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Hum Mutat. 2015 December ; 36(12): 1113–1127. doi:10.1002/humu.22904.**Pulmonary Arterial Hypertension: A Current Perspective on Established and Emerging Molecular Genetic Defects**

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Abstract

Pulmonary arterial hypertension (PAH) is an often fatal disorder resulting from several causes including heterogeneous genetic defects. While mutations in the bone morphogenetic protein receptor type II (*BMPR2*) gene are the single most common causal factor for hereditary cases, pathogenic mutations have been observed in approximately 25% of idiopathic PAH patients without a prior family history of disease. Additional defects of the transforming growth factor beta (TGF- β) pathway have been implicated in disease pathogenesis. Specifically, studies have confirmed activin A receptor type II-like 1 (*ACVRL1*), endoglin (*ENG*) and members of the *SMAD* family as contributing to PAH both with and without associated clinical phenotypes. Most recently, next-generation sequencing has identified novel, rare genetic variation implicated in the PAH disease spectrum. Of importance, several identified genetic factors converge on related pathways and provide significant insight into the development, maintenance and pathogenic transformation of the pulmonary vascular bed. Together, these analyses represent the largest comprehensive compilation of *BMPR2* and associated genetic risk factors for PAH, comprising known and novel variation. Additionally, with the inclusion of an allelic series of locus-specific variation in *BMPR2*, these data provide a key resource in data interpretation and development of contemporary therapeutic and diagnostic tools.

Keywords

BMPR2; *ACVRL1*; *ENG*; *SMAD1*; *SMAD4*; *SMAD9*; *CAV1*; *KCNA5*; *KCNK3*; *EIF2AK4*; haploinsufficiency; locus heterogeneity

Introduction

Heritable pulmonary arterial hypertension (HPAH) (*PPH1*; MIM# 178600) is a severe, progressive autosomal dominant vascular disorder, predominantly affecting the arterial circulation and, in particular, the pulmonary arterioles [Tuder et al., 2013]. Histopathological investigation reveals abnormal muscularization of these structures, which leads to a chronic elevation of pulmonary arterial pressure, often resulting in right heart failure 2-3 years post-

diagnosis in the absence of the contemporary treatment protocols [Vonk-Noordegraaf et al., 2013]. Identification of mutations in the *BMPR2* gene in probands with a family history of disease provided the first insight into the molecular pathogenesis of HPAH [Deng et al., 2000; Lane et al., 2000]. Subsequently, *BMPR2* mutations were identified in a cohort of idiopathic patients (IPAH) [Thomson et al., 2000]. Since, causal variation has been described in nine additional genes, in cases that include PAH associated with other conditions (APAH). Here, we describe molecular genetic analyses of the 10 functionally characterized genes that cause PAH (Figure 1) and provide a compilation of all mutations identified to date. The continuing identification of genetic factors, as explored in this report, provides unique insight to the genetic mechanisms driving disorders of pulmonary vascular function. Furthermore, these studies offer the foundation for the discovery and delivery of novel therapeutic options.

Key Components of the BMP Signaling Pathway

The *BMPR2* gene (MIM# 600799) encodes a type II receptor of the TGF- β family of signaling molecules. The mature polypeptide is composed of a signal peptide (encoded by exon 1), an extracellular domain (exons 2-3), a single transmembrane domain (exons 4-5), a highly conserved eukaryotic protein kinase region (exons 6-11) and an unusually large cytoplasmic tail (exons 12-13) amongst TGF- β receptors species [Liu et al., 1995]. In the canonical pathway, BMPR-II binds ligand in a heteromeric complex with a type I receptor, which may be activin receptor-like kinase 1 (ALK1), -2 (ALK2), -3 (ALK3/BMPR1A) or -6 (ALK6/BMPR1B), to initiate activation of intracellular partners within a cell-specific context [David et al., 2009; Rigueur et al., 2015]. Phosphorylation of the receptor SMAD proteins (R-SMADs) 1, 5 and 8 leads to their association with the nuclear chaperone SMAD4. This signaling complex translocates to the nucleus, where it acts in combination with transcriptional co-activators and -repressors to effect control of target gene expression (Figure 1). BMPR-II signaling has been established as essential to a multitude of fundamental cellular processes including proliferation, apoptosis, differentiation and migration [Shi and Massague, 2003].

Mutations of *BMPR2* Predispose to the Majority of Hereditary and Idiopathic Forms of PAH

Herein, we describe an additional 370 independent variants of *BMPR2* in patients either previously excluded from or ascertained since the last comprehensive mutation update in 2009 [Machado et al., 2009]. Of these, 108 were identified as part of this study and were generated by specialist PAH centers based in Germany, France, North America and the UK (Table 1, Table 2). The research was prospectively reviewed and approved by a duly constituted ethics committee for each center. Probands were assessed for point mutations and large gene abnormalities using multiple screening technologies including Southern blotting, denaturing high-performance liquid chromatography, multiplex ligation-dependent probe amplification (MLPA), dye-terminator and next-generation sequencing (NGS). All variants considered to be pathogenic were absent from a control population of at least 200 chromosomes and public variation databases including dbSNP v142 (<http://www.ncbi.nlm.nih.gov/SNP>) and the 1000 genomes project (<http://www.1000genomes.org>)

and/or have been previously demonstrated to have a functional impact. For a current estimation of population frequencies, we have additionally checked all point mutations reported here against the Broad Institute Exome Aggregation Consortium (ExAC) database v0.3 (<http://exac.broadinstitute.org>), comprising over 60,000 exomes derived from independent sequencing projects. Mutation nomenclature employs parameters set by the Human Genome Variation Society (<http://www.hgvs.org/mutnomen>). Taken together with previous reports [Machado et al., 2006; 2009], these data provide evidence of a total of 668 germline variants underlying PAH, thereby consolidating *BMP2* as the major causal gene for familial cases and subjects previously classified as IPAH. The spectrum and range of *BMP2* defects in this study comprise the major mutation categories which, in general, correlate with existing data [Machado et al., 2006; 2009]. Namely, we record missense variants leading to amino acid substitution (n=86, 23%), nonsense mutations (n=107, 29%), frameshift defects resulting from small insertions/deletions (n=79, 21%) and splice-site variation (n=33, 9%). However, and by contrast to earlier studies, we identified a significantly higher prevalence of major gene rearrangements (n=61, 16%) and single nucleotide mutations in the 5'-prime untranslated region (5' UTR) (Table 1, Table 2, Supp. Table S1). This, most likely, is a consequence of screening centers expanding the analysis of gene re-arrangements to include all exons of *BMP2*, combined with a growing recognition that mutation short-fall within cohorts is potentially explained by defects harbored within non-coding regions of *BMP2* [Machado et al., 2006]. For example, we report a total of four recurrent 5' UTR mutations resulting from a guanine to adenine change (c.-669G>A) likely to abolish specificity for an SP3 transcription factor binding site [Wang et al., 2009]. In combination, these genetic findings reinforce haploinsufficiency as the molecular mechanism for this disease [Machado et al., 2001; 2006; 2009]. Moreover, this report provides a comprehensive compilation of distinct variants (n=384) across the *BMP2* locus since the first identification of the gene (Supp. Table S2). A combination of genetic and functional studies have firmly established a large proportion of these to be likely pathogenic while others, although compelling, remain to be fully elucidated as disease-causing. These data have now been made available in the ClinVar database (<http://www.ncbi.nlm.nih.gov/clinvar>).

Distribution and Biological Significance of *BMP2* Variation

Although variation has been described across the entire coding structure of the gene, the mutation load differs significantly across exons, indicating both the likely existence of mutation hot-spots and potential regions of key functional importance. Taken together with previously reported findings, our analyses indicate that the majority of amino acid substitutions cluster in exons encoding the ligand-binding domain and key catalytic regions of the kinase domain, namely exons 2-3, 6-9 and 11 respectively. By contrast, exons 1, 4, 10 and 13, encoding receptor regions of uncertain importance to function, have a low frequency of missense mutation (Figure 2A). However, assessment of individual nucleotide defects within the *BMP2* open reading frame, relative to exon length, illustrates variant load by exon may be resultant on an abnormally high frequency of recurrence (e.g. exon 12) which corresponds to a low percentage of affected bases (Figure 2B). Conversely, exon 9 whilst harboring a relatively modest 6.7% of all reported coding variants, contains the highest

proportion of independent nucleotide defects (n=30) relative to exon size (148 bp). In combination with previous reports, the majority of mutations predict incorporation of a premature termination codon in the mRNA (n=483, 72%) and, as previous functional studies have demonstrated, result in degradation of the message through the nonsense-mediated decay pathway [Aldred et al., 2007; Nasim et al., 2008].

Cysteine substitutions comprise the majority of missense mutations in the extracellular ligand-binding domain and are concentrated on 9 of 10 conserved residues, which are essential for the formation of five disulfide bridges necessary to maintain the integrity of this highly ordered three-dimensional structure (Figure 3) [Greenwald et al., 1999]. Moreover, this analysis has indicated the existence of additional critical residues. Notably, mutation of an asparagine (p.N126S), adjacent to the frequently mutated cysteine residue (p.C123R, p.C123S), is observed on seven independent occasions [Machado et al., 2009] (Table 1, Supp. Table S1), highlighting its putative significance to extracellular domain function. The majority of tested mutations in the kinase domain of BMPR-II abolish catalytic function as determined by *in vitro* BMP/SMAD luciferase reporter gene assays. By contrast, the significance of mutations within the cytoplasmic tail remains enigmatic, as these receptors retain significant capacity for downstream signaling through the SMAD family. Yet, these defects appear to perturb non-canonical pathways which include signaling through the cytoskeleton-associated factors LIMK-1 and Tctex-1 [Foletta et al., 2003; Machado et al., 2003]. In addition, studies have suggested that missense variants present across all the functional domains of BMPR-II trigger constitutive up-regulation of p38MAPK indicating a perturbation of one or more SMAD-independent pathways yet to be fully investigated [Nishihara et al., 2002; Rudarakanchana et al., 2002].

Clinical Significance

Extracellular Domain Mutation Spectrum

Transient over-expression and subcellular localization of constructs harboring cysteine substitutions, specifically p.C60Y, p.C117Y, p.C118Y, p.C123R and p.C123S, have previously shown intracellular retention of these receptor species combined with a dramatic diminution of SMAD activation [Rudarakanchana et al., 2002]. Here, we report further cysteine substitutions likely to underlie structural variation in BMPR-II (p.C34R, p.C60G/R, p.C66G/R/Y, p.C84F/G/R, p.C94G/R, p.C99F/R/Y, p.C117R/S, p.C118W) (Figure 3). Utilization of these genetic observations with combinatorial functional studies facilitated an exploration of receptor rescue and restoration of signaling. By targeting the p.C118W mutant receptor with chemical chaperones, namely thapsigargin, glycerol or sodium 4-phenylbutyrate, a demonstrable and significant increase in plasma membrane localization of receptor species was observed concomitant with enhanced phosphorylation of SMADs 1 and 5. Rescued trafficking also led to an increase in the density of wild-type BMPR-II at the cell surface [Sobolewski et al., 2008]. This study provides an arresting example of how exploitation of genetic insights may lead to the potential development of future targeted therapeutic options in PAH.

Variants of Unknown Significance

Analyses of amino acid substitutions by PolyPhen, PROVEAN and SIFT bioinformatic tools [Kumar et al., 2009; Adzhubei et al., 2013; Choi and Chan, 2015] lead to ambiguous conclusions of pathogenicity in a proportion of observed missense variants reported herein. In the present dataset, a total of 13 variants are predicted by at least two algorithms to be non-damaging and/or benign (Table 2). In order to achieve greater confidence in assigning pathogenic status, it is important to assess such *in silico* predictions in the context of genomic data derived from substantive populations, for example the ExAC database. Comparison of missense variants against this combined cohort identified five variants with a population allele frequency greater than 0.000015, based on the most conservative measure of PAH prevalence in the literature (15 cases per million) [Archer et al., 2010]. Of interest, the two most commonly observed variants (p.V348I and p.Y589C) had been previously determined as damaging by at least two prediction methods and, therefore, had not been classified as variants of unknown significance (VUS). In the remaining three cases, the population data supported the *in silico* predictions which, taken together, provide further evidence for unclear pathogenicity (Table 2). One additional variant (p.T766A), previously designated VUS status by prediction algorithms, was present in the ExAC database but with a population allele frequency lower than our assigned threshold. Through these combined analytical techniques, we have identified a total of 15 variants that might be considered of uncertain significance from a genetic perspective (Table 2). While employing such analytical tools is of emerging value in mutation data interpretation, these analyses demonstrate that they must be treated with caution as: 1) the two approaches utilized may produce conflicting outputs, leading to ambiguity in interpretation; 2) large cohort datasets may contain study participants for whom the phenotype cannot be definitively assigned. Further, variants with a low population allele frequency in an apparently normal cohort may also be explained by the reduced penetrance of this condition. Together, this highlights a continuing role for functional studies, as a gold standard, to determine the true impact of observed variation in *BMP2*, which is of value to both diagnostic and basic science understanding of the physiological role of this receptor. In addition, these observations indicate the likely existence of as yet unexplored pathways underlying pathogenesis.

Uncommon TGF- β Family Variation in PAH

Mutations of Receptor Species and Functional Outcomes

PAH infrequently clinically co-presents with the autosomal dominant vascular disorder hereditary hemorrhagic telangiectasia (HHT), characterized by the presence of mucocutaneous telangiectasia and visceral arteriovenous malformations. PAH-associated HHT is caused by molecular defects in *ACVRL1*, encoding a type I receptor of the TGF- β family, and to a lesser extent by mutations of the *ENG* gene which encodes a type III, or accessory receptor (Figure 1). In rare instances, *ACVRL1* mutations have been identified in PAH patients without HHT but typically in early-onset disease thereby not precluding the development of the latter condition in later life [Harrison et al., 2003; Fujiwara et al., 2008]. Here we have compiled complete data on 66 mutations for both genes (*ACVRL1*, n=57; *ENG*, n=9), including 61 previously reported variants and 5 novel mutations underlying the development of PAH with and without HHT (Table 3). In *ACVRL1*, the majority of this

variation occurs within the vital kinase domain of the protein (exons 6-10) resulting in pathogenic amino acid substitutions (n=42, 74%) in marked contrast to the *BMPR2* pattern of predominantly truncating mutations and, indeed, the mutation spectrum in HHT alone. A recent study conducted in a cohort of 43 IPAH patients identified two missense variants in *BMPR1B* (p.S160N and p.F392L). However, contrary to previous reports, these variants induced SMAD9 signaling with concomitant induction of transcriptional activity [Chida et al., 2012a]. These studies suggest that further functional investigations are required for clarification of the pathogenic impact of these variants.

Mutations within Intracellular Partners of the BMP Signaling Pathway

Conventional functional candidate gene strategies conducted in Asian and European patient panels have subsequently identified independent mutations in the BMP-specific SMAD pathway, namely *SMAD1* (n=1), *SMAD4* (n=2) and *SMAD9* (n=3) [Shintani et al., 2009; Drake et al., 2011; Nasim et al., 2011] (Table 3). Of these, the *SMAD1* and *-4* defects have been described as VUS due to *in vitro* luciferase SMAD responsive elements reporter assays demonstrating an unclear impact on the canonical pathways [Nasim et al., 2011]. However, these analyses did not investigate SMAD-independent pathways, implicated in disease pathogenesis, leaving open the possibility that the identified variants may deleteriously affect other BMP related systems. By contrast, *SMAD9* mutations (*PPH2*; MIM# 615342) lead to a marked reduction of SMAD transcriptional activity and a down-regulation of the BMP target gene *Id1* [Shintani et al., 2009; Nasim et al., 2011]. Of interest, heterozygous *SMAD9* mutations have been observed to perturb non-canonical downstream pathways, in particular, micro-RNA (miRNA) processing. Examination of a human patient with a *SMAD9* nonsense mutation (p.R294*) indicated a modest reduction of *Id1* expression in contrast to a complete abrogation of *miR-21*. *In vitro* restoration of *miR-21* by over-expression led to a reversal of the hyperproliferative mutation-positive phenotype. These data suggest a specific role for SMAD8 in PAH pathogenesis and SMAD4-independent signaling [Drake et al., 2011].

Genotype-Phenotype Correlation in Risk Alleles of the BMP Pathway

In 53-86% of patients with familial aggregation and 14-35% of IPAH patients mutations in the *BMPR2* gene have been identified [Sztrymf et al., 2008; Girerd et al., 2010a; Pfarr et al., 2011; Liu et al., 2012; Kabata et al., 2013]. Patients who carry *BMPR2* mutations differ in several important aspects from IPAH patients who are *BMPR2*-negative [Soubrier et al., 2013]. Investigators have reported that HPAH patients with pathogenic variants in *BMPR2* develop this disorder at a younger age (38.53 ± 12.38 vs. 45.78 ± 11.32 years, $p < 0.001$) [Girerd et al., 2010b; Pfarr et al., 2011], and have a more severe clinical and hemodynamic phenotype at diagnosis [Koehler et al., 2004; Sztrymf et al., 2008; Austin et al., 2009a; Pfarr et al., 2011]. *BMPR2* mutation carriers undergo diagnostic catheterization almost 10 years earlier than patients with no identified *BMPR2* defect. Furthermore, compared with *BMPR2*-negative IPAH patients, *BMPR2*-positive patients have a higher pulmonary vascular resistance measured at diagnostic catheterization, are less likely to demonstrate acute vasoreactivity [Elliott et al., 2006; Rosenzweig et al., 2008], and are more likely to progress to death or lung transplantation [Sztrymf et al., 2008]. These observations all suggest that

BMPR2 mutations lead to a more severe PAH phenotype. Of note, patients with missense variants present with a higher degree of morbidity and mortality than those with truncating defects, suggestive of a more severe impact on the signaling pathway [Austin et al., 2009b]. However, *BMPR2* mutations are not associated with a worse exercise capacity and prognosis. The younger age of *BMPR2* mutation carriers may explain the similar survival and exercise capacity despite worse hemodynamics as compared with *BMPR2*-negative patients. Most recently, Girerd *et al.* indicated that *BMPR2* mutation position may influence clinical phenotype. Specifically, PAH patients with a point mutation within the cytoplasmic tail of *BMPR2* displayed a later age of onset, lower pulmonary vascular resistance and, of note, a higher proportion of acute vasodilator response by contrast to patients harboring mutations outside of this domain. In addition, *in vitro* assays suggested that cytoplasmic domain mutations tolerated activation of the Smad pathway, which is indicative of a lower degree of penetrance [Girerd et al., 2015].

These findings appear not to be influenced by gender [Girerd et al., 2010b]. However, there is a trend for more severe prognosis of the disease in males, particularly in male *BMPR2* mutation carriers. This observation is consistent with the observation that PAH mortality is most closely associated with male gender [Humbert et al., 2010]. Even though no significant impact of gender was observed on age at diagnosis and outcomes, it should be emphasized that PAH mostly occurs in females, irrespective of *BMPR2* status (sex ratio females:males = 2.4:1 in both *BMPR2* mutation carriers and non-carriers). To explain over-representation of female patients it has been suggested that estrogens and estrogen metabolism might be involved in the pathogenesis of PAH [West et al., 2008a; Austin et al., 2009a; Mair et al., 2015]. These studies support the hypothesis that altered estrogen metabolism could contribute to the penetrance of PAH in women and suggest Cytochrome P450 1B1 (*CYP1B1*) as a sex-specific modifier gene.

Similar findings are observed with *ACVRL1* mutations with a significant number of pediatric cases and a dismal prognosis [Girerd et al., 2010a]. In this study, *ACVRL1* mutation carriers were shown to be characterized by a younger age at PAH diagnosis (21.8 ± 16.7 years) than *BMPR2* mutation carriers and non-carriers (35.7 ± 14.9 and 47.6 ± 16.3 years, respectively; $p < 0.0001$). However, *ACVRL1*-positive patients had better hemodynamic status at diagnosis, but none responded to acute vasodilator challenge. Thus, despite less severe initial hemodynamics and similar management, these patients had a worse prognosis than other patients with PAH, suggesting more rapid disease progression.

Most recently, a ‘two-hit’ model has been proposed, wherein digenic mutations may account for earlier occurrence, increased severity and more rapid deterioration of PAH patients [Wang et al., 2014].

Expansion of the Genetic Architecture of PAH by Next-Generation Sequence Analysis

Caveolin 1 (CAV1)

Whole-exome sequencing was used to study one large family with six PAH cases across three generations with autosomal dominant transmission and without known mutation.

Specifically, the exomes of 4 of the 6 PAH patients were evaluated and following bioinformatic analyses 11 rare candidate variants were determined to be shared by all four patients in the family. Genetic analysis of an additional patient in the family supported the conclusion that a rare mutation in the *CAVI* gene (*PPH3*; MIM# 615343) was of pertinence to disease. The observed mutation in exon 3 (c.474delA; p.L159Sfs*22), impacts a highly conserved region and predicts deleterious functional consequences.

An additional 62 independent PAH families and 198 unrelated idiopathic PAH patients, all without detectable TGF- β gene mutations, were screened for *CAVI* mutations by Sanger sequencing. Of 260 patients one early-onset idiopathic patient harbored a *de novo* *CAVI* mutation in exon 3 of the gene (c.473delC; p.P158Hfs*23) (Table 4). Of note, identified variants were not present in over 1000 ethnically-matched Caucasian controls [Austin et al., 2012].

Potassium Channel, Subfamily K, Member 3 (*KCNK3*)

Most recently, whole-exome sequencing has led to the identification of *KCNK3* as a risk factor for familial and idiopathic disease (*PPH4*; MIM# 615344). By screening three affected subjects from an autosomal dominant family negative for mutation in *BMPR2*, *ACVRL1*, *ENG*, *SMAD9* and *CAVI*, Ma *et al.* detected a novel coding variant of *KCNK3* that was shared among all three subjects and predicted to be pathogenic by *in silico* bioinformatic tools. Subsequent Sanger sequencing across the extended family confirmed co-segregation of the disease with the c.608G>A (p.G203D) variant, which was absent from 100 ethnically matched control individuals [Ma *et al.*, 2013]. Analysis of exome sequence from 10 further HPAH probands identified two additional novel heterozygous variants, which also segregated with disease, providing strong evidence for a role of *KCNK3* in PAH pathogenesis. To assess the frequency of *KCNK3* variation in familial and idiopathic disease, an extended cohort of 82 HPAH and 230 IPAH cases were screened for mutation. Three novel heterozygous missense variants were detected in the idiopathic cohort, suggesting that *KCNK3* mutation accounts for 1.3% of IPAH cases and 3.2% of HPAH families [Ma *et al.*, 2013] (Table 4).

Eukaryotic Translation Initiation Factor 2 Alpha Kinase 4 (*EIF2AK4*)

Similarly, exome sequencing has revealed several disease causing mutations in the *EIF2AK4* gene in pulmonary veno-occlusive disease (PVOD) and pulmonary capillary hemangiomatosis (PCH), together classified as group 1' of PAH in the most recent diagnostic classification [Simonneau *et al.*, 2013]. Both conditions are inherited in an autosomal recessive manner (*PVOD2*; MIM# 234810) and are mainly characterized by proliferation of capillaries in the lung leading to an occlusion of pulmonary vasculature. Eyries *et al.* assessed five families with PVOD and focused on rare variants (minor allele frequency <0.1% in control populations), which were homozygous or compound heterozygous in affected children and heterozygous in unaffected parents. Using this analysis strategy, mutations in *EIF2AK4* were identified in all 13 families studied. The examination of 20 sporadic cases revealed *EIF2AK4* mutations in 5 additional patients [Eyries *et al.*, 2014]. Mutations were distributed throughout the gene and belonged to the

major mutation categories (Table 5). The range of mutations situated upon this locus underlines its functional importance in the development of PVOD.

In parallel, mutations in *EIF2AK4* were independently identified by Best *et al.* in patients with heritable PCH. The exomes of two affected brothers were sequenced and filtered for variants with a minor allele frequency of less than 1% in public repositories of polymorphic data resulting in the identification of two pathogenic variants in the gene. The unaffected parents and sister were confirmed to be heterozygous carriers of one of the two mutations [Best *et al.*, 2014]. Further sequencing of *EIF2AK4* in 10 patients with pathologically verified sporadic PCH and one familial case detected three additional mutations in two sporadic patients (Table 5). The genetic correlation between PVOD and PCH further supports the likelihood that these diseases define different clinical spectra of the same underlying disorder.

Most recently, *EIF2AK4* has also been investigated in an itinerant Iberian population with PAH [Tenorio *et al.*, 2015]. The authors identified a homozygous c.3344C>T (p.P1115L) missense mutation in five patients from five independent families with HPAH. Likely ancestral, this mutation co-segregated with a more severe phenotype than previously reported for other *EIF2AK4* mutations. The majority of affected subjects presented with an early onset, aggressive form of the disease resulting in an abnormally low survival rate post-lung transplantation (1.1 years).

Biological Significance of Mutations in Non-Canonical BMP Pathways

While rare, the biologic plausibility for *CAVI* mutations in PAH is strong. *CAVI* encodes Caveolin-1, a membrane protein required to form the flask-shaped invaginations of the cell membrane known as caveolae, abundant in lung endothelial and mesenchymal cells [Minshall *et al.*, 2003; Xu *et al.*, 2008]. Caveolae are critical to a number of cellular processes and receptor rich regions of the cell membrane [Nohe *et al.*, 2005; Mercier *et al.*, 2009; Chidlow and Sessa, 2010]. Intriguingly, mice haploinsufficient for *Cav1* display pulmonary vascular disease analogous to PAH [Drab *et al.*, 2001; Zhao *et al.*, 2002; Murata *et al.*, 2007; Maniatis *et al.*, 2008]. Moreover, Caveolin-1 protein staining is reduced in lung endothelial cells from human PAH patients [Zhao and Malik, 2009]. Nevertheless, although under careful scrutiny, the precise mechanism(s) by which *CAVI* mutations promote PAH remain unclear.

The *KCNK3* gene is located on chromosome 2p24 and encodes a pH-sensitive potassium channel with a role in regulation of resting membrane potential in a variety of cell types. Electrophysiological analyses demonstrated that all identified mutations lead to a loss of function as measured by current density. A subset of identified mutations (p.T8K, p.E182K and p.G203D) exhibited a significant increase in potassium-channel current when treated with the phospholipase inhibitor ONO-RS-082. The identification of this gene provides an additional avenue of treatment strategies for PAH.

The *EIF2AK4* gene encodes a kinase, which phosphorylates an initiation factor in protein synthesis that is primarily responsible for the translation of stress response proteins [Donnelly *et al.*, 2013]. Interactions between the kinase and various members of the BMPR-

II pathway have also been detected, but the exact link to the clinical manifestations of pulmonary veno-occlusive PH remains to be clarified [Eyries et al., 2014]. The EIF2AK4 protein belongs to a family of kinases that regulate angiogenesis in response to cellular stress. Of interest, these properties are compatible with the angiogenic pathology of PCH, a disorder characterized by uncontrolled proliferation of pulmonary microvessels.

Modifying Predisposition to PAH

Cerebellin 2 Precursor (CBLN2)

PAH demonstrates several complex traits including incomplete penetrance, sex bias and variable age of disease onset both within and across families. This has led to the hypothesis that modifier genes contribute to disease manifestation and/or progression. To examine the role of common variation in PAH predisposition, Germain *et al.* performed a genome-wide association study (GWAS) to identify susceptibility loci in IPAH and HPAH cases without *BMPR2* mutation. The discovery dataset comprised 340 PAH patients and 1,068 healthy controls, genotyped for ~470,000 variants, from which the 384 most significant variants were assessed in an independent replication cohort of 285 cases and 457 controls. This approach, which represents the best powered study to date, identified two variants 52 kb downstream of the *CBLN2* gene that were associated with a two-fold increased risk of disease [Germain et al., 2013].

CBLN2 encodes a secreted neuronal glycoprotein primarily expressed in the brain. However, real-time PCR studies demonstrated *CBLN2* mRNA expression in the whole lung, significantly higher in explants from PAH patients comparative to histologically normal lung tissue. Similar results were obtained for pulmonary arterial endothelial cells. Furthermore, an inhibition of pulmonary artery smooth muscle cell (PASMC) proliferation was observed when treated with increasing concentrations of CBLN2 peptide [Germain et al., 2013].

Potassium Channel, Voltage Gated Shaker Related Subfamily A, Member 5 (KCNA5)

Membrane potential is essential for contraction and vasodilation of PASMCs. Expression of potassium channels is regulated by BMP signaling *in vitro* and *in vivo* and knockouts in *Drosophila melanogaster* result in defects that are strikingly similar to phenotypes that result from disrupted TGF- β /BMP signaling [Young et al., 2006; Dahal et al., 2012]. Variation of the potassium channel *KCNA5* has been identified in IPAH patients [Remillard et al., 2007; Wang et al., 2014] suggesting a potential role in PAH development and penetrance. The potassium channel response in PASMCs of IPAH patients has been proven to be down regulated [Yuan et al., 1998] likely increasing pulmonary vasoconstriction and PASMC proliferation. Moreover, the channel, which is responsive to nitric oxide, is reduced in patients carrying a specific coding missense mutation [Remillard et al., 2007]. Mutations within *KCNA5* have been identified as a so-called ‘second hit’ in an index patient additional to a *BMPR2* missense mutation leading to an early onset and severe phenotype [Wang et al., 2014]. While these findings may represent a rare case of genetic modification in PAH, replication of this digenic genotype in an independent cohort has not been observed. Further, in the absence of comprehensive functional analysis, interpretation of the significance of this study requires caution.

Animal Models of PAH

Model systems of PAH serve a two-fold purpose in providing 1) a source of relevant, genetically modified cell types for *in vitro* studies, and 2) an *in vivo* platform for the analysis and refinement of direct therapeutic intervention. Several natural and engineered models of disease exist, for example, the fawn-hooded rat and the *Bmpr2* transgenic mouse over-expressing the p.R899* mutant allele, the latter being generated by introduction of a smooth muscle-specific doxycycline-inducible mutant transgene [West et al., 2008b; Ryan et al., 2011]. Further, knock-out models lacking exons 4 and 5 of *Bmpr2* generated by homologous recombination have been developed. These models develop mild to moderate disease phenotypes, often upon the application of environmental insults including exposure to hypoxia and 5-lipoxygenase as previously described [Machado et al., 2006]. Conditional targeting of mutant alleles to hallmark sites of damage in PAH, in particular the pulmonary artery endothelial or smooth muscle cell layers, has produced a more convincing *in vivo* reflection of the human disease state [West et al., 2004; Hong et al., 2008]. Most recently a heterozygous knock-in mouse model of the p.R899* mutation has been shown to develop age-related disease with close phenotypic relatedness to the human condition [Long et al., 2015]. Additionally a rat model of PAH (*BMPR2*^{140Ex1/+}), the first of its kind, has provided support to the hypothesis that endothelial-to-mesenchymal transition represents a pathophysiological process in PAH [Ranchoux et al., 2015]. Of note, mice representative of recently observed defects in previously uncharacterized genes, namely *Cav1* and *Smad9*, support the emerging concept of greater than anticipated genetic heterogeneity in PAH. Homozygous knock-out models of both genes develop spontaneous indications of PAH providing a powerful correlation to the human studies described herein [Zhao et al., 2002; Huang et al., 2009].

Genetic Counseling

Current guidelines recommend offering molecular genetic analysis and genetic counseling for HPAH patients [Badesch et al., 2007; McLaughlin et al., 2009]. Genetic counseling typically offers a combinatorial approach of dealing with putative outcomes and consequences of the analysis, based upon structured protocols determined by specialist centers evaluates the family history, educates patients about the causes of PAH, discusses the risks and benefits of genetic testing and supports patients and families through the process of genetic testing and disclosure of results. In addition to HPAH patients, IPAH patients and their relatives may also benefit from these analyses since up to 25% will have a *BMPR2* mutation; therefore, genetic testing in these populations should be considered [Badesch et al., 2007]. Current findings indicate that in patient populations with PVOD and PCH, autosomal recessive inheritance of the *EIF2AK4* gene is the most likely mode of transmission. Hence, within the PAH spectrum of disease, based on current classification, genetic counseling should take account of both autosomal dominant and recessive inheritance models, as well as the higher penetrance of bi-allelic mutations in patients carrying this genetic defect.

Following identification of an established molecular defect in the proband, at-risk asymptomatic family members should be offered the option of genetic counseling and

targeted mutation analysis within the parameters of full informed consent. Importantly, the data provided here offer an indication of the likely pathogenicity of identified variation. A significant factor to consider is the reduced penetrance of mutant alleles which must be addressed by informed pre-test counseling, comprising robust estimations of risk based on available population-specific epidemiological data and gender [Cogan et al., 2012; Larkin et al., 2012]. Of note, where a mutation is identified, implications for reproductive planning must be considered. In families harboring mutation, early mutational analysis of offspring is essential and should be combined with clinical assessment and initiation of treatment as deemed necessary by specialist centers. Additionally, a possible future avenue of medical care has been explored. Within a *BMP2*-positive family, pre-implantation genetic analysis of blastomeres following *in vitro* fertilization led to the successful implantation and delivery of a mutation-negative offspring [Frydman et al., 2012]. Although in its earliest stages this strategy is of significant clinical potential in the context of considered, long-term genetic evaluation.

Diagnostic Strategies

Molecular diagnostics for PAH have traditionally focused around dideoxy Sanger sequencing methods to screen the *BMP2*, *ACVRL1* and *ENG* protein-coding regions for heterozygous mutation. However, based on these and previous studies, for a complete exploration of deletion and duplication across these three genes, the application of MLPA or targeted comparative genomic hybridization (CGH) array technology is required. The HHT/PPH1 MLPA panel was introduced by MRC-Holland in the mid-2000s, with the current version containing 51 probes across *BMP2*, *ACVRL1* and *ENG*, and has led to the successful detection of numerous gene rearrangements that would otherwise not be identified by sequence analysis [Aldred et al., 2006; Cogan et al., 2006].

Despite the success of this combined approach to mutation detection in PAH, the recent expansion of candidate disease genes has resulted in traditional sequencing methods becoming more labor-intensive and less cost-effective. Indeed, to comprehensively screen the 10 genes detailed here would involve sequencing approximately 105 coding exons, totaling over 19 kb of DNA sequence. Custom capture and NGS are now becoming chosen methodologies across screening centers globally for the analysis of established candidate genes.

Future Prospects

It is clear that the most significant advance in the identification of risk factors for PAH over recent years has been the advent of exome sequencing, which has led to the rapid identification of multiple novel genes using relatively small sample sets. Whilst exome sequencing is undoubtedly a powerful method for detecting rare, highly penetrant genes in families with multiple affected individuals, there remain important caveats in applying these technologies to the identification of novel genes across cohorts of unrelated subjects and genetically divergent patient groups. In PAH, complexities such as locus heterogeneity, incomplete penetrance, *de novo* mutation and late onset of disease introduce significant challenges to the interpretation of exome sequence data, highlighting the need for large

homogeneous patient cohorts to detect pathogenic variation. In terms of technical limitations, exome sequencing also only focuses on the exonic regions. While these regions represent the most likely sites of functional mutations in PAH, there is increasing evidence supporting pathogenic variants in the promoter and 5' UTR of *BMP2*, suggesting this may also be true of other PAH genes. A recent report by Hinderhofer *et al.* has also identified an intronic mutation of *BMP2* that leads to aberrant splicing due to an insertion of an intronic Alu element 26 bp upstream from exon 6 [Hinderhofer *et al.*, 2014]. This points to a potential role for other intronic variants or regulatory elements such as intra- or intergenic enhancer and repressor motifs in the pathogenesis of PAH.

To address some of these issues, it is likely that future avenues will include the use of more NGS technologies, the pinnacle of which is whole-genome sequencing. Harnessing the complete genetic information of individuals affected with PAH will not only offer opportunities to identify the causative mutation but will also provide an important tool to correlate phenotypic information to individual genotype data, allowing for tailored approaches to the clinical management of disease, or so-called personalized or precision medicine. While this technology remains financially prohibitive to some centers, custom capture of genes with defined causal links to disease offers an alternative, yet powerful, means of analyses. These studies would, ideally, include non-coding sections of the genes to determine the presence of regulatory mutations increasingly implicated in PAH etiology as described herein. The catalogue of mutations described in this report provides an important tool for the determination of deleterious mutation, both within the context of conventional and indeed NGS analyses.

For future gene identification and follow-up GWAS studies, it is of particular importance in PAH, which exhibits traits of complex disease, that phenotype is precisely assigned for this disorder. This degree of rigor is most likely to lead to further advances in both the understanding and treatment of PAH at a significantly accelerated pace.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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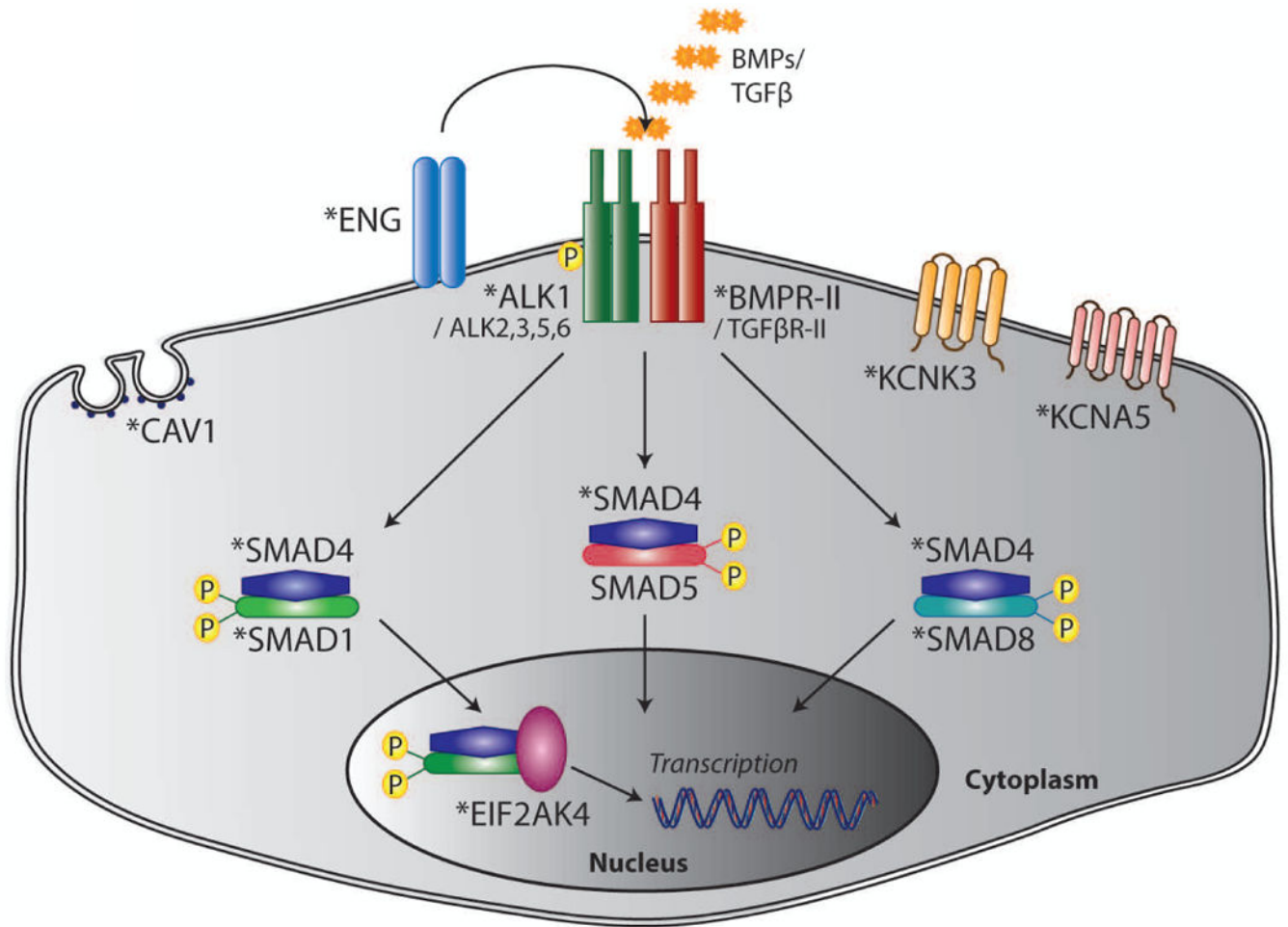


Figure 1. Schematic of canonical BMP signaling and additional pathways implicated in PAH pathogenesis by conventional and next-generation sequence analysis. Causal genes are indicated by the asterisks.

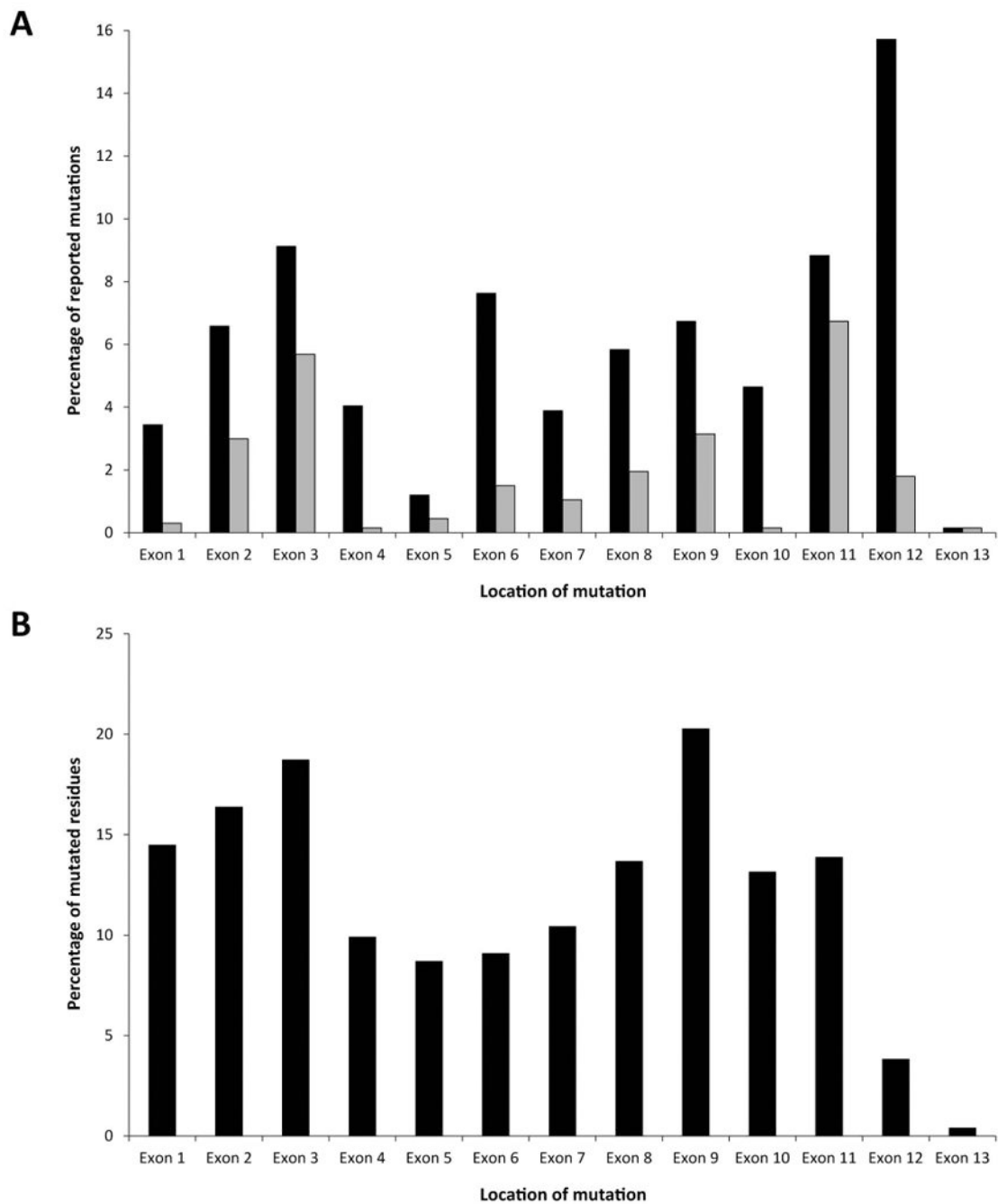


Figure 2.

A) Distribution of reported exonic mutations across the *BMPR2* gene. Black bars represent all mutation categories; grey bars indicate missense mutations only. This graph excludes data from non-coding regions and gene rearrangements for which start and/or end points have not been conclusively determined. B) Proportion of distinct point mutations relative to exon size. Multiple and/or recurrent variants at the same nucleotide were counted as a single event. The total number of mutated residues confined to the open-reading frame was calculated as a percentage of exon length in nucleotides.

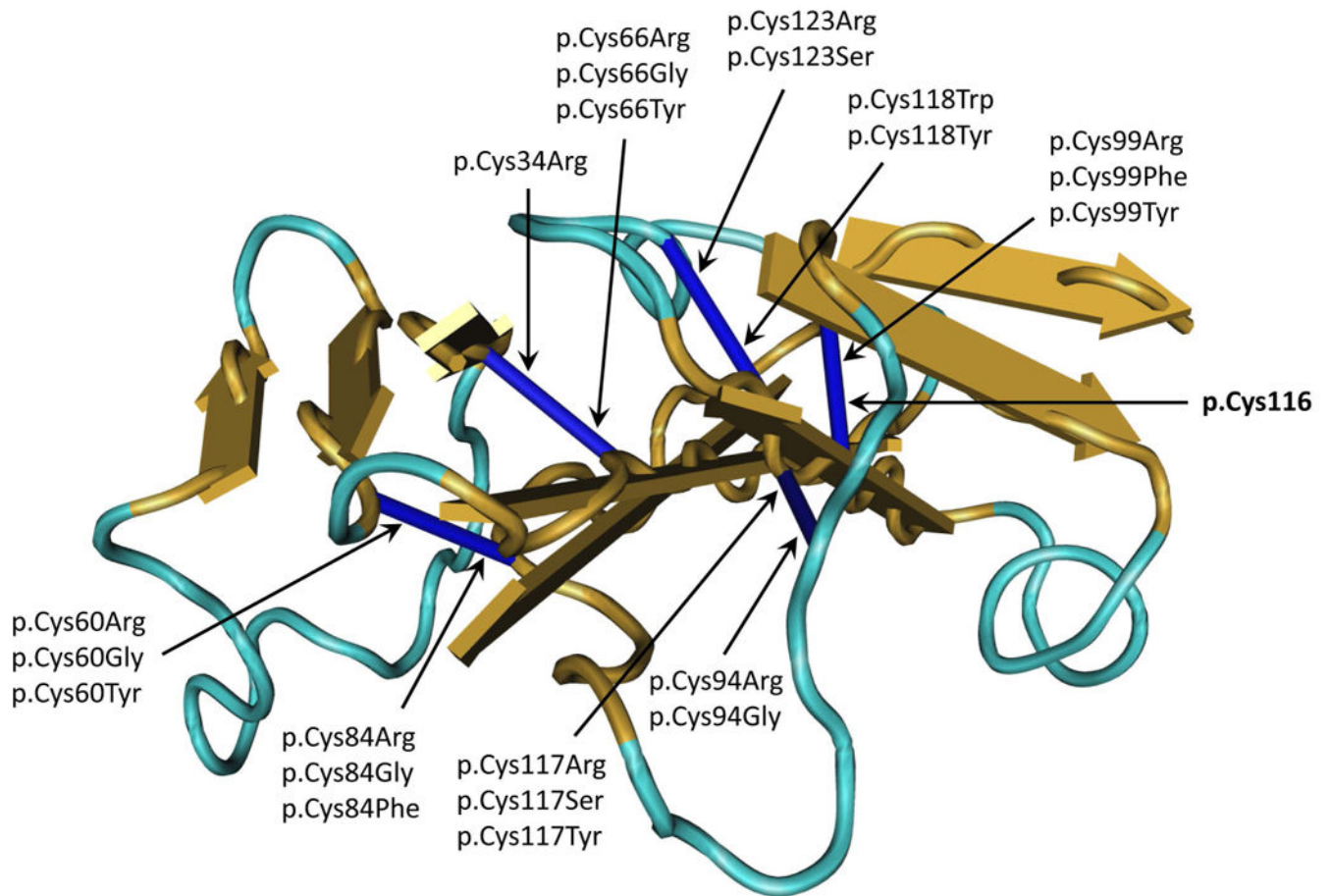


Figure 3.

Three-dimensional structure of the BMPR-II extracellular domain highlighting the location of substitutions impacting upon 9 of the 10 key cysteine residues responsible for disulfide bridge formation, indicated in dark blue. Defects in the Cys116 residue have not been identified in PAH thus far. Figure was reproduced from the crystal structure (PDB ID: 2HLQ) and processed using Cn3D v4.3 software.

Table 1

Novel pathogenic *BMPR2* mutations identified in this analysis

Location	Mutation category	Nucleotide change	Amino acid change	Frequency in this study	Clinical classification
5'UTR	Transition	c.-669G>A	p.?	3	I, NK, P
5'UTR to exon 1	Deletion	c.?-540_76+?del	p.?	1	NK
5'UTR to exon 13	Deletion	c? -540 3117+?del	p.?	1	NK
Exon 1	Deletion	c.1-?76+?del	p.?	[3]	I, NK (n=2)
Exon 1	Frameshift	c.9dupC	p.S4Lfs*34	1	NK
Exon 1	Nonsense	c.16C>T	p.Q6*	2	I, NK
Exon 1	Nonsense	c.38G>A	p.W13*	1	H
Exon 1	Nonsense	c.48G>A	p.W16*	1	I
Intron 1	Splice-site	c.76+1G>T	p.?	1	H
Intron 1	Splice-site	c.76+2T>C	p.?	1	NK
Intron 1	Splice-site	c.77-1G>A	p.A26Efs*9	1	H
Exons 2-5	Deletion	c.77-?_621+?del	p.?	1	H
Exons 2-9	Deletion	c.77-?_1276+?del	p.?	1	NK
Exon 2	Nonsense	c.82C>T	p.Q28*	1	NK
Exon 2	Missense	c.178D>C	p.C60R	1	NK
Exon 2	Missense	c.196D>G	p.C66G	1	NK
Exon 2	Frameshift	c.236_238delinsAAAAGGGGACA	p.L79Qfs*5	1	NK
Exon 2	Frameshift	c.246dupA	p.G83Rfs*15	1	H
Intron 2	Splice-site	c.247+1G>A	p.?	1	NK
Exon 3	Deletion	c.248-?_418+?del	p.?	[4]	I (n=3), NK
Exon 3	Duplication	c.248-?_418+?dup	p.?	1	H
Exon 3	Missense	c.280T>G	p.C94G	1	NK
Exon 3	Frameshift	c.339_340insAA	p.R114Nfs*39	1	H
Exon 3	Frameshift	c.345_346delCT	p.F115Lfs*4	1	NK
Exon 3	Missense	c.350G>A	p.C117Y	1	NK
Exon 3	Frameshift	c.353delG	p.C118Lfs*34	1	NK
Exon 3	Missense	c.354T>G	p.C118W	1	I
Exon 3	Missense	c.377A>G	p.N126S	1	NK

Location	Mutation category	Nucleotide change	Amino acid change	Frequency in this study	Clinical classification
Exon 4	Deletion	c.419-?_529+?del	p.?	1	NK
Exons 4-7	Deletion	c.419-?_967+?del	p.?	[2]	I, NK
Exons 4-10	Deletion	c.419-?_1413+?del	p.?	1	NK
Exon 4	Frameshift	c.435delT	p.F145Lfs*7	1	NK
Exon 4	Nonsense	c.439C>T	p.R147*	1	H
Exon 4	Nonsense	c.482T>A	p.L161*	1	I
Exon 5	Deletion	c.530-?_621+?del	p.?	1	NK
Exon 5	Nonsense	c.541C>T	p.Q181*	1	H
Exon 6	Nonsense	c.637C>T	p.R213*	1	H
Exon 6	Nonsense	c.642T>G	p.Y214*	1	NK
Exon 6	Frameshift	c.673_679delCGTCCAG	p.R225Lfs*3	1	H
Exon 6	Nonsense	c.727G>T	p.E243*	1	I
Exon 6	Frameshift	c.795_796delinsTT	p.E265 L1038delinsD	1	NK
Intron 6	Splice-site	c.853-2A>G	p.?	1	NK
Intron 6	Splice-site	c.853-1G>A	p.?	1	NK
Exon 7	Nonsense	c.860T>A	p.L287*	1	H
Exon 7	Nonsense	c.872T>G	p.L291*	1	NK
Exon 7	Nonsense	c.893G>A	p.W298*	1	NK
Exon 7	Frameshift	c.894_895dupGG	p.V299Gfs*2	1	H
Exon 7	Frameshift	c.961delC	p.R321Efs*14	1	NK
Intron 7	Splice-site	c.967+2T>C	p.?	1	H
Intron 7	Splice-site	c.968-3 C>G	p.?	1	NK
Intron 7	Splice-site	c.968-1G>T	p.?	1	NK
Exon 8	Nonsense	c.994C>T	p.R332*	1	NK
Exon 8	Frameshift	c.1011_1015delAAATG	p.R337Sfs*6	1	I
Exon 8	Frameshift	c.1060delC	p.L354Cfs*3	1	NK
Exon 8	Nonsense	c.1126G>T	p.E376*	2	I, NK
Exon 9	Frameshift	c.1129-1_1129dupGG	p.V377Gfs*13	1	I
Exon 9	Frameshift	c.1141dupA	p.R381Kfs*18	1	H
Exon 9	Missense	c.1156G>C	p.E386Q	1	NK
Exon 9	Missense	c.1220a>c	p.Y407S	1	NK

Location	Mutation category	Nucleotide change	Amino acid change	Frequency in this study	Clinical classification
Exon 9	Nonsense	c.1221Δg	p.Y407*	1	H
Exon 9	Frameshift	c.1268dupT	p.F424Lfs*24	1	NK
Exon 9	Missense	c.1276g>c	p.G426R	1	H
Intron 9	Splice-site	c.1277-9 A>C	p.?	1	H
Intron 9	Splice-site	c.1277-8a>g	p.?	1	NK
Exon 10	Frameshift	c.1279delG	p.E427Nfs*47	1	NK
Exon 10	Frameshift	c.1285_1286insGGATT	p.V429Gfs*47	2	I, NK
Exon 10	Frameshift	c.1293_1300delGTACCAGA	p.E431Dfs*14	1	NK
Exon 10	Frameshift	c.1371delA	p.K457Nfs*17	1	H
Exon 10	Nonsense	c.1398g>a	p.W466*	1	NK
Exon 10	Nonsense	c.1402g>t	p.E468*	1	H
Exon 11	Frameshift	c.1426_1450del	p.L476Gfs*22	1	NK
Exon 11	Nonsense	c.1441g>t	p.E481*	1	NK
Exon 11	Nonsense	c.1451g>a	p.W484*	1	H
Exon 11	Missense	c.1453g>a	p.D485N	1	P
Exon 11	Nonsense	c.1456c>t	p.Q486*	1	H
Exon 11	Frameshift	c.1477dupA	p.T493Nfs*6	1	NK
Exon 11	Missense	c.1486Δc	p.C496R	1	NK
Intron 11	Splice-site	c.1587-7_1587-4delCTTT	p.?	1	I
Exon 12	Nonsense	c.1629Δg	p.Y543*	1	I
Exon 12	Nonsense	c.1750c>t	p.R584*	1	NK
Exon 12	Nonsense	c.1789C>T	p.R597*	1	NK
Exon 12	Nonsense	c.1969C>T	p.Q657*	2	I, NK
Exon 12	Nonsense	c.1981g>t	p.E661*	1	NK
Exon 12	Frameshift	c.2291dupA	p.N764Kfs*49	1	H
Exon 12	Frameshift	c.2303_2309delAGCCCCG	p.E768Gfs*2	1	NK
Exon 12	Frameshift	c.2308delC	p.R770Gfs*2	2	NK (n=2)
Exon 12	Frameshift	c.2484delG	p.T829Qfs*10	1	H
Exon 12	Nonsense	c.2533G>T	p.E845*	1	I
Exon 12	Nonsense	c.2695C>T	p.R899*	3	H (n=2), NK
Exon 12	Nonsense	c.2730T>A	p.C910*	1	H

Location	Mutation category	Nucleotide change	Amino acid change	Frequency in this study	Clinical classification
Exon 12	Nonsense	c.2737C>T	p.Q913*	1	I

[†]GenBank reference sequence and version number for *BMP2*: NM_001204.6; numbering is from +1 as A of the ATG initiation codon

[‡]Total number of independent cases. Frequencies in square brackets denote chromosomal rearrangements for which the breakpoints are unknown and may therefore represent distinct mutations

Key to abbreviations: H: heritable pulmonary arterial hypertension; I: idiopathic pulmonary arterial hypertension; NK: not known; P: pediatric pulmonary arterial hypertension

Table 2

BMP2 variants of uncertain pathological significance

Location	Mutation category	Nucleotide change	Amino acid change	Clinical classification	Domain	Reference	Population frequency	a) PolyPhen-2 Prediction	Score	b) PROVEAN Prediction	Score	c) SIFT Prediction	Score
Exon 3	Missense	c.266G>C	p.G89A	HPAH	ECD	Liu et al., 2012	-	Possibly	0.638	Neutral	-1.785	Tolerated	0.137
Exon 3	Missense	c.276A>C	p.Q92H	HPAH	ECD	Kabata et al., 2013	0.0001071	Benign	0.001	Neutral	-1.388	Tolerated	0.311
Exon 3	Missense	c.292G>A	p.E98K	IPAH	ECD	Wang et al., 2010	-	Possibly damaging	0.515	Neutral	-1.792	Tolerated	0.092
Exon 4	Missense	c.461C>G	p.A154G	IPAH	TM	Pfarr et al., 2011	-	Benign	0.025	Neutral	-1.994	Damaging	0.032
Exon 6	Missense	c.818T>G	p.M273R	IPAH	KD	Pfarr et al., 2011	-	Benign	0.004	Deleterious	-2.716	Tolerated	0.213
Exon 7	Missense	c.901T>C	p.S301P	NK (n=2)	KD	Sztrymf et al., 2008; Girerd et al., 2010b	-	Benign	0.046	Neutral	-2.19	Tolerated	0.086
Exon 7	Missense	c.954A>C	p.E318D	HPAH	KD	This analysis	-	Benign	0.089	Neutral	-0.347	Tolerated	0.273
Exon 8	Missense	c.1042g>a	p.V348I	HPAH; IPAH (n=2)	KD	Wang et al., 2010; Liu et al., 2012	0.0004550	Possibly damaging	0.715	Neutral	-0.45	Damaging	0.023
Exon 8	Missense	c.1066a>t	p.M356L	IPAH	KD	Wang et al., 2010	-	Benign	0.023	Neutral	-0.497	Tolerated	0.417
Exon 8	Missense	c.1117g>c	p.A373P	IPAH	KD	Liu et al., 2012	-	Probably damaging	0.99	Neutral	-2.493	Tolerated	0.087
Exon 11	Missense	c.1516a>g	p.M506V	IPAH	CD	This analysis	0.0000412	Benign	0.008	Neutral	-0.896	Tolerated	0.244
Exon 12	Missense	c.1598a>g	p.H533R	IPAH	CD	Pfarr et al., 2011	-	Benign	0.267	Neutral	-2.049	Damaging	0.021
Exon 12	Missense	c.1766a>g	p.Y589C	CHD-APAH (exercise-induced) (n=2)	CD	Möller et al., 2010	0.0001320	Probably damaging	0.999	Deleterious	-3.436	Damaging	0.001
Exon 12	Missense	c.2296A>G	p.T766A	IPAH; CTEPH	CD	Liu et al., 2012; Feng et al., 2014	0.0000083	Benign	0	Neutral	-0.183	Tolerated	0.424
Exon 12	Missense	c.2618G>A	p.R873Q	NK (n=2)	CD	Sztrymf et al., 2008; Girerd et al., 2010b	0.0001153	Probably damaging	0.966	Neutral	-0.861	Tolerated	0.128

Population frequency data were obtained from the ExAC database (<http://exac.broadinstitute.org/gene/ENSG00000204217>). The likely pathogenicity of each missense variant was calculated by three *in silico* prediction methods, using the default parameters in each case. Ranges and cut-offs for output scores were as follows: a) PolyPhen-2 v2.2.2 (<http://genetics.bwh.harvard.edu/pph2>). Range: 0 – 1 [benign: 0 – 0.452; possibly damaging: 0.453 – 0.956; probably damaging: 0.957 – 1]; b) PROVEAN Human Protein v1.1 (http://provean.jcvi.org/protein_batch_submit.php?species=human). Cut-off: -2.5 [d

Table 3

PAH mutations identified in BMP pathway members

Gene name	Location	Mutation category	Nucleotide change	Amino acid change	Frequency in this study	Clinical classification	Reference(s)
ACVRL1	Exon 2	Frameshift	c.37delC	p.L13Cfs*2	1	PAH-HHT	Trembath et al., 2001; Girerd et al., 2010a
ACVRL1	Exon 3	Missense	c.199C>T	p.R67W	1	PAH-HHT	Chen et al., 2013
ACVRL1	Exon 3	Missense	c.293A>G	p.N98S	1	NK	This analysis
ACVRL1	Exon 4	Nonsense	c.430C>T	p.R144*	1	I	Machado et al., 2009
ACVRL1	Exon 5	Missense	c.536A>C	p.D179A	1	I	Harrison et al., 2003 [^]
ACVRL1	Exon 5	Missense	c.593T>A	p.V198E	1	PAH-HHT	Chen et al., 2013
ACVRL1	Exon 5	Missense	c.602A>G	p.Q201R	1	PAH-HHT	Girerd et al., 2010a
ACVRL1	Exon 6	Missense	c.632G>A	p.G211D	1	PAH-HHT	Harrison et al., 2003
ACVRL1	Exon 6	Missense	c.653_654inv	p.R218P	1	PAH-HHT	Chen et al., 2013
ACVRL1	Exon 6	Deletion	c.760_762delGAC	p.D254del	1	PAH-HHT	Trembath et al., 2001
ACVRL1	Exon 7	Missense	c.788A>G	p.D263G	1	PAH-HHT	This analysis
ACVRL1	Exon 7	Missense	c.818T>C	p.L273P	1	PAH-HHT	Smoot et al., 2009
ACVRL1	Exon 7	Missense	c.853C>T	p.L285F	1	NK	This analysis
ACVRL1	Exon 7	Missense	c.854T>C	p.L285P	1	P	Chida et al., 2012b
ACVRL1	Exon 7	Missense	c.936C>G	p.H312Q	1	P	Fujiwara et al., 2008 [^]
ACVRL1	Exon 7	Missense	c.950T>C	p.I317T	1	P	Pfarr et al., 2013
ACVRL1	Exon 7	Missense	c.955G>C	p.G319R	1	NK	This analysis
ACVRL1	Exon 7	Missense	c.1031g>a	p.C344Y	2	PAH-HHT (n=2)	Harrison et al., 2003
ACVRL1	Exon 8	Missense	c.1055c>a	p.A352D	2	PAH-HHT (n=2)	Smoot et al., 2009
ACVRL1	Exon 8	Missense	c.1120C>T	p.R374W	3	PAH-HHT (n=2), NK	Harrison et al., 2003; Abdalla et al., 2004; This analysis
ACVRL1	Exon 8	Missense	c.1121G>A	p.R374Q	2	PAH-HHT (n=2)	Harrison et al., 2003; Chen et al., 2013
ACVRL1	Exon 8	Missense	c.1124A>G	p.Y375C	1	PAH-HHT	Chen et al., 2013
ACVRL1	Exon 8	Missense	c.1142T>C	p.L381P	1	P	Fujiwara et al., 2008 [^]
ACVRL1	Exon 8	Missense	c.1195T>C	p.W399R	1	PAH-HHT	Chen et al., 2013
ACVRL1	Exon 8	Missense	c.1196G>C	p.W399S	1	PAH-HHT	Harrison et al., 2003

Gene name	Location	Mutation category	Nucleotide change	Amino acid change	Frequency in this study	Clinical classification	Reference(s)
ACVRL1	Exon 8	Missense	c.1196G>T	p.W399L	1	PAH-HHT	Ishiwata et al., 2014
ACVRL1	Exon 8	Missense	c.1231c>t	p.R411W	1	PAH-HHT	Trembath et al., 2001
ACVRL1	Exon 8	Missense	c.1232g>a	p.R411Q	1	PAH-HHT	Harrison et al., 2003
ACVRL1	Exon 9	Missense	c.1270c>a	p.P424T	1	P	Fujiwara et al., 2008 [^]
ACVRL1	Exon 9	Missense	c.1280a>t	p.D427V	1	PAH-HHT	Girerd et al., 2010a
ACVRL1	Exon 9	Missense	c.1324g>a	p.V442M	1	PAH-HHT	Girerd et al., 2010a
ACVRL1	Exon 10	Nonsense	c.1385C>G	p.S462*	1	PAH-HHT	Abdalla et al., 2004
ACVRL1	Exon 10	Frameshift	c.1388delG	p.G463Afs*2	1	PAH-HHT	Girerd et al., 2010a
ACVRL1	Exon 10	Frameshift; Nonsense	c.1388delG; c.1390delC	p.G463Afs*2; p.L464*	1	PAH-HHT (mosaic)	Eyries et al., 2012
ACVRL1	Exon 10	Missense	c.1433C>A	p.A478D	1	P	Chida et al., 2012b
ACVRL1	Exon 10	Nonsense	c.1435c>t	p.R479*	2	PAH-HHT (n=2)	Abdalla et al., 2004; Chen et al., 2013
ACVRL1	Exon 10	Missense	c.1436g>a	p.R479Q	1	P	Fujiwara et al., 2008 [^]
ACVRL1	Exon 10	Missense	c.1436g>c	p.R479P	1	I	Machado et al., 2009
ACVRL1	Exon 10	Missense	c.1450C>G	p.R484G	1	H	Jones et al., 2014
ACVRL1	Exon 10	Missense	c.1450c>t	p.R484W	2	PAH-HHT (n=2)	Trembath et al., 2001; Girerd et al., 2010a
ACVRL1	Exon 10	Frameshift	c.1450delinsTG	p.R484Wfs*10	1	PAH-HHT	Abdalla et al., 2004
ACVRL1	Exon 10	Missense	c.1451g>a	p.R484Q	7	P (n=2); PAH-HHT (n=3; 1 mosaic); NK (n=2)	Harrison et al., 2005 [^] ; Fujiwara et al., 2008 [^] ; Best et al., 2011; Chen et al., 2013; Pfarr et al., 2013; This analysis
ACVRL1	Exon 10	Missense	c.1460a>c	p.K487T	1	PAH-HHT	Harrison et al., 2003
ACVRL1	Exon 10	Nonsense	c.1468c>t	p.Q490*	1	PAH-HHT	Trembath et al., 2001; Girerd et al., 2010a
ENG	Exon 5	Missense	c.640G>A	p.G214S	1	P	Pfarr et al., 2013
ENG	Exon 5	Frameshift	c.682_686delTCGGC	p.S228Rfs*104	1	PAH-HHT	Harrison et al., 2003 [^]
ENG	Exon 6	Missense	c.788T>A	p.L263N	1	PAH-HHT	Chen et al., 2013
ENG	Exon 11	Frameshift	c.1334delIT	p.M445Rfs*46	1	PAH-HHT	Harrison et al., 2003 [^]
ENG	Exon 11	Frameshift	c.1410delIG	p.Q471Sfs*20	1	PAH-HHT + dexfenfluramine	Chaouat et al., 2004 [^]
ENG	Exon 12	Missense	c.1633g>a	p.G545S	1	CHD-PAH	Pfarr et al., 2013
ENG	Intron 13	Branch-site	c.1742-22T>C	p.C582 R618del	1	PAH-HHT	Harrison et al., 2005 [^] ; Mache et al., 2008 [^]

Gene name	Location	Mutation category	Nucleotide change	Amino acid change	Frequency in this study	Clinical classification	Reference(s)
ENG	Exon 14	Frameshift	c.1804delA	p.I602Sfs*38	1	PAH-HHT	Chen et al., 2013
ENG	Exon 15	Missense	c.1853G>T	p.R618L	1	I	This analysis
SMAD1	Exon 2	Missense	c.8T>C	p.V3A	1	I	Nasim et al., 2011
SMAD4	Exon 2	Missense	c.38A>G	p.N13S	1	I	Nasim et al., 2011
SMAD4	Intron 11	Splice-site	c.1448-6t>c	p.?	1	I	Nasim et al., 2011
SMAD9	Exon 2	Missense	c.127a>g	p.K43E	1	I	Nasim et al., 2011
SMAD9	Exon 3	Nonsense	c.606C>A	p.C202*	1	I	Shintani et al., 2009
SMAD9	Exon 5	Nonsense	c.880C>T	p.R294*	1	H	Drake et al., 2011

[‡] GenBank reference sequence and version number for *ACVRL1*: NM_000020.2; *ENG*: NM_001114753.2; *SMAD1*: NM_005900.2; *SMAD4*: NM_005359.5; *SMAD9*: NM_001127217.2

[‡]Total number of independent cases

[^] Previously reported in Machado et al., 2009

Key to abbreviations: H: heritable pulmonary arterial hypertension; I: idiopathic pulmonary arterial hypertension; NK: not known; P: pediatric pulmonary arterial hypertension; PAH-HHT: pulmonary arterial hypertension with hereditary hemorrhagic telangiectasia

Table 4

PAH mutations identified in non-canonical BMP pathways

Gene name	Location	Mutation category	Nucleotide change	Amino acid change	Frequency in this study	Clinical classification	Reference(s)
CAV1	Exon 3	Frameshift	c.473delC	p.P158Hfs*23	1	I	Austin et al., 2012
CAV1	Exon 3	Frameshift	c.474delA	p.L159Sfs*22	1	H	Austin et al., 2012
KCNA5	Exon 1	Missense	c.544G>A	p.G182R	2	I	Remillard et al., 2007
KCNA5	Exon 1	Missense	c.633G>C	p.E211D	2	I	Remillard et al., 2007
KCNA5	Exon 1	Frameshift	c.1448delA	p.Y483Sfs*4	1	H	Wang et al, 2014 [†]
KCNK3	Exon 1	Missense	c.23C>A	p.T8K	1	I	Ma et al., 2013
KCNK3	Exon 2	Missense	c.289G>A	p.G97R	1	H	Ma et al., 2013
KCNK3	Exon 2	Missense	c.544G>A	p.E182K	1	I	Ma et al., 2013
KCNK3	Exon 2	Missense	c.575A>G	p.Y192C	1	I	Ma et al., 2013
KCNK3	Exon 2	Missense	c.608G>A	p.G203D	1	H	Ma et al., 2013
KCNK3	Exon 2	Missense	c.661G>C	p.V221L	1	H	Ma et al., 2013

[†] GenBank reference sequence and version number for *CAV1*: NM_001753.4; *KCNA5*: NM_002234.3; *KCNK3*: NM_002246.2

[‡] Total number of independent cases

[^] Reported as a compound heterozygote with a *BMP2* c.1471C>T (p.R491W) mutation

Key to abbreviations: H: heritable pulmonary arterial hypertension; I: idiopathic pulmonary arterial hypertension

Table 5

PVOD/PCH mutations identified in *EIF2AK4*

Location	Mutation category	Nucleotide change	Amino acid change	Frequency in this study	Clinical classification	Reference(s)
Exon 3; Intron 9	Frameshift; Splice-site	c.354_355delTTG; c.1554-4C>A	p.C118Wfs*7; p.C519Dfs*17	1	PVOD-F	Eyries et al., 2014
Exon 5	Frameshift	c.560_564delAAGAA; c.560_564delAAGAA	p.K187Rfs*9; p.K187Rfs*9	1	PVOD-S	Eyries et al., 2014
Exon 5	Frameshift	c.567dupG; c.567dupG	p.K190Efs*8; p.K190Efs*8	1	PVOD-F	Eyries et al., 2014
Exon 7; Exon 12	Nonsense; Frameshift	c.745C>T; c.2136_2139dupCACT	p.R249*; p.S714Hfs*21	1	PVOD-F	Eyries et al., 2014
Intron 7; Exon 25	Splice-site; Nonsense	c.860-1G>A; c.3448C>T	p.?: p.R1150*	1	PCH	Best et al., 2014
Exon 9; Exon 28	Frameshift; Nonsense	c.1153dupG; c.3766C>T	p.V385Gfs*30; p.R1256*	1	PCH	Best et al., 2014
Exon 9	Nonsense	c.1387c>t; c.1387c>t	p.R463*; p.R463*	1	PVOD-F	Eyries et al., 2014
Exon 9; Exon 23	Nonsense; Nonsense	c.1387C>T; c.3244C>T	p.R463*; p.Q1082*	1	PVOD-F	Eyries et al., 2014
Exon 9	Frameshift	c.1392delT; c.1392delT	p.R465Vfs*38; p.R465Vfs*38	1	PCH	Best et al., 2014
Exon 9; Exon 28	Frameshift; Nonsense	c.1392delT; c.3802C>T	p.R465Vfs*38; p.Q1268*	1	PVOD-F	Eyries et al., 2014
Exon 11	Missense	c.1754g>a; c.1754g>a	p.R585Q; p.R585Q	1	PVOD-F	Eyries et al., 2014
Exon 12	Missense	c.1928t>g; c.1928t>g	p.L643R; p.L643R	1	PVOD-F	Eyries et al., 2014
Intron 13	Splice-site	c.2319+1G>A; c.2319+1G>A	p.?: p.?	1	PVOD-F	Eyries et al., 2014
Exon 15	Nonsense	c.2458C>T; c.2458C>T	p.R820*; p.R820*	1	PVOD-S	Eyries et al., 2014
Exon 19; Intron 25	Nonsense; Splice-site	c.2857C>T; c.3576+1G>T	p.Q953*; p.?	1	PVOD-S	Eyries et al., 2014
Exon 21	Splice-site	c.3159G>A; c.3159G>A	p.K975 K1053del; p.K975 K1053del	2	PVOD-F, -S	Eyries et al., 2014
Exon 23	Missense	c.3344C>T; c.3344C>T	p.P1115L; p.P1115L	5	H	Tenorio et al., 2014
Exon 24	Nonsense	c.3406C>T; c.3406C>T	p.R1136*; p.R1136*	1	PVOD-F	Eyries et al., 2014
Exon 25; Intron 36	Nonsense; Splice-site	c.3448C>T; c.4728+1_4728+13delinsTTCT	p.R1150*; p.?	1	PVOD-F	Eyries et al., 2014
Intron 29	Splice-site	c.4065+1G>C; c.4065+1G>C	p.?: p.?	1	PVOD-F	Eyries et al., 2014
Exon 31	Frameshift	c.4205dupT; c.4205dupT	p.S1403Kfs*45; p.S1403Kfs*45	1	PVOD-S	Eyries et al., 2014

[†] GenBank reference sequence and version number for *EIF2AK4*: NM_001013703.3

[‡] Total number of independent cases

Key to abbreviations: H: heritable pulmonary arterial hypertension; PCH: pulmonary capillary hemangiomatosis; PVOD: pulmonary veno-occlusive disease (-F: familial; -S: sporadic)