CLINICAL STUDY

SOX17 Loss-of-Function Mutation Underlying Familial Pulmonary Arterial Hypertension

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Summary

Pulmonary arterial hypertension (PAH) refers to a rare, progressive disorder that is characterized by occlusive pulmonary vascular remodeling, resulting in increased pulmonary arterial pressure, right-sided heart failure, and eventual death. Emerging evidence from genetic investigations of pediatric-onset PAH highlights the strong genetic basis underlying PAH, and deleterious variants in multiple genes have been found to cause PAH. Nevertheless, PAH is of substantial genetic heterogeneity, and the genetic defects underlying PAH in the overwhelming majority of cases remain elusive. In this investigation, a consanguineous family suffering from PAH transmitted as an autosomal-dominant trait was identified. Through whole-exome sequencing and bioinformatic analyses as well as Sanger sequencing analyses of the PAH family, a novel heterozygous SOX17 mutation, NM_022454.4: c.379C>T; p.(Gln127*), was found to co-segregate with the disease in the family, with complete penetrance. The nonsense mutation was neither observed in 612 unrelated healthy volunteers nor retrieved in the population genetic databases encompassing the Genome Aggregation Database, the Exome Aggregation Consortium database, and the Single Nucleotide Polymorphism database. Biological analyses using a dual-luciferase reporter assay system revealed that the Gln127*-mutant SOX17 protein lost the ability to transcriptionally activate its target gene NOTCH1. Moreover, the Gln127*-mutant SOX17 protein exhibited no inhibitory effect on the function of CTNNB1-encode β-catenin, which is a key player in vascular morphogenesis. This research firstly links SOX17 loss-of-function mutation to familial PAH, which provides novel insight into the molecular pathogenesis of PAH, suggesting potential implications for genetic and prognostic risk evaluation as well as personalized prophylaxis of the family members affected with PAH.

Key words: Congenital heart disease, Vascular morphogenesis, Medical genetics, Transcriptional regulation, Reporter gene assay

Pulmonary arterial hypertension (PAH), which is characterized by progressive occlusive remodeling of arterioles in the lung that leads to a significant increase in pulmonary vascular resistance and marked elevation in pulmonary artery pressure, refers to an uncommon but often fatal vascular disease, with an estimated annual incidence of one to two patients per million individuals. The prevalence of PAH is approximately 4.8 to 8.1 patients per million persons for pediatric-onset disease and 15 to 50 patients per million persons for adult-onset disease worldwide. In adult-onset PAH cases, there exists woman predominance, with a three- to four-fold higher disease prevalence in females compared with males, which is not observed in pediatric-onset PAH cases. Severe PAH may result in right ventricular hypertrophy, function failure, and, eventually, premature death. It is reported by PAH registries that the mortality rates of PAH cases at 1 and 3 years are 7%-32% and 23%-61%, respectively. During the past two decades, therapeutic approaches for PAH have made a pronounced progress, and currently, four types of drugs can be used for the treatment of PAH, including the prostacyclin analogs and receptor agonists.

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endothelin receptor antagonists, phosphodiesterase 5 inhibitors, and cysolic guanosine monophosphate activators. Despite the limited beneficial impacts on hemodynamics-related quality of life, disease progression, and survival, the currently available pharmaceutical therapies do not cure PAH, and the median survival rate remains less than 3 years from diagnosis, highlighting the urgent need for extensive researches to reveal the molecular mechanisms underlying PAH.

The pathogenesis of PAH is highly complex, and both environmental and genetic pathogenic factors can impair pulmonary vascular structure and function, thus leading to PAH. The well-established environmental risk factors encompass congenital heart disease (CHD), valvular heart disease, virus infection, connective tissue disease, chronic thromboembolism, chronic obstructive pulmonary disease, pulmonary tumor thrombotic microangiopathy, intake of chemical drugs or toxins, and long-term exposure to hypoxia. However, emerging evidence has demonstrated that genetic defects play a pivotal role in the pathogenesis of idiopathic PAH, especially for familial PAH, and deleterious mutations in multiple genes, including BMPR2, encoding a receptor of the transforming growth factor-β superfamily, TBX4, encoding a transcription factor, and KCNK3, as well as ABCB8 encoding potassium channels, have been found to cause PAH. In addition, genome-wide association studies have revealed that common genetic variations are associated with an enhanced susceptibility to PAH. Nevertheless, due to substantial genetic heterogeneity of PAH, the genetic determinants underpinning PAH in the overwhelming majority of patients remain to be identified.

Methods

Study subjects: In the current investigation, a three-generation consanguineous pedigree was identified, where idiopathic PAH was transmitted in an autosomal-dominant mode with complete penetrance. Available family members and 612 totally unrelated healthy volunteers used as controls were included in the study. All the study individuals were recruited from the Chinese Han population in the same geographic area. For each study participant, a comprehensive clinical evaluation was conducted, which included reviews of medical and familial histories, thorough physical examination, transthoracic echocardiography with color Doppler, chest X-ray radiography, standard 12-lead electrocardiography, and routine laboratory tests. In the affected family members, cardiac catheterization measurement was performed, as well as pulmonary angiography when strongly indicated. The diagnosis of PAH was made according to a revised criterion established by the World Health Organization: the concomitant presence of mean pulmonary arterial pressure (mPAP) > 20 mmHg (measured by right-heart catheterization at rest in the supine position), pulmonary arterial wedge pressure (PAWP) ≤ 15 mmHg, and pulmonary vascular resistance (PVR) ≥ 3 Wood units. This study project was fulfilled in conformity to the ethical principles of the Declaration of Helsinki. The study protocol was reviewed and approved by the healthcare ethics committee of Tongji Hospital, Tongji University School of Medicine, Shanghai, China. Prior to the commencement of the present investigation, written informed consent was obtained from the study individuals or from their legal guardians.

Whole-exome sequencing analysis: Peripheral venous whole blood samples were collected from all available family members and control individuals. Genomic DNA was isolated from whole blood leukocytes using the MagMAX™ DNA Multi-Sample Ultra 2.0 Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s protocols. Whole-exome sequencing (WES) was performed as previously described. Briefly, each exome library was constructed using 3 μg of genomic DNA and captured using the SureSelect™ Human All Exon V6 Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer’s protocol. The constructed exome libraries were enriched and sequenced on the Illumina HiSeq 2000 Genome Analyzer (Illumina, San Diego, CA, USA) using the HiSeq Sequencing Kit (Illumina) according to the manufacturer’s analysis of the WES data was conducted as described elsewhere. The minor allele frequency for each genetic variant was calculated according to such population genetic databases as the NHLBI Exome Sequencing Project database (https://evs.gs.washington.edu/evs/), the Single Nucleotide Polymorphism database (https://www.ncbi.nlm.nih.gov/snp/), and the Genome Aggregation Database (https://gnomad.broadinstitute.org/). The disease-causing potential of a novel genetic variation was predicted using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2), MutationTaster (http://www.mutationtaster.org), SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html), and PROVEAN (http://provean.jcvi.org/index.php). The candidate PAH-causative variants identified via WES were further filtered out or validated by Sanger sequencing and segregation analysis in the PAH pedigree. For the pathogenic genetic variant verified in the PAH family, 612 unrelated healthy persons were screened via Sanger sequencing analysis of the gene harboring the pathogenic variant.

Construction of expression plasmids and site-targeted mutagenesis: Extraction of total RNA from donated human heart tissue that otherwise would be discarded after the cardiac surgery and preparation of cDNA via reverse transcription-polymerase chain reaction (RT-PCR) were described previously. The wild-type cDNA of the human SOX17 gene (accession no. NM_022454.4) was generated via PCR using the PfuUltra high-fidelity DNA polymerase (Stratagene, Santa Clara, CA, USA) and a specific pair of primers (forward primer: 5'-CTGGCTAGCC GTCCGCGGGAGGTGTTGAG-3' and reverse primer: 5'-CT GCTCGAGCACTGTTCTGGCCTGAG-3'). For the construction of the eukaryotic expression plasmid SOX17-pcDNA3.1, the amplified cDNA of SOX17 was doubly digested with restriction enzymes NheI and XhoI (NEB, Hitchin, Herts, UK), purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and inserted at the NheI-XhoI sites into the pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA, USA). The Gln127*-mutant SOX17-pcDNA3.1 was yielded by site-directed mutagenesis using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with a complementary...
pair of primers (forward primer: 5’-GAGCGGCTGCGGCTGAGTCATGCAGAGCCTGTTCC-3’; reverse primer: 5’-GGTGCTAGCGTTCCTTCGCGCTCGCGG-3’), double cut with KpnI and NheI (NEB), and subcloned into the pGL3-Basic vector (Promega, Madison, WI, USA). The reporter plasmid TCF-luc, which expresses Firefly luciferase, a 1021-bp promoter region of the TCF gene (nucleotides from −941 to +80, with initial transcription nucleotide numbered +1; accession No. NC_000009.12) was amplified via PCR using a specific pair of primers (forward primer: 5’-GTGAGCTCTGCCAGAGCCTGTTCC-3’; reverse primer: 5’-GGTGCTAGCGTTCCTTCGCGCTCGCGG-3’), double cut with KpnI and NheI (NEB), and subcloned into the pGL3-Basic vector (Promega, Madison, WI, USA). The reporter plasmid TCF-luc, which expresses Firefly luciferase, was constructed as described previously.\(^5,^{35}\)

Cell transfection and dual-luciferase assay: COS-7 and 293T cells were cultivated and transiently transfected with various plasmids using the Lipofectamine 3000 reagent (Invitrogen) as described previously.\(^5,^{35}\) The pGL4.75 plasmid (Promega), which expresses the Renilla luciferase, and the pGL4.75 plasmid (Promega), which expresses the Firefly luciferase, a 1021-bp promoter region of the TCF gene (nucleotides from −941 to +80, with initial transcription nucleotide numbered +1; accession No. NC_000009.12) was amplified via PCR using a specific pair of primers (forward primer: 5’-GTGAGCTCTGCCAGAGCCTGTTCC-3’; reverse primer: 5’-GGTGCTAGCGTTCCTTCGCGCTCGCGG-3’), double cut with KpnI and NheI (NEB), and subcloned into the pGL3-Basic vector (Promega, Madison, WI, USA). The reporter plasmid TCF-luc, which expresses Firefly luciferase, was constructed as described previously.\(^5,^{35}\)

Identification of a SOX17 mutation causative of PAH: WES was fulfilled in the proband (III-2) and her affected father (II-3), sister (III-5), and aunt (II-8) as well as her unaffected mother (II-4) and uncle (II-7). A mean of 24-Gb bases of sequence for an individual sample was generated via paired-end sequencing. An average of 18,192 exonic variants (range 11,200—220×. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome. A mean percentage of 99% bases was mapped to the reference genome.
of the SOX17 gene was conducted on 612 unrelated control persons with the primers presented in Table III. Moreover, no potentially damaging variants were found. Besides, the truncating variation was absent from such population genetic databases as the Genome Aggregation Database, the Exome Aggregation Consortium database, and the Single Nucleotide Polymorphism database, which was retrieved again on September 26, 2020.

No transactivation function of the mutant SOX17 protein: As presented in Figure 2, 400 ng of wild-type SOX17-pcDNA3.1 expression plasmid and the same amount of Gln127*-mutant SOX17-pcDNA3.1 expression plasmid transcriptionally activated the NOTCH1 promoter by ~15-fold and ~1-fold, respectively (wild-type SOX17 versus Gln127*-mutant SOX17: t = 10.3412, \( \Delta P = 0.00049 \)). When 200 ng of wild-type SOX17-pcDNA3.1 expression plasmid was used in combination with the same amount of Gln127*-mutant SOX17-pcDNA3.1 expression plasmid,
the induced transcriptional activity was ~7-fold (wild-type SOX17 + empty pcDNA3.1 versus wild-type SOX17 + Gln127*-mutant SOX17: t = 5.39528, P = 0.00153).

**Diminished inhibitory effect of mutant SOX17 on CTNNB1:** As presented in Figure 3, CTNNB1 alone transcriptionally activated the TCF promoter by ~8-fold. In the presence of CTNNB1, the same amount of wild-type SOX17 and Gln127*-mutant SOX17 transcriptionally activated the TCF promoter by ~2-fold and ~8-fold, respectively (wild-type SOX17 versus Gln127*-mutant SOX17: t = 7.69876, P = 0.00153)

**Discussion**

In the current genetic study, a novel heterozygous SOX17 variant, NM_022454.4: c.379C>T; p.(Gln127*), was identified via WES in a family affected with idiopathic PAH. The variation, which co-segregated with the disease in the pedigree, was neither detected in the 424 reference chromosomes nor retrieved in such population genetic databases as the Genome Aggregation Database, the Exome Aggregation Consortium database, and the Sin犯al Residues database. Functional measurement revealed that Gln127*-mutant SOX17 lost the ability to transactivate the NOTCH1 promoter. Moreover, the Gln127*-mutant SOX17 protein demonstrated a diminished inhibitory effect on CTNNB1 (also termed β-catenin). Hence, it is very likely that the genetically compromised SOX17 gene contributes to PAH in this family.

The human SOX17 gene was mapped to chromosome 8q11.23, which codes for SRY-box transcription factor 17 (SOX17) with 414 amino acids, a member of the con-

### Table I. Phenotypic Features of the Living Family Members Affected with Idiopathic Pulmonary Arterial Hypertension

| Individual | Gender | Age (years) | Symptom | Electrocardiogram | Right heart catheterization |
|------------|--------|-------------|---------|-------------------|-----------------------------|
|            |        |             |         |                   | mPAP (mmHg) | PAWP (mmHg) | PVR (WU) |
| II-1 | M  | 35 | Exertional dyspnea and lightheadedness | Right deviation | 96 | RVH, RBBB | Enlarged pulmonary artery segment, RA and RV | 68 | 12 | 15 |
| II-3 | M  | 33 | Exertional dyspnea and fatigue | Right deviation | 85 | RVH, IRBBB | Enlarged pulmonary artery segment, RA and RV | 52 | 10 | 11 |
| II-8 | F  | 28 | Exertional dyspnea and fatigue | Right deviation | 80 | RVH, ASD | Enlarged pulmonary artery segment, RA and RV | 44 | 8 | 10 |
| III-2 | F  | 7  | Chest discomfort and fatigue | Normal | 92 | RVH | Enlarged pulmonary artery segment and RV | 32 | 5 | 7 |
| III-5 | F  | 3  | No symptom | Right deviation | 118 | RVH, ASD | Enlarged pulmonary artery segment and RV | 37 | 6 | 9 |

M indicates male; F, female; HR, heart rate; RVH, right ventricular hypertrophy; RBBB, right bundle branch block; IRBBB, incomplete right bundle branch block; ASD, atrial septal defect; RA, right atrium; RV, right ventricle; mPAP, mean pulmonary artery pressure; PAWP, pulmonary arterial wedge pressure; PVR, pulmonary vascular resistance, and WU, Wood units.

### Table II. A List of Nonsynonymous Mutations in the Candidate Genes for Pulmonary Arterial Hypertension Identified via Whole-Exome Sequencing and Bioinformatic Analyses

| Chr | Position | Ref | Alt | Gene | Variant |
|-----|----------|-----|-----|------|---------|
| 1   | 233,787,830 | G | A | DISP1 | NM_032890.5: c.3091G > A; p.(Gly1031Ser) |
| 2   | 234,750,188 | C | T | HJURP | NM_018410.5: c.1238C > T; p.(Pro413Leu) |
| 4   | 120,169,961 | G | C | USP53 | NM_001371395.1: c.296G > C; p.(Arg99Thr) |
| 6   | 2,679,632 | A | T | MYLK4 | NM_001012418.5: c.769A > T; p.(Asp257fs) |
| 8   | 55,371,689 | C | T | SOX17 | NM_022454.4: c.379C > T; p.(Gln127*) |
| 14  | 74,447,073 | G | T | TGF3B | NM_002339.5: c.164G > T; p.(Ser55Ile) |
| 15  | 102,190,236 | T | C | TM2D3 | NM_078474.3: c.1298T > C; p.(Arg99Thr) |
| 19  | 4,101,085 | T | A | MAP2K2 | NM_030662.4: c.637T > A; p.(Phe213Ile) |
| 22  | 44,322,946 | C | G | PNPLA3 | NM_025225.3: c.319C > G; p.(His107Thr) |

Chr indicates chromosome; Ref, reference; and Alt, alteration.
served SOX (SRY-related HMG-box) family of transcription factors. SOX17 is widely expressed during embryonic development and involved in Wnt/β-catenin and Notch signaling during development, playing a crucial role in cardiovascular morphogenesis and postnatal structural remodelling.66-68 In the embryonic vasculature, SOX17 is specifically expressed in arterial endothelial cells.69-72 Early investigations revealed that Sox17-deleterious mice did not exhibit obvious abnormalities in embryonic vasculature, which was explained at least partially by functional redundancy and compensatory roles of SOX17 and SOX18.73-75 Subsequent genetic researches revealed that gene compensation and phenotypic impacts depended on murine strain backgrounds.76 Moreover, recent studies revealed that the endothelial cell-specific knockout of Sox17 in murine embryo or postnatal retina led to impaired arterial specification and embryonic death or arteriovenous deformities, respectively.77 Additionally, in humans, several genome-wide association studies have associated SOX17 variants with intracranial aneurysms,78-80 and in an angiostenin II infusion mouse model, the endothelial cell-specific inactivation of Sox17 resulted in intracranial aneurysm pathology.79 Notably, the conditional disruption of Sox17 in mesenchymal progenitor cells revealed that Sox17 was essential for normal pulmonary vascular morphogenesis in utero and that Sox17 deficiency contributed to cardiovascular defects.81 Taken collectively, these results together with the current findings indicate that SOX17 haploinsufficiency is an alternative molecular mechanism underpinning PAH in a subset of patients.

The association between SOX17 variants and enhanced susceptibility to PAH has been revealed previously. Zhu and colleagues82 conducted WES analysis on 256 PAH-CHD patients and subsequently screened a separate cohort of 413 PAH patients without CHD for rare deleterious variants in the top association gene SOX17. As a result, rare deleterious variants of SOX17 were identified in approximately 3.2% of PAH-CHD cases and about 0.7% of PAH cases without CHD. Gräß and coworkers83 performed whole-genome sequencing in 1038 index cases with PAH and 6385 PAH-negative control persons. Case-control analyses revealed that rare pathogenic variants in SOX17 were significantly overrepresented in the PAH patients, with SOX17 variants identified in 9 of 1,038 PAH probands. Hiraihara and partners84 conducted WES in 12 Japanese patients with PAH and 12 asymptomatic family members in 6 families, as well as in 128 Japanese index cases with idiopathic PAH. Moreover, they identified four patients with PAH (of whom one had ASD and two had patent foramen ovale) and one asymptomatic family member with rare deleterious SOX17 variants. These data strongly identified SOX17 as a new causative gene responsible for PAH-CHD and PAH, although the functional effect of these identified PAH-associated variants remains unclear.

Cardiac morphogenesis during embryonic development is a complex biological process, necessitating precise temporal and spatial control of gene expression by such master transcription factors as GATA4, NKX2-5, TBX5, and MEF2C.85 Also, several signaling pathways, including WNT/β-catenin and NOTCH signaling cascades, have been demonstrated to induce cardiovascular development and differentiation.86-88 As a direct transcriptional target of GATA4, SOX17 co-localizes with GATA4 in the primitive endoderm and contributes to SOX17-positive endoderm from embryonic stem cells.89,90 Through direct protein interaction with β-catenin, SOX17 exerts a strong inhibitory effect on WNT/β-catenin signaling.91-93 Considering that β-catenin plays a key role in tissue development and remodeling and that inhibition of β-catenin signaling is beneficial in promoting vascular development, reducing abnormal vascular remodeling and preventing PAH,94 the diminished inhibitory effect on β-catenin by mutated SOX17 promotes the occurrence of PAH. In addition,
**CONFlicts of interest:**

Notch1 has recently been substantiated to be a direct transcriptional target of Sox17 during arterial development. Given that Notch1 has been implicated in vascular repairmainly by promoting the proliferation of lung vascular endothelial cells and recruitment of pulmonary arterial smooth muscle cells during vascular morphogenesis and remodeling, genetically defective Sox17 predisposes to PAH probably by reducing the capability of Notch1 to maintain normal vascular structure and function, though the exact role of Notch1 (pathogenic or beneficial) in the development of PAH remains elusive. Taken collectively, these studies support that the impaired functional interactions between Sox17 and these molecules give rise to CHD and PAH.

In conclusion, this study firstly reports that Sox17 loss-of-function variation contributes to PAH, which offers novel insight into the molecular mechanism of PAH, providing potential implications for genetic counseling, prognostic risk evaluation, and individualized management of patients suffering from PAH.

**Disclosure**

Conflicts of interest: None.

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