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***EIF2AK4* Mutations in Pulmonary Capillary Hemangiomatosis**

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Background: Pulmonary capillary hemangiomatosis (PCH) is a rare disease of capillary proliferation of unknown cause and with a high mortality. Families with multiple affected individuals with PCH suggest a heritable cause although the genetic etiology remains unknown.

Methods: We used exome sequencing to identify a candidate gene for PCH in a family with two affected brothers. We then screened 11 unrelated patients with familial (n = 1) or sporadic (n = 10) PCH for mutations.

Results: Using exome sequencing, we identified compound mutations in eukaryotic translation initiation factor 2 α kinase 4 (*EIF2AK4*) (formerly known as *GCN2*) in both affected brothers. Both parents and an unaffected sister were heterozygous carriers. In addition, we identified two *EIF2AK4* mutations in each of two of 10 unrelated individuals with sporadic PCH. *EIF2AK4* belongs to a family of kinases that regulate angiogenesis in response to cellular stress.

Conclusions: Mutations in *EIF2AK4* are likely to cause autosomal-recessive PCH in familial and some nonfamilial cases.

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Abbreviations: *EIF2AK4* = eukaryotic translation initiation factor 2 α kinase 4; IRB = institutional review board; PAH = pulmonary arterial hypertension; PCH = pulmonary capillary hemangiomatosis

Pulmonary capillary hemangiomatosis (PCH) is a rare disease characterized by a proliferation of multiple layers of capillaries that expand alveolar septa and often invade bronchial walls and the pleura.¹ The disorder is slowly progressive and ultimately fatal. A trial of antiangiogenic therapy followed by lung transplantation is often offered to affected individuals.^{2,3}

Symptoms, including progressive dyspnea, cough, hemoptysis, fatigue, and weight loss, are not specific and mimic other forms of pulmonary arterial hypertension (PAH). Echocardiography and cardiac catheterization may suggest PAH or pulmonary veno-occlusive disease,^{4,5} and the diagnosis may prove difficult because pathologic examination of lung tissue may show fea-

tures which suggest pulmonary veno-occlusive disease or pulmonary hemosiderosis.^{1,6} Furthermore, most patients with symptomatic PAH are not referred for lung biopsy due to risk of the procedure, unless there

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is a high suspicion of another underlying pathologic condition requiring a different treatment. Thus, PCH can challenge clinicians and pathologists, and the correct diagnosis is often delayed or not made until the time of lung transplant or death.^{1,6}

The cause of PCH remains unknown. Previous investigators have suggested that dysregulated angiogenesis

plays a role in the pathogenesis. Prior studies suggest that a variety of angiogenic factors including platelet-derived growth factor,⁷ basic fibroblast growth factor,⁸ vascular endothelial growth factor, and Ki-67⁹ may play a role in the pathobiology of PCH.

Although, typically, the family history is unremarkable, there have been reports of familial PCH with multiple affected family members.^{10,11} Families with affected siblings have suggested an autosomal-recessive mode of inheritance.

We used whole-exome sequencing to identify a novel gene for heritable PCH. We identified pathogenic mutations in eukaryotic translation initiation factor 2 α kinase 4 (*EIF2AK4*) (formerly known as *GCN2*) in familial PCH, as well as two cases of PCH without a family history of PCH.

MATERIALS AND METHODS

Study Participants

All participants provided written informed consent for genetic studies according to protocols approved by the institutional review boards (IRBs) of the participating institutions (Columbia University IRB #AAAA8979 and #AAAC0884; Intermountain Healthcare Urban Central Region IRB #1007044; Vanderbilt University IRB #9401).

We diagnosed PCH in two male siblings in a family (Fig 1). In 2004, PCH was diagnosed in the first sibling (II.1) at 20 years of age at the time of lung transplantation. Pathologic examination demonstrated extensive proliferation of pulmonary capillaries consistent with PCH (Fig 2). Clinical characteristics are provided in Table 1. In 2012, the second sibling (II.2) was diagnosed with PCH based upon lung biopsy at 33 years of age. The

two affected men, their sister, mother, and father provided blood samples from which genomic DNA was extracted. Ten additional patients with pathologically confirmed sporadic PCH and one patient with familial PCH consistent with autosomal-dominant inheritance were sequenced with dideoxy sequencing of all coding exons of *EIF2AK4*.

In addition to the pathologic findings, patients diagnosed with PCH had clinical features typical of the disease (Table 1). Presenting symptoms included marked dyspnea, cough, and fatigue. Pulmonary function studies demonstrated severe reduction of single-breath carbon monoxide diffusion capacity, and lung CT scans demonstrated bilateral diffuse nodular opacities. Cardiac catheterization showed pulmonary hypertension with normal pulmonary capillary wedge pressure measurements. In addition, the correct clinicopathologic diagnosis in the proband of family 1 (member II.1), and the proband in the unrelated family,¹² was not made until the diseased lungs were removed at the time of lung transplantation, even though both patients had undergone an extensive diagnostic evaluation including open lung biopsies before lung transplantation.

Exome Sequencing

Exome sequencing was performed using samples from the initial two male affected siblings. Exons were captured with the Agilent SureSelect kit (x7 Human all Exon U4; Agilent Technologies Inc) and sequenced with 2×100 base pair paired-end reads on an HiSeq2000 according to the manufacturer's recommendations (Illumina, Inc). Sequence was aligned to Hg19 using the Burrows-Wheeler Aligner (0.5.9),¹³ and variants were called with the Genome Analysis Toolkit (Version 1.6).¹⁴ More than 97% of bases sequenced had a quality score > 10 , and variants with a quality score < 10 were removed to avoid false-positives. Given the rarity of PCH, variants that had an allele frequency of $> 1\%$ in dbSNP, the 1000 Genomes Project, and the National Heart, Lung, and Blood Institute (NHLBI) Exome Variant Server were filtered out, leaving 792 variants present in at least one of the two brothers. Assuming an autosomal-recessive mode of inheritance, filtering for two shared variants in the two affected brothers reduced the number of variants to 87 and the number of possible genes to 37. Detailed review analysis of the 87 variants in these candidate genes revealed that only a single gene (*EIF2AK4*) contained two clearly pathogenic mutations.

Sanger Sequencing

Amplicons containing the two mutations in *EIF2AK4* in family 1 were amplified and Sanger sequenced in the nuclear members of family 1. To replicate the genetic findings, all coding exons and splice junctions of *EIF2AK4* were Sanger sequenced in 11 independent patients with PCH using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Life Technologies Corporation) on a 3730 DNA Analyzer (Applied Biosystems, Life Technologies Corporation). Primer sequences are available upon request.

RESULTS

Exome Sequencing

Through our filtering process, we identified a gene (*EIF2AK4*) containing two novel, clearly pathogenic mutations: c.1153dupG (p.Val385fs) and c.3766C>T (p.Arg1256X) shared by both affected brothers (Fig 1). Both variants were confirmed in each brother by Sanger sequencing. Both unaffected parents were heterozygous carriers of one of the two mutations in *EIF2AK4*. The mother carried the frameshift mutation, c.1153dupG, and the father carried the nonsense

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FIGURE 1. Pedigree of family 1. The c.1153dupG mutation and the c.3766C>T mutation segregate with pulmonary capillary hemangiomatosis (PCH) in the index family (family 1). The unaffected mother (I-2) and unaffected daughter (II-3) carried the c.1153dupG mutation, and the unaffected father (I-1) carried the c.3766C>T mutation. (+) denotes wild-type alleles and (-) denotes mutation. →, the proband; ○, unaffected females; ■, affected males; □, unaffected males; /, deceased family members.

mutation, c.3766C>T. The unaffected sister was heterozygous for c.1153dupG by Sanger sequencing only.

Case Series of Patients

We identified novel *EIF2AK4* mutations in two of the 10 additional patients with PCH without a family history (20%). One patient was homozygous for a frame-shift mutation c.1392delT (p.Arg465fs). The other patient was a compound heterozygote for a splice mutation (c.860-1G>A) and a nonsense mutation, c.3438C>T (p.Arg1150X). None of the identified mutations in *EIF2AK4* (Table 1) were observed in exome data from 112 patients recruited for an unrelated study. We did not identify *EIF2AK4* mutations in the proband from a second family, which is affected by PCH due to

autosomal-dominant transmission. None of the five *EIF2AK4* mutations is present in 1000 Genomes¹⁵ or in the available data from the NHLBI Exome Sequencing Project¹⁶ (ESP) Exome Variant Server.

DISCUSSION

We report the identification of a novel gene, *EIF2AK4*, as a likely cause of autosomal-recessive PCH characterized by proliferation of small pulmonary capillaries. Whole-exome sequencing allowed us to identify two loss-of-function mutations in *EIF2AK4* by analyzing only two affected brothers with PCH. We confirmed the role of *EIF2AK4* in PCH by identifying two of 10 additional patients with no family history

FIGURE 2. PCH of family 1, member II.1, as demonstrated in explanted lung. A, Note the low-power, somewhat nodular, appearance of the areas of increased alveolar thickness (hematoxylin and eosin, original magnification $\times 40$). B, The higher-power image shows that the increased thickness is due to a proliferation of capillaries. Special stains, including elastic stains and the endothelial marker CD31 confirmed this, but are not shown (hematoxylin and eosin, original magnification $\times 100$). C and D, Images are obtained from surgical biopsies of family 1, member II.2 (hematoxylin and eosin, original magnification $\times 15$). C, The thickened septa are less nodular in appearance and, D, somewhat mimic an interstitial pneumonia. Note the thickened pulmonary artery at 12 o'clock ($\leftarrow$), a feature typically observed in patients with capillary hemangiomatosis as a manifestation of pulmonary hypertension. See Figure 1 legend for expansion of abbreviation.

of PCH, each with two clear loss-of-function mutations. Previous reports suggested an autosomal-recessive inheritance pattern in a family affected by PCH.¹⁰ Our results provide molecular confirmation of the autosomal-recessive inheritance pattern described by Langleben et al.¹⁰

The absence of pathogenic mutations in *EIF2AK4* in a patient with familial PCH and in the other eight spo-

radic cases may reflect mutations in regions of *EIF2AK4* that we did not screen (ie, deep intronic regions, regulatory regions, or large exonic deletions/duplications) or more likely there may be additional genetic or non-genetic factors involved in the development of PCH. Etiologic and genetic heterogeneity is common in pulmonary vessel diseases including PAH, a disorder caused predominantly by mutations in bone morphogenetic

Table 1—Clinical and Molecular Characteristics of Four Patients With PCH and *EIF2AK4* Mutations

Characteristics	Family 1: Member II.1	Family 1: Member II.2	Sporadic 1	Sporadic 2
Sex	Male	Male	Female	Male
Age at diagnosis, y	19	33	22	15
Current age, y	Deceased at 20	35	33	17
Lung transplant	Yes	No	Yes	Yes
Presenting symptom	Dyspnea, fatigue	Dyspnea, cough	Dyspnea	Dyspnea, fatigue
WHO functional classification	IV	III	na	III
DLCO, % predicted	33	31	na	26
Lung CT scan	Diffuse micronodular pattern with septal thickening	Centrilobular nodules	Diffuse ground-glass opacities	Peribronchovascular and centrilobular nodules
Mean PA, mm Hg	54	40	77	50
PCWP, mm Hg	6	9	na	5
Mutation No. 1	c.1153dupG (p.Val385fs)	c.1153dupG (p.Val385fs)	c.860-1G>A	c.1392delT (p.Arg465fs)
Mutation No. 2	c.3766C>T (p.Arg1256X)	c.3766C>T (p.Arg1256X)	c.3438C>T (p.Arg1150X)	c.1392delT (p.Arg465fs)

DLCO = diffusion capacity of lung for carbon monoxide; *EIF2AK4* = eukaryotic translation initiation factor 2 α kinase 4; na = not available; PA = pulmonary artery pressure; PCH = pulmonary capillary hemangiomatosis; PCWP = pulmonary capillary wedge pressure; WHO = World Health Organization.

protein receptor type 2, and rarely caused by mutations in *ALK1*, *CAV1*, *SMAD9*, *KCNK3*, and likely other undiscovered genes.¹⁷ In addition, our initial family has a different mode of inheritance (autosomal recessive) from the second family (autosomal dominant), which may explain the absence of *EIF2AK4* mutations in the second family.

PCH is a well-recognized cause of pulmonary hypertension, but this disorder has proven difficult to understand and classify.¹⁸ Currently, PCH is classified in diagnostic group 1' and is combined with pulmonary veno-occlusive disease because the pathologic changes (ie, pulmonary hemosiderosis, interstitial edema, lymphatic dilation, and intimal fibrosis with or without medial hypertrophy in the small pulmonary arteries and/or veins) often overlap those seen with pulmonary veno-occlusive disease.¹⁸ Further studies, which include molecular analyses, may enhance the understanding and classification of PCH and possibly pulmonary veno-occlusive disease. However, there has been a paucity of data to date on the genetic and molecular factors causing PCH, hampering efforts to improve classification and to develop specific therapies for this rare disease.

The protein product of *EIF2AK4* belongs to a family of kinases that regulates angiogenesis. Specifically, these kinases phosphorylate the α subunit of eukaryotic translation initiation factor 2, a protein that down-regulates protein synthesis in response to varied cellular stresses.¹⁹ The α subunit of eukaryotic translation initiation factor 2 plays a critical role in tumor cell adaptations to stressful environments, whereby these cells induce angiogenesis, proliferate, and resist apoptosis.^{20,21} These properties are compatible with the angiogenic pathology of PCH, a disorder characterized by uncontrolled proliferation of pulmonary microvessels.

The two brothers diagnosed with PCH were found to have the same two pathogenic mutations in *EIF2AK4*, and yet the age of onset and the severity of the disease differed somewhat. The first brother presented at age 19 years with exertional dyspnea and severe precapillary pulmonary hypertension, having had bronchitis for much of his life. He died after lung transplantation at 20 years of age. His brother also had nonspecific respiratory complaints which began in his late teens. However, he did not present with exertional dyspnea and cough until 33 years of age. Initial evaluation of this brother also showed precapillary pulmonary hypertension, but his disease has progressed more slowly. These observations suggest that other genetic and/or environmental factors may modify the course and severity of PCH associated with pathogenic mutations in *EIF2AK4*.

If our observations are confirmed and expanded, then genetic counseling and genetic testing may prove helpful to patients and families affected by PCH, just

as they have for other heritable disorders, for example, cystic fibrosis. Genetic testing offers the possibility of earlier identification of asymptomatic patients who are at risk to develop PCH, providing opportunities for early interventions to delay the onset or treat heritable PCH. It may also serve as an additional diagnostic tool for select patients with PAH in whom there is a clinical suspicion of PCH.

CONCLUSIONS

In conclusion, we identified mutations in *EIF2AK4* as a novel genetic cause of PCH for some familial and sporadic patients with PCH. This finding provides a new opportunity to advance our understanding of the pathogenesis of PCH and pulmonary vascular biology.

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Dr Best: contributed to conception and design; acquisition, analysis, and interpretation of data; drafting of the submitted article; and revising it critically for important intellectual content.

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