Heterozygous germline mutations in *BMPR2*, encoding a TGF-β receptor, cause familial primary pulmonary hypertension

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Primary pulmonary hypertension (PPH), characterized by obstruction of pre-capillary pulmonary arteries, leads to sustained elevation of pulmonary arterial pressure (mean >25 mm Hg at rest or >30 mm Hg during exercise[1]). The aetiology is unknown, but the histological features reveal proliferation of endothelial and smooth muscle cells with vascular remodelling[2] (Fig. 1). More than one affected relative has been identified in at least 6% of cases[3] (familial PPH, MIM 178600). Familial PPH (FPPH) segregates as an autosomal dominant disorder with reduced penetrance and has been mapped to a locus designated *PPH1* on 2q33, with no evidence of heterogeneity[4–6]. We now show that FPPH is caused by mutations in *BMPR2*, encoding a TGF-β type II receptor (BMPR-II). Members of the TGF-β superfamily transduce signals by binding to heteromeric complexes of type I and II receptors, which activates serine/threonine kinases, leading to transcriptional regulation by phosphorylated Smads[7]. By comparison with *in vitro* studies, identified defects of BMPR-II in FPPH are predicted to disrupt ligand binding, kinase activity and heteromeric dimer formation[8–10]. Our data demonstrate the molecular basis of FPPH and underscore the importance *in vivo* of the TGF-β signalling pathway in the maintenance of blood vessel integrity.

To enable positional cloning of the FPPH gene, we verified and extended a published YAC map at 2q33 to anchor a BAC/PAC contig including genomic sequences available at GenBank[11]. This contig covers the entire 5.8-Mb *PPH1* region defined by recently detected recombination events limited by the polymorphic STS markers *D2S115* and *D2S1384* (ref. 12). We positioned 81 potential transcriptional units, which include 17 partially or completely characterized genes, within the 5.8-Mb interval by means of database and PCR-based screening. We established a panel of eight PPH kindreds for candidate gene mutational analysis. We identified no functionally significant sequence variants by direct sequencing in the following genes: *Casp10*, *CTLA4*, *CD28* and *PSMA2* (Fig. 2, and data not shown). An additional gene in the interval, *BMPR2*, was originally identified through a yeast two-hybrid screen using TGF-β family type I receptors as a bait[13–15]. Expression of TGF-β is upregulated in remodelling pulmonary arteries in PPH, and heterozygous mutations in *ENG* and *ACVRL1* (encoding components of the TGF-β receptor complex) cause hereditary haemorrhagic telangiectasia[16,17]. We therefore considered *BMPR2* to be both a biological and positional candidate for the FPPH gene.

We designed PCR primers for amplification of patient genomic DNA after determination of the intron/exon boundaries of *BMPR2* as predicted by the mouse *Bmpr2* genomic structure[18] (see Table 1, http://genetics.nature.com/supplementary_info/). We detected sequence variants in seven probands from the panel of eight kindreds studied. We found heterogeneous mutations, including two frameshift, two nonsense and three missense mutations, to be distributed across the gene (Fig. 3). We identified a frameshift mutation in individual US55 resulting from the deletion of a T residue within an ATT repeat sequence in exon 12. This, together with the exon 12 nonsense mutation R899X in US33, predicts premature truncation of the 1,038 amino acid protein, including the large cytoplasmic domain of BMPR-II. These mutations are likely to impede heteromeric receptor complex formation at the cell surface, a requirement for normal signal transduction[10]. The exon 2 nonsense mutation S73X (US35) and the exon 3 355delA frameshift (UK13) truncate the protein before the transmembrane domain and, if translated, may fail to reach the cell surface. Each of the amino acid substitutions occurs at a highly conserved and functionally important site of the BMPR-II protein (Fig. 4). One of the missense mutations, a cys-

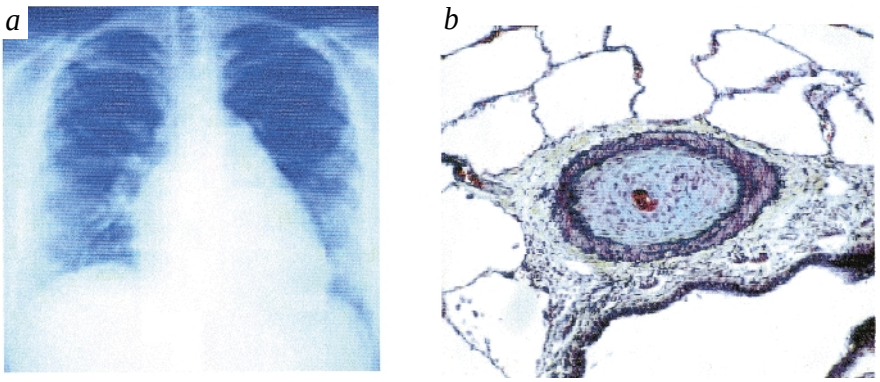

Fig. 1 Clinical and histological features of FPPH. ***a***, Chest radiograph showing increase in size of the cardiac silhouette due to right atrial and ventricular dilatation, central pulmonary artery dilatation and attenuation of the pulmonary arterial vascular markings. ***b***, Photomicrograph of a surgical lung biopsy from an individual with PPH, demonstrating occlusion of a pre-capillary pulmonary artery with severe concentric proliferation of the vascular intima and moderate hypertrophy of the media. Normal alveoli surround the vessel with a normal airway below.

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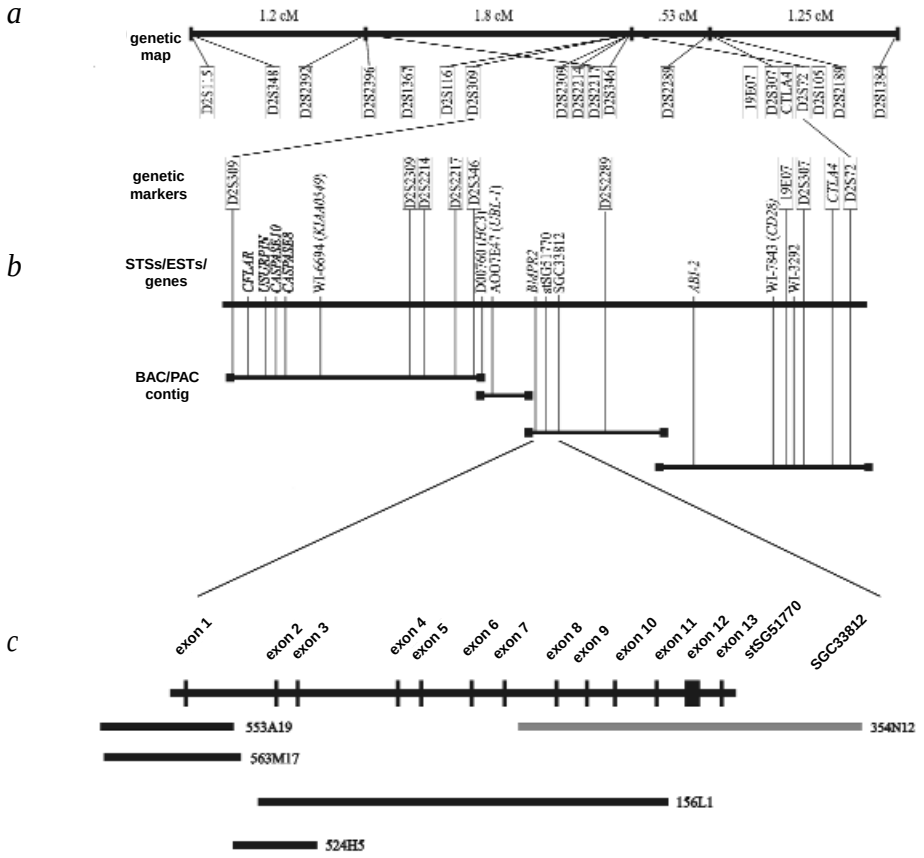

Fig. 2 Physical map of the *PPH1* interval at 2q33. ***a***, Position of microsatellite markers. ***b***, Physical map contig (BAC/PAC) of the region surrounding *BMPR2*, including other genes analysed here[12]. ***c***, *BMPR2* genomic structure, determined by analysis of available sequence data for BAC clone RP11-345N12 as well as sequence analysis of additional BAC clones identified by library screening as shown (not to scale). Comparison between the human and published mouse genomic organization shows strong conservation of the intron/exon boundaries[18].

teine-to-tryptophan substitution at position 118 within the extracellular domain (US14), is predicted to perturb ligand binding. Although TGF-β type II receptors differ in the amino acid sequence of the extracellular domain, they show identical spacing of cysteines in this region, a feature key to ligand-binding specificity[8] (Fig. 4). We found two further missense mutations, C347Y and D485G, in PPH families UK06 and NL01, respectively. Both of these amino acids are located within the kinase domain of BMPR-II and are conserved across TGF type II receptor homologues (Fig. 4). It is unknown whether these sequence changes alter constitutive phosphorylation of the type II receptor or lead to an inability of the kinase domain to phosphorylate the associated type I receptor, both of which are essential for normal receptor complex function. Expression of a recombinant mutant of the closely related TGF-β type II receptor harbouring an amino acid substitution at a position corresponding to residue 487 of BMPR2 fails to phosphorylate the type I receptor[9]. Together with the high degree of amino acid conservation throughout the kinase domain, these data suggest that the identified missense mutations are deleterious to the function of the type II receptor.

Either restriction endonuclease or sequence analysis of DNA from affected and unaffected first-degree relatives was used to show co-segregation of the mutations with the disease phenotype (including obligate gene carriers) in all but one of the pedigrees (Fig. 3). We screened 150 normal chromosomes derived from the same population as the affected families together with a panel of 64 chromosomes from normal, but ethnically diverse, control subjects. None of the mutations were detected in either panel. The predicted functional impact of these mutations, their absence in healthy controls and their co-segregation with the phenotype all provide strong support that they cause disease. We failed to detect a mutation in one of the eight families screened. It is likely that large deletions or gene rearrangements are not detected by the methods used here or, alternatively, further mutations may be located within the non-coding regions of *BMPR2*.

FPPH due to mutations at *PPH1* is inherited in a dominant manner[4]. The mutations observed here may impair BMPR-II function through either haploinsufficiency or a dominant-negative mechanism[19]. Although these may not be mutually exclusive, the precise mechanism will require further investigation. Reduced penetrance of FPPH, as well as the high frequency of apparent 'sporadic' PPH cases, suggests a requirement for exposure to additional triggers disrupting tissue specific growth control. One mechanism may include a second somatic mutational event within an unstable BMPR-II–specific signalling pathway[20]. The only other human germline mutation of a type II receptor to be reported is a deleterious amino acid substitution in the kinase domain of TGF-β type II. This mutation predisposes to colorectal cancer, with loss of the wild-type allele in the tumour tissue[21]. Using microdissection techniques, monoclonality of the endothelial cells was demonstrated in plexiform lesions from PPH cases[22]. Fetal and adult tissue northern-blot analyses show high levels of *BMPR2* transcripts in lung and heart, and studies to assess loss of the wild-type alleles in proliferating lesions of FPPH patients are now underway[13,15]. Further characterization of *BMPR2* mutations will provide insight into the molecular mechanisms that lead to the development of FPPH and will determine the contribution of defects in BMPR-II to the pathogenesis of 'sporadic PPH' and secondary forms of PH. These observations provide the platform for future therapeutic evaluation of treatments for PPH and related disorders, and have immediate implications for the management of FPPH.

Methods

Patients. We ascertained families in which at least two members had the typical manifestations of PPH after exclusion of known associated disorders as described[5]. We collected venous blood samples and extracted genomic DNA following informed consent. Obligate gene carriers are defined as those individuals who inherit and transmit the disease gene to an affected offspring but who themselves show no clinical manifestations of the disease.

Determination of the genomic structure of *BMPR2*. We compared available genomic sequence of BAC RP11-354N12 (http://www.ncbi.nlm.nih.gov/Genbank) with the published cDNA sequence (Genbank Z48923) and determined the intron/exon boundaries for the 3′ portion of the gene[14] (exons 8–13). To determine the intron/exon boundaries for exons 1–7,

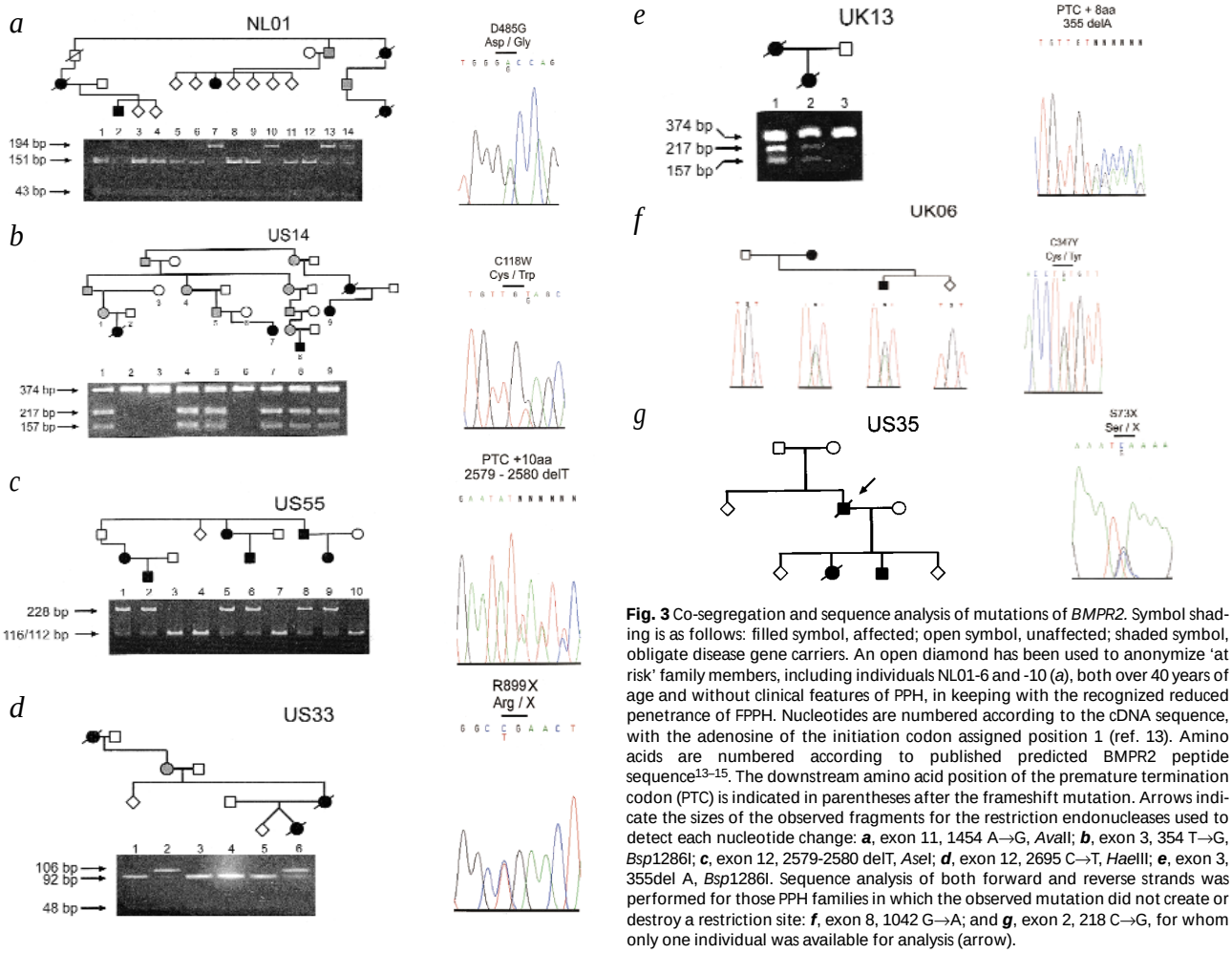

Fig. 3 Co-segregation and sequence analysis of mutations of *BMPR2*. Symbol shading is as follows: filled symbol, affected; open symbol, unaffected; shaded symbol, obligate disease gene carriers. An open diamond has been used to anonymize 'at risk' family members, including individuals NL01-6 and -10 (*a*), both over 40 years of age and without clinical features of PPH, in keeping with the recognized reduced penetrance of FPPH. Nucleotides are numbered according to the cDNA sequence, with the adenosine of the initiation codon assigned position 1 (ref. 13). Amino acids are numbered according to published predicted BMPR2 peptide sequence[13–15]. The downstream amino acid position of the premature termination codon (PTC) is indicated in parentheses after the frameshift mutation. Arrows indicate the sizes of the observed fragments for the restriction endonucleases used to detect each nucleotide change: ***a***, exon 11, 1454 A→G, *Ava*II; ***b***, exon 3, 354 T→G, *Bsp*1286I; ***c***, exon 12, 2579-2580 delT, *Ase*I; ***d***, exon 12, 2695 C→T, *Hae*III; ***e***, exon 3, 355del A, *Bsp*1286I. Sequence analysis of both forward and reverse strands was performed for those PPH families in which the observed mutation did not create or destroy a restriction site: ***f***, exon 8, 1042 G→A; and ***g***, exon 2, 218 C→G, for whom only one individual was available for analysis (arrow).

additional BAC clones were isolated by PCR screening of a human genomic BAC library (CITB B&C, Research Genetics) using both exon 1 and exon 3 STSs designed from the *BMPR2* cDNA. Direct sequence analysis of the BAC clones with primers predicted to be near intron/exon junctions, based on the mouse *Bmpr2* genomic structure, generated flanking intronic sequence for the remaining introns[18].

Mutational analysis. We screened the entire coding and intron/exon boundaries by direct sequencing of both forward and reverse strands on either an ABI 377 sequencer or an ABI 3700 DNA analyser (using the Applied Biosystems DyeDeoxy or BigDye terminator kit) and analysed the data using Sequence Analysis v3.2 or v3.6NT software (Perkin Elmer). The PCR primers for each exon are available on request.

Restriction endonuclease digestion. We confirmed segregation of the mutations within families and excluded the presence of the mutations in controls, including a panel from the DNA Polymorphism Discovery Resource, Coriell Cell Repositories, by PCR amplification of the relevant exon. This was followed by either mutation-specific restriction fragment length polymorphism (RFLP) analysis or direct sequencing as described[23]. Exon 12 (2579-2580delT) was PCR amplified using the following primers as a nested PCR reaction: 5′–ACCCAATATGCCAATGGGAC–3′, 5′–TTCGCCACCTTCTAGTGGCT–3′ followed by 5′–CATGTGGTAAACTGAAAAGCTCA–3′, 5′–TTGAGACCACTTTGATACACACA–3′. We digested an aliquot (10 µl) overnight at 37 ºC with the appropriate enzyme (10 U; Gibco) and separated the fragments on a 4% agarose gel.

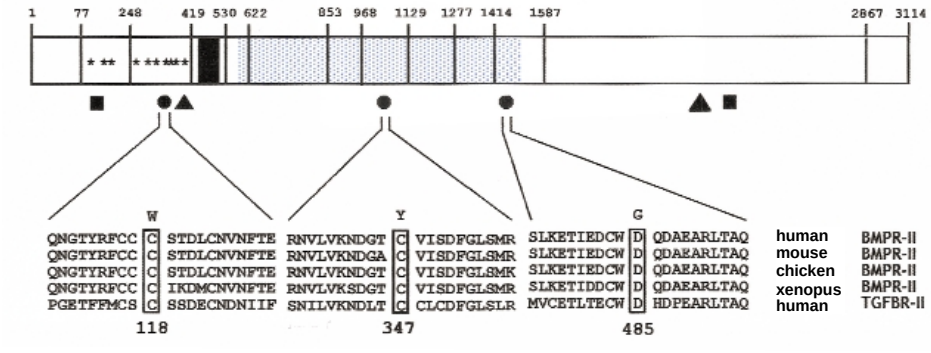

Fig. 4 *BMPR2* cDNA structure and location of FPPH mutations. The location of the exons are indicated by the nucleotide start position in the cDNA. The cysteine residues within the extracellular domain are each denoted by an asterisk. The filled box represents the transmembrane domain and the stippled area identifies the region encoding the receptor kinase domain. Mutations are depicted as follows: filled square, nonsense; filled circle, missense; filled triangle, frameshift. BLAST homology results showing protein similarity of human BMPR2 to receptors in other species and human TGF-β receptor type II (TGFBR-II). Amino acid positions are shown together with the codon substitutions of conserved amino acids (boxed).

GenBank accession number. *BMPR2* cDNA, Z48923.

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