Report

Familial Primary Pulmonary Hypertension (Gene *PPH1*) Is Caused by Mutations in the Bone Morphogenetic Protein Receptor–II Gene

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Familial primary pulmonary hypertension is a rare autosomal dominant disorder that has reduced penetrance and that has been mapped to a 3-cM region on chromosome 2q33 (locus *PPH1*). The phenotype is characterized by monoclonal plexiform lesions of proliferating endothelial cells in pulmonary arterioles. These lesions lead to elevated pulmonary-artery pressures, right-ventricular failure, and death. Although primary pulmonary hypertension is rare, cases secondary to known etiologies are more common and include those associated with the appetite-suppressant drugs, including phentermine-fenfluramine. We genotyped 35 multiplex families with the disorder, using 27 microsatellite markers; we constructed disease haplotypes; and we looked for evidence of haplotype sharing across families, using the program TRANSMIT. Suggestive evidence of sharing was observed with markers GGAA19e07 and D2S307, and three nearby candidate genes were examined by denaturing high-performance liquid chromatography on individuals from 19 families. One of these genes (*BMPR2*), which encodes bone morphogenetic protein receptor type II, was found to contain five mutations that predict premature termination of the protein product and two missense mutations. These mutations were not observed in 196 control chromosomes. These findings indicate that the bone morphogenetic protein–signaling pathway is defective in patients with primary pulmonary hypertension and may implicate the pathway in the nonfamilial forms of the disease.

Familial primary pulmonary hypertension (PPH [MIM 178600]) is a rare ($1/10^5$–$1/10^6$) autosomal dominant disorder that has reduced penetrance and that has been mapped to a 3-cM region on chromosome 2q33 (locus *PPH1*) (Morse et al. 1996, 1997; Nichols et al. 1997; Deng et al. 2000). It is characterized by monoclonal plexiform lesions of proliferating endothelial cells in pulmonary arterioles (Lee et al. 1998). These lesions lead to elevated pulmonary-artery pressures, right-ventricular failure, and death (Rich et al. 1987). The disease can occur from infancy throughout life; it has a mean age

at onset of 36 years, and the ratio of affected females to affected males is 2:1. Without intervention, the median survival is <3 years after diagnosis (D'Alonzo et al. 1991), although recent advances, such as long-term prostacyclin therapy (Barst et al. 1996) and transplantation (Pasque et al. 1995), have significantly improved both the quality of life and the chances of survival in some patients. Although familial PPH (FPPH) is rare, cases secondary to known etiologies are more common and include those associated with the appetite-suppressant drugs, including phentermine-fenfluramine (Douglas et al. 1981; Abenhaim et al. 1996).

We have collected a number of multiplex families with PPH, using experimental protocols approved by the institutional review board of the College of Physicians and Surgeons at Columbia University. Methods used for clinical examination, as well as the diagnostic criteria, have been described elsewhere (Morse et al. 1997). The families with FPPH were ascertained on the basis of phy-
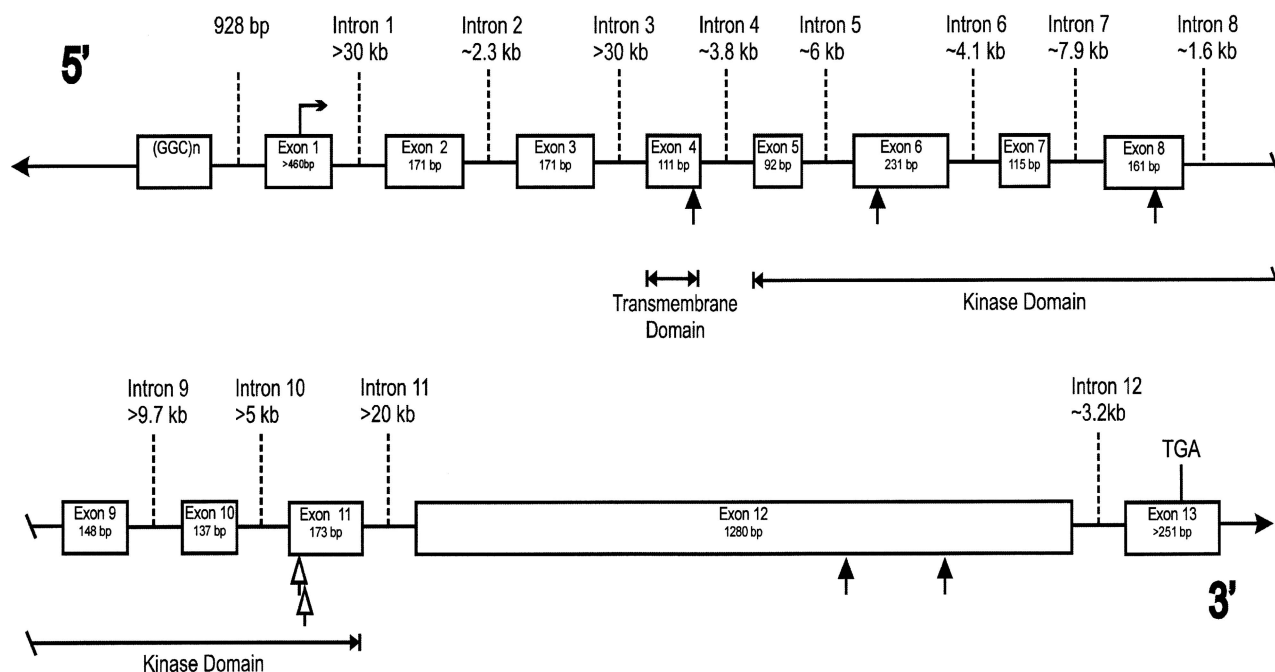
Figure 1 Intron/exon structure of the human *BMPR2* gene. Intron and exon sizes are as indicated. Protein start and stop codons are indicated by the horizontal arrow and "TGA," respectively. Mutations that cause premature termination of *BMPR2* are shown as blackened arrows; unblackened arrows denote mutations in Arg491. The transmembrane and kinase domains are encoded by the indicated exons.

sician referrals (meetings, literature, Internet, and e-mail), self-referrals via the Internet, and blood samples from the 1994 and 1996 Pulmonary Hypertension Association meetings (Stone Mountain, GA). The families were predominantly white, and most originated from the United States, Canada, and Europe. During the past 2–3 years, three individuals originally classified as sporadic PPH had to be reclassified as FPPH, when another family member developed the disease. Also, several asymptomatic individuals known to carry the *PPH1* gene have developed the disease within the past 4 years. Using DNA that was extracted from either whole-blood samples or formalin-fixed, paraffin-embedded tissue, we genotyped 35 of these families (72 affected individuals and 319 normal individuals and carriers), with 27 microsatellite markers located in the 3-cM minimal genetic region, as described elsewhere (Deng et al. 2000). With the genetic model and marker order determined by radiation-hybrid mapping (Deng et al. 2000), a 10-marker multipoint analysis using GENEHUNTER version 2.0 (Kruglyak et al. 1996) gave a nearly constant LOD score of 10, across the region (data not shown). At a recombination fraction of 0, the maximum LOD scores of the two-point analyses using MLINK from FASTLINK version 4.1p (Cottingham et al. 1993) were more variable, ranging from 0.6 to 8.6, with the higher scores clustering toward the telomeric end (data not shown). Given the low prevalence of the disorder and the consequent pos-

sibility that some of the families were from a common founder, we reconstructed, when possible, the 27-marker-microsatellite disease haplotype from each family and visually inspected these haplotypes in order to detect any shared segments. No obvious shared DNA segments were found, so we used the haplotype-analysis program TRANSMIT version 2.5 (1999) (available from the "David Clayton's Genetic Programs" Web page; also see Clayton 1999) to look in a more rigorous fashion. Suggestive evidence of sharing ($P = .07$) was found with the 345-/214-bp haplotype of markers GGAA19e07 and D2S307. Since these markers were in the telomeric cluster, we began our mutation scan in this region.

We investigated the genetic variation in the coding sequence of three nearby candidate genes by examining 22 individuals from 19 families with FPPH and 2 normal controls, using denaturing high-performance liquid chromatography (dHPLC) with a WAVE™ Nucleic Acid Fragment Analysis System (Transgenomics), according to the manufacturer's directions and as described elsewhere (Underhill et al. 1997; O'Donovan et al. 1998). These individuals were chosen on the basis of the amount of DNA available. PCR-amplification products (maximum size 602 bp) were run with as many as three melting profiles, for fragments with multiple melting domains. DNA sequence determination of fragments containing potential variants was performed by cycle sequencing using Big Dye™ terminators (Ap-

Table 1

BMPR2 Gene: Intron/Exon Boundaries, Known Size of Exons, and Approximate Sizes of Introns

Exon (Size)	Exon 3′	5′ Intron Sequence	Intron (Size)	3′ Intron Sequence	Exon 5′
1 (>460 bp)	GCT G Ala A	gtgagtagctccggc..	1 (>30 kb)	..tttcctttattttag	CT TCG la Ser
2 (171 bp)	CAA G Gln G	gcaagtgatactttc..	2 (~2.3 kb)	..catattgatttatag	GA TAT ly Cys
3 (171 bp)	CTC A Leu S	gtaagtaaagtaacc..	3 (>30 kb)	..tttgttttcttttag	GT CCA er Pro
4 (111 bp)	ACA G Thr G	gtaaaaattaccatt..	4 (~3.8 kb)	..ttcctgttcttatag	GA GAC ly Asp
5 (92 bp)	TTG GAG Leu Glu	gtaagtttgccgtta..	5 (~6 kb)	..ttaaaacacttgcag	CTG ATT Leu Ile
6 (231 bp)	CCC AAT Pro Asn	gtaagttcttcatag..	6 (~4.1 kb)	..ttttcctctatatag	GGA TCT Gly Ser
7 (115 bp)	GGA G Gly A	gtaagatagtcaata..	7 (~7.9 kb)	..aaattatccaaacag	AT CAT sp His
8 (161 bp)	AGC GAG Ser Glu	gtgagtgtatacaaa..	8 (~1.6 kb)	..actctaatttatcag	GTT GGC Val Gly
9 (148 bp)	CCA G Pro G	gtaaaaactactgtc..	9 (>9.7 kb)	..tctacaaatccacag	GG GAA ly Glu
10 (137 bp)	AGC CTG Ser Leu	gtaagaaaaaactaa..	10 (>5 kb)	..tactttgtcttacag	GCA GTG Ala Val
11 (173 bp)	GAA CG Glu Ar	gtaagaccctaaggg..	11 (>20 kb)	..ctttctttctttaag	C AAC g Asn
12 (1,280 bp)	CAG A Gln I	gtaagtggagggatc..	12 (~3.2 kb)	..cacttttattttcag	TA GGT le Gly
13 (>251 bp)					

plied BioSystems), and sequencing products were resolved on Long Ranger™ gels (BioWhittaker Molecular Applications) and were detected with an ABI model 377 DNA sequencer. DNA sequence traces were analyzed by Vector NTI suite 5.5 (Informax). The first two genes, *CD28* and *CTLA4,* were candidates because of their involvement in immune system regulation (Morse and Barst 1994). No variation in *CD28* was observed. In *CTLA4,* we found one previously unreported single-nucleotide polymorphism (SNP)—that is, 49A→G, with an allele frequency of .50—that causes a nonconservative change in protein structure (A17T). The polymorphism was homozygous in some of the patients and in one of the controls. Since it did not fit known inheritance patterns for the disease, this SNP was ruled out as a potential disease mutation.

The third positional candidate, the gene encoding the bone morphogenetic protein (BMP) receptor type II (BMPR-II [gene *BMPR2,* also known as "*T-ALK,*" "*CL4-1,*" and "*BRK-3*"]), a member of the transforming growth factor β (TGF-β)–receptor superfamily, was suggested on the basis of the role of the BMP-signaling pathway in lung morphogenesis (Warburton et al. 2000). The cDNA sequence of this ~4-kb gene encoding a 1,038-amino-acid protein has been described elsewhere (Kawabata et al. 1995; Liu et al. 1995; Nohno et al. 1995; Rosenzweig et al. 1995). To deduce the genomic structure of *BMPR2* (fig. 1 and table 1), we found ge-

nomic sequences homologous to exons 1 and 8–13, by querying the National Center for Biotechnology Information high-throughput genome sequence (HTGS) database, using BLAST (Altschul et al. 1990). The intron size and DNA sequence of the other intron-exon boundaries were determined by amplifying and sequencing PCR products by means of oligonucleotide primers designed to amplify either across neighboring exons or out to a nearby *Alu* repeat, with the structure of mouse *BMPR2* (Beppu et al. 1997) being used as a guide. We then designed oligonucleotide primers to amplify the exons from genomic DNA of the patients (table 2). These PCR fragments were screened by dHPLC, and the DNA sequences of those fragments containing apparent variation were determined. Primers to amplify exons 2–7 were designed prior to having the intron/exon structure, and hence a total of 204 bp of known exonic sequence was not screened.

In 9 of the 19 families screened, we observed mutations that are likely to disrupt the function of the receptor. Five of these mutations predict premature termination of BMPR-II, in exons 4, 6, 8, and 12, and each was seen in only one family (fig. 1 and table 3). In addition, in exon 11 in three families, an SNP that causes a nonconservative change in amino acid sequence—that is, from arginine, conserved in all known type II TGF-β–superfamily receptors (fig. 2), to tryptophan—was seen (fig. 1 and table 3). These three families do not

Table 2

Oligonucleotide Primer Sequences Used to Screen *BMPR2*

	PRIMER[a]		SIZE OF PCR PRODUCT
EXON	5′	3′	(bp)
1	AACTAGTTCTGACCCTCGCCCC	GGACGCATGGCGAAGGGCAA	602
2[b]	TAGCTTCGCAGAATCAAGAA	TGCCTTGTTTTACAAGATTT	177
3[b]	TAGGATGTTGGTCTCACATT	TACTGAGTGGTGTTGTGTCA	177
4[b]	TAGGTCCACCTCATTCATTT	TACCTGTCAACATTCTGTAT	117
5[b]	TAGGAGACCGTAAACAAGGT	TACCTCCAACAGTTTCAGAT	98
6[b]	CAGCTGATTGGCCGAGGTCG	TACATTGGGATAGTACTCCA	237
7[b]	TAGGGATCTTTATGCAAGTA	TACCTCCTCGTGGTAATTCT	121
8	GCAGAAAAATAATACTACTTCTATA	GATGTTTTAATTAAATTATCATTTC	319
9	AGAATATGCTACGTTCTCTC	ACACTAGATAGCAATGAACTAAAGG	336
10	GTATCAGAAATACCCCTGTT	TTAGGCAACTCCAAAAACTAT	328
11	GGTAAACTGAAAAGCTCAATAC	CATTGAACTATTAGGCTGGT	345
12-1	GATCCCCTTTCTTTCTTTAAGC	CTGTTTAAGAGAGTGCTCCATG	510
12-2	GAACCTCAAGGAAAGCTCTG	AGCATGGGAGTTAACACTGT	436
12-3	ACCTCATGTGGTGACAGTCA	ATTGGAATTAGTTCGGCCAC	316
12-4	ATTCCAGTCCTGATGAGCAT	AGTTATTTAAATGGCCCCAA	343
13	TTACATCCCTTACCCGTTAT	TTAAAGCAAGTCTTTGTTGC	454

  [a] Exonic DNA sequences are underlined.
  [b] Amplified by primers within the exon.

share a microsatellite marker haplotype. The same arginine was changed to glutamine (1472G→A) in the proband of another family, PPH019 (fig. 1 and table 3), but this proband's parents are genotypically normal. This proband (from whom we have a DNA sample) had both a son who died of PPH in childhood (presumptive; no postmortem was performed) and a deceased uncle with a history of portal hypertension and unspecified cardiac problems. Since the 1472G→A mutation had not been transmitted through either parent, more history on the "affected" uncle was obtained, and he was found to have longstanding alcoholism—and we think that this, rather than right-sided heart failure secondary to PPH, was responsible for his portal hypertension. The observation of this new mutation suggests that mutations in *BMPR2* might also cause sporadic cases of PPH.

Except for family PPH019 (see above), the pattern of mutations observed when all additional members of the other eight families were screened by dHPLC and DNA sequencing was identical to the segregation pattern of PPH. None of the putative mutations were observed in 96 additional samples (a total of 196 chromosomes total, including 4 from the screening). In both samples, we also observed a synonymous SNP (2811G→A) with a minor-allele frequency of .21. Since the nine mutations appeared to be functional, and since no such mutations were observed in the controls, we applied Fisher's exact test to the data, and we observed a significant difference ($P < .0001$), in mutation prevalence, between cases and controls.

The mutation in exon 4 is in the transmembrane domain, and those in exons 6, 8, and 11 are in the kinase domain, of this serine/threonine kinase receptor (fig. 1). In functional studies of the homologous TGF-β receptor type II (TβR-II), cell lines lacking endogenous TβR-II were transfected with TβR-II constructs lacking either the complete kinase domain or amino acids 490–508 (homologous to amino acids 452–471 in BPMR-II) (Wieser et al. 1993). These constructs were unable to restore the ability to inhibit growth, stimulate fibronectin production, and drive transcription from a TGF-β–responsive element, in response to exogenous TGF-β that was observed when the cell lines were transfected with the wild-type TβR-II (Wieser et al. 1993). Furthermore, the construct lacking amino acids 490–508 was unable to function as a kinase in vitro (Wieser et al. 1993). Therefore, at least three of these mutations (premature terminations in exons 4, 6, and 8) should encode a nonfunctional receptor that is unable both to phosphorylate a type I receptor and to propagate the signal from a BMP ligand, since they will lack this region of the kinase domain. The lack of kinase activity would be consistent with a disease model of haploinsufficiency. Alternatively, the prematurely terminated products could act as a dominant negatives, as has been observed for TβR-II (Wieser et al. 1993) and endoglin (Lux et al. 2000). Given that BMPR-II is likely to be present, on the cell surface, as a dimer (Gilboa et al. 2000), only 25% of such complexes might be functional.

The two mutations in exon 11 change Arg491. This arginine is highly conserved in all type II TGF-β–superfamily receptors (fig. 2) and appears to be homologous to the invariant Arg280 in subdomain XI in other protein kinases (Hanks et al. 1988). Mutation of the

Table 3

BMPR2 **Mutations Observed in PPH**

Family or Families	No. of A/C/U[a]	Exon	DNA Sequence Variation[b]	Protein Sequence Variation
PPH001, PPH008, PPH021	4/5/13	11	1471C→T	R491W
PPH010	2/0/1	8	1099–1103delGGGGA	E368fsX1
PPH015	6/1/8	12	2579delT	N861fsX10
PPH017	3/0/6	4	507–510delCTTTinsAAA	C169X
PPH018	3/2/4	12	2617C→T	R873X
PPH019	1/0/5	11	1472G→A	R491Q
PPH022	2/0/0	6	690–691delAGinsT	K230fsX21

[a] No. of DNA samples available for analysis of affected (A), known carrier (C), and unaffected (U) individuals in each family or set of families, determined by segregation pattern and DNA sequencing/dHPLC, except in the case of family PPH019 (in which only DNA sequencing was used; see text).

[b] Sequences are referenced to GenBank *BMPR2* cDNA sequence number NM_001204 (see NCBI GenBank Overview); the numbering is based on the use of "+1" to denote the A of the starting methionine codon.

homologous amino acid in transfected TβR-II (R582A) greatly reduces the ability of exogenous TGF-β to stimulate transcription of a TGF-β–responsive element in TβR-II–deficient cells (Brand and Schneider 1995). Last, arginine is the amino acid that is most frequently changed in disease mutations (see the "Statistics for Missense/Nonsense Mutations" Web page of the Institute of Medical Genetics, University of Wales College of Medicine, Cardiff). Taken together, these results strongly suggest that Arg491 is important to the function of BMPR-II. The mutations in exon 12 occur in the intracellular C-terminal domain, of unknown function, that is unique to BMPR-II. Possible functions for this portion of the molecule include the binding of downstream effector proteins or a role in dimerization and/or trafficking.

We screened 93% (2,913/3,117 bp) of the known publicly available coding sequence, the majority of the intron/exon boundaries, and 518 bp in the 5′ and 3′ UTRs
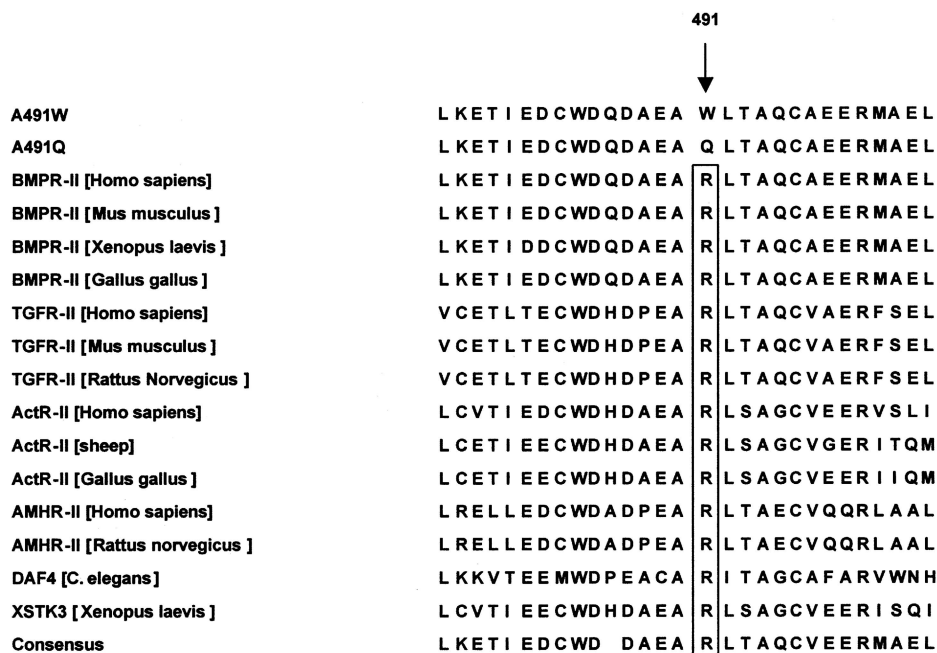
491

A491W	LKETIEDCWDQDAEA	W LTAQCAEERMAEL
A491Q	LKETIEDCWDQDAEA	Q LTAQCAEERMAEL
BMPR-II [Homo sapiens]	LKETIEDCWDQDAEA	R LTAQCAEERMAEL
BMPR-II [Mus musculus]	LKETIEDCWDQDAEA	R LTAQCAEERMAEL
BMPR-II [Xenopus laevis]	LKETIDDCWDQDAEA	R LTAQCAEERMAEL
BMPR-II [Gallus gallus]	LKETIEDCWDQDAEA	R LTAQCAEERMAEL
TGFR-II [Homo sapiens]	VCETLTECWDHDPEA	R LTAQCVAERFSEL
TGFR-II [Mus musculus]	VCETLTECWDHDPEA	R LTAQCVAERFSEL
TGFR-II [Rattus Norvegicus]	VCETLTECWDHDPEA	R LTAQCVAERFSEL
ActR-II [Homo sapiens]	LCVTIEDCWDHDAEA	R LSAGCVEERVSLI
ActR-II [sheep]	LCETIEECWDHDAEA	R LSAGCVGERITQM
ActR-II [Gallus gallus]	LCETIEECWDHDAEA	R LSAGCVEERIIQM
AMHR-II [Homo sapiens]	LRELLEDCWDADPEA	R LTAECVQQRLAAL
AMHR-II [Rattus norvegicus]	LRELLEDCWDADPEA	R LTAECVQQRLAAL
DAF4 [C. elegans]	LKKVTEEMWDPEACA	R ITAGCAFARVWNH
XSTK3 [Xenopus laevis]	LCVTIEECWDHDAEA	R LSAGCVEERISQI
Consensus	LKETIEDCWD DAEA	R LTAQCVEERMAEL

Figure 2 Sequence alignment of the type II TGF-β–superfamily receptors surrounding R491 in BMPR-II. The mutation in families PPH001, PPH008, and PPH021 was aligned with all known type II receptors (>50), and 14 of these are displayed. TGFR-II = TGF-β–receptor type II; ActR-II = activin receptor type II; AMHR-II = anti–mullerian hormone type II receptor; DAF-4 = development-regulatory growth factor type IV; XSTK3 = *Xenopus* activin receptor.

of *BMPR2,* but we failed to find a causative mutation in 10 of the 19 families. There are several possible explanations for why this may have occurred. The causative mutations may occur in known or currently unknown coding sequences, intronic or regulatory regions of *BMPR2,* or other genes in the BMP-signaling pathway. Several of the linked families are large enough to produce LOD scores suggestive of linkage to 2q33 (individual LOD scores >2), indicating that we may not have screened the entire gene. It is possible that some of the families have disease mutations in the 204 bp of known exonic sequence that we missed in our screen, and we currently are rescreening exons 2–7 in the 10 families. There is also the possibility that some of the coding sequence of *BMPR2* in lung tissue is currently unknown and that we therefore have not screened it. mRNA transcripts of 5, 6.5, 8, and 11.5 kb have been observed on northern blots, with the longest transcript predominating in lung tissue (Kawabata et al. 1995; Nohno et al. 1995; Rosenzweig et al. 1995), so we may have missed some alternatively spliced exons in our screen.

We have screened only a small portion of the intronic and regulatory sequences in these families, so mutations in these regions are possible. One regulatory region that we have examined is a $(GGC)_{8–16}$ trinucleotide repeat at the 5′ end of the gene, at positions −928 to −963, which we amplified by means of the oligonucleotide primers TGAGCGAATCACAACCCCCCG and GAGTTCCGTCAGGAGCCCAG. Using PCR, we have not observed evidence of either an apparent increase in homozygosity or expansion of the repeat, either of which would be consistent with the suggestion of anticipation in PPH (Loyd et al. 1995); but detection of this event might require a Southern blot, and we have a limited quantity of DNA from the affected individuals (many of whom are deceased) in many of our families.

Last, it is possible that the mutations in these 10 families occur in other genes in the BMP-signaling pathway. The microsatellite data are consistent—but not conclusive—with linkage to *PPH1* in all 19 families, but it is possible that the families with little linkage information could have no linkage to 2q33. An analogous situation has been observed in hereditary hemorrhagic telangectasia (HHT [MIM 187300]), another autosomal dominantly inherited vascular disorder with defects in the TGF-β–signaling pathway, where mutations have been observed in two genes, endoglin (HHT1) (McAllister et al. 1994) and the type I receptor ALK1 (HHT2) (Johnson et al. 1996).

So how do these mutations cause PPH? It is unlikely that they act as in a dominant-negative fashion, by inhibiting the apoptotic effect of the TGF-β pathway, because BMPR-II does not associate with type I receptors of the TGF-β family in transient-expression assays using

mammalian cells (Liu et al. 1995), even though this occurs in vitro (Kawabata et al. 1995; Liu et al. 1995; Nohno et al. 1995). It is also unlikely that these mutations completely abolish the BMP-signaling pathway, because mice homozygous for a mutation in the kinase domain of *BMPR2* die at day 9.5, prior to gastrulation (heterozygotes are grossly normal) (Beppu et al. 2000). This phenotype is very different from what we observe in PPH, and suggests that only 25%–50% of the function of the BMP pathway is required for it to perform a role in early development, given the analogy to TβR-II, which has been discussed above. The BMP pathway induces apoptosis in some cell types (Soda et al. 1998; Kimura et al. 2000), so a partial block of signal transmission might have a slow proliferative effect and could be caused by either dominant-negative protein interactions or reduced signal transmission due to haploinsufficiency of BMPR-II. As discussed above, both of these mechanisms have been observed with prematurely terminated protein products. Similarly, for both the missense mutations at Arg491 and the C-terminal mutations, either model is possible. In either case, both mechanisms lead to a partial block of BMP-signal transmission, which is likely to cause the development of PPH. This is similar to what has been observed for HHT (Lux et al. 2000), and determination of the mechanism of the mutations at *PPH1* will require study of BMPR-II expression.

BMP signaling may occur through both the "Smad" (Massague 1998) and mitogen-activated protein kinase (Kimura et al. 2000) cascades, and both are inhibited by Smad6, which can be induced by vascular shear stress (Topper et al. 1997). Either (*a*) the reduced apoptotic signals from the BMP pathway, caused by mutations in either *BMPR2* or other molecules in the signaling cascades, or (*b*) shear stress via Smad6, possibly after an initial nidus of vascular injury, might underlie many forms of PPH, including those associated with HIV or appetite-suppressant drugs.

Acknowledgments

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

BLAST, http://www.ncbi.nlm.nih.gov/BLAST (for search of HTGS database)

David Clayton's Genetic Programs, http://www.mrc-bsu.cam .ac.uk/pub/methodology/genetics (for TRANSMIT version 2.5 [1999])

Institute of Medical Genetics, University of Wales College of Medicine, Cardiff, http://www.uwcm.ac.uk/uwcm/mg/docs/ haha1.html (for disease mutations)

NCBI GenBank Overview, http://www.ncbi.nlm.nih.gov /Genbank/GenbankOverview.html (for *BMPR2* cDNA sequence number NM_001204)

Online Mendelian Inheritance in Man (OMIM), http://www .ncbi.nlm.nih.gov/Omim (for PPH [MIM 178600] and HHT [MIM 187300])

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