



Germline *BMP9* mutation causes idiopathic pulmonary arterial hypertension

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***BMP9* is a new culprit gene for IPAH ranking second to *BMPR2*. The rare deleterious mutations in *BMP9*, which lead to the reduction in *BMP9* secretion and impairment in *BMP9* function, account for 6.7% of IPAH cases.** <http://ow.ly/h0tS30mXr8j>

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ABSTRACT

Background: Idiopathic pulmonary arterial hypertension (IPAH) is a rare disease with high heritability. Although several predisposing genes have been linked to IPAH, the genetic aetiology remains unknown for a large number of IPAH cases.

Methods: We conducted an exome-wide gene-based burden analysis on two independent case-control studies, including a total of 331 IPAH cases and 10 508 controls. Functional assessments were conducted to analyse the effects of genetic mutations on protein biosynthesis and function.

Results: The gene encoding human bone morphogenetic protein 9 (*BMP9*) was identified as a novel genetic locus displaying exome-wide association with IPAH in the discovery cohort (OR 18.8; $p=1.9\times 10^{-11}$). This association was authenticated in the independent replication cohort ($p=1.0\times 10^{-5}$). Collectively, the rare coding mutations in *BMP9* occurred in 6.7% of cases, ranking this gene second to *BMPR2*, comprising a combined significance of 2.7×10^{-19} (OR 21.2). Intriguingly, the patients with *BMP9* mutations had lower plasma levels of *BMP9* than those without. Functional studies showed that the *BMP9* mutations led to reduced *BMP9* secretion and impaired anti-apoptosis ability in pulmonary arterial endothelial cells.

Conclusion: We identify *BMP9* as an IPAH culprit gene.

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Introduction

Idiopathic pulmonary arterial hypertension (IPAH), which accounts for 35–67% of pulmonary arterial hypertension (PAH) cases, is a rare but fatal disease with high heritability [1–4]. The genetic basis of IPAH has been studied extensively. Rare coding mutations in bone morphogenetic protein receptor type 2 (*BMPR2*) are found in ~70% of familial kindreds with PAH and 15–20% of sporadic IPAH cases [5–7]. Less frequently, rare mutations of large effect are present in the genes encoding caveolin-1 (*CAV1*), potassium channel two-pore domain subfamily K member 3 (*KCNK3*), SMAD family member 1 (*SMAD1*), SMAD family member 9 (*SMAD9*), T-box 4 (*TBX4*) and bone morphogenetic protein receptor type 1B (*BMPR1B*) [8–13]. Additionally, activin A receptor-like type 1 (*ACVRL1*) and endoglin (*ENG*) were identified as genetic loci implicated in the development of both PAH and hereditary haemorrhagic telangiectasia (HHT) [14, 15]. The biallelic recessive mutations in eukaryotic translation initiation factor 2 α kinase 4 (*EIF2AK4*) were shown recently to be a major cause of pulmonary veno-occlusive disease and/or pulmonary capillary haemangiomas, which are clinically and pathologically distinct forms of PAH [16]. Collectively, the mutations of the 10 genes could only explain a fraction (~20%) of IPAH cases, suggesting additional IPAH genes must exist [17].

The canonical approach to identify IPAH susceptibility genes involves the use of family-based genetics. To date, data generated from multigenerational PAH families have been commonly used to reveal mutations in one of these known PAH genes. The scarcity of IPAH makes it more difficult to identify new IPAH-related genes *via* targeting a larger number of cases for nonbiased whole-genome screening. Moreover, PAH stems from an interplay among many detrimental factors, including, but not limited to, drugs, environmental cues, age, sex and ethnic background. Thus, the genotypic data would not be the sole confounding factor for the clinical heterogeneity. With the aforementioned limitations, a large-scale analysis of genetic specimens with well-defined IPAH cases in conjunction with state-of-the-art methods is urgently needed to identify other IPAH susceptibility loci.

The analysis of data from next-generation sequencing (NGS) enables the identification of novel disease genes. Here, we aimed to identify IPAH causative genes by performing two-stage NGS in sporadic IPAH cases enrolled in two centres in China for >10 years. While the extent of the previously identified PAH genes (*e.g.* *BMPR2*) was verified, gene-based burden analysis was performed to screen IPAH susceptibility genes.

Methods

A detailed description of all methods can be found in the supplementary material.

Study design

IPAH patients were recruited from two national pulmonary hypertension referral centres in China: Shanghai Pulmonary Hospital of Tongji University in Shanghai (with patients mainly from south China) and FuWai Hospital of the Chinese Academy of Medical Sciences in Beijing (with patients mainly from north China). The study comprised two stages: “discovery” and “replication” stages. All of the IPAH cases were genotyped by NGS and screened for rare variants in 10 previously reported PAH genes (*BMPR2*, *ACVRL1*, *ENG*, *SMAD1*, *SMAD9*, *TBX4*, *BMPR1B*, *CAV1*, *KCNK3* and *EIF2AK4*). Gene-based burden analysis was then performed by comparing the exome data of cases against controls to identify novel IPAH susceptibility genes. These putative genes identified in the discovery cohort were subsequently evaluated in the replication cohort. The correlation between genotype and clinical phenotype was further analysed with the use of combined datasets. The research protocols were approved by the institutional review boards in Shanghai Pulmonary Hospital and FuWai Hospital. The study design is shown in figure 1.

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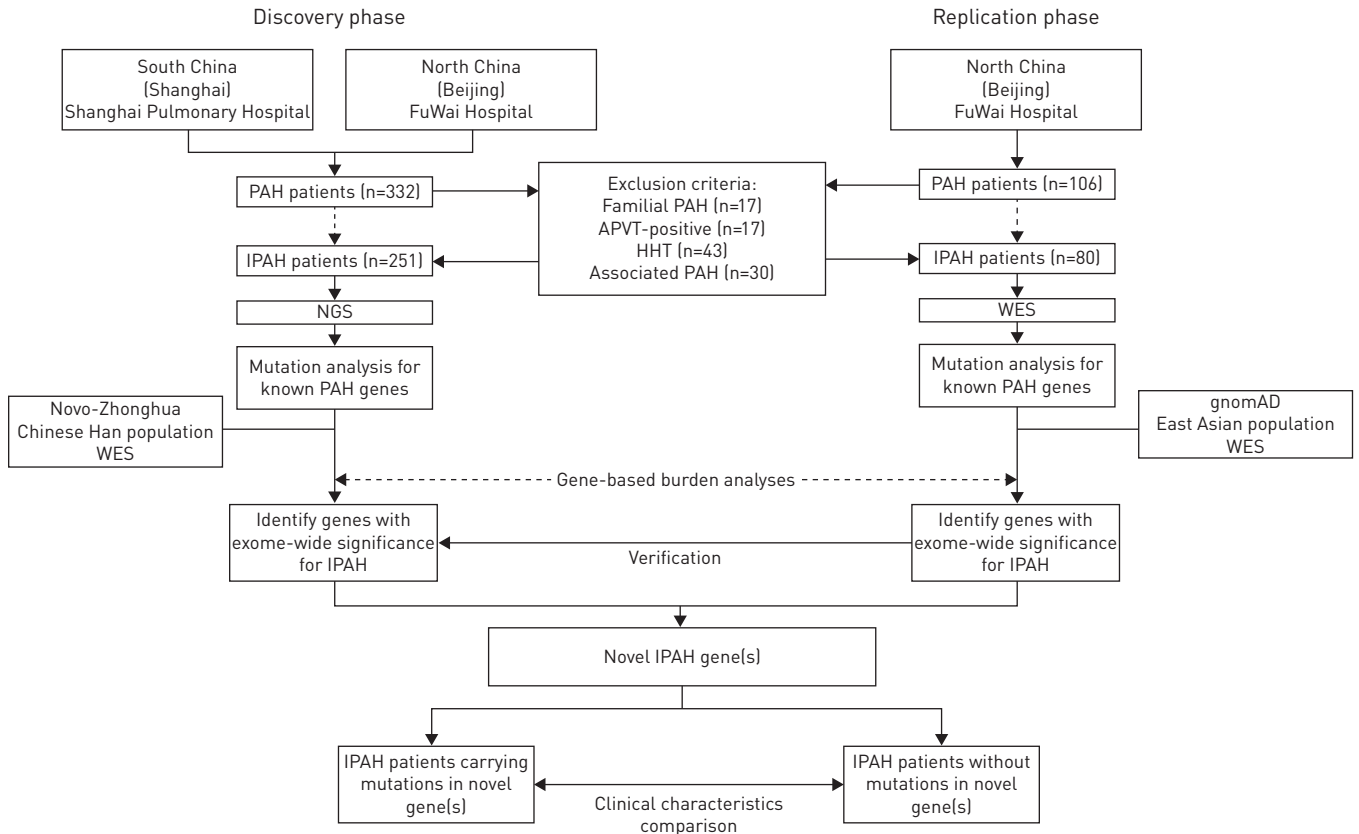


FIGURE 1 Flowchart of study design. PAH: pulmonary arterial hypertension; IPAH: idiopathic pulmonary arterial hypertension; APVT: acute pulmonary vasoreactivity testing; HHT: hereditary haemorrhagic telangiectasia; NGS: next-generation sequencing; WES: whole-exome sequencing; gnomAD: Genome Aggregation Database.

Statistical analysis

Results are reported as percentage, median (interquartile range (IQR)) or mean with standard deviation, as indicated. The normality of data distribution was assessed using Kolmogorov–Smirnov tests. A Chi-squared test or Fisher’s exact test was applied to compare qualitative variables and genotype/allele frequencies. For quantitative variables, statistical significance was determined using an unpaired t-test or one-way ANOVA. The Mann–Whitney U-test or Kruskal–Wallis test was performed to analyse nonnormally distributed variables. All statistical tests were two-tailed. Results were considered statistically significant at a level of $p < 0.05$. All analyses were performed with PASW Statistics version 18.0 (SPSS, Chicago, IL, USA).

Results

Study population

A total of 331 patients with IPAH were recruited from two national referral centres (figure 1). For the discovery cohort, 122 cases (15 paediatric patients) were recruited from Shanghai Pulmonary Hospital and 129 cases (33 paediatric patients) were recruited from FuWai Hospital. For the replication cohort, 80 incident cases (nine paediatric patients) were enrolled from FuWai Hospital. Demographic and clinical characteristics of the two IPAH cohorts are described in table 1.

Identification of BMP9 as an IPAH susceptibility gene

In the discovery stage, we genotyped 36 IPAH cases using whole-genome sequencing (WGS) and the other 215 cases using whole-exome sequencing (WES). WGS and WES achieved an average coverage of 37- and 128-fold on target, respectively. More than 92% of targeted bases were sequenced to a read depth of more than 20 times (supplementary table S1).

We first analysed the data for rare variants and mutations in the 10 previously reported PAH-predisposing genes. The rare heterozygous mutations of *BMP9* were found in 49 cases (19.5%). For the other PAH risk genes, we identified deleterious rare variants in *ACVRL1* (15 cases (6%)), *TBX4* (10 cases (4%)), *SMAD1* (two cases (0.8%)), *BMP9* (one case (0.4%)), *KCNK3* (one case (0.4%)) and *SMAD9* (one case (0.4%)). Four cases (1.6%) harboured biallelic *EIF2AK4* mutations: two cases carried homozygous

TABLE 1 Demographic characteristics of the patients in each cohort and the prevalence of bone morphogenetic protein 9 (*BMP9*) mutations

	Discovery cohort		Replication cohort:
	Shanghai Pulmonary Hospital	FuWai Hospital	FuWai Hospital
Subjects	122	129	80
Age at diagnosis years	27.4±9.0	27.0±12.7	30.7±10.9
Female	94 (77.0)	103 (79.8)	61 (77.2)
Right atrial pressure mmHg	9.3±6.3	8.1±4.5	8.2±4.5
Mean pulmonary arterial pressure mmHg	64.6±13.7	62.5±15.8	59.0±15.3
Pulmonary arterial wedge pressure mmHg	8.5±3.1	9.2±3.0	9.5±2.8
Cardiac index L·min ⁻¹ ·m ⁻²	2.6±1.2	2.3±0.7	2.5±0.6
Pulmonary vascular resistance Wood Units	17.4±9.4	15.7±6.5	12.6±5.0
Mixed venous oxygen saturation %	61.5±12.1	64.4±9.2	65.9±9.2
<i>BMP9</i> mutation carriers	7 (5.7)	10 (7.8)	5 (6.3)

Data are presented as n, mean±SD or n (%).

mutations and two cases carried heterozygous mutations (one missense and one frameshift in one case, and two missense mutations in the other case). Eight cases (3.2%) had double heterozygous mutations or rare variants in two different PAH risk genes. In total, rare variants and mutations were identified in 91 patients, corresponding to 36.3% of all cases (supplementary table S2). The details for all rare variants and mutations of the 10 PAH genes are provided in supplementary dataset S1.

To this end, the identified rare variants and mutations in genes known to be IPAH related confirmed the genetic background of the discovery cohort. We then performed a gene-based burden analysis utilising the exome data of 251 cases and 1884 controls (supplementary figure S1). Further analysis of the homogenous population structure demonstrated that all cases and controls were clustered with subjects of Chinese ancestry in the 1000 Genomes dataset (www.1000genomes.org) (supplementary figure S2). A total of 13 318 genes had more than one case in 251 IPAH patients with a potential deleterious genetic variant for the dominant model tested. After normalisation for multiple tests ($p < 3.8 \times 10^{-6}$ after accounting for the 13 318 genes examined), only three genes (*BMPR2*, *BMP9* (encoding bone morphogenetic protein 9) and *ACVRL1*) had an exome-wide significant enrichment of mutations in IPAH cases when compared with controls (table 2 and supplementary figure S3). Noticeably, besides the well-documented PAH causative *BMPR2* and *ACVRL1*, *BMP9* would be a new gene displaying exome-wide association with IPAH. Mutations in *BMP9* were found in 6.8% (17 out of 251) of cases and 0.4% (seven out of 1884) of controls (OR 18.8, 95% CI 7.4–53.9; raw $p = 1.9 \times 10^{-11}$, Bonferroni corrected $p = 2.6 \times 10^{-7}$). Under the dominant coding model, *BMP9* ranked second next to *BMPR2* (table 2 and supplementary figure S3). Specifically, *BMP9* mutations were found in 5.7% (seven out of 122) and 7.8% (10 out of 129) of cases from Shanghai Pulmonary Hospital and FuWai Hospital, respectively (table 1). The distribution of *BMP9* mutations did not significantly differ between the two centres ($p = 0.53$, Chi-squared test).

To further validate the risk effects of *BMP9*, we performed WES in an independent replication cohort of 80 IPAH cases. Rare variants and mutations in PAH-related genes were identified in 37 (46.3%) cases (summary in supplementary table S2; all rare variants and mutations are listed in supplementary dataset S1). Using the same pipeline of genetic burden analysis, we compared the 80 exomes of cases with the reference control exome sequencing data of 8624 East Asians from the Genome Aggregation Database (gnomAD-EAS; <http://gnomad.broadinstitute.org>). *BMPR2*, *BMP9* and *ACVRL1* were again identified as the top three disease-associated genes in the replication cohort (table 2). We identified five additional (6.3%) *BMP9* heterozygous mutations (table 1 and supplementary table S3). The prevalence of *BMP9* mutations was significantly higher than the reference controls of gnomAD-EAS (raw $p = 1.0 \times 10^{-5}$). In the combined discovery and replication datasets, mutations in the *BMP9* were found in 6.7% (22 out of 331) and 0.3% (34 out of 10508) of cases and controls, respectively, comprising a combined significance of 2.7×10^{-19} (OR 21.2, 95% CI 11.7–37.6). The prevalence of *BMP9* mutations was comparable between the discovery and replication cohorts (6.8% versus 6.3%). Therefore, this two-stage analysis strongly suggests that the *BMP9* locus is a novel contributor to the pathogenesis of IPAH.

BMP9 mutation characteristics

Among the 331 IPAH patients, we identified 22 cases carrying 21 distinct rare heterozygous mutations in *BMP9* (supplementary table S3). These mutations were spread throughout *BMP9* (figure 2a and b). All the

TABLE 2 Association of three genes with idiopathic pulmonary arterial hypertension (IPAH) in two independent case-control studies

	Case #	Control #	p-value [¶]	OR (95% CI)	Adjusted p-value [*]
Discovery cohort	IPAH	Novo-Zhonghua			
Subjects	251	1884			
<i>BMPR2</i>	56 (22.31)	23 (1.22)	1.98×10 ⁻³⁵	20.4 (12.2–35.1)	2.64×10 ⁻³¹
<i>BMP9</i>	17 (6.77)	7 (0.37)	1.92×10 ⁻¹¹	18.8 (7.4–53.9)	2.55×10 ⁻⁷
<i>ACVRL1</i> [§]	17 (6.37)	14 (0.74)	6.62×10 ⁻⁹	9.39 (4.3–20.7)	8.82×10 ⁻⁵
Replication cohort	IPAH	gnomAD-EAS			
Subjects	80	8624			
<i>BMPR2</i>	25 (31.25)	68 (0.79)	3.10×10 ⁻³⁰	46.7 (27.4–77.5)	2.47×10 ⁻²⁶
<i>BMP9</i>	5 (6.25)	27 (0.31)	1.02×10 ⁻⁵	20.5 (6.1–55.1)	8.12×10 ⁻²
<i>ACVRL1</i>	6 (7.50)	48 (0.56)	1.01×10 ⁻⁵	13.9 (4.8–33.1)	8.04×10 ⁻²
Combined cohorts	IPAH	Control			
Subjects	331	10508			
<i>BMPR2</i>	81 (24.47)	91 (0.87)	3.12×10 ⁻⁷⁶	32.1 (23.2–44.2)	4.38×10 ⁻⁷²
<i>BMP9</i>	22 (6.65)	34 (0.32)	2.67×10 ⁻¹⁹	21.2 (11.7–37.6)	3.74×10 ⁻¹⁵
<i>ACVRL1</i> [§]	23 (6.65)	62 (0.59)	6.15×10 ⁻¹⁶	12.1 (7.1–20.0)	8.62×10 ⁻¹²

Data are presented as n or n (%), unless otherwise stated. *BMPR2*: bone morphogenetic protein receptor type 2; *BMP9*: bone morphogenetic protein 9; *ACVRL1*: activin A receptor-like type 1; gnomAD-EAS: Genome Aggregation Database East Asian. #: the numbers denote alleles for rare deleterious mutations at conserved positions and the percentages in brackets denote the prevalence of mutation carriers in the cohort; ¶: raw p-value for comparing the alleles between cases and controls by two-tailed Fisher's exact test; *: adjusted p-value after Bonferroni correction for comparing the alleles between cases and controls; §: in the discovery stage, one patient carried two rare deleterious mutations in *ACVRL1* (therefore, the allele count of *ACVRL1* is 17, but the number of patients carrying the *ACVRL1* mutation is 16; in the combined dataset, the allele count of *ACVRL1* is 23, but the number of patients carrying the *ACVRL1* mutation is 22).

BMP9 mutations were confirmed by Sanger sequencing (supplementary figure S4). None of the *BMP9* mutation carriers had any deleterious mutations in the 10 previously reported PAH genes. 20 of these *BMP9* mutations were absent from more than 6512 exomes or genomes, including 301 Chinese samples from the 1000 Genome project, 1884 Novo-Zhonghua controls and 4327 East Asians in the Exome Aggregation Consortium database (ExAC-EAS; <http://exac.broadinstitute.org>). Only one mutation (p.G322R) was identified in these databases, with a prevalence of 0.0001–0.0002 (supplementary table S4). Of the 22 *BMP9* mutation carriers, loss-of-function (LOF) mutations were found in six cases (27.3%), including three nonsense mutations, two frameshifts and one mutation that would affect the canonical translational start site (supplementary table S4). In the two reference populations, no such *BMP9* mutations were found. The prevalence of *BMP9* LOF mutations in IPAH cases (six cases (1.8%)) was significantly higher than in the two reference populations ($p=1.1\times 10^{-5}$ for Novo-Zhonghua and $p=2.5\times 10^{-9}$ for gnomAD-EAS controls). Regarding the 15 *BMP9* missense mutations, *in silico* analyses suggested that these mutations would be deleterious (supplementary table S4) and located in the regions highly conserved among various species (figure 2c).

Clinical characteristics of *BMP9* mutation carriers

Supplementary table S3 shows the clinical, functional and haemodynamic characteristics of the 22 IPAH patients carrying the various *BMP9* mutations. Both males and females were affected, and a significant sex bias was evident with a female:male ratio of 4.5:1. Seven patients (31.8%) died during the follow-up.

We expanded the analyses of *BMP9* genotype and phenotypic outcomes in the combined datasets. Compared with the cases with or without *BMP9* mutations, there were no significant differences in age and haemodynamics (supplementary table S5). As a member of the BMP subgroup of the transforming growth factor- β superfamily proteins, *BMP9* is synthesised as a 429-amino-acid precursor protein (pre-pro-*BMP9*) and processed into pro-*BMP9* (100 kDa) and mature *BMP9* (25 kDa) (supplementary figure S5). The active form of *BMP9* circulates in the blood and is crucial for the regulation of vasculature tone [18]. To investigate whether the *BMP9* mutations affect the expression levels of *BMP9*, we analysed plasma *BMP9* levels in IPAH patients carrying *BMP9* mutations (n=19), age- and sex-matched healthy controls (n=87), and IPAH patients without *BMP9* mutations (n=38) (supplementary table S6). The *BMP9* levels were significantly different among the three groups ($p<0.0001$, Kruskal–Wallis test) (figure 3). Compared with healthy controls, the plasma *BMP9* level was lower in IPAH patients lacking *BMP9*

mutations (median (IQR) 19.8 (15.2–43.4) versus 36.2 (20.8–60.7) pg·mL⁻¹; $p=0.005$, Mann–Whitney test). Importantly, the BMP9 level was even lower in *BMP9* mutation carriers when compared with patients without mutations (median (IQR) 12.1 (8.6–21.8) versus 19.8 (15.2–43.4) pg·mL⁻¹; $p=0.002$, Mann–Whitney test) (figure 3). These results indicate that *BMP9* mutations may suppress BMP9 synthesis and/or secretion, resulting in decreased circulating BMP9 levels that may be causative of IPAH.

Functional studies of BMP9 mutations

To investigate the deleterious outcomes resulting from *BMP9* mutations, we selected six mutations for functional assessment based on the clinical presentations (supplementary figure S6). Three mutations

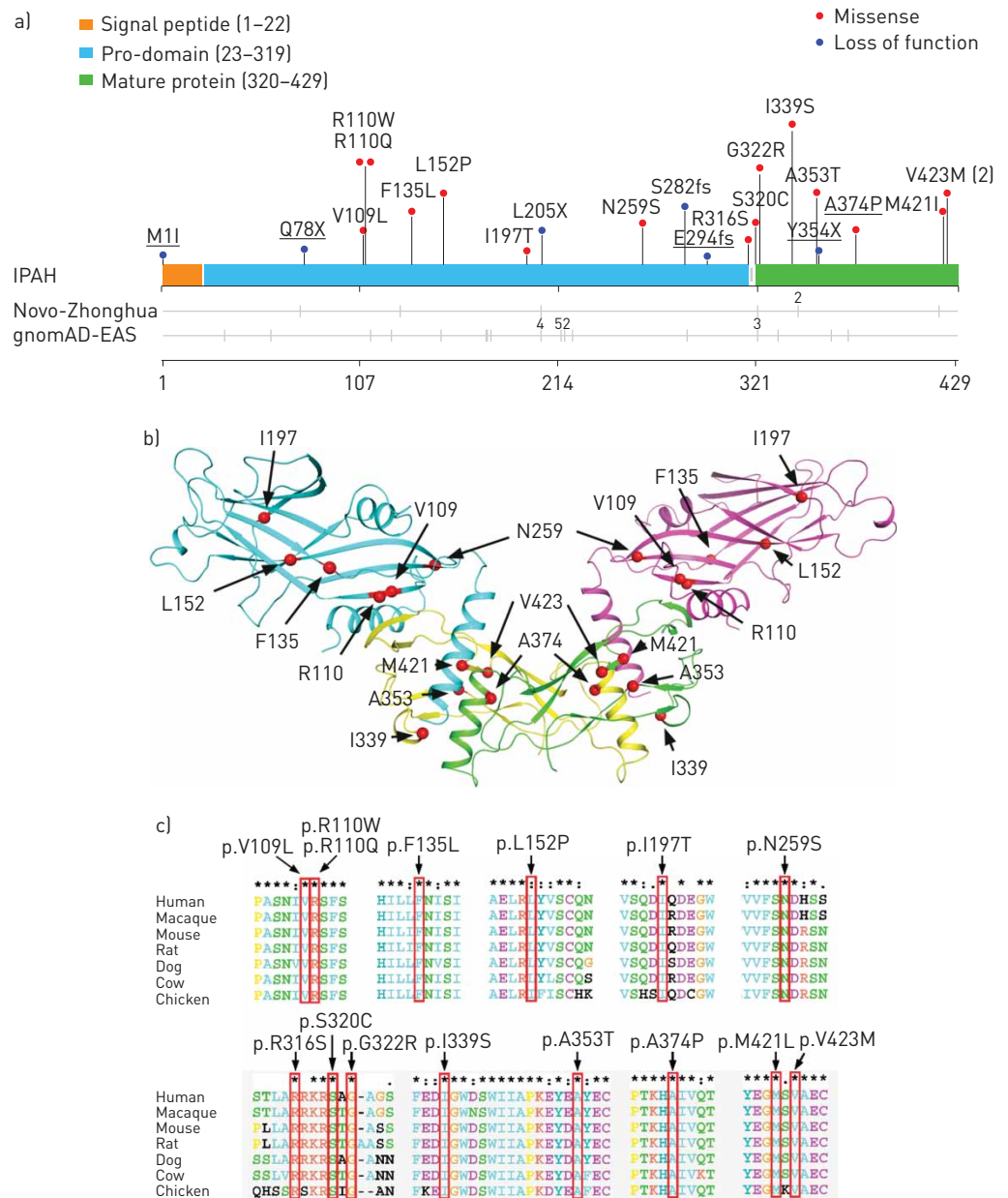


FIGURE 2 Bone morphogenetic protein 9 (*BMP9*) mutations in idiopathic pulmonary arterial hypertension (IPAH). gnomAD-EAS: Genome Aggregation Database East Asian. a) Spatial distribution of the mutations of *BMP9* found in patients with IPAH, along with the distribution of variants in healthy controls. Mutations identified in the replication cohort are underlined. The numbers of subjects carrying the same mutation are indicated. b) Distribution of 11 *BMP9* missense mutations that were found in IPAH patients displayed on the crystal structure of BMP9, with superimposition on growth factor dimers. The pro-domain ribbon is coloured blue and purple. The mature growth factor is coloured yellow and green. c) ClustalX (www.clustal.org) comparison of amino acid sequences shows high conservation of 15 missense *BMP9* mutations across different species. The mutations are indicated in red boxes.

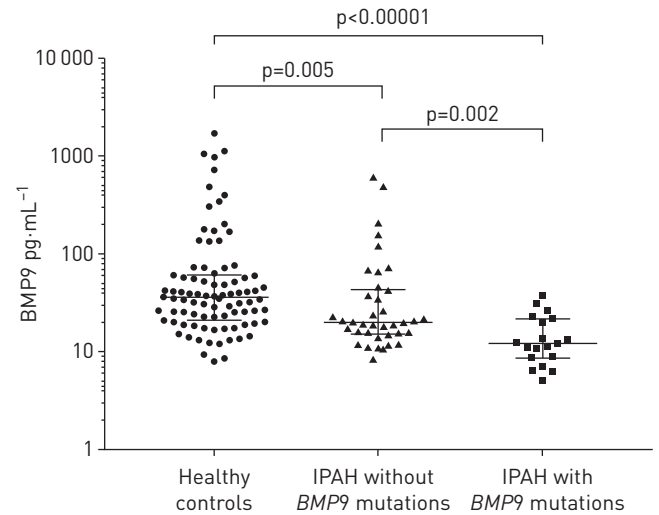


FIGURE 3 Bone morphogenetic protein 9 [BMP9] levels in healthy controls ($n=87$), idiopathic pulmonary arterial hypertension (IPAH) patients without *BMP9* mutations ($n=38$) and IPAH patients with *BMP9* mutations ($n=19$). BMP9 was decreased in the IPAH patients without *BMP9* mutations and lowest in *BMP9* mutation carriers. The data are presented as individual data points with median and interquartile range. Statistical analysis between two groups was performed using the Mann–Whitney test.

(V109L, S282fs and V423M) were chosen because patients with these mutations had early onset of disease. The other three mutations (R316S, S320C and A353T) were studied because the mutation carriers had severe clinical manifestations that decreased in a short period of time after diagnosis (supplementary table S3). Western blot demonstrated that the wild-type (WT) and five missense plasmids (V109L, V423M, R316S, S320C and A353T) expressed similar levels of pro-BMP9 protein either in cell lysis (supplementary figure S7) or in supernatants from human embryonic kidney HEK293E cells (figure 4a, right panel). However, a significant decrease in mature BMP9 was observed for all *BMP9* mutants (figure 4a, left panel). For the truncated mutation S282fs, neither pro-BMP9 nor mature BMP9 could be detected (figure 4a and supplementary figure S7). These results were confirmed by ELISA assay (figure 4b). The mature BMP9 level was significantly decreased in the culture supernatant of HEK293E cells transfected with V109L, R316S and S320C, and was almost undetectable for S282fs, A353T and V423M. We then used the conditioned media from V109L-, R316S- and S320C-transfected cells to examine their ability in activating the BMP signalling pathway (figure 4c). BRE (BMP response element)-luciferase activity assays revealed that R316S exhibited decreased activity compared with WT BMP9. Furthermore, pre-treatment of WT BMP9 conditioned media protected pulmonary arterial endothelial cells (PAECs) from apoptosis induced by tumour necrosis factor- α in the presence of cycloheximide. However, all of the three mutants were unable to prevent PAEC apoptosis (figure 4d).

Discussion

In the current study, we used NGS to identify that *BMP9* is a novel IPAH susceptibility gene. The rare *BMP9* mutations conferred a >21-fold greater odds of IPAH, ranking it second to *BMP2*. Functional studies confirmed that these *BMP9* mutations affected circulating BMP9 levels in IPAH patients. Furthermore, these mutants caused deleterious effects on protein expression and function in cultured cells. Taken together, these findings strongly suggest that mutations in *BMP9* contribute to the aetiology of PAH.

NGS involving exome sequencing has led to the identification of novel genes for heritable PAH (HPAH) within families having multiple affected individuals [10, 11]. However, challenges remain when applying this technology to identify novel genes among unrelated IPAH subjects. The difficulty stems from the recruitment of large homogeneous patient cohorts. In the current study, we recruited patients from two expert centres with consistent diagnostic procedures for IPAH. Almost all familial PAH (FPAH) patients carry a mutation of known PAH genes, and acute pulmonary vasoreactivity test-positive responders are different from IPAH patients with respect to the pathogenic mechanism and genetic aetiology [19]. Thus, we excluded these two subgroups of patients from the IPAH cohort to better decipher the genetic basis of IPAH. We enrolled 57 paediatric IPAH patients (mean \pm SD age 9.9 \pm 5.5 years), 274 adult patients (mean \pm SD age 31.8 \pm 7.7 years) and excluded cases >60 years old, resulting in a combined mean age of 28 years. In addition, all subjects participating in this study were officially registered as Chinese Han ethnicity.

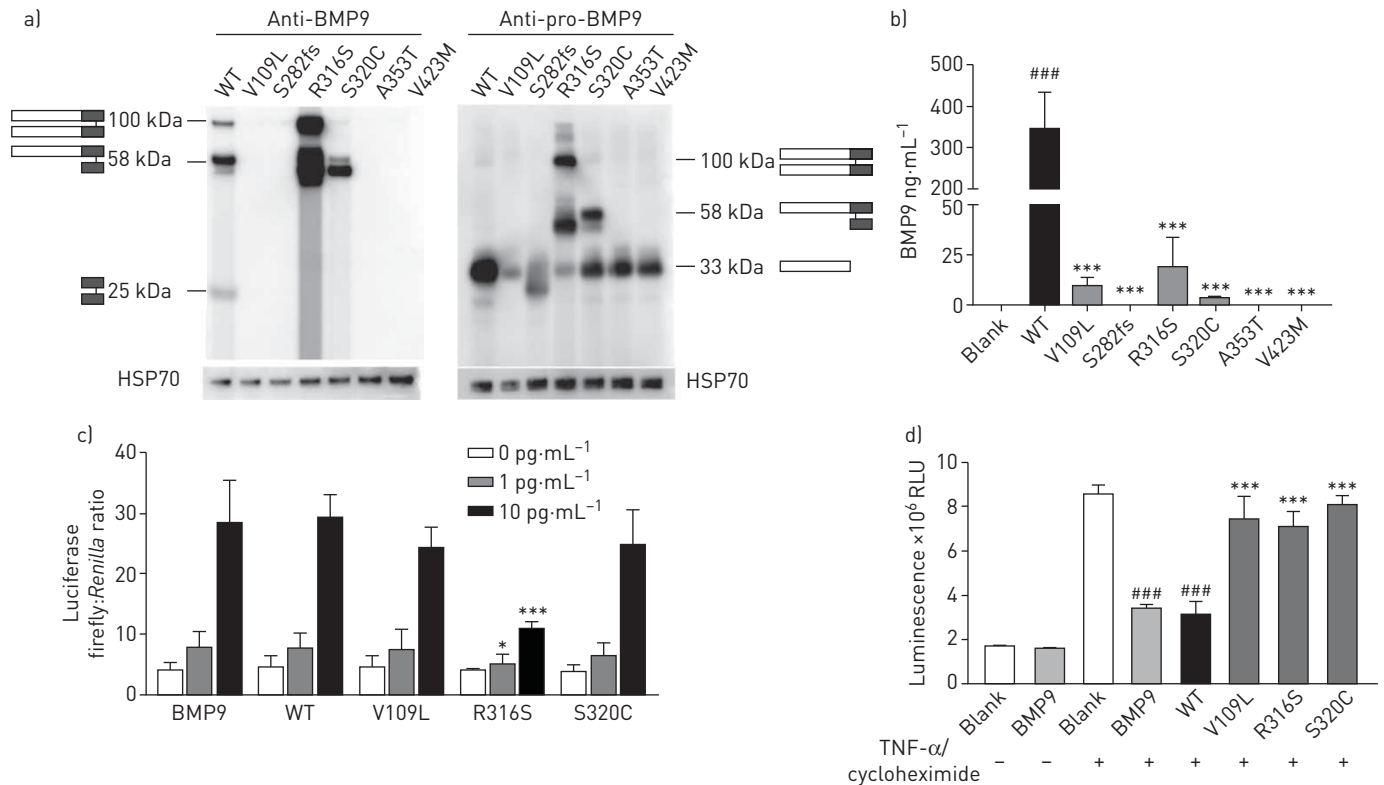


FIGURE 4 Functional study of bone morphogenetic protein 9 (*BMP9*) mutations. WT: wild-type; HSP: heat shock protein; RLU: relative light units; TNF: tumour necrosis factor; PAEC: pulmonary arterial endothelial cell. a) Human embryonic kidney HEK293E cells transfected with mutant *BMP9* showed a different expression pattern compared with WT. HEK293E cells were transfected with WT or *BMP9* mutant plasmids. The supernatant was harvested for immunoblotting with an antibody specific to mature *BMP9* or pro-*BMP9*. WT *BMP9* protein fractionated as three bands corresponding to pro-*BMP9* (100 kDa), partially processed pro-*BMP9* (58 kDa) and mature *BMP9* dimer (25 kDa). b) ELISA assay showed that the secretion of mature *BMP9* was significantly lower in the supernatant of HEK293E cells transfected with mutant *BMP9* than those from WT *BMP9*. ANOVA. ***: $p < 0.001$ compared with WT; ###: $p < 0.001$ compared with blank. c) The missense *BMP9* mutation R316S caused significantly reduced BRE (BMP response element) activity compared with WT *BMP9*. After co-transfection with human activin receptor-like kinase 1, BRE-luciferase and pTK-*Renilla*, C2C12 cells were treated with WT or mutant *BMP9* supernatant at 1 and 10 $\text{pg}\cdot\text{mL}^{-1}$, respectively. Commercial recombinant *BMP9* protein was used as a positive control. ANOVA. *: $p < 0.05$ compared with WT; ***: $p < 0.001$ compared with WT. d) *BMP9* mutations attenuated the anti-apoptosis effect of WT *BMP9* in PAECs. PAECs were pre-treated with WT or mutant *BMP9* supernatant at 5 $\text{ng}\cdot\text{mL}^{-1}$. The effect of the mutants of *BMP9* on PAEC apoptosis was assessed by measuring caspase-3/7 activity following stimulation with TNF- α and cycloheximide. ANOVA. ***: $p < 0.001$ compared with WT; ###: $p < 0.001$ compared with blank.

Therefore, the stringent selection criteria, precise clinical phenotype together with relatively young and homogeneous cohorts in the current study would generate convincing data on the IPAH-predisposing gene. As such, our data may also justify the higher mutation rate of the 10 previously identified PAH genes (38.7% versus 19.9%) and *BMP9* (6.8% versus 1.1%) in the current study than reported in a European cohort by GräF *et al.* [20].

The variations in genetic backgrounds among different populations and ethnicities result in the distinctive propensity of particular genes involved in rare diseases. For example, Factor V Leiden, an important risk factor in venous thromboembolism, has an average allele frequency of 4.4% in the European population, but is almost absent in the East Asian population [21]. Therefore, confirmation of putative genes involved in rare diseases between independent cohorts in different continents is crucial to identify the role of susceptible genes in the aetiology of the disease. Recently, GräF *et al.* [20] performed WGS in European Caucasian IPAH/HPAH patients and reported a significant overrepresentation of mutations in four genes (*ATP13A3* (ATPase 13A3), *AQP1* (aquaporin-1), *SOX17* (SRX-box 17) and *BMP9*). Among these genes, only *BMP9* was validated in our study. None of the other three genes (*ATP13A3*, *AQP1* and *SOX17*) achieved statistical significance in the combined cohorts (supplementary table S7). The mutations for the three genes are presented in supplementary dataset S2. Furthermore, among the 21 *BMP9* mutations described in the current study, only two of the *BMP9* mutations were reported previously [20]; the remaining 19 mutations were newly identified. Taken together, our results not only complement the previously reported European data by GräF *et al.* [20], but also reinforce the critical role of *BMP9* mutations in the genetic aetiology of IPAH.

BMP9 (also known as growth and differentiation factor 2) is predominantly expressed in the liver and constitutively secreted into the circulation. By binding to ACVRL1 (type 1 BMP receptor) and BMPR2 (type 2 BMP receptor), BMP9 plays an important role in regulating vascular biology and angiogenesis [18]. Because *ACVRL1* and *BMPR2* are highly expressed in pulmonary vascular endothelial cells, the optimum level of circulating BMP9 is essential to maintain the quiescence of the pulmonary vasculature [22]. Here, we provide compelling evidence indicating that *BMP9* is a PAH susceptibility gene through an exome-wide study. All of the 21 *BMP9* mutations identified are very rare, which is consistent with the low incidence of IPAH (six cases per million people per year). We have shown various *BMP9* mutations could negatively affect BMP9 function, including synthesis, secretion, processing and/or activation of *ACVRL1* signalling, which results in haploinsufficiency of a functional BMP axis in the pulmonary endothelium. Notably, the R316S mutation showed a unique protein expression pattern (figure 4a) and a more deleterious effect on *ACVRL1* signalling (figure 4c). These results might be caused by the special location of the R316S mutation in the highly conserved cleavage site of BMP9, which could interfere with the proteolytic cleavage. The molecular mechanisms underlying the pathogenesis of PAH elicited by these *BMP9* mutations deserve further investigation.

Of note, *ACVRL1* ranked third among all the IPAH-related genes in our two cohorts, with rare mutations occurring in 22 cases. This was seemingly contradictory to the previous studies showing that *ACVRL1* is a major disease-causing gene for HHT and PAH with HHT [23, 24]. To confirm our results, medical records and family histories were scrutinised to ensure that all these *ACVRL1* mutation carriers were indeed IPAH. *ACVRL1* mutation carriers might develop both PAH and HHT. In 78% patients carrying the *ACVRL1* mutation from the French PAH Network, PAH diagnosis preceded manifestations of HHT [25]. Moreover, because HHT has nearly complete penetrance at the age of 60 years, some *ACVRL1* mutation carriers might not exhibit clinical evidence of HHT when they are young. Given the younger age of the *ACVRL1* mutation carriers identified in our study (mean (range) age 25.7 (3–50) years), these patients might not present the clinical features of HHT. We will keep following these patients to confirm the phenotype.

Recently, a subgroup of PAH patients was found to carry more than one rare gene variant, leading to a double heterozygous genotype. For example, GRÄF *et al.* [20] reported that one PAH patient carried highly deleterious variants in both *BMPR2* and *SMAD9*. In the current study, we identified eight cases carrying double mutations or rare variants in *BMPR2/ACVRL1*, *BMPR2/BMPR1B*, *BMPR2/SMAD9*, *BMPR2/TBX4* and *KCNK3/TBX4*. The double heterozygosity for mutations or variants involving PAH risk genes highlights the complexity inherent to PAH. Whether the double variants contributed to PAH pathogenesis and clinical outcome warrants future study.

To the best of our knowledge, this is the first report to identify novel IPAH susceptibility genes in Asia using an exome-wide study based on a large population of IPAH patients. The identification of *BMP9* mutations strongly supports the hypothesis that an impairment of genes involved in BMP signalling leads to PAH susceptibility. Experimentally, administration of recombinant BMP9 to stimulate BMP signalling in the endothelium can prevent or reverse PAH in several genetic and nongenetic rodent models [26]. Data in figure 3 indicate that circulating BMP9 levels decreased significantly in patients carrying *BMP9* deleterious mutations. This finding suggests that IPAH patients carrying *BMP9* mutants could receive BMP9 supplemental therapy as part of their personalised treatment.

Study limitations

A caveat of our study is that all the patients involved in the two cohorts were sporadic cases. Due to the unavailability of DNA samples from the affected relatives, familial segregation with PAH has not been demonstrated. Whether *BMP9* mutations are associated with FPAH should be confirmed in pedigrees in the future. Another limitation is the relatively low number of patients in the replication cohort. Future studies among larger cohorts will be crucial to confirm further the association between *BMP9* and IPAH. Given that the circulating BMP9 levels were only measured at baseline for the IPAH patients with or without mutations, we were unable to test the variability and prognostic value of BMP9 over time or the relevance to different therapeutic strategies.

Conclusions

Using NGS exome sequencing on large homogenous cohorts, we have confirmed that mutations in human *BMP9* predispose to IPAH susceptibility.

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