Original Article

5′UTR Repeat Polymorphisms of the *BMPR2* gene in Children with Pulmonary Hypertension associated with Congenital Heart Disease

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The mutations of bone morphogenetic protein receptor type 2 (*BMPR2*) in patients with idiopathic pulmonary hypertension has been well defined. We investigated the occurrence of BMPR2 mutation and genetic polymorphisms in children with pulmonary hypertension associated with congenital heart disease (aPH/CHD) and correlated with the pulmonary haemodynamic and vasoreactivity.

Methods: ***BMPR2*** **mutation/polymorphisms were determined in 30 aPH/CHD children. All children underwent cardiac catheterisation to obtain baseline haemodynamic data. The 5′UTR containing promoter region and all the exons [1–13] of *BMPR2* gene were genotyped for possible genetic variants that may be related to the aPH/CHD.**

Results: **None of our 30 patients (median-age 90 months) with aPH/CHD (mean PAP 48 ± 17 mmHg, PVR 6.7 ± 4.2 WU m^2) has had any *BMPR2* mutation. Fifteen of them had single nucleotide polymorphism, rs1061157 and/or 5′UTR-polymorphism, specifically GGC repeat variant in seven patients; AGC repeat variant in one patient; and nine base pairs duplication (CTTCTTCGG) in one patient. The GGC repeat $\geq$13 was found in three out of six of children with aPH/CHD with normal PVR vs. two out of 24 children with aPH/CHD with high PVR. The odd ratio between these two subgroups of aPH/CHD is 0.09 (95% CI 0.02–0.34).**

Conclusions: **In our cohort, there was no *BMPR2* mutation in children with aPH/CHD while nine out of 30 of them have 5′UTR repeat polymorphisms. Our data suggests the occurrence of GGC repeat $\geq$13 at the 5′UTR region may have some protective effect towards pulmonary vasculopathy in children who have been exposed to high pulmonary blood flow due to CHD.**

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Keywords. Pulmonary hypertension; Congenital heart disease; BMPR2; Polymorphism; Children

Introduction

Pulmonary arterial hypertension (PAH) is a progressive pulmonary vasculopathy with significant morbidity and mortality in patients who had elevated pulmonary pressure or pulmonary hypertension (PH). The clinical spectrum of paediatric PH includes idiopathic PAH (IPAH), familial PAH (FPAH), as well as PH associated with congenital heart disease (CHD), and connective tissue disease. Despite the various causative mechanisms, the pathological evaluation of the PAH lung tissue consistently reveals obstruction of the pulmonary arterioles caused by proliferation of the endothelial cells and pulmonary artery smooth muscle cells (PASMCs), and fibroblast hypertrophy with an active vascular remodelling [1]. Several studies have shown that PAH in some cases have genetic determination. It is well documented that the bone morphogenetic protein receptor type 2 (*BMPR2*) gene mutation contributes to the pathogenetic mechanism of IPAH by down-regulation of the inhibitory influence of BMP on the PASMCs which leads to disturbed growth and differentiation of pulmonary circulation cells [2]. Previous studies have shown that IPAH and FPAH patients with *BMPR2* mutation appear to have more severe disease and are less likely to respond to pulmonary vasoreactivity testing [3,4]. In a mixed cohort of adult and children with PH associated with CHD (aPH/CHD), *BMPR2* mutations were found in 6% [5]. However, the impact of *BMPR2* mutation on severity and the clinical implication in this group of aPH/CHD patients has remained imprecise.

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The objectives of our study were to determine: (1) the frequency of *BMPR2* mutations/variants; (2) the correlation between the *BMPR2* mutations/variants and the pulmonary haemodynamics and vasoreactivity tests in our cohort of children with aPH/CHD.

Methods

We studied a cohort of consecutive paediatric patients with aPH/CHD at Ramathibodi Hospital, Mahidol University during 2007–2009. The diagnosis of their CHD was confirmed by transthoracic echocardiogram. These patients were confirmed with diagnosis of PH, according to the World Health Organization's Dana Point 2008 Pulmonary Hypertension Symposium consensus [6].

All patients underwent cardiac catheterisation to confirm the diagnosis of PH and also vasoreactivity testing [7]. None of them had idiopathic or familial PAH (IPAH/FPAH). Blood samples for genetic studies were obtained during cardiac catheterisation. Data has been presented as mean ± SD. Written informed consent was obtained according to the protocol approved by the institutional review board of Ramathibodi Hospital, Mahidol University.

Haemodynamics and Acute Vasoreactivity Testing

The patients were divided by their pulmonary vascular resistance (PVR) into two groups, specifically aPH/CHD with high PVR (PVR $\geq$ 3 WU m^2) and aPH/CHD with normal PVR (PVR < 3 WU m^2). Complete haemodynamic data were obtained during the right and left heart catheterisation at rest and during the pulmonary vasoreactivity testing. The substance used for acute pulmonary vasodilator testing included oxygen and aerosolised iloprost [8]. The acute response to the vasodilator testing is defined as the percentage of change in pulmonary vascular resistance (PVR) which is needed for patients with CHD in whom the intracardiac shunt remain present. The response to pulmonary vasodilator causes the decrease in PVR which is at the same time increased by the pulmonary blood flow, while the pulmonary artery pressure (PAP) usually remains unchanged. Therefore, patients who are responsive (responder) have the PVR decrease by $\geq$20% [9].

Genetic Analysis

Isolation of genomic deoxyribonucleic acid (DNA) and polymerase chain reaction (PCR) were performed following standard protocols. The noncoding exon 1, coding exons 2–13, and 5′ untranslated region (5′UTR; nucleotides −8 to −1128 from the first nucleotide of start codon) containing promoter of the *BMPR2* gene, were PCR amplified and directly sequenced. Program PRIMER 3 was used for primer design; http://www.Frodo.wi.mit.edu/cgi-bin/primer3 (Primer sequences are available per request or as supplementary data at the journal website). GenBank reference sequences were *BMPR2*: NT_005403 and NM_001204. Sequencing was performed on an ABI 3100 DNA sequencer after purification with QIAquick PCR Purification Kits (Qiagen®, USA). Fifty-four healthy control individuals (38 males and 16 females), aged ranging 18–60 years, who are regular blood donors at Ramathibodi Hospital were screened for any mutations/variants identified in the patients.

A synonymous single nucleotide polymorphism (SNP), c.2811G>A (rs1061157 or a silent mutation Arg937Arg), in exon 12 was identified. After identification of the c.2811G>A SNP in the first patient, we established a rapid DNA test using agarose gel electrophoresis, primer pairs Ex12 [3], and PCR-restriction digestion with endonuclease enzyme *Bsm*A1 (GTCTCN′). The SNP creates an additional *Bsm*A1 restriction site while the normal G-allele contains only one restriction site (Fig. 1).

Statistical Analysis

Data has been presented as mean ± SD or median with minimum and maximum range. Statistical analysis used was Student *t*-test for comparison of data with normal distribution and Mann–Whitney *U* test for comparison of data without normal distribution. Fisher exact test was used to compare variables (genetic variation status). *p*-Value < 0.05 was considered statistically significant.

Result

Clinical Data

The study population included 30 consecutive patients (15 females) with aPH/CHD. The median age was 72 months (range, 1–264 months). The cardiac diagnoses and demographic data have been shown in Table 1. All patients underwent cardiac catheterisation for haemodynamic evaluation and to confirm their diagnosis of PH. At baseline, the mean of mPAP was 48 ± 17 mmHg, the mean PVR was 6.7 ± 4.2 WU m^2, and the mean pulmonary-to-systemic blood flow ratio (Qp/Qs) was 2.8 ± 3.4. Twenty-four patients had aPH/CHD with high PVR (3 WU m^2), while the other six children have aPH/CHD with normal PVR. Comparison between these two groups, the aPH/CHD with high PVR group has significantly higher mPAP and PVR (mPAP 52.1 ± 16.7 mmHg vs. 31.8 ± 2.8 mmHg and PVR 7.8 ± 3.9 WU m^2 vs. 2.1 ± 0.6 WU m^2, $p < 0.05$) while the pulmonary-to-systemic blood flow ratio (Qp/Qs) is not significantly different (Qp/Qs 2.7 ± 3.7 vs. 2.9 ± 2.3, $p > 0.5$) (Table 2).

Genetic Data

The genetic analysis revealed no *BMPR2* mutation in our cohort. A SNP, c.2811G>A (rs1061157), was identified in nine patients (Table 3). An intronic variant in intron 13 (IVS13-19T>G) was identified in one patient. The extended analysis of the 5′UTR for possible mutation/SNPs disclosed three nucleotide repeat polymorphisms as follows: 12-GGC repeats at nt −928 to −963 upstream from the start codon in seven patients; 3-AGC repeats at nt −919 to −927 in one patient; and nine base pair (CTTCCTCGG) duplication of nt −908 and −916 in one patient (Fig. 2). The rs1061157 and GGC repeat variant were also observed in the control group (Table 4). One out of 53 control