

ARTICLE

NOTCH3 Mutations and CADASIL Phenotype in Pulmonary Arterial Hypertension Associated with Congenital Heart Disease

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ABSTRACT

Background: The etiology of pulmonary arterial hypertension associated with congenital heart disease (PAH-CHD) is complicated and the phenotype is heterogeneous. Genetic defects of *NOTCH3* were associated with cerebral disease and pulmonary hypertension. However, the relationship between *NOTCH3* mutations and the clinical phenotype has not been reported in CHD-PAH. **Methods:** We eventually enrolled 142 PAH-CHD patients from Fuwai Hospital. Whole exome sequencing (WES) was performed to screen the rare deleterious variants of *NOTCH3* gene. **Results:** This PAH-CHD cohort included 43 (30.3%) men and 99 (69.7%) women with the mean age 29.8 ± 10.9 years old. The pathogenic or likely pathogenic mutations of *NOTCH3* were identified in five cases. Patients 2, 5, 8 and 11 carried the same *NOTCH3* mutation c.1630C > T (pArg544Cys), which is the hot-spot mutation for cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). Patient 3 carried the *NOTCH3* mutation p.Arg75Gln that has also been reported to be associated with the CADASIL. Patients 2, 5, 8, 11 took the examination of the cerebral magnetic resonance imaging (MRI) and confirmed the phenotype of CADASIL. **Conclusions:** We first reported the *NOTCH3* rare mutations and CADASIL phenotypes in CHD-PAH patients. The *NOTCH3* rare variants were with a relatively high positive rate and CADASIL phenotypes were likely enriched in PAH-CHD patients. The preoperative neurological examination might be recommended for PAH-CHD patients to determine the surgical contraindications and reduce intraoperative neurological complications.

KEYWORDS

Pulmonary arterial hypertension; Congenital heart disease; *NOTCH3*; Cerebral autosomal dominant arteriopathy with subcortical infarcts and lesions (CADASIL)

1 Introduction

Congenital heart disease (CHD) is the most common birth defect with an incidence of 0.4%–1.5% in the total newborn infants every year. The morbidity and mortality of CHD are the first place of non-infectious diseases of children in the world [1,2]. Approximate 5%–10% of CHD adults develop pulmonary arterial



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hypertension (PAH) [3]. The etiology of PAH-CHD is complicated, and the phenotype is heterogeneous [4]. PAH stems from an interplay among many detrimental factors, including the sizes of the congenital cardiac defect, the delayed treatment and the defects of genetics or epigenetics factors [5]. The systemic pulmonary shunt leads to the increase of pulmonary blood flow and the blocked decrease of physiological pulmonary vascular resistance. In addition, the increased shear stress of pulmonary vessels leads to the damage of pulmonary vascular endothelium, the proliferation of smooth muscle cells and extracellular matrix and formation of intravascular thrombosis [6].

Genetic variants are closely associated with PAH-CHD [7,8]. Roberts et al. [9] screened the bone morphogenetic protein receptor 2 (*BMPR2*) gene in 40 adults and 66 children with PAH-CHD and demonstrated that 6% of the subjects have *BMPR2* mutations. Liu et al. [10] reported that *BMPR2* mutations were significantly more enriched in the CHD patients with PAH than in the CHD patients without PAH. These previous studies demonstrated that the pathogenic variants of *BMPR2* might predispose the susceptibility of PAH in CHD patients. However, whether the deleterious variants in other genes influence the development of CHD-PAH remains largely unknown.

NOTCH3 gene is a key member of NOTCH signaling pathway, which is evolutionarily conserved and involved in regulation of cell–cell interactions. NOTCH3 is predominantly expressed in the vascular smooth muscle cells (VSMCs), and is crucial for development of heart, pulmonary and brain vessels [11]. *NOTCH3* mutations have been reported previously in PAH patients [12] and were also linked to CADASIL [13]. However, the clinical phenotypes caused by *NOTCH3* mutations are diversified, including CADASIL and PAH [11,14]. Up to date, the relationship between *NOTCH3* mutations and clinical phenotypes has not been analyzed in CHD-PAH. We used whole exome sequencing to detect the rare variants of *NOTCH3* gene in 142 CHD-PAH patients, and we found *NOTCH3* mutations associated with the CADASIL phenotype in CHD-PAH patients for the first time.

2 Methods

2.1 Study Population and Clinical Data Collection

Patients who were first diagnosed with PAH-CHD were consecutively enrolled at Fuwai Hospital of Chinese Academy of Medical Sciences in Beijing from October 31, 2018, to October 25, 2019. CHD was diagnosed by echocardiography or cardiac CT scanning and PAH was diagnosed by right heart catheterization (RHC) or echocardiography according to the guideline³. All PAH-CHD patients had a moderately increased pulmonary artery pressure (pulmonary artery systolic pressure greater than 40 mmHg). Seven patients were excluded because they were unwilling to undergo genetic testing ($n = 4$) or combined left ventricular system lesions with congenital heart disease ($n = 2$) or acquired heart disease ($n = 1$). Finally, 142 patients were included in the study. The research proposal was approved by the Ethical Committee of National Center for Cardiovascular Diseases, Fuwai Hospital, CAMS&PUMC (Approval No. 2017-JQ-81425002). All the patients were of Chinese ancestry and provided written informed consent for DNA analysis.

2.2 Whole Exome Sequencing and NOTCH3 Mutations Screening

CHD-PAH patients were screened by whole exome sequencing (WES) to detect the rare variants in the coding regions and the intron/exon boundaries of *NOTCH3*. The pathogenic genes of pulmonary hypertension and congenital heart disease were also analyzed respectively. We used the QIAamp® DNA Kit (Qiagen, Germany) to extract DNA from whole blood according to standard operating methods. The DNA extracted from all blood samples was tested, and the OD_{260/280} was between 1.75–1.85, and the concentration was >30 ng/μL. The DNA libraries were prepared based on the protocols of Agilent SureSelect QXT Library Prep Kit (5500-0127). Exome capture was prepared based on the protocols of Agilent SureSelect QXT Target Enrichment for Illumina Multiplexed Sequencing. Then the DNA

libraries were mixed with capture probes of targeted regions using the SureSelect Human All Exon V6 kit. Illumina NovaSeq 6000 S2 Reagent Kit (300 cycles) was used for paired-end 2×150 bp sequencing on an Illumina NovaSeq 6000 System. A total of thirty-three exons of *NOTCH3* gene were detected, which encodes 2321 amino acids. The mutations of *NOTCH3* were checked with online database like HGMD (<http://www.hgmd.org/>) and the 1000 Genome project (<https://www.internationalgenome.org/>). The variants were confirmed by Sanger sequencing.

2.3 WES Data Analysis

The exome sequencing data was processed using the established bioinformatics pipeline [15]. In brief, paired-end reads were mapped and aligned to the human reference genome (version GRCh37/hg19) by BWA-MEM (Burrows-Wheeler Aligner). Picard Mark duplicates was used to identify and flag PCR duplicate reads. Varscan (version 2.4.3) and Samtools (version 1.9) were used to call genetic variants and estimate accuracy of variant calls respectively. To minimize potential technical artifacts, heuristic filters were used and the variants that met any of the following conditions: missingness $>10\%$, minimum read depth ≤ 15 reads, allele balance $\leq 20\%$, genotype quality <30 , mappability <1 (based on 150 bp fragments) were excluded. ANNOVAR was used to annotate the variants and aggregate information about allele frequencies (AF) and *in silico* predictions of deleteriousness. The population AF was obtained from Exome Aggregation Consortium (ExAC), 1000G Genomes Project, NHLBI Exome Sequencing Project (ESP) and gnomAD genome East Asian. Rare variants were defined by $AF < 0.5\%$ in four databases. We used REVEL (<https://sites.google.com/site/revelgenomics/>) as an ensemble method for predicting the pathogenicity of missense variants. The mutation is considered harmful to the protein structure when the REVEL score is greater than 0.5.

2.4 Statistical Analysis

Results were presented as percentages, median (interquartile range), or mean (standard error of mean or SD), as indicated. The frequency of rare variants was compared by χ^2 test or Fisher exact test. All the statistical testing was 2 sided and considered statistically significant at a level of $P < 0.05$. All analyses were performed with PASW Statistics, version 18.0 (SPSS Inc., Chicago, IL, USA).

3 Results

3.1 The Clinical Characteristics of PAH-CHD Patients

Totally 142 PAH-CHD patients were recruited in our study eventually, including 54 atrial septal defects (ASD 38.0%), 66 ventricular septal defects (VSD 46.5%), 7 patent ductus arteriosus (PDA 4.9%), and 15 (10.6%) cases had complex CHD. Forty-three cases (30.3%) were men and 99 cases (69.7%) were women. The mean age was 29.8 ± 10.9 years old. The mean value of mPAP (mean Pulmonary Arterial Pressure) and sPAP (systolic Pulmonary Arterial Pressure) in the cohort were 65.2 ± 15.7 mmHg and 97.1 ± 22.3 mmHg, respectively (see Table 1). Furthermore, the patients demonstrated decreased SiO_2 , which indicated the reduced cardiopulmonary function.

3.2 NOTCH3 Rare Variants of PAH-CHD Patients

Whole exome sequencing was performed in the 142 PAH-CHD patients. Pathogenic or likely pathogenic mutations of PAH and CHD related to pathogenic gene were not found in all patients. We identified 11 (7.7%) patients carrying rare *NOTCH3* missense mutants (see Table 2). The full list of PAH and CHD related variants of 11 patients were shown in Table S1. Among these patients, five (3.4%) cases carried the pathogenic or likely pathogenic mutations of *NOTCH3*. Patients 2, 5, 8 and 11 carried the same mutation *NOTCH3* c.1630C>T (pArg544Cys), which has been reported to be a hot-spot mutant in CADASIL [15–17]. In addition, patient 5 also carried c.515G>A, which was with an uncertain significance by ACMG criteria but REVEL indicated harmful to protein structure with a score of 0.893.

Patient 3 carried a likely pathogenic mutation c.224G>A (p.Arg75Gln) in *NOTCH3*, which was also associated with CADASIL. These rare *NOTCH3* variants (c.1630C>T, c.224G>A and c.515G>A) were confirmed by Sanger sequencing (Fig. 1). All the variants have an allele frequency of <0.5% in the gnomAD genome East Asian. *NOTCH3* rare variants c.224G>A and c.515G>A were found only in the VSD patients. The variant c.1630C>T was not significantly different among the four groups patients (ASD, VSD, PDA and complex CHD) ($P = 0.849$).

Table 1: Clinical characteristics of PAH-CHD patients

| Items | CHD-PAH (Mean ± SD) | (Median, IQR) |
|-------------------------|---------------------|---------------|
| Gender | / | / |
| Male (%) | 46 (31.29) | / |
| Female (%) | 101 (68.71) | / |
| Age (Year) | 29.84 ± 10.94 | (39.5, 12.8) |
| mPAP (mmHg) | 65.15 ± 15.71 | (34, 23) |
| sPAP (mmHg) | 97.12 ± 22.31 | (53, 26.6) |
| CTR | 0.52 ± 0.09 | (0.49, 0.1) |
| SiO ₂ (mmHg) | 92.08 ± 3.69 | (91.2, 4.7) |
| PVR (WoodU) | 9.69 ± 5.76 | (3.12, 8.19) |

Note: INR, Interquartile range; mPAP, Mean pulmonary artery pressure; sPAP, systolic pulmonary artery pressure; CTR, Cardiac thoracic rate; SiO₂, Oxygen saturation (arteria femoralis); PVR, Pulmonary vascular resistance.

Table 2: The 11 PAH-CHD patients with rare variants in the *NOTCH3*

| No. | Gender | Age (Years) | Clinical diagnosis | Revel score ^a | 1000 Genomes ^b | gnomAD genome east asian ^c | Coding nucleotide | Amino acid change | Inheritance | ACMG grade |
|-----|--------|-------------|--------------------|--------------------------|---------------------------|---------------------------------------|-------------------|-------------------|-------------|------------------------|
| 1 | Male | 29 | ASD, PAH | 0.054 | 0.000199681 | 0.001010 | c.4791T>A | p.Asn1597Lys | AD | Uncertain significance |
| 2 | Male | 41 | ASD, PAH | 0.574 | 0.000199681 | 0.003967 | c.1630C>T | p.Arg544Cys | AD | Pathogenic |
| 3 | Female | 38 | VSD, PAH | 0.421 | – | 0.001781 | c.224G>A | p.Arg75Gln | AD | Likely Pathogenic |
| 4 | Female | 27 | VSD, PAH | 0.173 | – | 0.001616 | c.4793A>T | p.Asp1598Val | AD | Likely benign |
| 5 | Female | 20 | VSD, PAH | 0.574 | 0.000199681 | 0.003967 | c.1630C>T | p.Arg544Cys | AD | Pathogenic |
| | | | | 0.893 | 0.00001652 | 0.0001658 | c.515G>A | p.Gly172Asp | AD | Uncertain significance |
| 6 | Female | 50 | ASD, PAH | 0.29 | 0.000199681 | 0.001655 | c.3299G>A | p.Arg1100His | AD | Uncertain significance |
| 7 | Male | 39 | VSD, PAH | 0.753 | 0.0007987 | 0.002356 | c.709G>A | p.Val237Met | AD | Uncertain significance |
| 8 | Female | 31 | VSD, PAH | 0.574 | 0.000199681 | 0.003967 | c.1630C>T | p.Arg544Cys | AD | Pathogenic |
| 9 | Female | 21 | VSD, PDA, PAH | 0.334 | 0.000199681 | 0.001332 | c.4348G>A | p.Ala1450Thr | AD | Uncertain significance |
| 10 | Female | 32 | ASD, PAH | 0.173 | – | 0.001616 | c.4793A>T | p.Asp1598Val | AD | Likely benign |
| 11 | Female | 21 | PDA, PAH | 0.574 | 0.000199681 | 0.003967 | c.1630C>T | p.Arg544Cys | AD | Pathogenic |

Note: CHD, Congenital heart disease; ASD, Atrial septal defect; PAH, Pulmonary hypertension; VSD, Ventricular septal defect; PDA, Patent ductus arteriosus; AD, Autosomal dominant; ACMG, The American College of Medical Genetics and Genomics.

^a REVEL is an ensemble method for predicting the pathogenicity of missense variants based on a combination of scores from 13 individual tools: MutPred, FATHMM v2.3, VEST 3.0, PolyPhen-2, SIFT, PROVEAN, MutationAssessor, MutationTaster, LRT, GERP++, SiPhy, phyloP, and phastCons. The REVEL score for an individual missense variant can range from 0 to 1, with higher scores reflecting greater likelihood that the variant is disease-causing. Protein prediction: REVEL>5 indicates harmful.

^b Allele frequency in the 1000 Genomes Project Database.

^c The Genome Aggregation Database (East Asian).

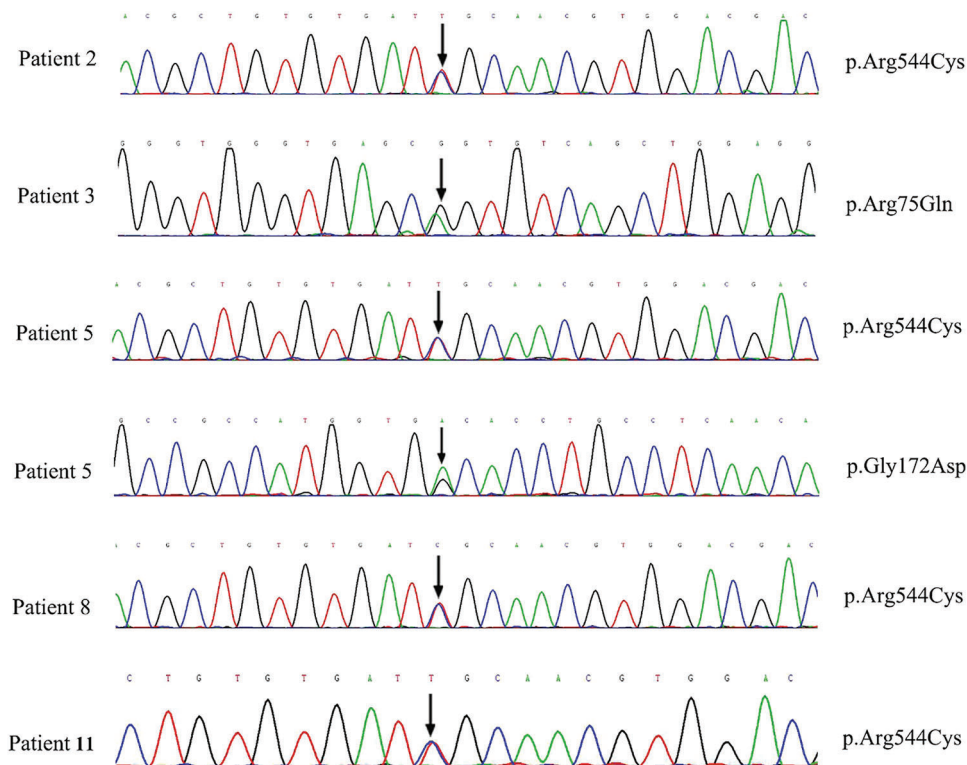


Figure 1: The sanger sequencing results of *NOTCH3* c.1630C>T, c.224G>A and c.515G>A carried by five patients

3.3 Clinical Phenotype of Five PAH-CHD Patients

Five patients (ID: 2, 3, 5, 8, 11) carrying the pathogenic variants of *NOTCH3* were suspected as CADASIL. To confirm the cerebral pathogenic phenotype, four patients (ID: 2, 5, 8, 11) undertook MRI. Patient 3 who had no neurologic symptoms refused to take the examination because of the short hospitalization time and the lack of MRI equipment in the local community. Although these patients denied suffering from Seizure, migraine and other neurological symptoms, Surprisingly, the cerebral MRI results of four cases showed that the presence of white matter changes, which conformed the CADASIL phenotype (Fig. 2). Patient 2 had a few white matter lesions in bilateral paraventricular and central posterior gyrus (Fig. 2A). Patient 5 had white matter scattered ischemic lesions in the cerebral hemisphere (Fazekas I) (Fig. 2B). Multiple lacunar lesions and mild white matter demyelination changes were presented in the bilateral basal ganglia and semi-oval central of Patient 8 (Fig. 2C). Patient 11 had a few white matter lesions in the left occipital lobe and the right radiating crown (DWML I) (Fig. 2D). The results of pulmonary vascular hemodynamic examination of five patients are shown in Table 3. All of five patients had severe pulmonary hypertension.

For Patient 3, the average pulmonary artery pressure was estimated by echocardiogram. For Patients 2, 5, 8, 11, the pulmonary vascular hemodynamic were assessed by right heart catheterization.

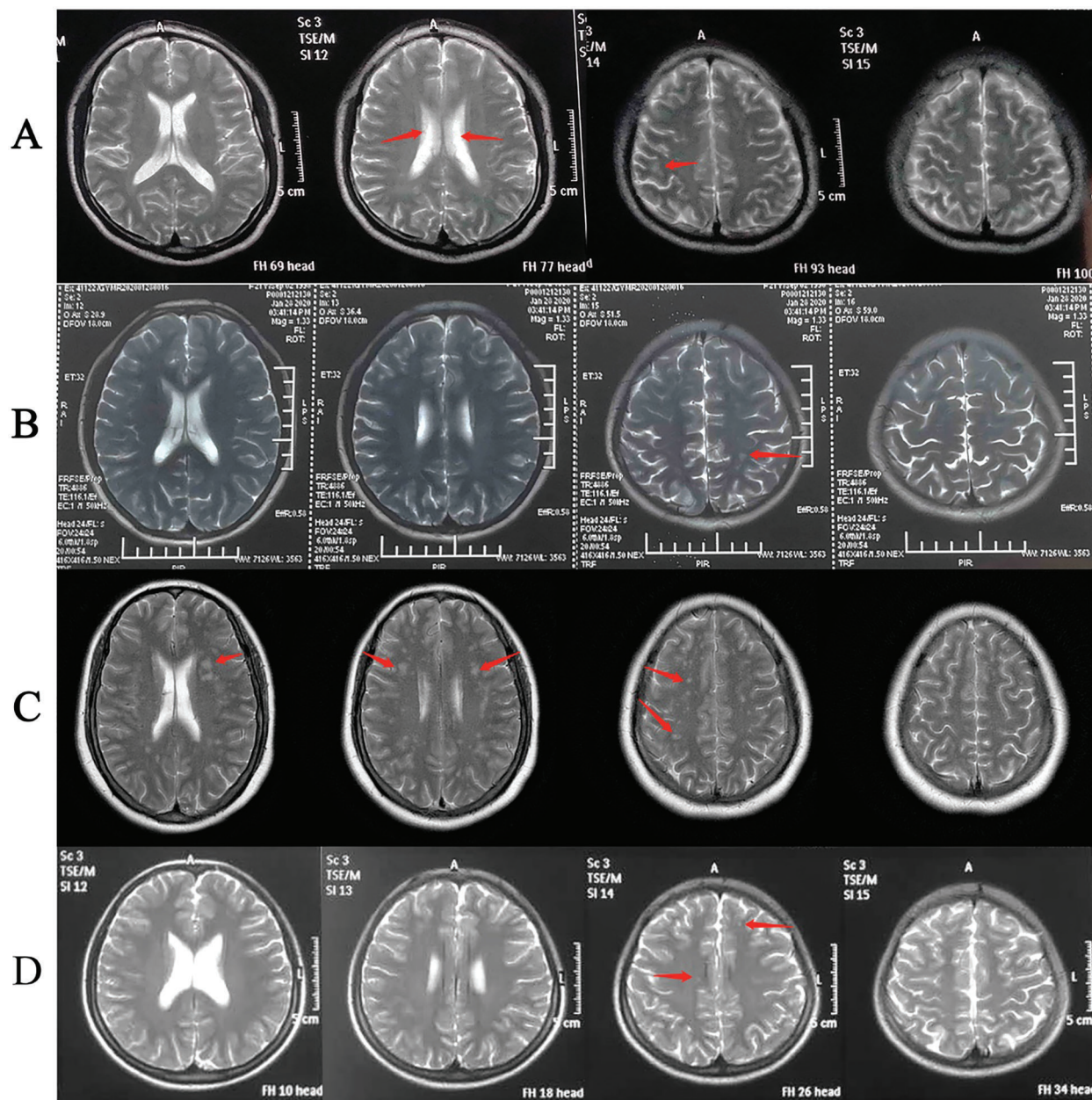


Figure 2: The cerebral MRI of Patients 2,5,8, and 11

4 Discussion

To our knowledge, the etiology of PAH-CHD is complicated and affected by many factors, and the phenotype is heterogeneous [4]. Defining the cause is essential for the treatment of PAH-CHD patients [18]. CADASIL is the most prevalent inherited cause of cerebral small-vessel disease, cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy, a disorder linked to mutations in *NOTCH3* [19,20]. The pathophysiology of CADASIL mainly includes cerebral infarction, cerebral hemorrhage, and white matter changes, etc. [19,21]. In our study, four out of five patients carried the same *NOTCH3* mutation (c.1630C>T, p.Arg544Cys). One patient carried *NOTCH3* c.224G>A, which

has been reported in patients with a CADASIL phenotype [20]. Moreover, *NOTCH3* c.224G>C mutation in the same position has been indicated to be pathogenic in several studies [22–25]. According to American College of Medical Genetics and Genomics (ACMG) guideline [26], *NOTCH3* c.224G>A was a likely pathogenic mutation. Patients 2, 5, 8 and 10 displayed CADASIL image characteristics of cerebral MRI (Fig. 2), which conformed to the CADASIL phenotype.

Table 3: Pulmonary vascular hemodynamic characteristics of five PAH-CHD patients carrying rare deleterious *NOTCH3* variants

| No. | sPAP | sPAP ^a | dPAP | dPAP ^a | mPAP | mPAP ^a | PA/BP | PA/BP ^a | QP:QS | QP:QS ^a | PVR | PVR ^a |
|-----|------|-------------------|------|-------------------|------|-------------------|-------|--------------------|-------|--------------------|-------|------------------|
| 2 | 55.5 | 49.5 | 34 | 31 | 43 | 39 | 0.44 | 0.41 | 1.095 | 1.64 | 6.805 | 4.54 |
| 3# | 97 | / | / | / | / | / | / | / | / | / | / | / |
| 5 | 96 | 95 | 67 | 54 | 79 | 72 | 0.89 | 0.96 | 0.91 | 1.71 | 19.37 | 12.16 |
| 8 | 127 | 110 | 62 | 57 | 86 | 77 | 0.98 | 0.95 | 1.22 | 1.73 | 10.16 | 4.69 |
| 11 | 109 | 106 | 42 | 40 | 73 | 66 | 1.04 | 1.08 | 1.36 | 1.75 | 6.47 | 2.09 |

Note: sPAP, Pulmonary arterial systolic pressure; dPAP, Diastolic pulmonary artery pressure; mPAP, Mean pulmonary artery pressure; PA/BP, Pulmonary artery pressure/blood pressure; QP/QS, Pulmonary blood flow/systemic blood flow; PVR, Pulmonary vascular resistance.

^a After oxygen inhalation.

For Patient 3, the average pulmonary artery pressure was estimated by echocardiogram. For Patients 2, 5, 8, 11, the pulmonary vascular hemodynamic were assessed by right heart catheterization.

Genetic testing finds that the pathogenic mutation of *NOTCH3* can be diagnosed as CADASIL [19]. Except being the well-known pathogenic gene for CADASIL, *NOTCH3* was also involved in pulmonary hypertension pathogenesis [27,28]. Although *NOTCH3* mutations were found to be pathogenic in CADASIL and PAH diseases, it has been assumed that *NOTCH3* has distinct function in these two diseases. However, in our study, there is a possibility that the same *NOTCH3* mutation could cause both CADASIL and PAH-CHD pathogenesis or at least predispose to the development of PAH-CHD, which needs to be subsequently confirmed in the large cohort and functional studies. Furthermore, whether PAH-CHD patients need to receive genetic testing before surgery is still elusive. A neurological examination is needed to determine whether CADASIL is present for PAH-CHD patients without genetic testing. The symptoms of CADASIL are affected by age, drug use, and other factors [29]. Therefore, even though PAH-CHD patients have a CADASIL phenotype, they do not necessarily show obvious neurological symptoms clinically.

Three patients with p.Arg544Cys mutation were treated with surgery in this study. No neurological complications and abnormalities were found in the surgical patients, but the MRI results suggested that the CADASIL was indeed present. The reason might be that most patients with PAH-CHD were young, the onset time was not long for cerebrovascular disease and the condition was not serious in our study. Although the number of cases in this study was small, however, 11 (7.7%) PAH-CHD patients had *NOTCH3* rare mutations, including 5 (3.5%) patients who were diagnosed with CADASIL which was a relatively high prevalence, indicating that the importance of neurological examination before undergoing cardiac surgery for PAH-CHD patients. In addition, the small number of cases may also be one of the reasons that no obvious neurological complications were observed in PAH-CHD patients.

PAH-CHD patients undergoing the neurological examination before surgery is not a clinical routine requirement [30], unless the patient has a history of neurological symptoms, such as headache, dizziness, or peripheral atherosclerosis that is more serious. In addition, there is a great risk of surgery if the neurological examination is not completed or related interventions are not taken, which has an important impact on the postoperative treatment of PAH-CHD patients with CADASIL. Patients undergoing systemic anticoagulation during cardiac surgery and some patients who require postoperative oral

warfarin anticoagulation and blood pressure fluctuations may confer an increased risk of cerebral hemorrhage [31]. Hypoperfusion of cerebral infarction area under cardiopulmonary bypass may also increase the risk of cerebral ischemia and hypoxia [32,33]. What's more, PAH-CHD patients also have headaches and dizziness due to the presence of hypoxia and the use of targeted drugs to reduce lung pressure [34,35], which will mask the mild symptoms of CADASIL and make it difficult to have obvious clinical prompts.

The results of this study suggest that the CADASIL has a relatively high incidence in CHD-PAH patients. Clinically, attention should be paid to investigate the nervous system problems of CHD-PAH patients, determine whether the patients are contraindicated in surgery, and reduce intraoperative neurological complications, especially for elder patients. The symptoms of headaches and dizziness originally caused by CHD-PAH should also be noted. It is crucial to avoid increasing the risk of surgery due to missed diagnosis of CADASIL in PAH-CHD patients. Nevertheless, the impact of postoperative drug use on CADASIL should also be concerned if surgical treatment is allowed in PAH-CHD patients with some obvious abnormal symptoms of CADASIL.

In this study, we first reported the relationship between *NOTCH3* mutations and the CADASIL phenotype in PAH-CHD patients. We found that PAH-CHD patients have CADASIL with a relatively high prevalence. This suggests that PAH-CHD patients should be recommended to undergo a neurological examination before surgery to clarify the surgical contraindications, which is also important for the treatment of patients during and after surgery. However, our research has some limitations. There are no obvious clinical symptoms of CADASIL in the five patients, long-term follow-up observation study is needed to pay attention to the changes in CADASIL symptoms and the effects of cardiac surgical treatment on CADASIL. The sample size of this study is relatively small, and subsequent large sample clinical studies are needed to further confirm the importance of neurological examination before surgery in PAH-CHD patients. In addition, the *NOTCH3* gene causing CADASIL is not only related to the pathogenicity of CADASIL and PAH, but also may be associated with the pathogenic of PAH-CHD patients, and the large cohort and functional studies are in progress.

5 Conclusions

In conclusion, the *NOTCH3* rare mutations and CADASIL phenotype in CHD-PAH patients were first reported. The results strongly implicate that the *NOTCH3* rare variants and CADASIL phenotype were likely enriched in PAH-CHD patients. Preoperative neurological examination might be recommended for PAH-CHD patients to determine the surgical contraindications and reduce intraoperative neurological complications. However, we have only screened for rare variants of *NOTCH3* of small number of patients. Larger cohort studies are needed to confirm the relationship among the *NOTCH3*, CADASIL phenotype and PAH-CHD.

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Authorship: Rui Jiang, Kaisheng Lai and Xiaojian Wang had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the results. Jianping Xu, Xiang Feng and Shaoye Wang collected data and critical revision of the manuscript for important intellectual content. Rui Jiang and Kaisheng Lai drafted of the manuscript. Kaisheng Lai and Zhe Liu detected the samples and conduct data analysis.

Data Availability Statement: Data are available on reasonable request.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Table S1: The full list of PAH and CHD related variants in 11 patients

| No. | Gene | Coding nucleotide | ACMG grade |
|-----|----------------|-------------------|------------------------|
| 1 | <i>WDR35</i> | c.3130C>A | Uncertain significance |
| | <i>GLIS3</i> | c.2053G>A | Uncertain significance |
| | <i>FBN2</i> | c.6662A>G | Uncertain significance |
| | <i>DCHS1</i> | c.7645C>T | Uncertain significance |
| | <i>ABCC9</i> | c.3622G>A | Uncertain significance |
| | <i>NOTCH3</i> | c.4791T>A | Uncertain significance |
| | <i>GLI3</i> | c.58A>G | Uncertain significance |
| | <i>CACNA1C</i> | c.6050G>A | Uncertain significance |
| | <i>LBR</i> | c.1331C>A | Uncertain significance |
| 2 | <i>NOTCH3</i> | c.1630C>T | Pathogenic |
| | <i>DDX58</i> | c.781A>G | Uncertain significance |
| 3 | <i>NOTCH3</i> | c.224G>A | Likely Pathogenic |
| | <i>AFF4</i> | c.2104C>T | Uncertain significance |
| | <i>DCHS1</i> | c.2953C>T | Uncertain significance |
| | <i>B3GAT3</i> | c.974A>G | Uncertain significance |
| | <i>WDR60</i> | c.3088A>G | Uncertain significance |
| | <i>LRP5</i> | c.290C>T | Uncertain significance |
| 4 | <i>ACVRL1</i> | c.213C>G | Uncertain significance |
| | <i>LRP2</i> | c.7516G>A | Uncertain significance |
| | <i>NOTCH2</i> | c.3143G>A | Uncertain significance |
| | <i>NOTCH3</i> | c.4793A>T | Likely benign |
| 5 | <i>NOTCH3</i> | c.1630C>T | Pathogenic |
| | <i>NOTCH3</i> | c.515G>A | Uncertain significance |
| | <i>SAMD9</i> | c.170T>C | Uncertain significance |
| | <i>KYNU</i> | c.275A>G | Uncertain significance |
| | <i>IFT172</i> | c.1541T>C | Uncertain significance |
| 6 | <i>NOTCH1</i> | c.2743C>G | Uncertain significance |
| | <i>NOTCH3</i> | c.3299G>A | Uncertain significance |
| | <i>GLI3</i> | c.1843A>T | Likely benign |
| | <i>PKD1L1</i> | c.6013C>T | Uncertain significance |
| | <i>DCHS1</i> | c.9679C>T | Uncertain significance |
| | <i>PRKD1</i> | c.1202G>A | Uncertain significance |
| | <i>CCDC114</i> | c.1540G>A | Uncertain significance |

(Continued)

| Table S1 (continued) | | | |
|-----------------------------|----------------|-------------------|------------------------|
| No. | Gene | Coding nucleotide | ACMG grade |
| 7 | <i>FLNB</i> | c.2183C>T | Uncertain significance |
| | <i>NOTCH1</i> | c.844C>T | Uncertain significance |
| | <i>HSPA9</i> | c.516G>A | Uncertain significance |
| | <i>CRB2</i> | c.3448T>C | Uncertain significance |
| | <i>NOTCH3</i> | c.709G>A | Uncertain significance |
| 8 | <i>NOTCH3</i> | c.1630C>T | Pathogenic |
| | <i>AQP1</i> | c.244G>A | Uncertain significance |
| | <i>FLNA</i> | c.7544G>A | Uncertain significance |
| | <i>IFIH1</i> | c.2232T>A | Uncertain significance |
| | <i>ELN</i> | c.1358-6C>T | Uncertain significance |
| 9 | <i>KMT2D</i> | c.7327C>T | Uncertain significance |
| | <i>KMT2D</i> | c.4682T>G | Likely benign |
| | <i>ESCO2</i> | c.1468G>A | Uncertain significance |
| | <i>LONP1</i> | c.1315T>A | Uncertain significance |
| | <i>SOS2</i> | c.1187G>A | Uncertain significance |
| | <i>HTR2B</i> | c.166T>C | Uncertain significance |
| | <i>CHD7</i> | c.6401A>G | Uncertain significance |
| | <i>KAT6B</i> | c.3752T>C | Uncertain significance |
| | <i>TBX4</i> | c.175G>A | Uncertain significance |
| | <i>NOTCH3</i> | c.4348G>A | Uncertain significance |
| | <i>BCOR</i> | c.757G>A | Uncertain significance |
| 10 | <i>DNMT3A</i> | c.407G>C | Uncertain significance |
| | <i>MYPN</i> | c.2417A>C | Uncertain significance |
| | <i>CHRM3</i> | c.1766C>A | Uncertain significance |
| | <i>EIF2AK4</i> | c.1306G>A | Uncertain significance |
| | <i>NOTCH3</i> | c.4793A>T | Likely benign |
| 11 | <i>NOTCH3</i> | c.1630C>T | Pathogenic |
| | <i>KAT6A</i> | c.409G>A | Uncertain significance |
| | <i>ASXL2</i> | c.3175A>C | Uncertain significance |