High Frequency of *BMPR2* Exonic Deletions/Duplications in Familial Pulmonary Arterial Hypertension

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Rationale: Previous studies have shown that approximately 55% of patients with familial pulmonary arterial hypertension (FPAH) have *BMPR2* coding sequence mutations. However, direct sequencing does not detect other types of heterozygous mutations, such as exonic deletions/duplications.

Objective: To estimate the frequency of *BMPR2* exonic deletions/duplications in FPAH.

Methods: *BMPR2* mRNA from lymphoblastoid cell lines of 30 families with PAH and 14 patients with idiopathic PAH (IPAH) was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) and sequencing. Sequencing of genomic DNA was used to identify point mutations in splice donor/acceptor sites. Multiplex ligation-dependent probe amplification (MLPA) was used to detect exonic deletions/duplications with verification by real-time PCR.

Measurements and Main Results: Eleven (37%) patients with FPAH had abnormally sized RT-PCR products. Four of the 11 patients were found to have splice-site mutations resulting in aberrant splicing, and exonic deletions/duplications were detected in the remaining seven patients. MLPA identified three deletions/duplications that were not detectable by RT-PCR. Coding sequence point mutations were identified in 11 of 30 (37%) patients. Mutations were identified in 21 of 30 (70%) patients with FPAH, with 10 of 21 mutations (48%) being exonic deletions/duplications. Two of 14 (14%) patients with IPAH exhibited *BMPR2* point mutations, whereas none showed exonic deletions/duplications.

Conclusions: Our study indicates that *BMPR2* exonic deletions/duplications in patients with FPAH account for a significant proportion of mutations (48%) that until now have not been screened for. Because the complementary approach used in this study is rapid and cost effective, screening for *BMPR2* deletions/duplications by MLPA and real-time PCR should accompany direct sequencing in all PAH testing.

Keywords: dosage; genetics; multiplex ligation-dependent probe amplification

Pulmonary arterial hypertension (PAH) is a disease of the pulmonary vasculature defined by mean pulmonary artery pressure ≥ 25 mm Hg at rest or ≥ 30 mm Hg during exercise (1, 2).

Patients with PAH display characteristic vascular remodeling of the small pulmonary arteries, which results in increased pulmonary resistance and subsequent right heart failure (3). Until recently, the mean survival time after PAH diagnosis was 2.8 yr (4). However, several studies suggest that recent advances in treatment have significantly lengthened survival time (5). The ratio of affected females to males is approximately 2:1. At least 6% of all PAH cases are familial (FPAH) in origin and display autosomal dominant inheritance with incomplete penetrance. The remainder of PAH cases are idiopathic (IPAH) and seem to have the same clinical presentation and presumably the same pathogenesis as FPAH (6).

Several studies have reported mutations in the bone morphogenetic protein receptor, type II gene (*BMPR2*) as causal for PAH in patients with FPAH and IPAH (7–13). Bone morphogenetic protein receptor type II is a member of the transforming growth factor- β superfamily of cell signaling molecules critical in cell differentiation and cell growth (14). More than 140 *BMPR2* mutations have been identified in approximately 55% of FPAH cases and from 11 to 40% of IPAH cases (6). Due to the enormous size of *BMPR2* (13 exons spanning over 180 kb), the method used to identify these *BMPR2* mutations has been almost exclusively direct sequencing of the coding portion of the gene and the intron/exon boundaries. Recently, a study by Cogan and colleagues (15) used a combination of Southern blot analysis and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of leukocyte mRNA to identify novel *BMPR2* exonic deletions/duplications in 3 of 14 patients with FPAH in whom *BMPR2* coding sequence mutations could not be detected. These data suggest that exonic deletions/duplications could account for a significant proportion of *BMPR2* mutations in patients with PAH not harboring a coding sequence mutation.

In an effort to better understand the role of *BMPR2* DNA dosage mutations in PAH, we have investigated 30 families with FPAH and 14 individuals with IPAH. Using a combination of RT-PCR, multiplex ligation-dependent amplification (MLPA), and real-time PCR, *BMPR2* exonic deletions/duplications were identified in 33% of the patients with FPAH. This type of mutation was not detected in the IPAH cases. *BMPR2* mutations (including point mutations, small insertions/deletions, and exonic deletions/duplications) were identified in 70% of FPAH cases and in 14% of IPAH cases. Our study illustrates the limitations of earlier studies that looked only at *BMPR2* coding sequence via sequence analysis and suggests that mutations in *BMPR2* play a larger role in the cause of PAH than previously reported. Some of the results of this study have been previously reported in the form of abstracts (16–18).

METHODS

Patients

Study subjects included 26 patients with FPAH plus four obligate carriers without disease from 30 different families and 14 patients with IPAH. Patients with FPAH were treated at centers throughout the

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United States, and some patient data are therefore incomplete. All patients with FPAH had a well-documented family history of PAH and had hemodynamic data confirming the presence of pulmonary hypertension or were receiving therapy commonly used for the treatment of PAH strongly indicative of the diagnosis of pulmonary hypertension. The 14 patients with IPAH were diagnosed and treated at Vanderbilt University, were thoroughly evaluated to exclude other causes of pulmonary hypertension, and were diagnosed with IPAH according to the criteria established by the NIH Primary Pulmonary Hypertension (PPH) registry (4). Available data on demographics, hemodynamics, treatment, and outcomes for patients with FPAH and IPAH are provided in Tables E1 and E2, respectively, in the online supplement. The study was approved by the institutional review boards at Cincinnati Children's Hospital Medical Center and Vanderbilt University Medical Center, and written, informed consent was obtained from all study subjects.

RNA Analysis

Previously established lymphoblastoid cell lines of patients with PAH were grown in RPMI 1640 containing 15% fetal bovine serum, 100 μ g penicillin/ml, and 100 μ g streptomycin/ml in T-25 flasks. Cells were grown to approximately 1×10^6 cells/flask and incubated in the presence or absence of puromycin (100 μ g/ml) for 16 h before harvesting (19). Cells were harvested using RNeasy Mini Kit including the optional DNase treatment (Qiagen, Inc., Valencia, CA). Generation of *BMPR2* RT-PCR products and their sequencing was performed as previously described with the addition of a second forward primer that corresponded to nucleotides 333–352 (5'-CCTCCCGGCTGTTTCTCCGC-3') (15).

DNA Sequencing of *BMPR2* Exons

Genomic DNA was isolated from the blood of patients with PAH using the Roche MagNA Pure LC DNA purification system (Roche Molecular Biochemicals, Indianapolis, IN). Primers described in the Exon Locator and eXtractor for Resequencing database (<http://mutation.swmed.edu/ex-lax/user/query-11291365541/>) were used to amplify the *BMPR2* exons (including the intron/exon boundaries) of interest (20). After an initial denaturation at 95°C for 5 min, PCR was performed for 35 cycles (15 s at 95°C, 45 s at 60°C, and 45 s at 72°C) followed by a 10-min extension at 72°C. The resulting amplification products were visualized by ethidium bromide staining on a 3% agarose gel. PCR products were purified using ExoSAP-IT (USB Corp., Cleveland, OH) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA). The sequencing reaction products were separated on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

MLPA Analysis

MLPA was performed with 100 ng of genomic DNA according to the manufacturer's instructions using the P093 Salsa MLPA HHT/PPH1 probe set (MRC-Holland, Amsterdam, The Netherlands) (21). Probe amplification products were run on an ABI 3730xl DNA Analyzer using GS500 size standard (Applied Biosystems). MLPA peak plots were visualized using Genemapper software v3.7 (Applied Biosystems). Non-normalized values for peak height and peak area were exported from Genemapper software v3.7 to an Excel template. Normalization of data and calculation of dosage ratios was performed as described at www.mrc-holland.com/MLPA%20analysis.htm. Due to variation in assay performance, we used dosage ratio values of ≤ 0.7 and ≥ 1.35 as our boundaries for deletions and duplications, respectively.

Real-Time PCR Analysis

Applied Biosystems' Assay by Design service was used to design fam-labeled TaqMan gene expression assays for each exon of *BMPR2*. Genomic DNA samples were quantitated by Pico Green fluorescence in triplicate with the Quant-iT PicoGreen dsDNA Kit (Molecular Probes, Eugene, OR). After quantitation, 50 ng of genomic DNA was used in a real-time absolute quantitation assay for the *BMPR2* exon in question performed on the 7300 Real Time PCR System (Applied Biosystems). Assays were performed as 25- μ l reactions in triplicate, with each genomic DNA sample being done in duplicate for the *BMPR2* exon in question. After real-time PCR, data were analyzed with the ABI Se-

quence Detection software, RQ Study upgrade, version 1.2.3 (Applied Biosystems). Quantitation of target amount in DNA samples was accomplished by measuring the threshold cycle (Ct) value and comparing it with a standard curve. Quantitations for patient samples were compared with those of normal control samples for evidence of exonic deletions/duplications.

RESULTS

Using the complementary techniques of RT-PCR, MLPA, and real-time PCR, we identified *BMPR2* mutations in 21 of 30 (70%) unrelated families with PAH and 2 of 14 (14%) of IPAH cases (Tables 1 and 2). Of the 23 *BMPR2* mutations, 10 (43%) were exonic deletions or duplications due to presumed recombination events, 4 (17%) were donor/acceptor splice site mutations, 6 (26%) were small insertions/deletions resulting in frameshifts, 2 (9%) were nonsense resulting in premature termination codons (PTCs), and 1 (4%) was missense. No patient was identified carrying more than one mutation in the *BMPR2* gene, and each patient identified with a mutation was heterozygous for the respective mutation.

Mutations Due to Alterations in DNA Dosage or mRNA Splicing

To identify *BMPR2* mutations in patients with PAH, we initially performed RT-PCR using total RNA isolated from lymphoblastoid cell lines. To prevent potential nonsense-mediated decay (NMD), the lymphoblastoid cell lines were grown with puromycin, which prevented premature degradation of transcripts to enable detection of them by RT-PCR (22). Full-length *BMPR2* cDNAs generated by RT-PCR were directly sequenced. Abnormally sized *BMPR2* mRNAs were detected in 11 patients with FPAH, all from unrelated families (Table 1). Five of these abnormally sized mRNAs resulted from deletions of single exons, two resulted from deletions of multiple exons, two resulted from duplications of single exons, and two resulted from partial deletions of exon 2. These observed alterations of transcript size were predicted to be the result of an exonic deletion/duplication at the genomic DNA level or a mutation in a splice donor/acceptor site.

To determine how many of these 11 altered transcripts were due to exonic deletions or duplications, MLPA analysis was performed on each of the 11 FPAH cases. MLPA analysis showed that 7 of the 11 abnormally sized *BMPR2* mRNA transcripts resulted from DNA dosage changes (Table 1). These included two deletions of exon 2 (Families 12 and 108), two deletions of exon 3 (Families 92 and 95), one deletion of exons 4 and 5 (Family 20), one duplication of exon 2 (Family 65), and one duplication of exon 10 (Family 5). The exon 2 deletions seen in Family 12 (Figure 1) and Family 108 are predicted to result in an in-frame loss of amino acids 26–82 of the 1,038-amino acid *BMPR-II* protein. Conversely, the duplication of exon 2 in Family 65 is predicted to result in an in frame duplication of amino acids 26–82 of the mature protein. The deletion of exon 3 in Families 92 and 95 would presumably lead to an in-frame loss of amino acids 83–139. The deletion of exons 4–5 seen in Family 20 and the duplication of exon 10 observed in Family 5 were previously identified by Southern analysis and RT-PCR (15). However, by MLPA analysis, we have confirmed that these are DNA dosage mutations. The deletion of exon 4–5 is an out-of-frame deletion beginning at codon 140 with a PTC at codon 151. The exon 10 duplication in Family 5 results in alteration of the coding frame at codon 472 leading to a PTC at codon 519. The mutant transcripts for the exon 4–5 deletion and the exon 10 duplication were not detected in RNA from non-puromycin-treated cells and thus were subject to NMD. For confirmation

TABLE 1. *BMPR2* DNA DOSAGE/SPLICING MUTATIONS

Location	Family	Nucleotide Change	Amino Acid Change	Detection Methods	Previous Study
Exon 1	30	?_IVS1 del	Unknown	MLPA/Real-Time	(9, 15)
Exon 1	110	?_IVS1 del	Unknown	MLPA/Real-Time	
Exons 2–13	57	IVS1_? Del	Unknown	MLPA/Real-Time	(9, 15)
Exon 2	12	IVS1_IVS2 del	del aa26–82	RT-PCR/MLPA/Real-Time	(15)
Exon 2	108	IVS1_IVS2 del	del aa26–82	RT-PCR/MLPA/Real-Time	
Exon 2	65	IVS1_IVS2 dup	dup aa26–82	RT-PCR/MLPA/Real-Time	
Exon 2	76	IVS 2 247+2delC	del aa47–82, del aa63–82	RT-PCR/Genomic	
Exon 2	68	IVS 2 247+6T > G	del aa47–82, del aa63–82	RT-PCR/Genomic	
Exon 3	92	IVS2_IVS3 del	del aa83–139	RT-PCR/MLPA/Real-Time	(9, 15)
Exon 3	95	IVS2_IVS3 del	del aa83–139	RT-PCR/MLPA/Real-Time	(9)
Exons 4–5	20	IVS3_IVS5 del	S140fs (+ 11 amino acids)	RT-PCR/MLPA/Real-Time	(15)
Exons 8–9	67	IVS 8 1128+1G > T	del aa323–425	RT-PCR/Genomic	(9, 15)
Exon 9	59	IVS 8 1129–3C > G	V377fs (+ 47 amino acids)	RT-PCR/Genomic	(9, 15)
Exon 10	5	IVS9_IVS10 dup	A472fs (+ 47 amino acids)	RT-PCR/MLPA/Real-Time	(15)

Definition of abbreviations: MLPA = multiplex ligation-dependent probe amplification; RT-PCR = reverse transcriptase–polymerase chain reaction.

of all *BMPR2* exonic deletions/duplications found by MLPA analysis, absolute and/or relative quantitative real-time PCR was performed in all patients except those determined to have a *BMPR2* coding sequence point mutation (Table 2).

The remaining four families with abnormally sized *BMPR2* mRNAs for which DNA dosage mutations were not detected by MLPA analysis were screened for potential splicing mutations by direct sequencing of PCR products containing the exon(s)

Figure 1. *BMPR2* cDNA sequence and multiplex ligation-dependent amplification (MLPA) analysis of Family 12 showing deletion of exon 2. (A) The absence of exon 2 in the mutant allele with splicing of exon 1 directly to exon 3 detected by sequencing of the gel-purified mutant reverse transcriptase–polymerase chain reaction product. (B) MLPA tracing for an affected individual from Family 12 (top tracing) demonstrating a reduction in the exon 2 peak (asterisk) as compared with a normal control (bottom tracing). This approximate 50% reduction in peak intensity is indicative of a heterozygous deletion of exon 2 at the genomic DNA level.

TABLE 2. *BMPR2* CODING SEQUENCE MUTATIONS

Location	Family	Nucleotide Change	Amino Acid Change	Detection Methods	Previous Study
Exon 4	71	516C > G	Y172X	RT-PCR/Genomic	
Exon 6	66	631C > T	R211X	RT-PCR/Genomic	
Exon 6	64	673C > T + 690–91delAGinsT	R225C + K230fs (+ 21 amino acids)	RT-PCR/Genomic	
Exon 6	135	796–799delAGAG	R266fs (+ 11 amino acids)	RT-PCR/Genomic	
Exon 6	124	804delT	A269fs (+9 amino acids)	RT-PCR/Genomic	
Exon 7	119	872delT	L291fs (+ 0 amino acids)	RT-PCR/Genomic	
Exon 10	61	1346T > G	M449R	RT-PCR/Genomic	(9, 15)
Patient					
Exon 3	IPAH303	277insG	E93fs (+ 4 amino acids)	RT-PCR/Genomic	
Exon 12	IPAH88	2504delC	T835fs (+ 3 amino acids)	RT-PCR/Genomic	

Definition of abbreviation: RT-PCR = reverse transcriptase–polymerase chain reaction.

and surrounding intron/exon junctions determined by RT-PCR to be altered. Sequencing of the RT-PCR products from Families 68 and 76 had indicated two mutant species of *BMPR2* transcripts: one lacking codons 63–82 and the other missing codons 47–82 within exon 2 (Figure 2). DNA sequencing of an affected individual from Family 68 identified a heterozygous thymine to guanine transversion at the 6th nucleotide of intron 2 (247+6 T > G). We hypothesized that this alteration resulted in alternative use of two upstream GT cryptic donor sites located within exon 2. The more commonly observed mutant species resulted from use of an exon 2 cryptic donor site 60 bp upstream from the inactivated wild-type site and led to the loss of amino acids 63–82. The less commonly observed mutant species resulted from use of a second exon 2 cryptic splice site 108 bp upstream, which resulted in loss of amino acids 47–82. Sequencing of the exon 2/intron 2 boundary from the affected individual of Family 76 identified a deletion of the second nucleotide of IVS2 (247+2 del C), changing the GC of the donor splice site to a GA. Alteration of the wild-type donor splice site resulted in use of the same two upstream GT cryptic donor sites located within exon 2.

Analysis of *BMPR2* transcripts from Family 67 identified a heterozygous deletion of exons 8–9, but MLPA analysis did not detect deletions of exon 8 or 9 within the genomic DNA. Sequencing of exons 8 and 9 from genomic DNA, including the exon/intron boundaries, revealed a G-to-T transversion of the first base of intron 8 (1128+1 G > T) (Figure 3). The +1 G nucleotide is over 99% conserved in eukaryotic species, and alteration of this nucleotide inactivated the donor splice site, accounting for the altered splicing seen by RT-PCR. Deletion of exons 8–9 results in an in-frame deletion of codons 323–425. Sequencing of transcripts from non-puromycin-treated cells confirmed that this transcript was stable and not subject to NMD.

Family 59 showed a deletion of exon 9 by RT-PCR and sequencing of *BMPR2* mRNA, but no deletion of exon 9 was detected by MLPA. Sequencing of exon 9 and the surrounding intronic sequence revealed an IVS8 C-to-G transversion at the –3 position of the acceptor site (1129–3 C > G). This position is 95% conserved (C or T). Deletion of exon 9 is predicted to result in a frameshift at codon 377 and generation of a PTC 47 codon downstream. Sequencing of *BMPR2* transcripts using non-puromycin-treated RNA failed to detect the mutant mRNA and confirmed that this mutation was subject to NMD.

Point Mutations and Small Insertions/Deletions

Sequencing of *BMPR2* RT-PCR products identified point mutations or small insertions/deletions in 7 of 30 (23%) FPAH samples and in 2 of 14 (14%) IPAH samples (Table 2). All point mutations and small insertions/deletions were confirmed by direct sequencing of genomic DNA. One missense mutation, a

T-to-G transversion in codon 449, was identified in exon 10 of Family 61 and introduced a nonconservative amino acid change of Met to Arg (M449R). This methionine is located within the kinase domain of *BMPR-II* and is highly conserved across species.

Nonsense mutations were identified in Family 71 and Family 66, both of which were found to be subject to NMD. In Family 71, a C-to-G transversion was detected at codon 172 in exon 4 resulting in a PTC (Y172X). The mutation in Family 66 was a C-to-T transition in exon 6, which changed codon 211 from Arg to a PTC (R211X).

Six subjects (4 with FPAH and 2 with IPAH) were found to have small insertions and/or deletions in exons 3, 6, 7, or 12. All six mutations caused a frameshift leading to premature termination and were subject to NMD (Table 2). Patients from Families 124 and 119 were found to have a deletion of a single thymine in exons 6 and 7, respectively. The Family 124 exon 6 delT altered the amino acid sequence starting at codon 269 and caused a PTC at codon 278. The Family 119 exon 7 delT changed the amino acid sequence at codon 291 converting a TTA (Leu) to a TAA (Stop). Family 135 was found to have a 4-bp deletion (AGAG) in exon 6, which caused a shift in the amino acid sequence starting with codon 266 and resulted in a PTC at codon 277.

Family 64 had a complex mutation in exon 6 involving a C-to-T mutation at position 673 (R225C) along with an AG-to-C mutation 17 bp downstream. This resulting frameshift generated a PTC 21 codon downstream. Sequencing of *BMPR2* mRNA from the non-puromycin-treated cells from this patient failed to detect either of the sequence changes identified in the puromycin-treated RNA, confirming that the sequence changes detected were on the same allele and that it was subject to NMD.

IPAH Patients 303 and 88 had insertion of a guanine in exon 3 and deletion of a cytosine in exon 12, respectively. Insertion of a G in exon 3 alters the amino acid sequence at codon 93 and leads to premature termination at codon 97. The delC in exon 12 alters the amino acid sequence starting at codon 835 and causes premature termination at codon 838. Comparison of puromycin- and non-puromycin-treated cells indicated that both mutations resulted in *BMPR2* transcripts that were subject to NMD.

Analysis to Identify *BMPR2* DNA Dosage Deletions/Duplications in Exons 1 and/or 13

Twelve of 30 (40%) FPAH samples and 12 of 14 (86%) IPAH samples were found to be negative for *BMPR2* mutations by sequencing of RT-PCR products. Because heterozygous deletions of exon 1 or exon 13 would fail to be detected by RT-PCR due to nonannealing of the PCR primers, MLPA analysis was

Figure 2. Splicing mutations in Family 68 and Family 76 resulting in partial deletions of exon 2 due to use of cryptic donor sites. (A) Heterozygous G-to-T substitution at the 6th position of intron 2 (247+6T > G) detected in the genomic DNA of an affected individual of Family 68. (B) Heterozygous deletion of the 2nd base of intron 2 (247+1delC) detected in Family 76. (C) The sequence of exon 2 (uppercase) and a portion of the sequence of IVS2 (lowercase). The normal IVS2 splice donor site (gc) is indicated by the green arrow. The positions of the IVS2 donor splice-site mutations are boxed for Families 68 (+6T > G) and 76 (+2delC). The exon 2 cryptic donor splice sites used as a result of the splice donor mutations are marked by red arrows and blue arrows. There is a cryptic donor site with a score of 0.85 (blue arrow) that seems to be used over the mutated donor splice site. A second cryptic donor site upstream of the first with a score of 0.65 (red arrow) also seems to be used, though to a lesser extent. Cryptic GT splice sites were scored using NNSPLICE (www.fruitfly.org/seq_tools/splice.html).

performed in these 24 samples to detect DNA dosage mutations involving these exons. Three of the 12 FPAH samples were determined to have deletions that were confirmed by real-time PCR (see Table 1). These included two deletions of exon 1 (Families 30 and 110) and one deletion encompassing exons 2–13 (Family 57; Figure 4). Of the 12 IPAH samples negative for *BMPR2* mutations by RT-PCR sequencing, none was found to be positive for exonic deletions or duplications by MLPA analysis.

Additional Studies of Patients Negative for *BMPR2* Mutations

The first and last exons (1 and 13) of *BMPR2* were PCR amplified from the genomic DNA of nine familial and 12 idiopathic patients found to be negative for *BMPR2* mutations by RT-PCR

and DNA dosage analysis. Direct sequencing of these PCR products did not identify any mutations in the 5' or 3' untranslated regions of these patients. In addition, six familial and eight idiopathic *BMPR2* mutation-negative patients found to be homozygous by RT-PCR for known exonic SNPs were screened for the two most common SNPs (14% heterozygosity each) at the genomic DNA level by Taqman assay. The SNPs were an A/C (Leu to Leu) at codon 200 and a G/A (Arg to Arg) at codon 937 (rs1061157). We reasoned that any patients found to be homozygous for SNPs at the RNA level but heterozygous for the SNPs at the DNA level could have promoter mutations that prevented expression of one allele. All patients homozygous at the RNA level were also homozygous at the DNA level, so we

Figure 3. *BMPR2* cDNA (A) and genomic DNA sequence (B) of Family 67. As seen in A, cDNA sequencing identified a mutant transcript lacking exons 8 and 9. Genomic DNA sequencing (B) revealed a heterozygous G > T substitution at the first base of intron 8 (1128+1G > T), causing the aberrant splicing.

were unable to discern potential promoter mutations by this method.

DISCUSSION

Of the more than 140 *BMPR2* mutations reported in patients with PAH, the vast majority are coding sequence point, non-sense, or frameshift mutations and several splice donor/acceptor site mutations. These mutations account for approximately 60% of FPAH cases and 10 to 40% of patients with IPAH (6). There are few reports of *BMPR2* exonic deletions or duplications in FPAH or IPAH, and no large-scale studies have been performed to detect this type of mutation (9, 23). The aim of our study was to estimate the frequency of *BMPR2* exonic deletions/duplications in patients with FPAH using complementary methods. This would enable us to obtain a more accurate estimate of the overall frequency of *BMPR2* mutations in subjects with FPAH. Patients having a diagnosis of PAH or being an obligate carrier of the disease and having lymphoblastoid cell lines available were selected for this study. This allowed us to confirm any DNA dosage or potential splicing mutations at the mRNA level.

This report includes analysis of 30 unrelated families with PAH. Eighteen of these families have had no previous mutation analysis reported (see Table E1). Of the 18 new families analyzed, *BMPR2* mutations were identified in 11 (61%), with 8 (73%) of these being point mutations (six coding and two splice) and three (27%) being exonic deletions/duplications. The remaining

12 had previously been included in studies to detect *BMPR2* mutations by use of direct sequence analysis of genomic DNA or by Southern analysis (9, 15). Ten of the previously reported 12 families had no mutation identified by direct sequence analysis, but Families 12 and 92 indicated a possible mutation after Southern analysis. Of these 10 families, the methods described here enabled the detection of a *BMPR2* dosage mutation in five (50%) of them. Point mutations that had gone undetected in previous studies were identified in three families. In addition, our report includes two families (5 and 20) from a previous study in which Southern analysis followed by RT-PCR of *BMPR2* transcripts was used to identify patients with heterozygous *BMPR2* deletions/duplications (15). However, MLPA and real-time PCR analysis of these two families in this study confirmed the presence of the deletions/duplications at the DNA level. Figure 5 shows a summary of the distribution of the mutations identified in the 30 families between the 18 for which no previous mutation analysis had been reported and the 12 that had been included in previous studies (9, 15).

Our study indicates that *BMPR2* exonic deletions/duplications account for a significant proportion of mutations in patients with FPAH. Of the 30 families analyzed in this article, mutations were identified in 21 (70%). Exonic deletions or duplications accounted for 10 of these 21 (48%) mutations. Previous mutation detection methods have used direct sequence analysis of the coding region and intron/exon boundaries or denaturing HPLC (7–10, 13). Relying on these previously used methods would only have enabled detection of 11 of 21 (52%) mutations in these 30 families with PAH. This study indicates that the combination of direct sequencing of the *BMPR2* coding region and intron/exon boundaries with DNA dosage analysis can detect mutations in up to 70% of familial PAH. In addition, two of the 14 (14%) patients with IPAH were found to have mutations; one was a single base insertion, and the other was a single base deletion. No exonic deletions/duplications were detected in patients with IPAH.

An added advantage of our study was the availability of lymphoblastoid cell lines from all 30 patients with FPAH and 14 patients with IPAH selected for analysis. These cell lines were used to prepare total RNA, which was used in RT-PCR studies of *BMPR2* transcripts. Although all 23 *BMPR2* mutations identified in our study were detectable by analysis of genomic DNA using direct sequencing or MLPA/real-time PCR, RT-PCR analysis was necessary to confirm the effects of putative splicing mutations identified at the DNA level. Four potential splicing mutations were identified that involved point mutations in splice donor/acceptor sites. Two different point mutations in the splice donor site of IVS2, one a thymine-to-guanine substitution at nucleotide 6 (Family 68) and the other a single base deletion of the second nucleotide (Family 76), resulted in the same deleterious effect on the *BMPR2* mRNA. Families 68 and 76 were found to have two different aberrant mRNA species that resulted from the use of two cryptic splice donor sites in exon 2. This is the first report of *BMPR2* splicing mutations resulting in the use of cryptic donor or acceptor sites in FPAH, and these mutations were detectable because of the availability of mRNA from the lymphoblastoid cell lines. Putative mutations in the splice donor and splice acceptor sites of intron 8 were confirmed as splicing mutations when mRNA studies showed skipping of exons 8 and 9 due to the intron 8 donor splice mutation (Family 67) and skipping of exon 9 as a result of the intron 8 splice acceptor mutation (Family 59). RT-PCR studies of lymphoblastoid RNA also enabled confirmation of 7 of the 10 DNA dosage mutations detected in this study. Three of the mutations that involved deletion of the first (exon 1) or last (exon 13) exon of *BMPR2* were not detectable by RT-PCR

Figure 4. MLPA analysis of Family 57 indicating a heterozygous deletion of *BMPR2* exons 2–13. The MLPA tracing of an affected individual from Family 57 (top) shows a reduction in peak height/area of all exons except exon 1 as compared with the tracing from a normal individual (bottom).

because the mutant transcripts, if present, were not amplifiable due to lack of the primer annealing site(s). These mutations were therefore discerned only through the use of MLPA/real-time PCR analysis.

Figure 5. Distribution of *BMPR2* mutations identified in 30 families with FPAH. The types of mutations identified are represented by colored circles within the outer circle representing the families analyzed. The shaded circle represents the 12 families previously analyzed. The area outside of the shaded circle represents the 18 families not previously analyzed. Numbers in parentheses indicate the total number of patients in each labeled category. Numbers in the circles show the distribution within each mutation type.

Messenger RNAs containing PTCs are generally targeted for degradation through nonsense-mediated mRNA decay (NMD) (24). To prevent NMD and to enable the detection of these abnormal messages by RT-PCR, it is often necessary to grow the lymphoblastoid cells in the presence of a protein synthesis inhibitor such as puromycin (22). In this study, patient cell lines were grown in the presence and absence of puromycin to enable detection of any abnormal *BMPR2* mRNAs that were subject to NMD. Of the 23 *BMPR2* mutations identified in our study, 11 (48%) were predicted to contain PTCs and therefore would potentially be subject to NMD. Sequencing of RT-PCR products derived from non-puromycin-treated cells failed to detect the presence of the mutant mRNA species for 11 of these mutations, which suggested loss of the mutant species due to NMD.

Of the 10 dosage mutations identified in this study, nine involved exons 1, 2, 3, or 4 of *BMPR2*. Six of these mutations were accounted for by three apparently similar mutations, each occurring in two families. Deletion of exon 1 was identified in Families 30 and 10, deletion of exon 2 was identified in Families 12 and 108, and deletion of exon 3 was identified in Families 92 and 95. Deletion of exons 2–13 (Family 57), duplication of exon 2 (Family 65), deletion of exons 4–5 (Family 20), and duplication of exon 10 (Family 5) were each identified in a single family. Although the breakpoints for these deletions/duplications have not been determined, they are presumed to occur in the introns flanking the exon(s) deleted/duplicated and result from recombination errors. Because 9 of the 10 dosage mutations identified are deletions involving exons 1, 2, 3 or 4, these exons may be hotspots for recombination errors due to the large introns (IVS1 > 80 kb, IVS3 > 40 kb), which flank these exons on the 5' or the 3' end.

Because the breakpoints for the dosage mutations are unknown, the origin(s) of the apparently similar mutations

Figure 6. Recommended workflow for identification of *BMPR2* mutations in patients with PAH. Steps using genomic DNA are shown in blue, and those requiring RNA are indicated in red. Labeled circles indicate actions within the workflow. Text accompanying arrows indicates rationale for flow from one step to the next. Possible outcomes from actions within the workflow are indicated in text at the ends of arrows.

occurring in more than one family cannot be determined. Given the large size of the introns involved in these deletions, it would not be surprising for them to have different breakpoints and therefore represent two separate mutations. It is also possible that these large introns (IVS1 and IVS3) contain hotspots for the same deletion to recur. Haplotype analysis of families carrying the same mutation is often used to determine whether a mutation has occurred via independent mutational events or is on a common genetic background. Although haplotype analysis of these families could identify a common haplotype between them, it would not indicate whether they were carrying the same mutation. Genealogic studies of these families, some dating back to the mid-1700s, do not indicate any common ancestry between those carrying the apparently similar deletion mutations.

A recommended work flow for the analysis of *BMPR2* mutations in patients with FPAH and IPAHA is illustrated in Figure 6. Most studies are limited to the use of genomic DNA for mutation detection. Genomic DNA sequencing enables the detection of coding sequence point mutations and small insertions/deletions in addition to potential intronic splice site mutations. MLPA analysis and real-time PCR are also used with genomic DNA to detect dosage errors, such as deletions or duplications encompassing entire exons or multiple exons, as were identified in several families in this study. Our study had the added advantage of the availability of lymphoblastoid cell lines for all patients analyzed. This enabled the extraction and analysis of RNA not only to determine the effects of intronic splice mutations on the *BMPR2* transcript but to study NMD in those transcripts harboring point mutations. Therefore, although all mutations can be identified by sequencing or MLPA/real-time PCR using genomic DNA, RNA is necessary to confirm any potential splice-site mutations.

In summary, this study using DNA sequence analysis, MLPA analysis, and RT-PCR analysis is the most comprehensive screen for *BMPR2* mutations in a panel of patients with FPAH and IPAHA to date. Our study indicates that exonic deletions and/or duplications of *BMPR2*, which until now have not been screened for in a patient cohort, may account for a significant proportion of mutations in FPAH. Of 30 families studied, nearly half of the mutations identified (10 of 21, 48%) were exonic deletions/duplications. Without the use of MLPA/real-time PCR analysis, these mutations would have gone undetected. RT-PCR analysis of RNA prepared from lymphoblastoid cell lines detected 7 of the 10 deletion/duplication mutations but not the three that involved a deletion of the first or last (exon 13) exon of *BMPR2*. Any potential splicing mutations identified in splice donor/acceptor sites at the genomic DNA level (four in this study) could be confirmed only through the use of RT-PCR studies as done here. Identification of the heterozygous deletion of *BMPR2* exons 2–13 in Family 57 supports haplo-insufficiency as a disease mechanism as previously suggested (9). Taken together, our data suggest that screening for *BMPR2* mutations in familial or idiopathic PAH should include not only direct sequencing but also MLPA analysis of genomic DNA. In addition, any potential mutations identified by MLPA should be confirmed by an alternative method, such as real-time PCR. Although previous studies have detected mutations in 55 to 60% of families with PAH (7–9) and 10 to 40% of patients with IPAHA (10–13), the combination of methods used in this study would enable *BMPR2* mutations to be detected in 70%, and possibly more, of all FPAH cases and a greater percentage of patients with IPAHA.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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