHILDREN

As stated above, because this is a family study and families are ascertained through a child with a diagnosis of PPB or cystic nephroma, the recruitment of women, men and children from all race/ethnic groups around the world is intrinsic to the study design. Our contact with the children in the study is handled through one or both parents or the designated guardian. Jennifer Ivanovich or the P.I. contacts the family of each patient to discuss the study and obtains verbal and written informed consent to participate. For cases in which the proband is a young child, consent is obtained from the parent and assent is obtained from the child (if appropriate).