High frequency of \textit{BMPR2} exonic deletions/duplications in familial pulmonary arterial hypertension

Joy D. Cogan\textsuperscript{1,*}, Michael W. Pauciulo\textsuperscript{3,*}, Amy P. Batchman\textsuperscript{3}, Melissa A. Prince\textsuperscript{1}, Ivan M. Robbins\textsuperscript{2}, Lora K. Hedges\textsuperscript{1}, Krista C. Stanton\textsuperscript{1}, Lisa A. Wheeler\textsuperscript{2}, John A. Phillips III\textsuperscript{1}, James E. Loyd\textsuperscript{2} and William C. Nichols\textsuperscript{3,4}

Divisions of \textsuperscript{1}Medical Genetics and \textsuperscript{2}Pulmonary Biology, Vanderbilt University Medical Center, Nashville, TN, and \textsuperscript{3}Division of Human Genetics, Cincinnati Children’s Hospital Medical Center and \textsuperscript{4}Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH

*These authors contributed equally to this work

Address correspondence and requests for reprints to:
William C. Nichols, Ph.D.
Associate Professor of Human Genetics
Division of Human Genetics
Cincinnati Children’s Hospital Medical Center
3333 Burnet Avenue
1469 TCHRF
Cincinnati, OH 45229
513 636-2438 (Phone)
513 636-3486 (Fax)
bill.nichols@cchmc.org

This work was supported by HL61997 (WCN), HL72058 (JDC, JAP, JEL), RR15534 (IMR), and RR000095 (GCRC VUMC)

Running title: \textit{BMPR2} deletions/duplications in FPAH

Word count: 4602

Descriptor number: 92 – Pulmonary Hypertension – Primary

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

Copyright (C) 2006 by the American Thoracic Society.
Abstract

Rationale: Previous studies have shown that ~55% of familial pulmonary arterial hypertension (PAH) patients 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 PAH families and 14 idiopathic PAH patients was subjected to 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%) FPAH patients had abnormally-sized RT-PCR products. Four of the 11 were found to have splice site mutations resulting in aberrant splicing, and exonic deletions/duplications were detected in the remaining seven. Additionally, MLPA identified 3 deletions/duplications which were not detectable by RT-PCR. Coding sequence point mutations were identified in 11 of 30 (37%). Overall, mutations were identified in 21 of 30 (70%) FPAH patients with 10 of 21 mutations(48%) being exonic deletions/duplications. Additionally, 2 of 14 (14%) IPAH patients exhibited $BMPR2$ point mutations while none showed exonic deletions/duplications.

Conclusions: Our study indicates that $BMPR2$ exonic deletions/duplications in FPAH patients account for a significant proportion of mutations (48%) which to now have not been screened for. Since the complementary approach used in this study is both rapid and cost effective, screening for $BMPR2$ deletions/duplications by MLPA and real-time PCR should accompany direct sequencing in all PAH testing.
Word Count: 248  
Key Words: genetics, MLPA, dosage
Introduction

Pulmonary arterial hypertension (PAH) is a disease of the pulmonary vasculature defined by mean pulmonary artery pressure $\geq 25$ mm Hg at rest or $\geq 30$ mm Hg during exercise(1, 2). PAH patients 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 only 2.8 years(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 familial PAH(6).

Several studies have reported mutations in the bone morphogenetic protein receptor, type II gene ($BMPR2$) as causal for PAH in both FPAH and IPAH patients(7-13). Bone morphogenetic protein receptor, type II is a member of the transforming growth factor $\beta$ (TGF-$\beta$) superfamily of cell signaling molecules critical in both cell differentiation and cell growth(14). To date, more than 140 $BMPR2$ mutations have been identified in approximately 55% of FPAH cases and from 11-40% 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 et al.(15) used a combination of Southern blot analysis and reverse transcriptase PCR of leukocyte mRNA to identify novel $BMPR2$ exonic deletions/duplications in 3 of 14 FPAH patients in whom $BMPR2$ coding sequence mutations could not be detected. These data
suggested that exonic deletions/duplications could account for a significant proportion of 
*BMPR2* mutations in PAH patients 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 FPAH families and 14 individuals with IPAH. Using a combination of reverse 
transcriptase PCR, multiplex ligation-dependent amplification (MLPA), and real-time PCR, 
*BMPR2* exonic deletions/duplications were identified in 33% of FPAH patients. This type of 
mutation was not detected in the IPAH cases. Overall, *BMPR2* mutations (including point 
mutations, small insertions/deletions, exonic deletions/duplications) were identified in 70% of 
FPAH and 14% of IPAH cases. Our study illustrates the limitations of earlier studies that only 
looked 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 abstracts(16-18).

**Materials and 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 United States and some patient data is therefore incomplete. However, all FPAH 
patients had a well documented family history of PAH and either had hemodynamic data 
confirming the presence of pulmonary hypertension or were receiving therapy commonly used 
for treatment of PAH strongly indicative of the diagnosis of pulmonary hypertension. The 14 
IPAH patients 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 PPH registry(4). Available data on demographics,
hemodynamics, treatment and outcomes for FPAH and IPAH patients is provided in Tables E1
and E2, respectively in the online data supplement. The study was approved by the IRBs 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 PAH patients were grown in RPMI 1640
containing 15% FBS, 100µg penicillin/ml, and 100µg streptomycin/ml in T-25 flasks. Cells
were grown to approximately 1x10^6 cells/flask and incubated in the presence or absence of
puromycin (100µg/ml) for 16 hrs prior to harvesting(19). Cells were harvested using RNAeasy
Mini Kit including the optional DNase treatment (Qiagen Inc, Valencia CA). Generation of
*BMPR2* RT-PCR products and their sequencing was carried out 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 PAH patients using the Roche MagNA Pure LC
DNA purification system (Roche Molecular Biochemicals, Indianapolis, IN). Primers described
in the Exon Locator & eXtractor for Resequencing (ELXR) 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). Following an initial denaturation at
95°C for 5 minutes, PCR was carried out for 35 cycles (15s at 95°C, 45s at 60°C and 45s at 72°C) followed by a 10 min extension at 72°C. The resulting amplification products were visualized by etidium 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 then separated on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions.

MLPA Analysis
MLPA was performed with 100 ng of genomic DNA according to 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, Foster City, CA). MLPA peak plots were visualized using Genemapper Software v3.7 (Applied Biosystems, Foster City, CA). Non-normalized values for peak height and peak area were then 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 \( \leq 0.7 \) and \( \geq 1.35 \) 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). Following quantitation, 50ng 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, Foster City, CA). Assays were performed as 25ul reactions in triplicate, with each genomic DNA sample being done in duplicate for the BMPR2 exon in question. Following real-time PCR, data were analyzed with the ABI Sequence Detection Software, RQ Study upgrade, v. 1.2.3 (Applied Biosystems, Foster City, CA). Quantitation of target amount in DNA samples was accomplished by measuring the Ct value and comparing it to a standard curve. Quantitations for patient samples were then compared to 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 PAH families and 2 of 14 (14%) of IPAH cases (See Tables 3 and 4). 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 (PTC), 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 \textit{BMPR2} mutations in PAH patients, we initially performed reverse transcriptase PCR (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\cite{22}. Full length \textit{BMPR2} cDNAs generated by RT-PCR were then directly sequenced. Abnormally-sized \textit{BMPR2} mRNAs were detected in 11 FPAH patients, all from unrelated families (See Table 1). Five of these abnormally sized mRNAs resulted from deletions of single exons, two were due to deletions of multiple exons, two resulted from duplications of single exons, and two were due to partial deletions of exon 2. These observed alterations of transcript size were predicted to be the result of either 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 \textit{BMPR2} mRNA transcripts resulted from DNA dosage changes (See Table 1). These included 2 deletions of exon 2 (families 12 and 108), 2 deletions of exon 3 (families 92 and 95), 1 deletion of exons 4-5 (family 20), 1 duplication of exon 2 (family 65), and 1 duplication of exon 10 (family 5). The exon 2 deletions seen in family 12 (See Figure 1) and family 108 are predicted to result in an in frame loss of amino acids 26-82 of the 1038 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 now 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 both the exon 4-5 deletion and the exon 10 duplication were not detected in RNA from nonpuromycin-treated cells and thus were subject to NMD. For confirmation 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 (See below, Table 2).

The remaining 4 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) 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 (See 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 utilization 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 nuleotide of IVS2 (247+2 del C) changing the GC of the donor splice site to a GA. As above, alteration of the wild type donor splice site resulted in utilization 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 1st base of intron 8 (1128+1 G>T) (See 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 codons downstream. Sequencing of BMPR2 transcripts using nonpuromycin-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 \textit{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 non-conservative 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 FPAH and 2 IPAH) were found to have small insertions and/or deletions in exons 3, 6, 7, or 12. All 6 mutations caused a frameshift leading to premature termination and were subject to NMD (See 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 codons downstream. Sequencing of BMPR2 mRNA from the nonpuromycin 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 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 nonpuromycin 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. Since heterozygous deletions of either exon 1 or exon 13 would fail to be detected by RT-PCR due to non-annealing of the PCR primers, MLPA analysis was performed in these 24 samples in order to detect DNA dosage mutations involving these exons. Three of the 12 FPAH samples were determined to have deletions which 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).
(See Figure 4). Of the 12 IPAH samples negative for BMPR2 mutations by RT-PCR sequencing, none were 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 9 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’ UTRs of these patients. In addition, 6 familial and 8 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 potentially have promoter mutations that prevented expression of one allele. Unfortunately, all patients homozygous at the RNA level were also homozygous at the DNA level so we were unable to discern any potential promoter mutations by this method.

**Discussion**

Of the more than 140 BMPR2 mutations reported in PAH patients, the vast majority are coding sequence point, nonsense, or frameshift mutations as well as several splice donor/acceptor site mutations. These mutations account for approximately 60% of FPAH cases and 10–40% of IPAH patients(6). To date, there are few reports of BMPR2 exonic deletions or duplications in FPAH or IPAH, although no large scale studies have been performed to detect this type of
The aim of our study was to estimate the frequency of \textit{BMPR2} exonic deletions/duplications in FPAH patients using complementary methods. This would enable us to obtain a more accurate estimate of the overall frequency of \textit{BMPR2} mutations in FPAH subjects. Patients having either 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 in the online data supplement). Of the 18 new families analyzed, \textit{BMPR2} mutations were identified in 11 (61\%) with 8 (73\%) of these being point mutations (6 coding and two splice) and the remaining 3 (27\%) being exonic deletions/duplications. The remaining 12 had previously been included in studies to detect \textit{BMPR2} mutations either by use of direct sequence analysis of genomic DNA or by Southern analysis\textsuperscript{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 \textit{BMPR2} dosage mutation in five (50\%) of them. Interestingly, point mutations which had previously gone undetected in previous studies were identified in three families. Additionally, our report includes two families (5 \& 20) from a previous study in which Southern analysis followed by RT-PCR of \textit{BMPR2} transcripts was used to identify patients with heterozygous \textit{BMPR2} deletions/duplications\textsuperscript{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 which had been included in previous studies (9, 15).

Our study indicates that *BMPR2* exonic deletions/duplications account for a significant proportion of mutations in FPAH patients. Of the 30 families analyzed in this report, mutations were identified in 21 (70%) with exonic deletions or duplications accounting for 10 of these 21 (48%) mutations. Previous mutation detection methods have employed 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 PAH families. Taken together, 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 of which was a single base insertion, the other a single base deletion. No exonic deletions/duplications were detected in IPAH patients.

An added advantage of our study was the availability of lymphoblastoid cell lines from all 30 FPAH and 14 IPAH patients selected for analysis. These cell lines were used to prepare total RNA which was used in RT-PCR studies of *BMPR2* transcripts. While all 23 *BMPR2* mutations identified in our study were detectable by analysis of genomic DNA using either 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 which 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. Both families 68 and 76 were each found to have two different aberrant mRNA species which 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 only detectable due to the availability of mRNA from the lymphoblastoid cell lines. Putative mutations in both 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 which involved deletion of either the first (exon 1) or last (exon 13) exon of **BMPR2** were not detectable by RT-PCR because the mutant transcripts, if present, were not amplifiable due to lack of the primer annealing site(s). These mutations were therefore only discerned through the use of MLPA/real-time PCR analysis.

Messenger RNAs containing PTCs are generally targeted for degradation through nonsense-mediated mRNA decay (NMD)(24). To prevent NMD and 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 both 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 of them 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 both 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. While 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. Since nine of the ten 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 either the 5’ or 3’ end.

Because the breakpoints for the dosage mutations are unknown, the origin(s) of the apparently similar mutations 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. However, 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. While haplotype analysis of these families could potentially identify a common haplotype between
them it still would not indicate whether they were carrying the same mutation. Genealogic studies of these families, some dating back to the mid 1700’s, does not indicate any common ancestry between those carrying the apparently similar deletion mutations.

A recommended work flow for the analysis of BMPR2 mutations in familial and idiopathic PAH patients is illustrated in Figure 6. Most studies will be 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. As already stated, our study had the added advantage of the availability of lymphoblastoid cells 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 nonsense mediated decay in those transcripts harboring point mutations as well. Therefore, while 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 familial and idiopathic PAH patients 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 familial PAH. 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 3 which involved a deletion of either the first or last (exon 13) exon of **BMPR2**. However, any potential splicing mutations identified in splice donor/acceptor sites at the genomic DNA level (4 in this study) could only be confirmed through the use of RT-PCR studies as done here. Of note, identification of the heterozygous deletion of **BMPR2** exons 2-13 in Family 57 supports haploinsufficiency as a disease mechanism as previously suggested(9). Taken together, our data suggest that screening for **BMPR2** mutations in either 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. While previous studies have only detected mutations in 55-60% of PAH families(7-9) and 10-40% of IPAH patients(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 as well as a greater percentage of IPAH patients.
Acknowledgments

We thank the patients and their families for participation in the study.
References


16. Pauciulo MW, Batchman AP, and Nichols WC. Use of real-time quantitative PCR to detect exonic deletions/duplications of BMPR2 in familial primary pulmonary hypertension[abstract]. Presented at The 55th Annual Meeting of American Society of Human Genetics; October 2005; Salt Lake City, Utah, USA.


Figure 1: BMPR2 cDNA sequence and MLPA analysis of family 12 showing deletion of exon 2. Panel A demonstrates 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 RT-PCR product. Panel B shows the MLPA tracing for an affected family 12 individual (top tracing) demonstrating a reduction in the exon 2 peak (asterisk) as compared to 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.

Figure 2: Splicing mutations in family 68 and family 76 resulting in partial deletions of exon 2 due to use of cryptic donor sites. Panel A shows the 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. Panel B demonstrates the heterozygous deletion of the 2nd base of intron 2 (247+1delC) detected in family 76. Panel C illustrates 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 and blue arrows. There is a cryptic donor site with a score of 0.85 (blue arrow) that appears 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 appears to be used, though to a lesser extent. Cryptic GT splice sites were scored using NNSPLICE (www.fruitfly.org/seq_tools/splice.html)
**Figure 3:** *BMPR2* cDNA (Panel A) and genomic DNA sequence (Panel B) of family 67. As seen in Panel A, cDNA sequencing identified a mutant transcript lacking exons 8 and 9. Genomic DNA sequencing (Panel B) revealed a heterozygous G>T substitution at the first base of intron 8 (1128+1G>T) causing the aberrant splicing.

**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 to the tracing from a normal individual (bottom).

**Figure 5:** Distribution of *BMPR2* mutations identified in 30 FPAH families analyzed. 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.

**Figure 6:** Recommended work flow for identification of BMPR2 mutations in PAH patients. Those steps utilizing genomic DNA are shown in blue while those requiring RNA are indicated in red. Labeled circles indicate actions within the work flow. Text accompanying arrows indicates rationale for flow from one step to the next. Possible outcomes from actions within the labeled circles are indicated in text at the ends of arrows.
Table 1: *BMPR2* DNA Dosage/Splicing Mutations

<table>
<thead>
<tr>
<th>Location</th>
<th>Family</th>
<th>Nucleotide Change</th>
<th>Amino Acid change</th>
<th>Detection Methods</th>
<th>Previous Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1</td>
<td>30</td>
<td>?_IVS1 del</td>
<td>unknown</td>
<td>MLPA/RealTime</td>
<td>(9, 15)</td>
</tr>
<tr>
<td>Exon 1</td>
<td>110</td>
<td>?_IVS1 del</td>
<td>unknown</td>
<td>MLPA/RealTime</td>
<td></td>
</tr>
<tr>
<td>Exons 2-13</td>
<td>57</td>
<td>IVS1_? del</td>
<td>unknown</td>
<td>MLPA/RealTime</td>
<td>(9, 15)</td>
</tr>
<tr>
<td>Exon 2</td>
<td>12</td>
<td>IVS1_IVS2 del</td>
<td>del aa26-82</td>
<td>RT-PCR/MLPA/RealTime</td>
<td>(15)</td>
</tr>
<tr>
<td>Exon 2</td>
<td>108</td>
<td>IVS1_IVS2 del</td>
<td>del aa26-82</td>
<td>RT-PCR/MLPA/RealTime</td>
<td>(15)</td>
</tr>
<tr>
<td>Exon 2</td>
<td>65</td>
<td>IVS1_IVS2 dup</td>
<td>dup aa26-82</td>
<td>RT-PCR/MLPA/RealTime</td>
<td>(15)</td>
</tr>
<tr>
<td>Exon 2</td>
<td>76</td>
<td>IVS 2 247+2delC</td>
<td>del aa47-82, del aa63-82</td>
<td>RT-PCR/Genomic</td>
<td></td>
</tr>
<tr>
<td>Exon 2</td>
<td>68</td>
<td>IVS 2 247+6T&gt;G</td>
<td>del aa47-82, del aa63-82</td>
<td>RT-PCR/Genomic</td>
<td></td>
</tr>
<tr>
<td>Exon 3</td>
<td>92</td>
<td>IVS2_IVS3 del</td>
<td>del aa83-139</td>
<td>RT-PCR/MLPA/RealTime</td>
<td>(9, 15)</td>
</tr>
<tr>
<td>Exon 3</td>
<td>95</td>
<td>IVS2_IVS3 del</td>
<td>del aa83-139</td>
<td>RT-PCR/MLPA/RealTime</td>
<td>(9)</td>
</tr>
<tr>
<td>Exons 4-5</td>
<td>20</td>
<td>IVS3_IVS5 del</td>
<td>S140fs (+ 11 amino acids)</td>
<td>RT-PCR/MLPA/RealTime</td>
<td>(15)</td>
</tr>
<tr>
<td>Exons 8-9</td>
<td>67</td>
<td>IVS 8 1128+1G&gt;T</td>
<td>del aa323-425</td>
<td>RT-PCR/Genomic</td>
<td>(9, 15)</td>
</tr>
<tr>
<td>Exon 9</td>
<td>59</td>
<td>IVS 8 1129-3C&gt;G</td>
<td>V377fs (+ 47 amino acids)</td>
<td>RT-PCR/Genomic</td>
<td>(9, 15)</td>
</tr>
<tr>
<td>Exon 10</td>
<td>5</td>
<td>IVS9_IVS10 dup</td>
<td>A472fs (+ 47 amino acids)</td>
<td>RT-PCR/MLPA/RealTime</td>
<td>(15)</td>
</tr>
</tbody>
</table>
Table 2: *BMPR2* Coding Sequence Mutations

<table>
<thead>
<tr>
<th>Location</th>
<th>Family</th>
<th>Nucleotide Change</th>
<th>Amino Acid change</th>
<th>Detection Methods</th>
<th>Previous Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 4</td>
<td>71</td>
<td>516C&gt;G</td>
<td>Y172X</td>
<td>RT-PCR/Genomic</td>
<td></td>
</tr>
<tr>
<td>Exon 6</td>
<td>66</td>
<td>631C&gt;T</td>
<td>R211X</td>
<td>RT-PCR/Genomic</td>
<td></td>
</tr>
<tr>
<td>Exon 6</td>
<td>64</td>
<td>673C&gt;T + 690-91delAGinsT</td>
<td>R225C + K230fs (+ 21 amino acids)</td>
<td>RT-PCR/Genomic</td>
<td></td>
</tr>
<tr>
<td>Exon 6</td>
<td>135</td>
<td>796-799delAGAG</td>
<td>R266fs (+ 11 amino acids)</td>
<td>RT-PCR/Genomic</td>
<td></td>
</tr>
<tr>
<td>Exon 6</td>
<td>124</td>
<td>804delT</td>
<td>A269fs (+9 amino acids)</td>
<td>RT-PCR/Genomic</td>
<td></td>
</tr>
<tr>
<td>Exon 7</td>
<td>119</td>
<td>872delT</td>
<td>L291fs (+ 0 amino acids)</td>
<td>RT-PCR/Genomic</td>
<td></td>
</tr>
<tr>
<td>Exon 10</td>
<td>61</td>
<td>1346T&gt;G</td>
<td>M449R</td>
<td>RT-PCR/Genomic</td>
<td>(9, 15)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Location</th>
<th>Patient</th>
<th>Nucleotide Change</th>
<th>Amino Acid Change</th>
<th>Detection Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 3</td>
<td>IPAH303</td>
<td>277insG</td>
<td>E93fs (+ 4 amino acids)</td>
<td>RT-PCR/Genomic</td>
</tr>
<tr>
<td>Exon 12</td>
<td>IPAH88</td>
<td>2504delC</td>
<td>T835fs (+ 3 amino acids)</td>
<td>RT-PCR/Genomic</td>
</tr>
</tbody>
</table>
Figure 1

A.

```
ACGCGCTGGATGTGCT
```

Exon 1 → Exon 3

B.

```
Exon #  1 7 8 2 9 3 10 4 11 5 12 6 13
```

*
Figure 2

A.

\[
\begin{align*}
\text{A C A A G} & \quad g \quad c \quad a \quad a \quad g \quad g \quad g \quad a \quad t \quad a \quad c \quad t \quad t \quad c \quad e \\
\text{Exon 2} & \quad \longrightarrow \quad \text{IVS 2}
\end{align*}
\]

B.

\[
\begin{align*}
\text{G T A A A C A A G} & \quad g \quad c \quad a \quad a \quad g \quad t \quad g \quad a \quad t \quad a \quad c \\
\text{g a a g t g a t a c t} & \quad \text{Exon 2} \quad \longrightarrow \quad \text{IVS 2}
\end{align*}
\]

C.

\[
\begin{align*}
\text{CTTCGCAATCAAGAAACGGCTATGCGTTCAGGATTCAACAGCAAGACCTTGGGATTAGGAGAGTGAATCTC} \\
\text{TCAATGAAATGGGACGATATTTATGCTCGAAAGGAGCACCTGTGCTGGCTTTGGGAGAAATCAAAAGGGGACATAAAT} \\
\text{CTTGTAACAAGGAaagTgatactttccctacgtaaatgactgtgttttatataaattgtatattttactaaa}
\end{align*}
\]
Figure 3

A.

```
C C A C G A G A G G G A A T C C G T

Exon 7 ← Exon 10
```

B.

```
C A T A A G C G A G g t g a g t g t a t

Exon 8 ← IVS 8
```
Figure 4
Figure 5

Splice mutations (4)

All families analyzed (30)

Dosage mutations (10)

Mutations not identified (9)

Families not previously analyzed (18)

Families previously analyzed (12)

Coding mutations (7)
High frequency of *BMPR2* exonic deletions/duplications in familial pulmonary arterial hypertension


**Online Data Supplement**
Table E1: Demographic and Clinical Data on and Patients and Obligate Carriers with FPAH

<table>
<thead>
<tr>
<th>Family</th>
<th>Gender</th>
<th>Age at diagnosis</th>
<th>Survival (years)</th>
<th>mPAP (mmHg)</th>
<th>PVR (units)</th>
<th>Treatments</th>
<th>Status</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>female</td>
<td>66, obligate</td>
<td>27</td>
<td>57</td>
<td>7.9</td>
<td>CCB, bosentan</td>
<td>alive</td>
<td>(E1)</td>
</tr>
<tr>
<td>12</td>
<td>female</td>
<td>61, obligate</td>
<td>12*</td>
<td>73</td>
<td>23.4</td>
<td>bosentan, PGI₂</td>
<td>alive</td>
<td>(E1)</td>
</tr>
<tr>
<td>20</td>
<td>female</td>
<td>27, obligate</td>
<td>27</td>
<td>57</td>
<td>7.9</td>
<td>PGI₂, Tx</td>
<td>dead</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>30</td>
<td>female</td>
<td>27, obligate</td>
<td>1</td>
<td>73</td>
<td>23.4</td>
<td>PGI₂, Tx</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>43</td>
<td>male</td>
<td>28</td>
<td>12*</td>
<td>----</td>
<td>----</td>
<td>PGI₂, Tx</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>45</td>
<td>female</td>
<td>37</td>
<td>10</td>
<td>62</td>
<td>10.4</td>
<td>CCB</td>
<td>dead</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>54</td>
<td>female</td>
<td>51, obligate</td>
<td>44</td>
<td>4</td>
<td>-----</td>
<td>PGI₂</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>56</td>
<td>male</td>
<td>26</td>
<td>9</td>
<td>60</td>
<td>8.1</td>
<td>PGI₂, sildenafil</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>59</td>
<td>female</td>
<td>32</td>
<td>10*</td>
<td>61</td>
<td>17.8</td>
<td>CCB, PGI₂</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>61</td>
<td>female</td>
<td>32</td>
<td>18</td>
<td>77</td>
<td>20.9</td>
<td>PGI₂</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>62</td>
<td>male</td>
<td>36</td>
<td>3</td>
<td>----</td>
<td>----</td>
<td>PGI₂</td>
<td>dead</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>63</td>
<td>male</td>
<td>----</td>
<td>----*</td>
<td>----</td>
<td>----</td>
<td>PGI₂</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>64</td>
<td>male</td>
<td>33</td>
<td>3</td>
<td>54</td>
<td>17.6</td>
<td>CCB, PGI₂</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>65</td>
<td>female</td>
<td>36</td>
<td>9</td>
<td>----</td>
<td>----</td>
<td>PGI₂</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>66</td>
<td>female</td>
<td>42</td>
<td>7</td>
<td>----</td>
<td>----</td>
<td>PGI₂</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>67</td>
<td>male</td>
<td>35</td>
<td>8</td>
<td>49</td>
<td>15.2</td>
<td>PGI₂</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>68</td>
<td>female</td>
<td>63, obligate</td>
<td>21</td>
<td>7*</td>
<td>----</td>
<td>PGI₂</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>71</td>
<td>female</td>
<td>41</td>
<td>11</td>
<td>84</td>
<td>12</td>
<td>PGI₂</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>82</td>
<td>female</td>
<td>7</td>
<td>11</td>
<td>----</td>
<td>----</td>
<td>treprostinil</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>84</td>
<td>female</td>
<td>33</td>
<td>7</td>
<td>74</td>
<td>12.2</td>
<td>CCB, PGI₂</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>92</td>
<td>male</td>
<td>59</td>
<td>2</td>
<td>52</td>
<td>----</td>
<td>CCB, PGI₂</td>
<td>dead</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>95</td>
<td>female</td>
<td>44</td>
<td>2</td>
<td>50</td>
<td>17.5</td>
<td>PGI₂</td>
<td>alive</td>
<td>(E2)</td>
</tr>
<tr>
<td>108</td>
<td>female</td>
<td>45</td>
<td>1</td>
<td>58</td>
<td>13.2</td>
<td>sildenafil</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>109</td>
<td>female</td>
<td>25</td>
<td>12</td>
<td>----</td>
<td>----</td>
<td>CCB</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>110</td>
<td>female</td>
<td>28</td>
<td>9</td>
<td>----</td>
<td>----</td>
<td>PGI₂</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>119</td>
<td>female</td>
<td>57</td>
<td>3</td>
<td>----</td>
<td>----</td>
<td>PGI₂</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>124</td>
<td>female</td>
<td>57</td>
<td>1</td>
<td>58</td>
<td>10.9</td>
<td>CCB, ambrisentan</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
<tr>
<td>135</td>
<td>female</td>
<td>31</td>
<td>2</td>
<td>----</td>
<td>----</td>
<td>PGI₂</td>
<td>alive</td>
<td>(E1, E2)</td>
</tr>
</tbody>
</table>

*=patient underwent lung transplantation. CCB=calcium channel blockers; PGI₂=epoprostenol 
mPAP=mean pulmonary artery pressure; PVR=pulmonary vascular resistance
### Table E2: Demographic and Clinical Data on Patients with IPAH

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age at diagnosis</th>
<th>Survival (years)</th>
<th>mPAP (mmHg)</th>
<th>PVR (units)</th>
<th>Treatments</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>04</td>
<td>female</td>
<td>26</td>
<td>12</td>
<td>58</td>
<td>14.6</td>
<td>CCB, sildenafil</td>
<td>alive</td>
</tr>
<tr>
<td>12</td>
<td>female</td>
<td>28</td>
<td>9</td>
<td>56</td>
<td>9.8</td>
<td>PGI₂, bosentan</td>
<td>alive</td>
</tr>
<tr>
<td>14</td>
<td>female</td>
<td>40</td>
<td>6</td>
<td>61</td>
<td>17.2</td>
<td>PGI₂, sildenafil</td>
<td>alive</td>
</tr>
<tr>
<td>46</td>
<td>female</td>
<td>54</td>
<td>4</td>
<td>54</td>
<td>17.7</td>
<td>PGI₂, bosentan, sildenafil</td>
<td>alive</td>
</tr>
<tr>
<td>70</td>
<td>female</td>
<td>57</td>
<td>10</td>
<td>57</td>
<td>21.0</td>
<td>PGI₂, sildenafil</td>
<td>alive</td>
</tr>
<tr>
<td>78</td>
<td>female</td>
<td>59</td>
<td>3</td>
<td>72</td>
<td>20.4</td>
<td>PGI₂, sildenafil</td>
<td>alive</td>
</tr>
<tr>
<td>87</td>
<td>female</td>
<td>31</td>
<td>3</td>
<td>61</td>
<td>19.6</td>
<td>PGI₂, sildenafil</td>
<td>alive</td>
</tr>
<tr>
<td>88</td>
<td>female</td>
<td>33</td>
<td>3</td>
<td>52</td>
<td>22.4</td>
<td>PGI₂, sildenafil</td>
<td>alive</td>
</tr>
<tr>
<td>104</td>
<td>female</td>
<td>45</td>
<td>15</td>
<td>50</td>
<td>12.5</td>
<td>CCB, ambrisentan</td>
<td>alive</td>
</tr>
<tr>
<td>137</td>
<td>female</td>
<td>39</td>
<td>3</td>
<td>61</td>
<td>19.9</td>
<td>sildenafil, bosentan</td>
<td>alive</td>
</tr>
<tr>
<td>195</td>
<td>female</td>
<td>62</td>
<td>5</td>
<td>58</td>
<td>16.7</td>
<td>PGI₂, sildenafil</td>
<td>alive</td>
</tr>
<tr>
<td>253</td>
<td>male</td>
<td>24</td>
<td>9*</td>
<td>102</td>
<td>24.8</td>
<td>CCB, PGI₂</td>
<td>alive</td>
</tr>
<tr>
<td>278</td>
<td>female</td>
<td>64</td>
<td>9</td>
<td>53</td>
<td>16.1</td>
<td>PGI₂, sildenafil</td>
<td>alive</td>
</tr>
<tr>
<td>303</td>
<td>male</td>
<td>38</td>
<td>3</td>
<td>66</td>
<td>16.5</td>
<td>bosentan, iloprost</td>
<td>alive</td>
</tr>
</tbody>
</table>

*=patient underwent lung transplantation. CCB=calcium channel blockers; PGI₂=epoprostanol; mPAP=mean pulmonary artery pressure; PVR=pulmonary vascular resistance
References
