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Exome Sequencing in Children With Pulmonary Arterial Hypertension Demonstrates Differences Compared With Adults

See Editorial by Cirulis and Ryan

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BACKGROUND: Pulmonary arterial hypertension (PAH) is a rare disease characterized by pulmonary arteriole remodeling, elevated arterial pressure and resistance, and subsequent heart failure. Compared with adult-onset disease, pediatric-onset PAH is more heterogeneous and often associated with worse prognosis. Although *BMPR2* mutations underlie ≈70% of adult familial PAH (FPAH) cases, the genetic basis of PAH in children is less understood.

METHODS: We performed genetic analysis of 155 pediatric- and 257 adult-onset PAH patients, including both FPAH and sporadic, idiopathic PAH (IPAH). After screening for 2 common PAH risk genes, mutation-negative FPAH and all IPAH cases were evaluated by exome sequencing.

RESULTS: We observed similar frequencies of rare, deleterious *BMPR2* mutations in pediatric- and adult-onset patients: ≈55% in FPAH and 10% in IPAH patients in both age groups. However, there was significant enrichment of *TBX4* mutations in pediatric- compared with adult-onset patients (IPAH: 10/130 pediatric versus 0/178 adult-onset), and *TBX4* carriers had younger mean age-of-onset compared with *BMPR2* carriers. Mutations in other known PAH risk genes were infrequent in both age groups. Notably, among pediatric IPAH patients without mutations in known risk genes, exome sequencing revealed a 2-fold enrichment of de novo likely gene-damaging and predicted deleterious missense variants.

CONCLUSIONS: Mutations in known PAH risk genes accounted for ≈70% to 80% of FPAH in both age groups, 21% of pediatric-onset IPAH, and 11% of adult-onset IPAH. Rare, predicted deleterious variants in *TBX4* are enriched in pediatric patients and de novo variants in novel genes may explain ≈19% of pediatric-onset IPAH cases.

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Key Words: mutation ■ pediatrics ■ primary pulmonary hypertension ■ whole exome sequencing

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CLINICAL PERSPECTIVE

Pulmonary arterial hypertension (PAH) is a rare disease with high mortality despite advances in treatment. Symptoms of PAH usually manifest in early- to mid-adulthood but can occur in children, albeit less frequently. Pediatric-onset PAH is more frequently associated with other clinical conditions, including congenital heart disease, and is less likely to respond to medical treatment. Mutations in genes involved in the TGF-β (transforming growth factor-β) signaling pathway, primarily within bone morphogenetic protein receptor 2 (*BMPR2*), have been shown to be important in familial and idiopathic forms of PAH in adults, but few genetic studies of PAH have been performed in children. We report a significant contribution of mutations in T-box4 (*TBX4*) variants, encoding a transcription factor widely expressed during development, in pediatric-onset PAH with a 20-year earlier age of disease onset compared with *BMPR2* mutation carriers. Further, de novo variants in *TBX4* and other genes occurred statistically more frequently than expected by chance, suggesting that de novo variants may explain ≈19% of pediatric-onset idiopathic PAH.

Pulmonary arterial hypertension (PAH) is a rare disease with high mortality despite advances in treatment. The disease is etiologically heterogeneous, including familial PAH (FPAH), sporadic/idiopathic PAH (IPAH), hereditary PAH (which includes FPAH and IPAH with identified mutations) and PAH associated with other medical conditions (ie, congenital heart disease, connective tissue disorders, portal hypertension, and others). PAH manifests in early to midlife with an estimated prevalence of 4.8 to 8.1 cases per million for pediatric-onset[1] and 15 to 50 cases per million for adult-onset.[2] Although less prevalent than adult-onset, pediatric-onset disease is more frequently associated with other clinical conditions and less likely to respond to medical treatment. The untreated natural history of IPAH in children is poor with a historical median survival after diagnosis of 10 months compared with 2.8 years for adults.[3] Further, the female predominance observed in adult-onset PAH (3.6:1 female:male ratio) is less pronounced in pediatric-onset cases (1.7:1 female:male ratio).[4] Genetics play an important role in the pathogenesis of PAH, including both FPAH and IPAH. However, the genetic basis of pediatric disease has not been widely investigated.

Previous genetic studies of PAH have been conducted primarily in adult cohorts. Germline mutations in the bone morphogenetic protein receptor 2 (*BMPR2*) gene, encoding a member of the TGF-β (transforming growth factor-β) superfamily of receptors, have been identified as the major genetic cause for FPAH (≈70% of patients) and, to a lesser extent, IPAH (10% to 40% of patients).[5] Mutations in other TGF-β family member genes, activin A, receptor type II-like 1 (*ACVRL1*) and endoglin (*ENG*) cause hereditary hemorrhagic telangiectasia (HHT) and hereditary PAH.[5] Mutations in the BMP receptor type IA and type 1B (*BMPR1A* and *BMPR1B*, also called activin receptor-like kinase 6 [*ALK6*]), caveolin-1 (*CAV1*), eukaryotic initiation translation factor 2 alpha kinase 4 (*EIF2AK4*), potassium two-pore-domain channel subfamily K member 3 (*KCNK3*), SMAD family members 4 and 9 (*SMAD4* and *SMAD9*), and T-box 4 (*TBX4*) have all been identified as less frequent or rare causes of PAH.[5–8] Finally, common variants in cerebellin-2 (*CBLN2*) increase risk for PAH ≈2-fold.[9]

In children, *BMPR2* mutations have been evaluated with inconsistent results, but the number of children studied previously was relatively small. One study of 13 children with IPAH failed to identify *BMPR2* mutations.[10] Another study of 18 pediatric IPAH cases identified 4 mutations in *ACVRL1*, *ENG*, or *BMPR2*.[11] A larger, retrospective study reported significantly lower 5-year survival rates among *BMPR2* mutation carriers compared with noncarriers in a cohort of 54 children.[12] Mutations in other genes including *TBX4*, *BMPR1B*, and neurogenic locus notch homolog 3 (*NOTCH3*) have been reported to cause PAH in children.[6,8,13]

We have previously used exome sequencing to identify *CAV1*, *KCNK3*, and *EIF2AK4* as novel genetic causes of FPAH, IPAH, or pulmonary capillary hemangiomatosis/pulmonary veno-occlusive disease.[5,14–16] In the current study, we describe and compare the frequency and spectrum of genes with mutations in pediatric- versus adult-onset PAH using a genomic approach.

METHODS

The data, analytic methods, and study materials are available on request from the corresponding author, for purposes of reproducing the results or replicating the procedure.

Patients

We sought to identify rare inherited and de novo genetic variants causing pediatric- and adult-onset PAH and to compare the distribution and frequency of genetic mutations in the 2 age groups. Patients were recruited from pulmonary hypertension centers at Columbia University and Children's Hospital Colorado. The patients were collected over a 22-year period, from 1993 to 2015, largely recruited from a single center at Columbia University. Patients were diagnosed according to the World Health Organization pulmonary hypertension group I classification[17] and included primarily FPAH and IPAH cases. All patients included in the analyses were unrelated. Patients with PAH associated with congenital heart disease, which is much more prevalent among pediatric-onset cases, were excluded

to reduce heterogeneity and are the focus of a separate report. The diagnosis of PAH was confirmed by medical record review including right heart catheterization. Pediatric-onset was defined by diagnosis before 19 years of age, although subgroup comparison of the rate of de novo mutations considered age-of-onset within the pediatric group (ie, ≤5 versus 5–18 years). Written informed consent (and assent when appropriate) was obtained under a protocol approved by the institutional review board at Columbia University Medical Center and Children's Hospital Colorado.

FPAH cases were screened for *BMPR2* and *ACVRL1* mutations by Sanger sequencing and multiplex ligation-dependent probe amplification. Then, FPAH cases without mutations in *BMPR2* and *ACVRL1*, and all IPAH cases, were analyzed by exome sequencing (Figure I in the Data Supplement).

Whole Exome Sequencing

Whole exome sequencing (WES) was performed as a family-based analysis when possible and included 48 pediatric trios (proband and 2 biological parents), 24 pediatric-/10 adult-onset duos (proband and 1 biological parent), 60 pediatric-/185 adult-onset singleton probands, 10 pediatric-/13 adult-onset families with multiple affected individuals for a total of 350 probands (Table 1). DNA was extracted from peripheral blood leukocytes, and WES was performed in collaboration with the Regeneron Genetics Center or the PAH Biobank at Cincinnati Children's Hospital Medical Center using standard procedures (Materials in the Data Supplement).

WES Data Analysis

The workflow is outlined in Figure I in the Data Supplement. We used a previously established bioinformatics procedure to process and analyze WES data. Specifically, we used BWA-MEM (Burrows-Wheeler Aligner)[18] to map and align paired-end reads to the human reference genome (version GRCh37/hg19), Picard MarkDuplicates to identify and flag polymerase chain reaction duplicated reads, GATK[19,20] HaplotypeCaller (version 3) to call genetic variants and GATK variant quality score recalibration to estimate accuracy of variant calls. To guard against technical artifacts, we excluded variants that met any of the following conditions: minimum read depth ≤8 reads, allele balance ≤20%,[21] genotype quality <30, mappability <1 (based on 150 bp reads), genomicSuperDups segmental duplication similarity ≥95%, or variant quality score recalibration >99.7. All candidate variants were confirmed with Sanger sequencing and tested for disease segregation when family DNA samples were available.

We used ANNOVAR[22] to annotate the variants and aggregate information of allele frequency and in silico predictions of deleteriousness. We used population allele frequencies from internal databases and public data from the Exome Aggregation Consortium[23] and Genome Aggregation Database to define rare variants with a population allele frequency ≤0.1%. We used multiple bioinformatics prediction algorithms including PolyPhen 2, Mutation Taster, SIFT, PROVEAN, metaSVM,[24] and Combined Annotation Dependent Depletion[25] to predict variant deleteriousness but ultimately focused on metaSVM (damaging) to define damaging missense variants (D-Mis) for enrichment analyses. We identified de novo variants as described previously, using trios composed of proband and unaffected biological parents, and manually inspected all candidate de novo variants using the Integrative Genomics Viewer[26] to reduce the false-positive rate. Finally, we inferred copy number variants from WES data using the CLAMMS algorithm (Copy Number Estimation using Lattice-Aligned Mixture Models).[27]

Table 1. Pulmonary Arterial Hypertension Patient Population

	Pediatric	Adult
Idiopathic, n (%)	130 (83.9)	178 (69.3)
Familial, n (%)	25 (16.1)	79 (30.7)
Total, n	155	257
Female:male ratio (n)	1.6:1 (96:59)	4.1:1 (207:50)*

*Pediatric- vs adult-onset, *P*=4.9e-05 by Fisher exact test.

Identification of Pathogenic/Likely Pathogenic Variants in Established PAH Risk Genes and Novel Candidate Risk Genes

Variants identified in known PAH risk genes were classified as pathogenic, likely pathogenic, or of uncertain significance according to the American College of Medical Genetics and Genomics guidelines.[28] We compared deleterious rare or de novo variants with mutations reported in the literature and in genetic databases (Online Mendelian Inheritance in Man database, Human Genome Mutation Database,[29] and ClinVar[30]). Variants in established PAH genes of uncertain significance by American College of Medical Genetics and Genomics guidelines and variants in novel candidate risk genes were classified based on predictions of deleteriousness. We defined 3 levels of deleterious variants (1) high, likely-gene-disrupting (LGD) variants (including premature stop-gain, frameshift indels, canonical splicing variants, and deletion of exons) and missense variants predicted to be damaging by MetaSVM and Combined Annotation Dependent Depletion (Phred-scale) score of ≥15; (2) medium, missense variants predicted to be damaging by MetaSVM or Combined Annotation Dependent Depletion score of ≥15; and (3) low, all others.

Statistical Analysis

To compare the frequency of rare deleterious variants between pediatric- and adult-onset PAH cases in known risk genes, we used a binomial test, with a null hypothesis that the frequency of patients carrying rare deleterious variants in a known risk gene (*BMPR2* or *TBX4*) or gene set (*ACVRL1*, *BMPR1A*, *BMPR1B*, *CAV1*, *EIF2AK4*, *ENG*, *KCNK3*, *SMAD4*, and *SMAD9*) is the same between pediatric- and adult-onset patients.

To assess the enrichment of rare variants in known risk genes in cases, we obtained WES data of 2426 unaffected parents from a congenital heart disease study by the Pediatric Cardiac Genetics Consortium[31] as controls, and selected subjects (cases and controls) of European ancestry using principal components analysis implemented in PLINK version 1.9.[32,33] We used an exact binomial test to test the significance of enrichment of rare variants in cases compared with controls, with a null hypothesis that the number of rare variants

observed in cases follows a binomial distribution, given the total number of such variants in cases and controls, and a rate determined by the number of cases over the total number of cases and controls.

To estimate the burden of de novo variants in cases, we used previously calibrated background mutation rate[34,35] to calculate the expected number of de novo variants of various types (eg, LGD, missense, or synonymous) by chance in several cases in all genes or a gene set. For a certain type of de novo variant in a gene set, if the observed number in cases is m1, the expected number is m0, then we estimated the enrichment rate by (m1/m0), and test the significance by an exact Poisson test using m0 as the expectation.

We used fisher.test, binom.test, and poisson.test functions in R to perform statistical tests described above.

RESULTS

There were 155 pediatric PAH patients diagnosed before age 19 (25 FPAH, 130 IPAH) and 257 adult-onset patients (79 FPAH, 178 IPAH). The female to male ratio was 1.6:1 (96/59) in the pediatric-onset and 4.1:1 (207/50) in the adult-onset groups (Table 1), and the difference is statistically significant (Fisher exact test *P* value=4.9e-05). We performed targeted genetic analysis of *BMPR2* (Sanger sequencing and multiplex ligation-dependent probe amplification) and *ACVRL1* (sequencing) in 104 FPAH cases and identified pathogenic or likely pathogenic variants in 63 cases according to American College of Medical Genetics and Genomics guidelines. Next, WES was performed for the 42 *BMPR2/ACVRL1* mutation-negative FPAH and all 308 IPAH patients, for a total of 350 samples (Figure I in the Data Supplement). The full set of rare pathogenic, likely pathogenic, or predicted deleterious variants in established PAH genes are provided in Table I in the Data Supplement (pediatric-onset) and Table II in the Data Supplement (adult-onset).

Similar BMPR2 Mutation Frequencies in Pediatric- and Adult-Onset PAH Patients

The combined analysis of Sanger sequencing/multiplex ligation-dependent probe amplification/WES resulted in the identification of 84 rare, predicted deleterious *BMPR2* mutations in FPAH/IPAH probands (Tables I and II in the Data Supplement). Altogether, there were 58 LGD variants, 25 D-Mis variants, and 1 in-frame deletion of 7 amino acids. Fourteen of the 84 mutations are novel and all are depicted in Figure II in the Data Supplement. Similar *BMPR2* mutation frequencies were observed in pediatric- and adult-onset FPAH (14/25, 56% pediatric and 43/79, 54.4% adult) and IPAH (13/130, 10% pediatric and 14/178, 7.9% adult) patients (Table 2). However, there was a difference in the distribution of LGD and D-Mis variant types between the 2 age groups: LGD variants occurred more frequently in patients with an older age of onset (31.3±14.0 years, mean±SD) compared with D-Mis variants (19.3±12.5 years; *P*=0.0006, Mann–Whitney *U* test). The distribution of variant locations was similar with almost all of the D-Mis variants in both age groups located within the conserved activin or protein kinase domains and most of the LGD variants located in the first half of the protein that contains the 2 domains (Figure II in the Data Supplement).

Table 2. Enrichment of Rare Genetic Variants in Known Pulmonary Arterial Hypertension Genes* in Pediatric- and Adult-Onset Patients of European Ancestry

	Mutation Type†	Observed (Cases)	Observed (Controls, n=1319)	Enrichment Rate	*P* Value
Pediatric-onset (n=88)	SYN	4	30	2.00	0.16
	LGD	3	2	22.48	2.22E-03
	MIS	9	69	1.96	0.06
	D-Mis	5	29	2.58	0.06
Adult-onset (n=160)	SYN	5	30	1.37	0.42
	LGD	8	2	32.98	6.90E-07
	MIS	10	69	1.19	0.59
	D-Mis	2	29	0.57	0.77

**ACVRL1, BMPR1A, BMPR1B, BMPR2, CAV1, EIF2AK4, ENG, KCNK3, SMAD4, SMAD9*, and *TBX4*.

†D-Mis, damaging missense predicted by MetaSVM; LGD, likely gene-damaging; MIS, all missense; and SYN, synonymous.

Substantial Contribution of TBX4 Mutations to Pediatric-Onset PAH

We identified 13 likely pathogenic/predicted highly deleterious *TBX4* variants in 13 probands (12 pediatric- and 1 adult-onset; Tables I and II in the Data Supplement). The variants included 9 LGD and 3 D-Mis variants, including 1 intragenic deletion encompassing exons 3 to 9. All of these variants are novel and most reside within the conserved T-box domain (Figure 1). Based on the 12 probands with at least 1 parental sample available, we determined the inheritance pattern of a subset of variants: 1 de novo, 4 inherited from mothers without PAH, and 4 inherited from fathers without PAH. The observed inheritance of *TBX4* variants from an unaffected parent in at least 8 of 13 cases is consistent with incomplete penetrance that is also observed for *BMPR2* mutation carriers,[36] suggesting that the effect of any single risk variant on development of disease is likely dependent on genetic background, somatic mutations, environmental effects, or some combination thereof. Notably, pediatric-onset IPAH cases were significantly enriched for *TBX4* mutations compared with adult-onset patients (10/130, 7.7% versus 0/178, *P* value<0.0001; Figure 2A). Although a much smaller sample size, there was a similar trend for FPAH cases

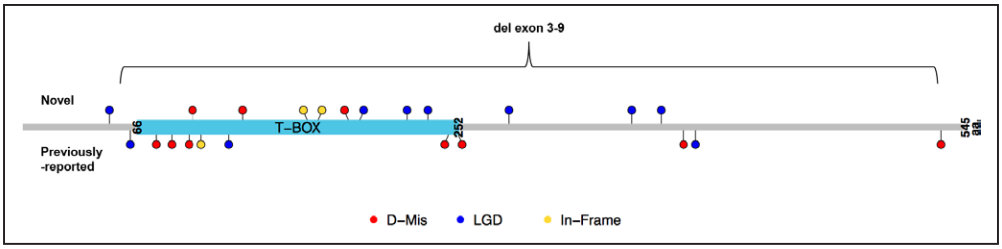

Figure 1. Novel and previously-reported genetic mutations in pulmonary arterial hypertension (PAH) risk gene *TBX4*.

Novel mutations identified by whole exome sequencing (WES) are indicated above the protein schematic. Previously reported mutations not identified in this study are indicated below the schematic. D-Mis, damaging missense mutations predicted by MetaSVM (red), LGD, likely gene-damaging (blue), and In-frame insertion/deletions (yellow).

(Figure 2A). Additionally, *TBX4* carriers had a 20-year younger age-of-onset (7.9±9.0, mean±SD; range 0.6–33 years) compared with *BMPR2* carriers (28.2±15.4; range 1.5–72 years; $P<0.0001$; Figure 2B).

Rare Genetic Causes of PAH

We identified a small number of patients with rare deleterious mutations in recently identified genes for PAH, PAH associated with HHT (PAH-HHT), and genes involved in the BMP and TGF-β signaling pathway (Tables I and II in the Data Supplement). Two pediatric-onset patients were observed to carry *KCNK3* mutations: a novel paternally-inherited 2 amino acid insertion (c.641_642insGCAGAC:p.214insQT; IPAH) and a previously-reported heterozygous missense variant (c.G544A:p.E182K; FPAH).[15] A novel heterozygous frameshift mutation in *CAV1* (c.471delC; p.D157fs) was found in an adult patient with a pediatric-onset daughter who died at 9 years old. Compound heterozygous LGD variants in *EIF2AK4* (c.C3766T:p.R1256X and c.1150dupG:p.S383fs) were identified in a familial case with pulmonary capillary hemangiomatosis. A previously-reported homozygous nonsense mutation in *EIF2AK4* (c.C1387T:p.R463X)[37] was present in an adult FPAH patient whose brother had PAH and died at age 33 and was not available for testing.

In our total PAH cohort, 9 patients had a diagnosis of PAH-HHT. *ACVRL1* and *ENG* are known risk genes for PAH-HHT and targeted Sanger sequencing/WES identified 3 pediatric- and 5 adult-onset patients with mutations in *ACVRL1* as well as 1 adult patient with an *ENG* mutation (Tables I and II in the Data Supplement). Interestingly, one patient with isolated PAH without HHT carried a paternally-inherited missense variant in *ACVRL1* (c.C1199T:p.A400V) as well as a paternally-inherited missense variant in *BMPR2* (c.A1509C:p.E503D).

Finally, 3 candidate pathogenic variants were identified in genes in the BMP and TGF-β signaling pathway. A deleterious missense variant and a nonsense variant were identified in *SMAD9* and a deleterious missense variant in *BMPR1B* (Tables I and II in the Data Supplement). In total, the rare genetic cause mutations had similar frequencies in pediatric and adult PAH cases: 3.8% (5/130) of IPAH cases and 16% (4/25) of FPAH pediatric-onset patients and 1.7% (3/178) of IPAH and 12.7% (10/79) of FPAH adult-onset cases (Table 2).

A

Gene		Pediatric (n=155)	Adult (n=257)	Enrichment rate	p-value
BMPR2	IPAH, n (%)	13 (10%)	16 (9%)	1.0	0.64
	FPAH, n (%)	14 (56%)	42 (53.2%)	1.1	0.84
TBX4	IPAH, n (%)	10 (7.7%)	0	N/A	<0.0001
	FPAH, n (%)	2 (8%)	1 (1.3%)	6.2	0.04
Other*	IPAH, n (%)	5 (3.8%)	3 (1.7%)	2.2	0.07
	FPAH, n (%)	4 (16%)	10 (12.7%)	1.4	0.52

*Other: *ACVRL1, BMPR1A, BMPR1B, CAV1, EIF2AK4, ENG, KCNK3, SMAD4* and *SMAD9*

B

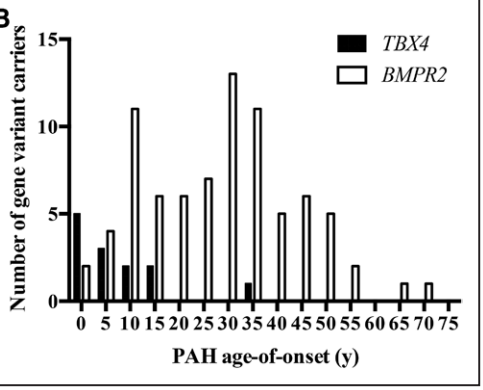

Figure 2. Role of *TBX4* in pediatric-onset pulmonary arterial hypertension (PAH).

A, Enrichment of rare, predicted deleterious variants in *TBX4*, but not other known risk genes, in pediatric-onset cases. *P* values were calculated by binomial tests. **B**, Younger age of disease onset for *TBX4* variant carriers compared with *BMPR2* variant carriers ($P<0.0001$, Mann–Whitney *U* test).

Excess of Rare Deleterious Variants in Known PAH Risk Genes Because of *BMPR2* and *TBX4*

We then evaluated the burden of rare variants in all 11 established PAH-associated risk genes from WES data. We used 1319 unaffected parents from a congenital heart disease cohort in the Pediatric Cardiac Genetics Consortium[31] as population controls for allele frequency estimates. To avoid spurious association because of population stratification, we inferred ethnicity of all subjects using principal components analysis,[33] and selected 88 pediatric-onset and 160 adult-onset PAH cases of European ancestry for this analysis. We observed a significant burden of rare LGD variants in both the pediatric- (enrichment rate=22, *P* value=0.002) and adult-onset cases (enrichment rate=33, *P* value=6.9e-7) in this established PAH gene set (Table 3). There was also a trend toward enrichment of rare, predicted deleterious missense variants in pediatric-onset cases (odds ratio=2.6, *P* value=0.06), but not in adult-onset cases. The enrichment was not observed when *BMPR2* and *TBX4* were removed from the analysis (Table III in the Data Supplement).

Burden of De Novo Variants in Pediatric-Onset Patients

To further explore the genetic underpinnings of PAH, we performed a genome-wide burden test for de novo variants. Forty-six pediatric-onset cases for which we had ascertained parental DNA were available for trio WES. We were unable to perform parallel trio analyses in adult patients because of the lack of available parental DNA samples. Excluding the cases with rare deleterious variants in the 11 known risk genes, 36 pediatric-onset IPAH cases were used to compare the frequency of de novo variants in cases with expectation from the background mutation rate.[34,35] We detected a 2.35-fold enrichment (*P*=0.01) of de novo D-Mis variants in the pediatric IPAH cases (Table 3). Subgroup analysis revealed that very early-onset cases (≤5 years of age) were enriched for LGD variants (6/16 patients) compared with older-onset cases (>5–18 years, 0/18 patients; *P*=0.01; Table IV in the Data Supplement). A list of all de novo mutations/genes identified in this study, excluding synonymous variants, is provided in Table V in the Data Supplement. As expected, 8 of these patients harbored deleterious de novo mutations in ≥2 genes. Overall, the data suggest that ≈25% of pediatric-onset IPAH without mutations in known PAH genes, or 19% of all pediatric-onset IPAH cases, may be explained by de novo LGD or deleterious missense variants in novel genes.

Table 3. Enrichment of Rare De Novo Mutations in Pediatric-Onset Idiopathic Pulmonary Arterial Hypertension Trios (n=36)

Mutation Type*	Observed	Expected by Chance	Enrichment	*P* Value
SYN	11	11.12	0.99	1
LGD	6	3.42	1.75	0.46
MIS	29	24.58	1.18	0.36
D-Mis	11	4.68	2.35	0.009
LGD & D-Mis	17	8.04	2.11	0.004

*D-Mis, damaging missense predicted by MetaSVM; LGD, likely gene-damaging; MIS, all missense; and SYN, synonymous.

For patients without rare, deleterious variants in known PAH risk genes, we also performed a genome-wide burden test for rare, deleterious variants excluding the set of 11 known risk genes. We again used the unaffected individuals from the Pediatric Cardiac Genetics Consortium[31] as controls and selected 66 pediatric- and 120 adult-onset PAH cases of European ancestry for this analysis. We observed no enrichment of rare, deleterious variants in either pediatric (Table VI in the Data Supplement) or adult (Table VII in the Data Supplement) cohorts.

DISCUSSION

In a cohort of 412 PAH patients, we identified new rare, deleterious mutations in known PAH genes, candidate causal de novo variants in novel genes, and compared the results of genetic analyses in pediatric- versus adult-onset disease. Fourteen novel mutations were identified in the major PAH risk gene, *BMPR2*, 13 in *TBX4*, and 12 in other known PAH risk genes. Most of the mutations are located in conserved protein functional domains. Using a genome-wide de novo screen, we identified 6 LGD and 10 deleterious missense variants in novel candidate disease genes. Although the frequencies of *BMPR2* mutations were similar for pediatric- and adult-onset PAH cases, the frequency of *TBX4* mutations among pediatric-onset patients was significantly greater than that of adult-onset patients, with significantly younger age-of-onset. Furthermore, we identified an enrichment of LGD and deleterious missense de novo variants in pediatric IPAH patients without mutations in known PAH risk genes.

BMPR2 and Other Genes in the TGF-β Pathway in PAH

The TGF-β superfamily includes the TGF-β/BMP ligands, receptors, accessory proteins, activins, and downstream signaling mediators SMADs and NOTCH3.[38] In this study, we identified rare, deleterious mutations in known PAH risk genes *BMPR2, BMPR1B*, *ACVRL1*, *ENG*, and *SMAD9* for a total yield of 48 variants in pediatric-onset (20/25, 80% FPAH and 28/130, 21.5% IPAH) and 74 in

adult-onset (53/79, 67.2% FPAH and 19/178, 10.7% IPAH) patients. Additionally, we identified rare, deleterious variants in other candidate genes in this pathway: *BMPR1A*, *ACVR2B*, *GDF2*, *SMAD6, TGFB*, and *TGFBR2*. *BMPR2* mutations accounted for ≈55% of FPAH cases in both children and adults. Although the contribution of *BMPR2* mutations to PAH in this study was slightly lower than in previous reports, it is likely that patients with clinically-identified *BMPR2* mutations chose not to enroll in this genetic study. Exploration of the novel candidate risk genes in additional pediatric and adult PAH patients, along with functional characterization of potentially pathogenic variants, will help further our understanding of this pathway and how its dysregulation contributes to the pathophysiology of PAH.

TBX4 and Pediatric PAH

TBX4 endcodes a transcription factor in the T-box gene family expressed in the atrium of the heart, the limbs, and the mesenchyme of the lung and trachea. TBX4, jointly with TBX5, has been shown to interact with FGF10 during lung growth and branching.[39] *TBX4* was first suggested as a candidate PAH risk gene because of its localization within chromosome 17q microdeletions of patients presenting with severe developmental delays sometimes associated with pulmonary hypertension.[40,41] Then, Kerstjens-Frederikse et al[8] performed sequencing of *TBX2* and *TBX4*, both located within the deletions, in PAH patients and identified 3 rare *TBX4* mutations and 3 novel 17q22q23.2 microdeletions in 20 pediatric patients as well as one rare *TBX4* mutation among 49 adults; no mutations in *TBX2* were identified. Heterozygous loss of function mutations in *TBX4* have been reported to cause small patella syndrome (MIM No. 147891)[42] and 5 of the *TBX4* variant carriers reported by Kerstjens-Frederikse were retrospectively found to have clinical features of small patella syndrome.[8] More recent studies have identified 3/40 *TBX4* mutation carriers in a children's PAH cohort[43] and 3/136 adult carriers in a Spanish PAH cohort.[44] Our study is the largest to date, confirming a role for rare, predicted deleterious *TBX4* variants in PAH, especially pediatric-onset disease. Of the 13 PAH patients carrying LGD or D-Mis *TBX4* variants, only 1 was diagnosed with small patella syndrome; however, no radiological records of knees, pelvis, or feet were available for retrospective assessment of the others. The clinical phenotypes of the *TBX4* mutation carriers ranged from mild/moderate PAH to persistent, progressive PAH unresponsive to vasodilators and fatal or requiring lung transplantation. Notably, we observed a significant enrichment of rare, predicted deleterious *TBX4* variants among pediatric- compared with adult-onset patients. Further, *TBX4* variant carriers had a 20-year earlier mean age-of-onset compared with *BMPR2* carriers.

Gender Bias of PAH Presentation Is Modified by Age of Disease Onset

In studies performed primarily in adult cohorts, PAH has been shown to occur ≈3-fold more frequently in females than males. However, a previous study of pediatric PAH reported that females represented only ≈60% of patients.[4] In our study of both pediatric- and adult-onset patients, the female gender bias was significantly lower in pediatric compared with adult patients (Table 1). Genetically, *BMPR2*, *ACVRL1*, and *ENG* mutations occurred more frequently in females, whereas *TBX4* mutations, which exhibited enrichment in the pediatric-onset patients, occurred with equal frequency among both genders. Mutations in other known PAH genes occurred less frequently and effects of age and gender could not be determined.

Pediatric- Versus Adult-Onset PAH Genetics

A comparison of the genetic causes of pediatric- and adult-onset PAH as suggested from our study is depicted in Figure 3. Although the contribution of *BMPR2* mutations was similar between both groups of patients, mutations in *TBX4* made a significantly greater contribution to pediatric- versus adult-onset PAH patients. The contribution of other known PAH risk genes was rare in both age groups. Analysis of de novo mutations in pediatric IPAH patients and their unaffected relatives revealed an enrichment of D-Mis de novo mutations, whereas vary early age-of-onset patients had an enrichment of LGD de novo mutations. Of note, a pediatric-onset patient who died in childhood carried a D-Mis mutation in *MAPK6* (mitogen-activated protein kinase 6). *MAPK6* encodes ERK3 (extracellular signal-regulated kinase 3) and mice carrying null alleles exhibit intrauterine pulmonary hypoplasia and early neonatal death.[45] *AMOT* (angiomotin) encodes an angiostatin-binding protein involved in embryonic endothelial cell migration and tube formation as well as endothelial cell tight junctions and angiogenesis[46–48]; the frameshift insertion mutation carried by pediatric-onset patient JM0004 (diagnosed by 1 year of age) would result in the loss of 2 coiled-coil motifs, a PDZ protein-interaction domain as well as the angiostatin-binding motif. *KEAP1* (Kelch-like ECH associated protein 1), encoding an E3 ubiquitin ligase, regulates cellular responses to oxidative stress through interactions with NRF2. Disruption of KEAP1–NRF2 interaction ameliorated oxidative stress and inhibited apoptosis in murine vascular cells,[49] and endothelial-specific deletion of *NRF2* reduced endothelial cell sprouting in vivo.[50] *NUCB1*, nucleobindin-1, encodes a protein with multifunctional domains, and the frameshift variant at amino acid 189 would disrupt a DNA-binding site and cause loss of 2 Ca^{+}-binding

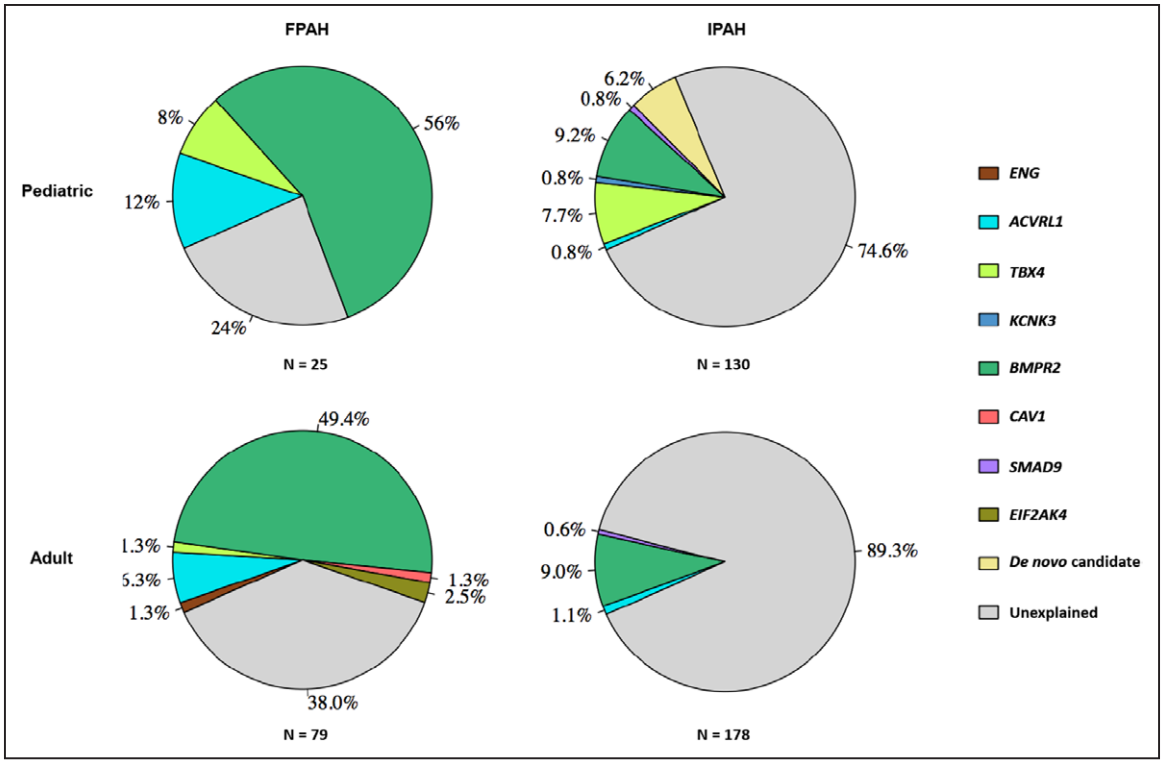

Figure 3. The genetic architecture of pulmonary arterial hypertension (PAH) in pediatric- versus adult-onset patients.
The percentage of total rare, deleterious mutations identified by Sanger sequencing or whole exome sequencing (WES) attributable to known PAH risk genes, de novo candidates, or unexplained is depicted as pie charts for pediatric- and adult-onset familial PAH and idiopathic PAH.

sites and a leucine zipper motif.[51] These and other de novo variants identified in this study may provide novel insights into the pathophysiology underlying PAH.

In summary, differences in the genetic basis of PAH in children compared with adults include an enrichment of *TBX4* mutations, likely contributing to the earlier onset of disease. Notably, de novo mutations account for a significant fraction of PAH in children and could provide an important strategy to identify novel PAH genes in this patient group. The genetic assessment of larger pediatric study cohorts could provide an important opportunity to identify novel genes, elucidate the pathogenic mechanisms of PAH, and provide targets for future therapies.

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