BMPR2 Mutations in Six Taiwanese Patients with Idiopathic Pulmonary Arterial Hypertension

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Background: Mutations in bone morphogenetic protein receptor type II (*BMPR2*) and activin-like kinase 1 (*ALK1*), have been implicated in the pathogenesis of heritable or idiopathic pulmonary arterial hypertension (HPAH/IPAH). However, there was still a lack of information regarding the underlying genetic factors in Taiwanese PAH patients.
Methods: A total of 6 patients diagnosed with IPAH were enrolled in this study. The entire protein-coding region and intron/exon boundaries of the *BMPR2* and *ALK1* genes were amplified by polymerase chain reaction and analyzed by direct sequencing.
Results: We identified 3 patients with *BMPR2* heterozygous exonic mutations. One was a missense mutation, *R491W*. The second was a 2-base pair (bp) TG deletion at positions 1446 and 1447 relative to the translation start site. The third was a 2-bp CA deletion replaced by a single nucleotide T insertion at positions 991 and 992. The latter two mutations are novel and expected to result in frame shifts and premature termination. None of these 6 patients carried exonic mutations in the *ALK1*gene.
Conclusion: A substantial portion of Taiwanese IPAH patients carry *BMPR2* mutations. Since mutations in *BMPR2* may be heritable and are associated with poor prognosis, genetic screening for *BMPR2* mutations may be necessary for Taiwanese IPAH patients.

Key Words: *BMPR2* • Mutation • Pulmonary arterial hypertension

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a rare syndrome resulting from restricted pulmonary arterial circulation, which leads to pathological increases in pulmonary vascular resistance and, ultimately, to right heart failure and mortality.[1,2] Both genetic and environmental factors play an important role in the changes in pulmonary vascular structure and function.[1] Mutations in 2 genes in the transforming growth factor beta (TGF-β) receptor pathway, bone morphogenetic protein receptor type II (BMPR2) and activin-like kinase 1 (ALK1), have been implicated in the pathogenesis of PAH. *BMPR2*, a type II receptor of the *TGF*-β superfamily, modulates vascular cell growth by activating the intracellular pathways of *SMAD* and *LIM* kinases.[3-5] *BMPR2* mutations, which lead to loss of function in the *SMAD* signaling pathway, are prevalent in heritable PAH (prevalence ~75%).[3,4,6,7] Additionally, 11% to 40% of apparent sporadic cases have been reported to have mutations in *BMPR2*.[7-12] An *ALK1* mutation was detected in a group of patients with hereditary hemorrhagic telangiectasia (HHT) and PAH.[5] In the Updated Clinical Classification of Pulmonary Hypertension (Dana Point, 2008), all patients with *BMPR2* mutations have a heritable disease; consequently, the term "familial PAH" has been replaced

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with the term "heritable PAH." Heritable forms of PAH (HPAH) include idiopathic PAH (IPAH) with germline mutations, and familial cases with or without identified germline mutations.[13]

Until recently, there remained a lack of information regarding the underlying genetic factors of Taiwanese PAH patients. Therefore, we conducted a *BMPR2* and *ALK-1* gene survey in 6 Taiwanese patients with PAH.

MATERIALS AND METHODS

Study population

A total of 6 patients with PAH were enrolled in the study. PAH was diagnosed based on the criteria of the Updated Clinical Classification of Pulmonary Hypertension (Dana Point, 2008).[13] IPAH corresponds to sporadic disease in which there is neither a family history of PAH nor an identified risk factor. The occurrence of HPAH was excluded in all patients on account of the absence of a family history of PAH upon entering the study. One patient had previously received surgical correction for a small ventricular septal defect at the age of 10, who later developed severe PAH. All 6 patients with IPAH received right heart catheterization and an acute vasoreactivity test with nitric oxide inhalation from 20 to 80 ppm. The details of the study subjects have been previously reported[14] and are briefly summarized in Table 1. The study protocol was approved by the Ethics Committee of the Chang Gung Memorial Hospital and informed consent was obtained from all subjects.

Genomic DNA extraction

Genomic DNA of patients was isolated from the peripheral blood leukocytes using a Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA).

Mutation analysis of the BMPR2 gene

The entire protein-coding region and intron/exon boundaries of the *BMPR2* gene were amplified by polymerase chain reaction (PCR) using DNA samples from affected individuals and primer pairs specific for all 13 exons, as described in the literature.[3] Automated sequencing of both strands of the PCR products was performed using a commercial sequencing service with an ABI 3700 DNA analyzer (Applied Biosystems, Foster City, CA, USA).

Genotyping of the BMPR2 promoter G-699A mutation

Genotyping of the G-699A mutation in the promoter region of the *BMPR2* gene was performed as previously described.[15]

Mutation analysis of the ALK1 gene

The entire protein-coding region and intron/exon boundaries of the *ALK1* gene were amplified by PCR using DNA samples from affected individuals and primer pairs specific for all 10 exons except exon 1, as described by Berg et al.[16] The coding region of *ALK-1* is contained within these 9 exons. Automated sequencing of both strands of the PCR products was performed using a commercial sequencing service with an ABI 3700

Table 1. Patient characteristics

Patient No.	Age (onset years)	Sex	Diagnosis	6MWD (meter)	mPAP (mm Hg)	PCWP (mm Hg)	PVR (Dynes x sec/cm^{-5})	AVT	Treatment
1	25	Male	HPAH	423	81	9	1811	Nonresponse	Bosentan + iloprost inhalation
2	53	Female	IPAH	324	63	5	1352	Nonresponse	Bosentan + sildenafil
3	54	Female	IPAH	260	46	7	1177	Response	Bosentan + sildenafil
4	36	Female	HPAH	468	60	10	1280	Nonresponse	Bosentan + sildenafil
5	27	Female	HPAH	330	61	6	1433	Nonresponse	Bosentan
6	20	Male	IPAH*	450	99	11	1408	Nonresponse	Bosentan + iloprost

* Surgical correction of a small ventricular septal defect at the age of 10 years.

AVT, acute vasoreactivity test; HPAH, heritable pulmonary arterial hypertension; IPAH, idiopathic pulmonary arterial hypertension; mPAP, mean pulmonary artery pressure; PCWP, pulmonary capillary wedge pressure; PVR, pulmonary vascular resistance; 6MWD, 6-minute walking distance.

DNA analyzer (Applied Biosystems).

RESULTS

The *BMPR2* mutations identified in this study are summarized in Table 2. DNA mutation numbering is based on +1 as the A of the ATG translation start site in the cDNA sequence. In the 6 cases of PAH examined, we found 3 cases with *BMPR2* heterozygous exonic mutations. One was a missense mutation and the remaining two were novel deletion/insertion (del/ins) mutations, which were expected to result in frame shift mutations and premature termination. The first patient had a single nucleotide substitution (C>T transversion) at nucleotide position 1471 within exon 11, which converted an arginine into a tryptophan residue (c.1471C>T, pArg491Trp, Figure 1). In this case, genomic DNA samples from both parents were also examined but the mutation was not observed. The second patient had a 2-base pair (bp) TG deletion within exon 11 at nucleotide positions 1446 and 1447, and the third patient had a 2-bp CA deletion replaced by a single nucleotide T insertion within exon 8 at nucleotide positions 991 and 992. Both mutations are predicted to result in frame shifts, leading to premature termination (1446-1447delTG, C483fsX15 and 991-992delCAinsT, H331fsX4; Figures 2, and 3, respectively); however, genomic DNA samples from neither set of parents were available for examination.

Additionally, we sequenced the promoter region of the *BMPR2* gene to detect the -699G>A mutation, which has previously been identified in a Chinese patient with heritable PAH.[15] None of our 6 patients carried this mutation.

We found a single nucleotide polymorphism (SNP) rs2071218 in intron 3 of the *ALK-1* gene in 3 patients. One of these 3 patients had 2 additional SNPs, rs2277382 and rs56379428, located in the 5′-untranslated region and exon 7, respectively. None of the 6 individuals tested carried mutations in the entire protein-coding region or the intron/exon boundaries of the *ALK-1* gene.

DISCUSSION

We examined 6 Taiwanese PAH patients thought to be IPAH for germline mutations in the *BMPR2* and *ALK-1* genes. *BMPR2* heterozygous exonic mutations were identified in 3 of the 6 (50%) patients. Therefore, according to a recently developed classification system,[13] the patients were reclassified as having HPAH. One of the 3 patients had a R491W mutation, whereas the other two patients had mutations that, to the best of our knowledge, are novel del/ins resulting in frame shifts. The R491W mutation of the *BMPR2* gene was first reported by Deng et al.[3] in a family with primary pulmonary hypertension, and was subsequently observed in several Caucasian FPPH families[3,4,8,17-20] and in a Chinese family.[21] The 2 novel del/ins mutations resulting in a frame shift and premature termination are located in

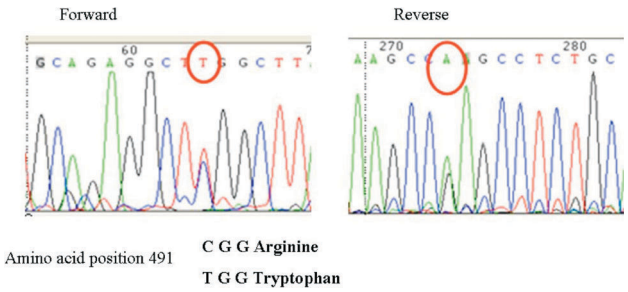

Figure 1. *Sequence analysis of the heterozygous BMPR2 c.1471C>T (R491W) mutation in case PAH1.*

Table 2. Bone morphogenetic protein receptor 2 (BMPR2) mutations

Patient No.	Exon	DNA Sequence variation	Protein sequence variation	Familial screening
1	11	1471C > T	R491W	Absent in both parents
2	-	-	-	ND
3	-	-	-	ND
4	11	1446-1447delTG	C483fsX15	ND
5	8	991-992delCAinsT	H331fsX4	ND
6	-	-	-	ND

ND, not done.

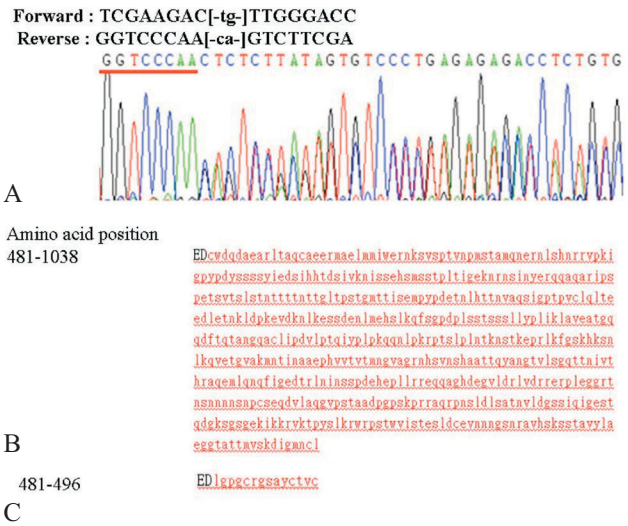

Figure 2. *Sequence analysis of the heterozygous BMPR2 c.1446-1447delTG mutation in case PAH4. (A) indicates the reverse sequence comprising the mutation site. (B) indicates the BMPR2 gene amino acid sequence from codon 481 to the end of the coding sequence. (C) indicates a frame shift amino acid sequence from codon 483 followed by premature termination.*

exons 8 and 11, respectively, which is a region comprising the kinase portion of *BMPR2*.

Compared to other previously studied Asian populations, we found a greater percentage (50%) of IPAH patients possessing the *BMPR2* mutation. Morisaki et al. studied 34 Japanese PAH patients, which included 4 familial and 30 sporadic cases, and identified *BMPR2* mutations in all 4 HPAH cases and 12 (40%) of the IPAH cases.[22] Two recent studies examined the Chinese Han population (72 and 290 IPAH, 4 and 15 HPAH patients in the respective studies), and reported the mutation detection rates were 16.6 and 14.5% for IPAH, in all 4 cases and 53.3% for HPAH, respectively.[23,24] It has been suggested that many cases of apparent IPAH are in fact "heritable," and it is only the low penetrance of the gene that causes these cases otherwise thought to be sporadic. Our finding may have resulted from underdiagnosis of heritable PAH since only 1 family was genetically analyzed. At least 1 of the mutations in the 3 patients with a *BMPR2* mutation was shown to be de novo and not transmitted from either parent; however, in the remaining 2 patients, we were unable to analyze the transmission status because samples from their parents were not available. Nevertheless, our results could still be important for guiding treatment strategies for Taiwanese patients with PAH. Previous results have shown

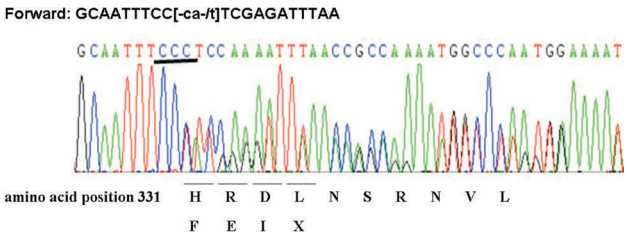

Figure 3. *Sequence analysis of the heterozygous BMPR2 c.991-992delCAinsT mutation in case PAH5. The mutation leads to a shift in the reading frame and premature termination.*

that PAH associated with *BMPR2* mutations may represent a subgroup of patients with a more severe disease who are less likely to show vasoreactivity than those without *BMPR2* mutations.[12,25,26] Accordingly, genetic screening for *BMPR2* may be indicated for Taiwanese patients with PAH. However, initially, the prevalence of *BMPR2* mutations should be examined in a larger PAH population in Taiwan. Moreover, identification of a proband as a *BMPR2* mutation carrier increases the probability that children and siblings carry the affected gene. Therefore, identifying a familial mutation can be valuable in reproductive planning and identifying family members who are not mutation carriers, and thus will not require lifelong surveillance. Nevertheless, the value of routine genetic screening for patients with IPAH is still controversial. In fact, it is not recommended by most experts and guidelines.

Three SNPs of the *ALK-1* gene were identified in 3 of the 6 patients examined in this study. Rs2071218 and rs2277382 are common SNPs, with minor allele frequencies greater than 1% according to a PubMed database. No functional study of these SNPs has been reported previously. The rs56379428 SNP is also considered to be a nonfunctional polymorphism because it is a synonymous substitution. Thus, *ALK-1* gene mutation might not play an important role in Taiwanese patients with IPAH.

In this study, we failed to identify a causative mutation in the *BMPR2* gene for 3 of the 6 cases. There are several possible explanations for this. First, we did not perform quantitative multiplex PCR experiments to exclude the possibility of a large size deletion in the *BMPR2* gene for these 3 cases. Second, causative mutations may occur in currently unknown coding sequences, intronic or regulatory regions of *BMPR2*, or other genes in the *TGF*-β cell signaling pathway. Major limitations

of this study include the small sample size and lack of complete family gene screening for every patient. Thus, information regarding the correlations between genotype and phenotype, outcome, and responsiveness to treatment could not be obtained. Therefore, we believe that a multicenter survey of the *BMPR2* and *ALK-1* genes in Taiwanese PAH patients will provide more comprehensive information.

Despite the small sample size in this study, our results suggest that a substantial portion of Taiwanese PAH patients carry *BMPR2* mutations. The importance of genetic screening for *BMPR2* mutations can be used to determine hereditary transmission. In conclusion, *BMPR2* gene mutations may contribute to the genetic background of IPAH patients in Taiwan, and we suggest routine genetic analysis of the *BMPR2* gene for Taiwanese PAH patients.

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