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A Novel Mutation in *BMPR2* in Patients with Congenital Heart Disease and Pulmonary Arterial Hypertension

Meili Wei[1], Bo Han[1]*, Fengqin Liu[1], Laicheng Wang[2] and Jin Sun[1]

[1]*Division of Cardiology, Department of Pediatrics, Provincial Hospital Affiliated to Shandong University, Jinan, Shandong, PR China*
[2]*Central Laboratory of Shandong Provincial Hospital, Jinan, Shandong, PR China*

Abstract

Background: Pulmonary arterial hypertension (PAH) is caused by intensive remodeling of small pulmonary arteries. The main pathological characteristic is proliferation of endothelial and smooth muscle cells. PAH is clinically characterized by a sustained increase in pulmonary arterial pressure, right-sided heart failure and death. Genetic studies in patients of familial PAH (FPAH), idiopathic pulmonary arterial hypertension (IPAH) and congenital heart disease with pulmonary arterial hypertension (CHD/PAH) have identified heterozygous mutations in the bone morphogenetic protein type receptor II (*BMPR2*) gene. To date, only six distinct missense mutations have been identified in patients with CHD/PAH.

Methods: The protein-coding region and intron/exon boundaries of the *BMPR2* gene were amplified by PCR using DNA samples from 80 Chinese Han patients with CHD/PAH and 80 matched controls. Direct sequencing of PCR products was conducted on both the sense and antisense strands. Mutations were excluded if present in a panel of chromosomes from 80 normal individuals.

Results: A novel missense mutation, a G-to-A transition at position 1042 in exon 8, which encodes a Val348Ile mutation, of the *BMPR2* gene, was identified in a female pediatric patient with atrioventricular septal defect/anterior mitral valve cleft/pulmonary arterial hypertension (AVSD/ AMVC/PAH). A single nucleotide polymorphisms (SNP), c. 2811G>A, in the *BMPR2* gene was identified in nine patients and ten controls. However, no significant difference was found in the frequency distribution of the SNP between patients with CHD/PAH and controls.

Conclusions: We identified a novel missense mutation occurring at a valine located in the kinase domain of *BMPR2*. The Val348Ile mutation may be responsible for the development of CHD/PAH.

Keywords: Pulmonary arterial hypertension; Congenital heart disease; Bone morphogenetic protein receptor type II; Gene; Mutations

Introduction

For the first time in 1954, Dresdale detected a genetic predisposition for pulmonary arterial hypertension (PAH) [1]. In 1997, Nichols et al. located susceptibility genes for familial pulmonary arterial hypertension (FPAH) in 2q31-2q32 based on collected home linkage analysis [2]. In 2000, Lane et al. from the international collaborative group of PAH found that mutant *BMPR2* was pathogenic gene for partial Western Caucasian FPAH [3]. *BMPR2* mutations are pathogenically important in FPAH and idiopathic pulmonary arterial hypertension (IPAH). These mutations exist in about 70% of patients with FPAH and 10%-40% of patients with IPAH [4]. In 2004, Roberts reported that *BMPR2* mutations were pathogenic factors for some Western patients with CHD/PAH [5]. However, no relevant reports about mutations in exon 8 of *BMPR2* in patients with CHD/PAH have been published. We conducted mutation screening in the coding regions and intron/exon boundaries of *BMPR2* to identify mutations in Chinese Han patients with CHD/PAH.

Materials and Methods

Study subjects

This research project was approved by the Ethics Committee of Provincial Hospital affiliated with Shandong University. Eighty patients with CHD/PAH were recruited from the Pediatric Cardiology Department and the Cardiac Surgery Department of Provincial Hospital affiliated with Shandong University from January 2000 to March 2011. There was no consanguineous relationship among them. All patients belonged to the Han nationality: 44 males and 36 females, ranging in age from 29 days to 48 years old, and included 66 children (age <18 years) and 14 adults (age >18 years old). Among them, 25 patients had ventricular septal defect (VSD), five patients had atrial septal defect (ASD), 18 patients had patent ductus arteriosus (PDA) and 32 patients had complicated congenital heart diseases. The categories of congenital heart disease are described in Table 1. The diagnosis of PAH was confirmed by measuring mean pulmonary arterial pressure with right heart catheterization (>25 mmHg) [6]. Pulmonary artery pressure was classified into mild (30-40 mmHg), moderate (40-70 mmHg) and severe (≥70 mmHg) according to the pulmonary artery systolic pressure [7]. The subgroups of pulmonary artery pressure in 80 patients with CHD/PAH are listed in Table 2. Each patient and control underwent echocardiography and the CHD category of 19 patients was confirmed by cardiac catheterization.

Mutation detection

Genomic DNA was isolated from lymphocytes using a Pure Gene DNA Isolation Kit. The protein-coding region and intron/exon

***Corresponding author:** Bo Han, Division of Cardiology, Department of Pediatrics, Provincial Hospital Affiliated to Shandong University, Jinan, Shandong, PR China, Tel:+86-531-85187889; E-mail: hanbo35@163.com

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boundaries of the *BMPR2* gene were amplified by polymerase chain reaction (PCR) using DNA samples from 80 patients and a panel of 80 chromosomes from blood of normal donors with matched age and gender. The normal donors were from the same ethnic/regional origin as the patients. Primer pairs specific for all 13 exons and amplification conditions are described in Table 3 [8,9]. PCR reactions were performed in 50 μL reaction system containing 5 μL magnesium chloride, 4 μL deoxynucleoside triphosphate, 0.5 μL primer, 0.25 μL HotStar Taq polymerase and 50 ng genomic DNA. The amplification condition were 95°C for 4 min, followed by 35 cycles of 95°C for 30 sec, 51.2-58.9°C for 30 sec, 72°C for 1min and a final extension at 72°C for 10 min. All PCR products were confirmed by 2% agarose gel electrophoresis. The PCR products were purified and analyzed by direct sequencing using an ABI 3730 (Applied Biosystems, CA, USA). Peak charts of sequencing were analyzed with Sequencher 4.7 Demo software, and compared with the sequence of the *BMPR2* gene in GeneBank (ID: 659; reference sequence: NM-001204.6). All novel detected mutations were excluded if present in a panel of 80 chromosomes from normal controls.

Statistical analysis

Differences between groups were examined for statistical significance using the chi-square test (χ^2-test). A P-value <0.05 denoted the presence of a statistically significant difference.

Results

Genetic findings

To detect variants in the protein-coding region and intron/exon boundaries of the *BMPR2* gene, *BMPR2* was amplified in 80 patients and controls. Direct sequencing of the PCR products revealed that one patient with AVSD/AMVC/PAH carried a novel heterozygous substitution mutation, G1042A, in exon 8 of *BMPR2,* which encodes the kinase domain (responsible for phosphorylation). No other mutations were found in any of the 13 exons. This mutation converted a valine at codon 348 to an isoleucine (Figure 1). This mutation was not detected in the other CHD/PAH patients or the panel of 80 chromosomes from normal individuals.

CHD Category	Children	Adults	Total
VSD	25	0	25
ASD	5	11	16
PDA	16	2	18
VSD/ASD	7	0	7
VSD/ASD/PDA	2	0	2
VSD/ASD/AMVC/MI	1	0	1
VSD/ASD/MVC/ TI	1	0	1
VSD/AC	2	0	2
VSD/PDA/AC/BAV	1	0	1
VSD/MVP	2	0	2
ASD/PDA/MVP	0	1	1
PAPVC/ASD/PDA	1	0	1
PAPVC/CTA/ASD	1	0	1
TAPVC/ASD	2	0	2
TOTAL	66	14	80

Ventricular septal defect (VSD), Atrial septal defect (ASD), Patent ductus arteriosus (PDA), Mitral insufficiency (MI), Anterior mitral valve cleft (AMVC), Mitral valve cleft (MVC), Tricuspid insufficiency (TI), Aortic coarctation (AC), Bicuspid aortic valve (BAV), Mitral valve prolapsed (MVP), Cor triatriatum (CTA), Partial anomalous pulmonary venous connection (PAPVC), Total anomalous pulmonary venous connection (TAPVC)

Table 1: Category of congenital heart disease (CHD) in 66 children and 14 adults with pulmonary arterial hypertension.

Control (<30 mmHg)	**Cases**			**Total**
	Mild (30-40 mmHg)	**Moderate** (40-70 mmHg)	**Severe** (≧70 mmHg)	
80	9	54	17	160

Table 2: Pulmonary artery pressure subgroups in 80 cases with CHD/PAH.

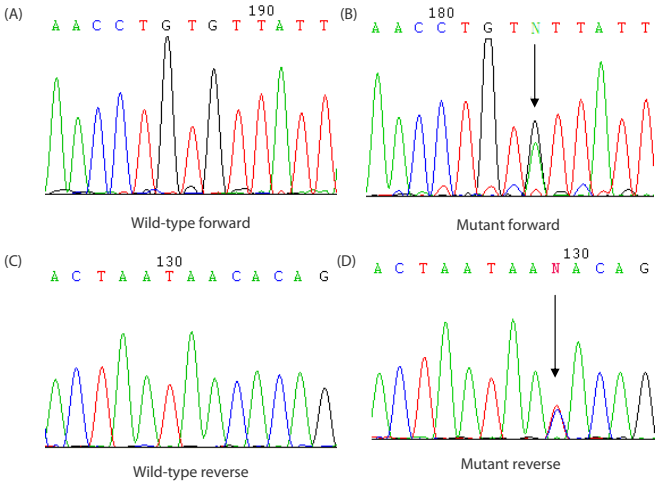

Figure 1: A and **C** indicate the forward and reverse sequence analyses of exon8 coding region in *BMPR2* gene in a healthy control individual, respectively. The G-1042A mutation present in the children with partial atrioventricular septal defect, anterior mitral valve cleft and pulmonary arterial hypertension is shown **B** and **D**, which indicate the *BMPR2* forward and reverse sequence analyses.

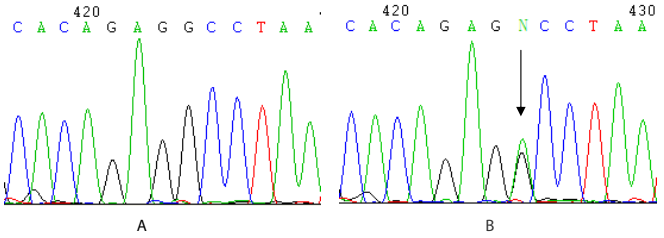

Figure 2: A and **B** indicate the homozygous A / A and heterozygous G / A sequence of c. 2811G> A polymorphism in exon 12 coding region of BMPRII gene, respectively.

Clinical findings

The patient was a girl aged 3 years and 4 months from Liangshan County, Shandong Province. She came to our hospital because of cyanotic lips after activity for one and a half years. Systolic ejection murmur of level 3/6 could be heard between her second and third rib along the left sternal edge, with P2 hyperfunction and fixed splitting. Twelve-lead electrocardiogram showed right axis deviation and right ventricular hypertrophy. Chest X-ray revealed prominent pulmonary arterysegment, peripheral hypovascularity, and right ventricular enlargement. Echocardiography reported (1) that the diameter of her left atrium was 2.65 cm, right atrium was 3.61 cm and right ventricle was 2.48 cm; (2) that the diameter of the main pulmonary artery was expanded to 2.55 cm; (3) atrioventricular septal defect; (4) anterior

mitralvalve cleft with a large number of reflux; (5) that the pulmonary artery systolic pressure was 38 mmHg. The diagnosis was partial AVSD/AMVC/PAH.

Polymorphism at nucleotide 2811(c.2811G>A) in *BMPR2* may be not associated with PAH

A SNP, c.2811G>A (rs1061157), in *BMPR2* was identified in nine patients and 10 healthy controls (Figure 2). The SNP was in the coding region, but it had no effect on the encoded amino acid. Two adult patients were diagnosed with VSD and VSD/PDA/mitral valve prolapse (MVP). The pulmonary arterial pressure was moderate and mild. The other seven patients were children. Among them, two were diagnosed with VSD, three with PDA, one with VSD/ASD and one with VSD/ASD/mitral valve cleft (MVC)/tricuspid regurgitation (TR). Pulmonary artery pressure was mild in two patients, moderate in four patients and severe in one patient. The difference in frequency distributions for alleles G and A and genotypes GG, AG and AA at nucleotide 2811 of the coding sequence for *BMPR2* was compared by χ^2-test in two groups (Table 4). Frequency distribution of the genotypes was consistent with Hardy-Weinberg equilibrium in the overall study population ($\chi^2 = 0.638$, $P = 0.425$).

Discussion

The total length of the *BMPR2* is about 190 kb. It is located on chromosome 2q33, consists of 13 exons and 12 introns, and encodes a protein of 1038 amino acids. The ligand of *BMPR2* is bone morphogenetic protein (*BMP*) [10,11]. *BMPR2* belongs to the transforming growth factor-β receptor superfamily, with serine/threonine kinase activity. The TGF-β/BMPR/BMP signal transduction pathway contributes to proliferation, differentiation and apoptosis of pulmonary artery endothelial cells and smooth muscle cells [10]. Atkinson et al discovered that mutations in *BMPR2* can result in disorder of signal transduction and excessive hyperplasia of pulmonary artery endothelial cells and smooth muscle cells, which led to plexiform lesions and pulmonary hypertension [12]. In 2004, Roberts conducted *BMPR2* gene sequencing in 106 patients of CHD with PAH. He found that six patients carried *BMPR2* missense mutations in exons 2, 3, 5 and 11. Among them, two patients had complete atrioventricular canal defect [5]. The present study revealed a missense mutation in exon 8 of *BMPR2* in a 3 years and 4 months old girl with AVSD/AMVC/PAH. Although it was different from the mutations Roberts reported, the category of CHD in the patient was similar, suggesting that this mutation may be the etiology of PAH in the girl.

In addition, *BMP2* and *BMPR2* play an important role during the process of differentiation and heart development. The ventricles become small and thin and the myocardial trabeculae are lost when the *BMP2* pathway is inhibited by mutation of Smand6 or *BMPR2*. Thus, the *BMP2* signal affects not only the migration and integration, but also the differentiation of myocardial cells [13]. Delot et al. cultivated a knockout mouse model that expressed a *BMP* type II receptor lacking half of the ligand-binding domain. This altered receptor was expressed at comparable levels with the wild-type allele, but the transduction capability was reduced. Mutants died at midgestation with cardiovascular and skeletal defects, demonstrating that the development of these organs required wild-type levels of *BMP* signaling. The most striking defects occurred in the outflow tract of the heart, with the absence of septation of the conotruncus below the valve level and interrupted aortic arch. In addition, semilunar valves did not come into being in the mutants, while the atrioventricular valves appear unaffected [14]. The missense mutation in exon 8 of *BMPR2* led to AVSD. We speculate that *BMPR2* mutations may interfere with, or inhibit, the *BMP2* signaling pathway. The migration and integration of the heart, differentiation of myocardial cells, endocardial cushion and valve formation were affected as a result.

The present study detected a novel *BMPR2* mutation in a Chinese Han pediatric patient with CHD/PAH. The mutation was different from cases in foreign literature. It may be attributed to differences in the category of CHD and ethnicity.

We are not sure whether the exon 8 missense mutation in *BMPR2* was responsible for PAH and AVSD individually or simultaneously in the female pediatric patient. Thus, our colleagues are performing further

EXON	Sense primer	Antisense primer	Annealing Tm
EXON1	5'-TCATCAGCCATTTGTCCTTTCA-3'	5'- GGAAGTGGGGATAGGAAAATACA -3'	58.4 (DMSO5%)
EXON2	5'-TCATGAACAGAAGAACGTCATT-3'	5'-CACAGTCATTTCAGGTAAGAA -3'	58.9
EXON3	5'-GAAACTCCGCCTCAATAA -3'	5'-GAGACGGGATTTCACCAG-3'	51.2
EXON4	5'- CTAAAGGGCAGTCTGTCAGT -3'	5'- TACTATTGAGGCTGGGTGTAT -3'	58.0
EXON5	5'-TGGCTTTCATGCTATTCTGC-3'	5'-CCCCTTTTCATCACTTTCTTAT-3'	51.2
EXON6	5'-TGTAAGCAACAGAGAGCTGT-3'	5'-ACCACACCTGGCCTCAGTT-3'	58.0
EXON7	5'TGTTTAAATTCCCCTTTCCATC--3'	5'-TTTGAACCCACATGAGTGTCA-3'	58.0
EXON8	5'–GAGTTGAAATTCCGATTTCTCT3'	5'-ACACCTGGCCAGTAGATGTT-3'	58.0
EXON9	5'-TTAATGACATGGTTAGGGTCAA-3'	5'-GTTAGGTACTATAGGTAGAGAA-3'	56.0
EXON10	5'TGGTATCAGAAATACCCCTGT-3'	5'-ATTTGTGGCATTAGGCAACTC-3'	58.0
EXON11	5'-ATGGTTTGACATGTACTTTGTC-3'	5'-ATCTTGCACTTGTACCAAACAA-3'	58.0
EXON12-1	5'-TGTACGTTTGGAAGAAAATG -3'	5'-ATTGTATTCATCTTGGCAACTC -3'	51.2
EXON12-2	5'-GCAACTGGACAGCAGGACTT-3'	5'-AATAGTTATTTAAATGGCCCCA-3'	51.2
EXON13	5'-AGTTACATCCCTTACCCGTTA-3'	5'-CACTCCATAGGCTTGAAAACA-3'	58.0

Table 3: Primers used for PCR amplication.

Groups	Genotype frequency (%)			Allele frequency (%)	
	GG	AG	AA	G	A
Case group	71(88.75%)	9(11.25%)	0(0%)	151(94.38%)	9 (5.62%)
Control group	70(87.50%)	10(12.50%)	0(0%)	150(93.75%)	10 (6. 25%)

Two groups of genotype comparison, χ^2=0.0597, P=0.80697; Comparison of allele frequencies, χ^2=0.0560, *P*=0.812933

Table 4: Genotype and allele frequency distribution of the BMPR2 nucleotide 2811 in the case and control groups.

studies on the child's pedigree. We are trying to find similar patients and the same mutation in the pedigree to analyze the relationship between the missense mutation and occurrence of AVSD and PAH.

Meanwhile, a SNP, c.2811G>A was detected through sequence analysis of *BMPR2* in the CHD patients and controls. However, no correlation was found between the SNP and CHD occurrence in the patients by correlation analysis.

In addition, we need a large sample of genetic research in order to identify whether the patients with CHD/PDA present other genetic mutations. Further detection is under way on the specific function of the signaling pathway of TGF-β/BMP/BMPR members. The relationship between the signaling pathway, CHD/PAH and pulmonary vascular obstructive disease will be clarified. Animal experiments are also being carried out. These studies will provide a greater theoretical basis for the early diagnosis, treatment and long-term prognosis for CHD.

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