article

Gross *BMPR2* gene rearrangements constitute a new cause for primary pulmonary hypertension

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Purpose: Approximately 50% of patients with familial primary pulmonary hypertension (FPPH) have been reported to have mutations within the bone morphogenic protein receptor type 2 (*BMPR2*) gene. The vast majority of these mutations were identified by PCR amplification and sequencing of individual exons. The aim of our study was to determine if additional *BMPR2* mutations not found by exon sequencing alone could account for a significant portion of these negative cases. **Methods:** We examined DNA samples from 12 families, previously found to be negative for *BMPR2* mutations, to identify any large *BMPR2* gene rearrangements. **Results:** Southern blot analysis found large gene rearrangements in four (33%) unrelated kindreds. Further analysis by reverse transcriptase PCR (RT-PCR) of *BMPR2* transcripts from two of these kindreds found one to be heterozygous for a exon 10 duplication and the second to be heterozygous for a deletion of exons 4 to 5. Nonhomologous recombination is believed to be the cause of these large insertions/deletions. **Conclusion:** Our results demonstrate the inherent problems associated with exon-by-exon sequencing and the importance of other screening methods such as Southern blot and RT-PCR in the identification of *BMPR2* mutations. ***Genet Med* 2005:7(3):169–174.**

Key Words: *primary pulmonary hypertension, bone morphogenic receptor type 2, Southern blot, reverse transcriptase PCR, gross gene rearrangements*

Primary pulmonary hypertension (PPH [MIM 178600]) is characterized by obstruction of the pulmonary arteries due to proliferation of endothelial and smooth muscle cells, leading to elevated pulmonary artery pressure and right heart failure.[1,2] Familial PPH (FPPH) is an autosomal-dominant disorder with reduced penetrance, a 2:1 (female to male) sex ratio, and variable age of onset.[3,4] Approximately 6% of PPH is familial, and we currently have more than 110 families with multiple affected members.

Mutations in the bone morphogenic protein type II receptor (*BMPR2* [MIM 600799]) gene have been shown to play a major role in FPPH.[5–7] BMPR2 is a member of the transforming growth factor β superfamily and plays a vital role in the regulation of cell growth and differentiation. The *BMPR2* gene has 13 exons and is over 180,000 bp in length with 98% of the gene being composed of intronic sequence. Due to the enormous size of the introns within this gene, the majority of *BMPR2* mutations have been identified by PCR amplification and sequencing of individual exons (including the intron/exon boundaries). Because of this, the majority of mutations reported have been within exons or at splice junctions. This method however can miss other disease-causing mutations including, but not limited to, duplications, deletions, and inversions.

We have identified *BMPR2* mutations in 24 out of 53 (45%) unrelated kindreds with familial PPH by PCR amplification and sequencing of individual exons. To determine what proportion of remaining patients have *BMPR2* defects due to gene rearrangements, we examined DNA and/or RNA from 12 families that were negative for mutations by exon sequencing. Using a combination of segregation analysis, Southern blotting, and reverse transcriptase PCR, we demonstrate that a significant proportion of these 12 families have *BMPR2* mutations that would be missed using exon-by-exon sequencing alone.

MATERIALS AND METHODS

Patient recruitment

Patients were recruited into this study through the Pulmonary Hypertension Association or after being referred to Vanderbilt by outside physicians. Families were identified by the presence of at least two members with typical clinical manifestations of PPH after exclusion of other causes of pulmonary hypertension.[3] Twelve of fourteen subjects included in the current study have developed PPH. Hemodynamic data confirming the presence of pulmonary hypertension is available for

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nine patients; the other three patients are receiving therapy commonly used for treatment of PPH strongly indicative of the diagnosis of pulmonary hypertension.

Ethical considerations

The study was approved by the IRB at Vanderbilt University Medical Center, and written informed consent was obtained from all study subjects.

Southern blot analysis

Southern blot analysis was performed on high molecular weight genomic DNA from members of 12 PPH families with no previously identified *BMPR2* mutation, 4 PPH families with known *BMPR2* mutations (T354G; C2695T; 169delCTTTinsAAA; C1471T), and 20 normal controls.[5–7] Ten-microgram aliquots of DNA from each individual was digested with restriction endonucleases *Eco*RI, *Bam*HI, *Hind*III, or *Bgl*II for 8 hours, then subjected to gel electrophoresis on a 0.8% agarose gel at 30 V for 14 hours and 50 V for 3 hours. After depurination with HCl and denaturation with NaOH, the DNA was transferred from gel to filter by vacuum. The filters were hybridized with a 3.2-kb *Eco*RI/*Xho*I ^{32}P random-primed labeled fragment containing the *BMPR2* cDNA.[8] Hybridization was done using Hybrisol I solution (Chemicon International) at 45°C for 62 hours. Excess unbound probe was removed by washing 3 times at RT in 0.1X SSC/0.1% SDS and 1 hour at 54°C, and autoradiography was performed at −70°C for 3 to 7 days.

RNA analysis

Whole blood was collected directly into PAXgene blood RNA tubes (2.5 mL draw volume/tube), and total RNA was isolated approximately 24 hours after blood draw using the PAXgene Blood RNA Kit (Qiagen) according to manufacturers instructions, including an optional DNAse treatment. Approximately 3 μg of total RNA was then used as a template for cDNA synthesis using reverse transcriptase PCR (RT-PCR). First strand cDNA synthesis was performed using the Superscript First-Strand System (Invitrogen Life Technologies, Carlsbad, CA) with 3 μg of total RNA and an oligo-dT primer. One-tenth volume of the first strand reaction was then used as a template for PCR amplification using the Elongase Amplification System (Invitrogen Life Technologies, Carlsbad, CA). The forward and reverse primers corresponded to nucleotides 238 to 259 (5'-atgaaagctctgcagctaggtc-3') and the complement of 3586 to 3562 (5'-catcatttaaacatgcagaagatgt-3') based on published *BMPR2* mRNA sequence (accession no. NM_001204.3). The PCR reaction mixture was denatured for 30 seconds at 94°C, cycled 55 times (94°C, 30 seconds; 58°C, 30 seconds; 68°C, 3 minutes 30 seconds), followed by a 5-minute extension at 68°C. The resulting 3349-bp cDNA products were purified by filtration with a Microcon 50 microconcentrator (Amicon Corp, Danvers, MA) and then visualized by ethidium bromide staining on a 1% agarose gel.

DNA sequencing of RT-PCR products

The *BMPR2* RT-PCR amplification products generated in the previous section, were sequenced directly to detect mutations. The *BMPR2* RT-PCR products were then used as templates for an internal PCR containing the mutated region using the Elongase Amplification System (Invitrogen Life Technologies, Carlsbad, CA). The forward and reverse primers were described previously by Gobbi et al. and correspond to nucleotides 1522 to 1542 (5'-GCAGCCATAAGCGAGGTTGGC-3') and the complement of 1993 to 2014 (5'-GCCTATTATGTGACAGGTTGCG-3') for family 5, and 746 to 767 (5'-ACCGTTTCTGCTGTTGTAGCAC-3') and the complement of 1195 to 1216 (5'-CTGCAGTGACTCTCTCATCTCC-3') for family 20 based on published *BMPR2* mRNA sequence (accession no. NM_001204.3).[9] The PCR reaction mixture was denatured for 30 seconds at 94°C, cycled 15 times (94°C, 30 seconds; 58°C, 30 seconds; 68°C, 1 minute), followed by a 5-minute extension at 68°C. The resulting cDNA products were purified by filtration with a Microcon 50 microconcentrator (Amicon Corp, Danvers, MA) and then visualized by ethidium bromide staining on a 3% low melt agarose gel. The normal and variant allelic bands were then excised and purified using β-agarase according to manufacturers instructions (New England Biolabs). The purified PCR products were sequenced using the BigDye Terminator v3.1 Sequencing kit (Applied Biosystems), and the same primers used in the PCR reactions. The sequencing reaction products were then examined on an ABI Prism 3100-*Avant* Genetic Analyzer (Applied Biosystems) according to manufacturers instructions.

Electronic database information

Accession numbers and URLs for data in this article are as follows: NCBI National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov/ and Online Mendelian Inheritance in Man (OMIM) http://www.ncbi.nlm.nih.gov/Omim.

RESULTS

Demographics

Demographics for the 14 study subjects are presented in Table 1. Ten patients and two obligate carriers were included in the study. Six patients were male and six were female, and patient ages at diagnosis ranged from 7 to 58 years with a median age of 31 years. Mean pulmonary artery pressure was 57 $\pm$ 12 (mean $\pm$ SD) mm Hg in the nine patients in whom hemodynamic data are available. This value is similar to that reported in the NIH PPH Registry.[3] Survival, with medical therapy, has ranged from 3 to 25 years, and three patients have undergone lung transplantation. No differences in disease severity or survival were found between families with gene rearrangement mutations and those in whom we were unable to identify a mutation or families with previously identified point mutations. Additionally, all exhibit incomplete penetrance and genetic anticipation.

Table 1
Patient demographics

Patient	Family	No. of identified cases in family	Sex	Age at diagnosis (y)	Status	Survival after dx (y)
1	5	8[a]	female	65, unaffected[b]	alive	N/A
2	12	8	male	93, unaffected[b]	alive	N/A
3	20	12	female	28	alive	25
4	20	12	female	36	alive	12
5	30	5	male	34	alive	8
6	57	2	male	26	alive[c]	9
7	59	2	female	30	alive[c]	11
8	61	3	female	32	alive[c]	17
9	67	3	male	35	alive	9
10	82	2	male	10	alive	7
11	82	2	female	7	alive	8
12	84	2	female	33	alive	6
13	92	2	male	58	dead	3
14	103	2	male	7	alive	6

[a]Only 5 confirmed by hemodynamic testing; [b]Obligate carrier; [c]Underwent lung transplantation.

Southern blot studies

Unique bands were observed on Southern blots representing DNA from 4 of 12 (33%) PPH families in which no *BMPR2* mutation had been previously characterized (Fig. 1). These unique bands were not shared between families but were family specific and were not detected in the DNA from 20 control specimens or in the DNA from 4 PPH families in which *BMPR2* mutations had been previously characterized. In family 92, a unique band was observed after digestion of genomic DNA with *Bgl*II, *Hind*III, and *Eco*RI. Similarly, in family 12, unique bands were observed with multiple restriction endonucleases including *Bgl*II, *Bam*HI, and *Hind*III. However, in family 5, a unique band was only seen after digestion of genomic DNA with *Eco*RI. In family 20, a unique band detected after digestion of nuclear DNA with *Eco*RI was associated with reduced intensity of an unaltered *Eco*RI-specific band in addition to the diminished intensity of a *Bam*HI-derived band. Further, these family-specific bands were not observed in the DNAs from four PPH families with known *BMPR2* mutations nor were they detected in 20 normal control DNA specimens. In 1/12 (8%) PPH families (family 67), a band was deleted in DNA from one affected member after digestion with *Bgl*II; however, this band was also completely lost in one control DNA specimen (Fig. 1). *BMPR2* exon–specific PCR amplification coupled with sequence analysis of this patient and the control (C*) DNA specimen did not identify a base substitution that would alter a *Bgl*II recognition sequence. Together, these results suggest that this alteration is likely not associated with PPH in family 67, but rather represents homozygosity for a benign polymorphism within an intronic sequence. In addition, this band is half as intense in the other simultaneously analyzed control (C) DNA specimen, which probably reflects the heterozygous state of this intronic polymorphism. Although this polymorphism results in the loss of a *Bgl*II restriction site, the alternatively created restriction fragments are not visible on the blot, suggesting that these new *Bgl*II restriction fragments are not apparent because they either comigrate with other bands or are < 2.3 kb in length and have migrated off of the gel or are not detected by this assay.

Our finding family-specific, unique banding patterns that were most often detected with more than one restriction endonuclease and were not observed in control DNA specimens suggested that these patterns were associated with PPH causing mutations. To test this hypothesis, we blindly analyzed DNAs from many family members of a large PPH kindred (family 20). In all PPH-affected family members and obligate carriers of a *BMPR2* mutation studied, a unique *Eco*RI pattern was observed (Fig. 2). In contrast, the unique band was not seen in Southern blotting patterns of DNAs from unaffected family members or from the spouse of a PPH-affected family member that was simultaneously analyzed.

RT-PCR studies

To determine the effects of the altered patterns seen in Southern blots, we performed reverse transcriptase PCR (RT-PCR) using total RNA isolated from whole blood. RT-PCR from Patient 5 yielded PCR products of 3349 bp (wild-type allele) and 3486 bp (mutant allele), whereas leukocyte RNA from Patient 20 generated products of 3349 bp (wild-type allele) and 3146 bp (mutant allele) (see Fig. 3). Sequencing of the individual alleles showed that Patient 5 had an allele with a tandem duplication of exon 10 (Fig. 4) and Patient 20 had an

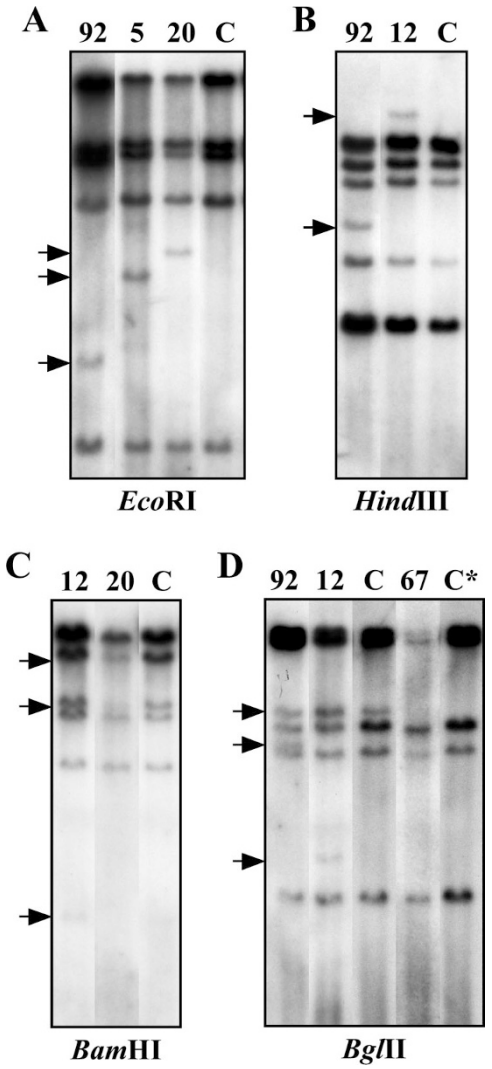

Fig. 1. Southern blot analysis of genomic DNA from PPH patients and normal controls after digestion with restriction endonucleases *Eco*RI (A), *Hind*III (B), *Bam*HI (C), or *Bgl*II (D) and hybridization with a ^{32}P-labeled *BMPR2* gene cDNA probe. Numerical listing for each lane corresponds to the Vanderbilt University Medical Center PPH cohort family number. Normal control DNA simultaneously analyzed for each restriction endonuclease digestion is denoted with a C. Normal control DNA demonstrating homozygosity for the loss of a *Bgl*II digestion site, as seen in PPH family 67, is noted by a C*. Arrows denote novel bands detected in PPH patients suggestive of *BMPR2* gene rearrangements.

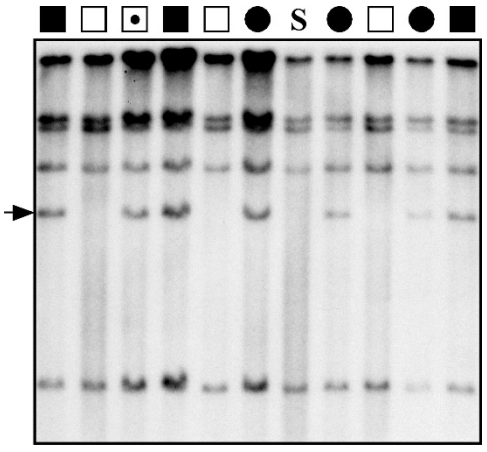

Fig. 2. Southern blot analysis of genomic DNA from Vanderbilt University Medical Center PPH cohort family 20 after digestion with restriction endonuclease *Eco*RI and hybridization with a ^{32}P-labeled *BMPR2* gene cDNA probe. Noted are PPH patients (filled symbol), obligate carriers (dotted symbol), unaffected family members (open symbol), and a spouse (S) to an affected member of the family. Arrow indicates the unique *Eco*RI band segregating with PPH in this family.

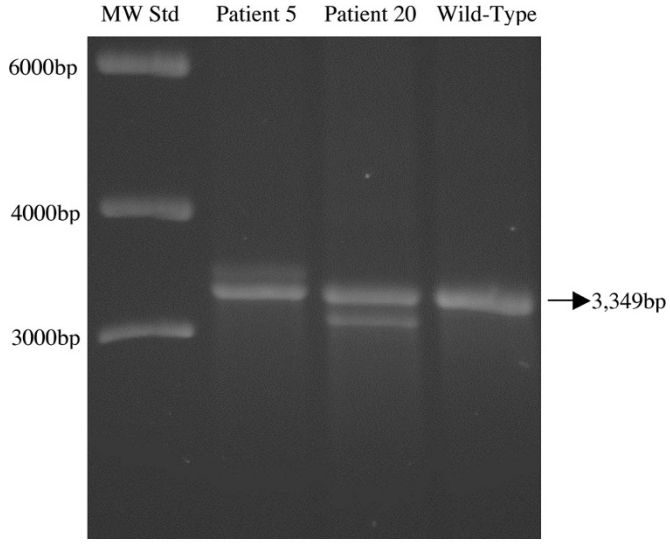

Fig. 3. Electrophoretic analysis of *BMPR2* RT-PCR products derived from total RNA isolated from whole blood of patients with familial PPH. The 3349bp product corresponds to the full-length (wild-type) *BMPR2* cDNA.

allele with a deletion of exons 4 to 5 (Fig. 5). Duplication of exon 10 in Patient 5 causes a frame shift and leads to the production of a premature stop codon (TGA) generated by nucleotides 5 to 8 of exon 11. Premature stop codons can cause increased degradation of transcripts due to nonsense-mediated mRNA decay and could explain why fewer transcripts of the mutated allele, compared to wild-type, were detected by RT-PCR (see Fig. 3). We hypothesized that duplication of exon 10 was most likely due to unequal, homologous recombination between introns 9 and 10 of the *BMPR2* gene. Deletion of exons 4 to 5 in Patient 20 also causes a frame shift and leads to the production of a premature stop codon (TAA) generated by nucleotides 33 to 35 of exon 6. This premature stop codon also appears to be subject to nonsense-mediated decay. We hypothesized that deletion of exons 4 to 5 was also likely to be the result of unequal, homologous recombination, this time between introns 3 and 5 of the *BMPR2* gene.

DISCUSSION

Familial primary pulmonary hypertension (FPPH) is caused by mutations in the *BMPR2* gene.[5] PCR primers designed to amplify the entire protein-coding region of *BMPR2* as well as intron/exon boundaries have been used to screen families with FPPH to characterize *BMPR2* disease-causing mutations segregating in each family for risk assessment during genetic counseling. Missense, nonsense, splice-site, and frameshift

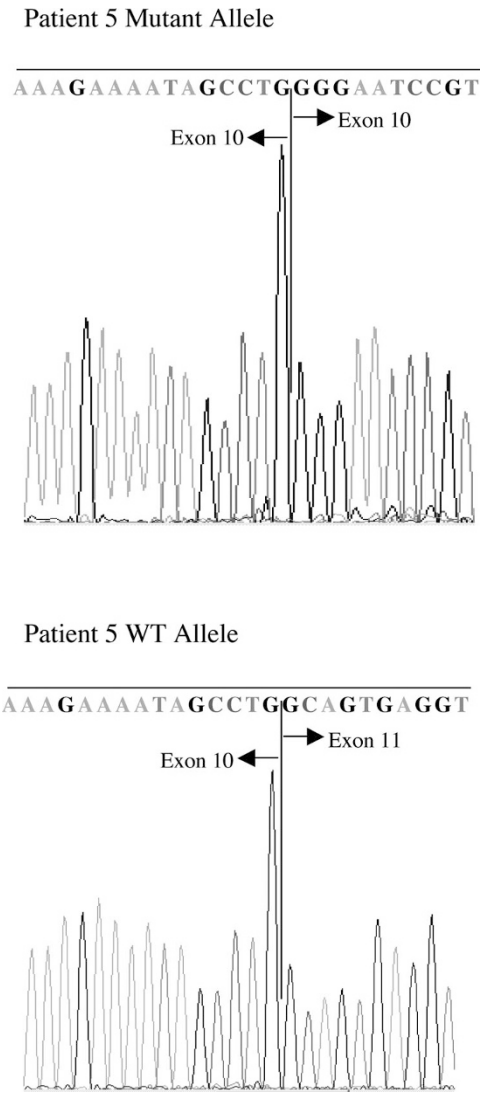

Fig. 4. Comparison of *BMPR2* cDNA sequences of Patient 5 mutant (top) and wild-type (bottom) alleles. Mutant allele sequence shows a tandem duplication of exon 10.

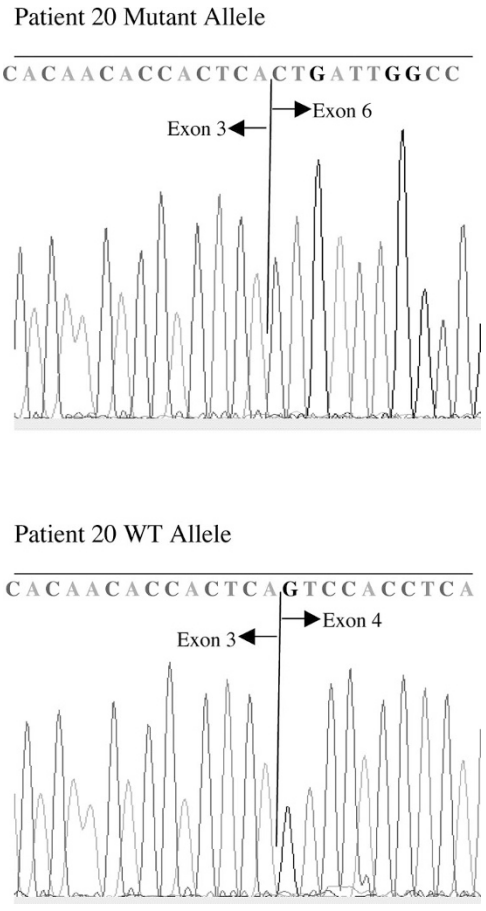

Fig. 5. Comparison of *BMPR2* cDNA sequences of Patient 20 mutant (top) and wild-type (bottom) alleles. Mutant allele sequence has exons 4 to 5 deleted (i.e., exon 3 is contiguous with exon 6).

mutations have been identified in FPPH kindreds from around the world.[5,7,10–13] Mutations are heterogeneous and widely dispersed throughout the *BMPR2* gene. BMPR2 is a member of the transforming growth factor-β cell signaling family, and in vitro, different mutations have been shown to result in a heterogeneous array of functional differences in the translated BMPR2 proteins.[14] Although BMPR2 proteins with missense mutations affecting the cysteine residues in the extracellular or kinase domains exhibit altered intracellular trafficking and are unable to escape the cytoplasm and therefore unable to transduce BMP-mediated signaling pathways, a mutation in the cytoplasmic tail of the protein retained most of its biological activity yet, as compared to wild-type BMPR2, less efficiently phosphorylated the intracellular signal-transducing molecule, Smad5.[14]

BMPR2 mutations have only been identified in about 50% of PPH families.[7] This suggests that *BMPR2* mutations in some FPPH families reside outside of *BMPR2* protein-encoding regions and intron/exon boundaries and may be within upstream regulatory regions, intronic sequences, or 3' untranslated regions. Alternatively, mutations may occur at a second locus, PPH2, distinct from *BMPR2* on 2q31.[15] Because gross genomic rearrangements of *BMPR2* would be missed by conventional PCR amplification and sequencing of *BMPR2* exons, we used a ^{32}P-labeled *BMPR2* cDNA probe and Southern blot analysis of genomic DNA to rescreen 12 *BMPR2* mutation-negative FPPH kindreds. We identified unique bands suggestive of large *BMPR2* gene rearrangements in 4/12 (33%) *BMPR2* mutation negative FPPH families (Fig. 1). By devising a RT-PCR assay using RNA from peripheral blood and sequence analysis of amplified products, we were able to identify previously unreported gross rearrangements of *BMPR2* as a cause of FPPH (Figs. 3–5). Our studies identified the disease-causing mutation in some *BMPR2* negative PPH families and indicate that previously reported *BMPR2* negative FPPH families from other cohorts likely have *BMPR2* gene rearrangements that have escaped detection by standard screening methods used by other investigators.

Our identification of a deletion involving exons 4 and 5 in family 20 and the duplication of exon 10 in family 5 suggests that these mutations likely arose from unequal recombination

between flanking intronic sequences. Hotspots for genetic recombination accounting for gross rearrangements of other human genes are well documented. Perhaps the most noted example is that for hemophilia A. For years, PCR amplification and sequence analysis was used to screen the FVIII genes of patients with hemophilia A, yet in 50% of patients with severe disease, the mutation was not identified. However, in 1993, Lakich et al.[16] reported common reoccurring inversion mutations that resulted from unequal crossing over between homologous sequences lying 5' to the FVIII gene and one contained within intron 22 of the gene itself. This mutation was detected by Southern blot analysis and, once identified, enabled direct mutation analysis for at risk carrier females in these families that otherwise were dependent on less accurate laboratory screening assays and DNA linkage analysis.[17] Although approximately 15% to 20% of FPPH mutations and close to 50% of hemophilia A mutations are caused by gross rearrangements in their respective genes, molecular rearrangement events usually represent a small percentage of mutations causing a genetic disorder. In contrast, 70% of patients with Charcot-Marie Tooth disease type IA result from a duplication of the *PMP22*, and close to 90% of patients with hereditary neuropathy with liability to pressure palsies result from a deletion of the *PMP22* gene caused by unequal crossing over events between 1.7 kb repeats in this gene.[18] This hotspot contains a *mariner* transposon-like element that is believed to increase the likelihood of homologous recombination between these sequences.

In conclusion, Southern blot studies and RT-PCR analysis should be included in routine *BMPR2* mutation detection screening assays to complement existing methodologies and increase the detection rate of *BMPR2* mutations. These methods may be utilized at the time of the initial screening for *BMPR2* mutations in new PPH patients to alleviate the need of multiple PCR amplification and DNA sequencing steps used for screening of this complex gene. Although these methods could enable detection of previously undetected *BMPR2* mutations, they may not be applicable to families in which only archived tissue from family members is available for analysis because 10 μg of high molecular weight DNA would be not available for Southern blot analysis nor could large cDNA products be generated from extracted RNA. Further, by identifying these mutations in approximately 15% to 20% of PPH patients, we can provide genetic counseling and offer presymptomatic testing to a greater number of at risk family members. In addition, knowledge of the precise mutation within each patient may aid in the future development of mutation-specific targeted therapy. Importantly, characterization of the breakpoints causing these gross rearrangements, we can unravel the mechanism by which these mutations have occurred and add to our understanding of the DNA sequence motifs and/or chromatin structure that promotes genetic recombination for both inherited disease and cancer.[19,20] It is possible that these intronic sequences represent hot spots for recurring mutations and may also be responsible for some sporadic PPH cases as well.

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