Association of Rare PTGIS Variants With Susceptibility and Pulmonary Vascular Response in Patients With Idiopathic Pulmonary Arterial Hypertension

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IMPORTANCE Idiopathic pulmonary arterial hypertension (IPAH) is a fatal disease with high heritability; however, the bone morphogenetic protein receptor 2 (BMPR2) gene only accounts for 17% of IPAH. The genetic basis of IPAH needs further investigation.

OBJECTIVE To identify novel IPAH susceptibility genes other than BMPR2.

DESIGN, SETTING, AND PARTICIPANTS This 2-stage, case-control genetic association study enrolled 230 patients with IPAH from 2 referral pulmonary hypertension centers in China. Eligible patients had no BMPR2 variants and were compared with 968 healthy control participants. Data were collected from January 1, 2000, to July 31, 2015, and analyzed from August 1, 2015, to May 30, 2018.

EXPOSURES PTGIS rare variants.

MAIN OUTCOMES AND MEASURES Whole-genome sequencing was performed to identify putative IPAH genes in a discovery cohort, with validation in an independent referral cohort. Correlation of genotype and hemodynamic characteristics was then evaluated at baseline and after pulmonary vasodilator testing. Functional assessments were conducted to analyze the effects of identified genetic variants on transcript splicing, enzymatic activity, and endothelial cell phenotypes.

RESULTS Among 230 patients with IPAH (164 female [71.3%]; mean [SD] age, 34 [18] years), an enrichment of rare variants in a gene encoding prostacyclin synthase (PTGIS) was identified in the discovery cohort. The association of PTGIS rare variants with IPAH was confirmed in the replication cohort. In the combined data set, PTGIS rare variants were found in 14 of 230 cases (6.1%) and 8 of 968 controls (0.8%) (odds ratio, 7.8; 95% CI, 3.2-18.8; P = 5 x 10^{-6}, logistic regression). Compared with patients without PTGIS variants, inhaled iloprost induced a more significant decrease of pulmonary vascular resistance (difference in the least square mean, −21.7%; 95% CI, −31.4% to −12.0%; P < .001, linear regression model) and an increase of cardiac index (difference in the least square mean, 18.3%; 95% CI, 8.8%-27.8%; P < .001, linear regression model) in patients with PTGIS variants. The minigene assay indicated that the c.521 + 1G>A variant resulted in aberrant messenger RNA transcripts. The functional studies showed that the 2 missense rare variants (R252Q and A447T) resulted in a decrease in prostacyclin production and increased cell death of pulmonary microvascular endothelial cells.

CONCLUSIONS AND RELEVANCE This study identified 3 rare loss-of-function variants in the PTGIS gene from 2 independent cohorts with IPAH. The genetic variants of PTGIS predispose pulmonary vascular responses to the iloprost stimulation. These findings suggest that PTGIS variants may be involved in the pathogenesis of IPAH.
Pulmonary arterial hypertension (PAH) is a rare disease with an estimated prevalence of 15 to 50 cases per 1 million adults. Approximately 40% of PAH occurs in individuals without any family history or predisposing conditions, when it is termed idiopathic PAH (IPAH). The pathogenesis of IPAH is complicated and largely unknown. The prognosis is still poor, with a 5-year survival rate at 50%.

Genetic variants are closely associated with PAH. To date, at least 17 PAH risk genes have been reported. However, all known predisposing genes, such as BMPR2, account for only 17% of patients with IPAH. Taking the most predominant causal gene, bone morphogenetic protein receptor type 2 (BMPR2), as an example, its variants only account for 17% of patients with IPAH. For other known predisposing genes, such as CAV1 and KCNK3, the genetic variants are rare in patients with IPAH.

In addition to the unexplained etiology of IPAH, the reported PAH disease genes are not relevant to any pathways corresponding to important therapeutic targets. For example, the dysregulation of the prostacyclin metabolic pathway in patients with PAH has been long documented. The production of prostacyclin is significantly decreased during the onset of PAH. Four drugs (epoprostenol sodium, iloprost, treprostinil sodium, and beraprost sodium) targeting the prostacyclin pathway have been developed and recommended for the treatment of advanced PAH. However, whether the genetic variants in the prostacyclin pathway influence the initiation and development of IPAH is largely unknown. Thus, large-scale genetic analyses using cutting-edge technology are crucial for identification of additional IPAH-susceptible loci, especially those involved in clinical management of IPAH.

Liu et al have previously reported a mutation rate of 14.5% for BMPR2 in Chinese patients with IPAH. In the present study, we sought to perform whole-genome sequencing (WGS) in a cohort of patients with IPAH and without the BMPR2 variants to identify novel genetic variants associated with IPAH, with subsequent evaluation of pulmonary vasodilator responsiveness in patients with the identified variants, followed by functional characterization of the genetic variants.

Methods

Study Population

The study participants were patients with incident IPAH who were recruited from 2 pulmonary hypertension referral centers in China: Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, and FuWai Hospital, Chinese Academy of Medical Sciences, Beijing. Baseline hemodynamic measurements and pulmonary vasodilator testing were performed in patients with IPAH before starting pulmonary vasodilator therapy. Pulmonary arterial hypertension was diagnosed as a mean pulmonary arterial pressure of at least 25 mm Hg at rest, a pulmonary artery wedge pressure of no greater than 15 mm Hg, and pulmonary vascular resistance of greater than 3 Wood units. The diagnosis of IPAH was made by at least 2 experienced PAH experts (X.-Q.X., X.J., L.W., X.-C.J.). Patients with known causes of PAH listed in the classification of the current guideline were excluded, including hereditary hemorrhagic telangiectasia, drug-induced PAH, connective tissue disease, congenital heart disease, and chronic thromboembolic pulmonary hypertension. The rare variants of the BMPR2 gene appear to contribute higher susceptibility to idiopathic pulmonary arterial hypertension, and screening of PTGIS variants may help improve personalized treatment of these patients.

Data were collected from January 1, 2000, to July 31, 2015, and analyzed from August 1, 2015, to May 30, 2018. Results were recorded as percentages, median (interquartile range), or
mean (standard error of mean or SD), as indicated. The normality of data distribution was assessed using Kolmogorov-Smirnov test. A χ² test or Fisher exact test was applied to compare qualitative variables and genotype/allele frequencies. For quantitative variables of clinical characteristics between the discovery and replication cohorts, statistical significance was determined using an unpaired t test or 1-way analysis of variance. The association between the genetic variants and disease status was assessed using logistic regression. The effect of genetic variants on clinical phenotype was analyzed by the linear regression model, which had genotype and baseline measurement as predictors. All the statistical testing was 2 sided. Results were considered statistically significant at a level of P < .05. All analyses were performed with PASW Statistics, version 18.0 (SPSS, Inc).

Results

Study Patients

In the discovery cohort, 42 patients with IPAH (including 10 pediatric patients) were recruited from Shanghai Pulmonary Hospital and FuWai Hospital. For the replication cohort, 188 patients (including 27 pediatric patients) were enrolled. In total, 230 patients with IPAH were recruited from the 2 national referral centers (Table I) (164 female [71.3%] and 66 male [28.7%]; mean [SD] age, 34 [18] years).

Whole-Genome Sequencing

Whole-genome sequencing was performed in the discovery cohort. To identify new pathogenic variants under the dominant model, we analyzed the genome data and screened out potential deleterious heterozygous variants with the minor allele frequencies of less than 0.5% in 4 variant databases and absent in the Chinese population from the 1000 Genomes Project (eTable 2 in the Supplement). A total of 1386 rare variants were predicted to affect 1772 candidate genes. Most of these genes ([551 [87.5%]) harbored 1 variant in a single case. Only 15 genes (0.8%) harbored rare variants shared by 3 or more individuals (eTable 2 in the Supplement). Four genes, including PTGIS (Gene ID 5740), MACF1 (Gene ID 23449), GTF3C1 (Gene ID 2979), and ABCA3 (Gene ID 21), are abundantly expressed and absent in the Chinese population from the 1000 Genomes Project. For the known PAH-related genes, we identified 14 patients (including 2 pediatric cases) carrying rare variants in the known PAH-related genes.

In the replication cohort, we screened all exons and splicing sites of PTGIS by Sanger sequencing (eTable 3 in the Supplement) and detected another 6 cases carrying the variant A447T.

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In the replication cohort, we screened all exons and splicing sites of PTGIS by Sanger sequencing (eTable 3 in the Supplement) and detected another 6 cases carrying the variant A447T. The prevalence for PTGIS A447T variant was comparable between the discovery (2 of 42 [4.8%]) and replication (6 of 188 [3.2%]) cohorts (χ² = 0.0013; P = .97, continuity-adjusted χ² test). In addition, we detected another rare missense PTGIS variant, c.755G>A (p.Arg252Glu [R252Q]; rs759344518) in 5 cases in the replication cohort (eFigures 2A and 3 in the Supplement). Given the variant R252Q passed the population frequency filters (minor allele frequency, <0.5%, and absent in Chinese population from the 1000 Genomes Project), it was included in further analysis. The copy number variations and structure variations of PTGIS were analyzed by in silico tools in the discovery cohort and by real-time polymerase chain reaction analysis in the replication cohort. No such variant was found (eFigure 4 in the Supplement). In total, we identified 14 patients (including 2 pediatric cases) carrying PTGIS rare variants. No significant difference was observed in the genetic variant rate between pediatric and adult cases with PAH (2 of 37 [5.4%] and 12 of 193 [6.2%], respectively; P > .99, continuity-adjusted χ² test).

Table 1. Demographic, Clinical, and Invasive Hemodynamic Characteristics of the 2 IPAH Cohorts

<table>
<thead>
<tr>
<th>Demographic characteristics</th>
<th>IPAH cohort*</th>
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<tr>
<td></td>
<td>WGS discovery (n = 42)</td>
<td>Replication (n = 188)</td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis, y</td>
<td>23 (10)</td>
<td>37 (18)</td>
<td></td>
</tr>
<tr>
<td>Female, No. (%)</td>
<td>34 (81.0)</td>
<td>130 (69.1)</td>
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<tr>
<td>6-min walking distance, m</td>
<td>425 (96)</td>
<td>379 (139)</td>
<td></td>
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</table>

Abbreviations: IPAH, idiopathic pulmonary arterial hypertension; mPAP, mean pulmonary artery pressure; PAWP, pulmonary artery wedge pressure; PVR, pulmonary vascular resistance; RAP, right atrial pressure; SvO₂, mixed venous oxygen saturation; WGS, whole-genome sequencing; WHO, World Health Organization.

*Unless otherwise indicated, data are expressed as mean (SD).

bCompared with the WGS discovery cohort, P < .05, unpaired t test.

cRange from I to IV, with higher numbers indicating greater functional limitations.
homogeneous between cases with IPAH and controls (eFigure 5 in the Supplement). The kinship analysis was performed using the exome data from 42 cases and 508 controls. More than 99.9% of the kinship values between cases with IPAH and controls were less than 0.0884, and the maximum kinship value was 0.1400, suggesting that participants in the study were unrelated. For the 188 cases and 460 controls who did not have the exome data, we carefully checked the recorded ancestry information and confirmed that they were unrelated individuals. In total, we identified 6 participants carrying the variant A447T and 2 with the variant R252Q, and no splicing variant was detected in the control cohort. The R252Q A allele (5 of 230 [2.2%] vs 2 of 968 [0.2%]; P = .005, logistic regression) and A447T A allele (8 of 230 [3.5%] vs 6 of 968 [0.6%]; P = .001, logistic regression) were associated with a higher risk of IPAH (Table 2). In terms of the genetic burden, the rare variant frequency was 6.1% (14 of 230) in the patients with IPAH compared with 0.8% (8 of 968) in the control group (odds ratio, 7.8; 95% CI, 3.2-18.8; P = 5.0 × 10⁻⁶, logistic regression) (Table 2). These results strongly suggest that the PTGIS locus may contribute to the pathogenesis and pathophysiology of IPAH.

All 3 PTGIS variants were located in the conserved region (eFigure 2B in the Supplement) and were predicted to be deleterious by in silico analysis (eTable 4 in the Supplement). According to the crystal structure of PTGIS, A447 is located at the highly conserved N-terminus of the alpha L helix of the protein, which is within 3.5 Å of the active site (eFigure 2C in the Supplement). The A447T produces a larger side chain, which could hinder the ligand-binding space. Moreover, a swap of nonpolar alanine for polar threonine will alter the electrostatics of the binding site, which is likely to cause a change in the PTGIS catalytic activity (eFigure 2D in the Supplement).

### Clinical Phenotypes of Cases With PTGIS Rare Variants

The clinical, functional, and hemodynamic characteristics of the 14 patients with IPAH carrying the PTGIS variants are shown in eTable 5 in the Supplement. Noticeably, these patients were diagnosed with IPAH at a young age (median, 26 [range, 17-70] years). Significant sex bias was also observed in these 14 patients, with a female-to-male ratio of 6:1.

To determine whether the identified PTGIS variants were correlated with different responses to prostacyclin, we compared the acute hemodynamic response to iloprost, a synthetic analogue of prostacyclin, between 12 patients with PTGIS variants (mean [SD] age, 32 [19] years; female-to-male ratio, 10:2) and 36 age- and sex-matched patients without PTGIS variants (mean [SD] age, 32 [8] years; female-to-male ratio, 31:5). Two patients with PTGIS variants were excluded from this analysis because they did not undergo iloprost testing at baseline. The changes from baseline were analyzed by a linear regression model, which had genotype and baseline measurement as predictors. As shown in Figure 1 and eTable 6 in the Supplement, the pulmonary vascular resistance decreased (difference in the least square mean, −21.7%; 95% CI, −31.4% to −12.0%; P < .001, linear regression model), and the cardiac index increased (difference in the least square mean, 18.3%; 95% CI, 8.8% to 27.8%; P < .001, linear regression model) more significantly in patients with PTGIS variants than those without. Thus, genetic variants of PTGIS predisposed pulmonary vascular responses to the iloprost stimulation.

### Interference of PTGIS Rare Variants With Enzyme Function

It has been shown previously that PTGIS is downregulated in the lung vasculature of patients with severe PAH.¹⁸ To confirm this finding, we examined the expression level of Ptgis in 3 experimental PAH rat models. Compared with healthy control rats, Ptgis messenger RNA levels significantly decreased in the monocrotaline-treated lungs (50% reduction; P = .03, analysis of variance), lungs with hypoxia (41% reduction; P = .04, analysis of variance), and lungs with sugen treatment (SU-5416) plus hypoxia (55% reduction; P = .02, unpaired t test) (eFigures 6 and 7 in the Supplement). These results were consistent with the decrease of PTGIS in the lungs of patients with PAH, indicating that an optimal level of PTGIS is necessary for functional homeostasis of the pulmonary vasculature.

The rare variant c.521 + 1G>A replaced the almost invariant GU (guanine-uracil) with an AU (adenine-uracil). To determine the effect of c.521 + 1G>A on the PTGIS transcription, we performed a minigene assay (Figure 2 and eFigure 8 in the Supplement). When transfected into the HEK293T cells, the mutant plasmid produced 2 distinct transcripts, including 1 with exon 4 skipping (dim band in Figure 2, right panel) and 1 resulting from an activation of a cryptic splice site in the intron 4 (bright band in Figure 2, middle panel). Exon 4 skipping caused an in-frame deletion of 144 base pairs (bp) in the PTGIS transcript (Figure 2, right panel) that was predicted to have a 48-amino acid deletion (p.Thr127_Arg174del) within the region homologous to cytochrome P450 superfamily according to the CATH database, which could lead to a compro-

<table>
<thead>
<tr>
<th>Nucleotide change</th>
<th>Discovery (n = 42)</th>
<th>Replication (n = 188)</th>
<th>Combined cases (n = 230)</th>
<th>Controls (n = 968)</th>
<th>P value*</th>
<th>OR (95% CI)*</th>
</tr>
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<tbody>
<tr>
<td>c. 521 + 1 G&gt;A</td>
<td>1</td>
<td>0</td>
<td>1 (0.4)</td>
<td>0</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>c. 755 G&gt;A</td>
<td>0</td>
<td>5</td>
<td>5 (2.2)</td>
<td>2 (0.2)</td>
<td>.005</td>
<td>10.7 (2.1-55.7)</td>
</tr>
<tr>
<td>c. 1339 G&gt;A</td>
<td>2</td>
<td>6</td>
<td>8 (3.5)</td>
<td>6 (0.6)</td>
<td>.001</td>
<td>5.8 (2.0-16.8)</td>
</tr>
<tr>
<td>Combined</td>
<td>3</td>
<td>11</td>
<td>14 (6.1)</td>
<td>8 (0.8)</td>
<td>5 × 10⁻⁶</td>
<td>7.8 (3.2-18.8)</td>
</tr>
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**Abbreviations:** NE, not estimable; OR, odds ratio; PTGIS, prostacyclin synthase.

* Calculated with the logistic regression model.
mised enzymatic activity. Furthermore, 1 cryptic donor 371 bp downstream of exon 4 was activated, resulting in a premature termination codon immediately following the transcript product from exon 4 (Figure 2, middle panel). Therefore, the rare variant c.521 + 1G>A resulted in 2 types of aberrant messenger RNA transcript, and both transcripts may be translated into loss-of-function PTGIS protein.

In the lung, PTGIS is predominantly localized in pulmonary endothelial cells, where it plays a critical role in regulating endothelial cell viability, apoptosis, and integrity. To investigate the consequences of the 2 missense PTGIS rare variants, we transfected the pulmonary microvascular endothelial cells (PMECs) with PTGIS wild-type (WT) and variant plasmids and analyzed the effect of the PTGIS variants on the enzyme activity and function by measuring 6-keto-prostaglandin F1α production. Immunoblot analysis showed similar protein levels in the total lysates of PMECs expressing the WT or 2 missense variant subunits, indicating that the variant proteins had similar stability to the WT protein (eFigure 9 in the Supplement). Both R252Q and A447T variants had significantly reduced PTGIS activities. At the baseline level, both variants generated only 57% (P < .001) and 50% (P < .001) of 6-keto-prostaglandin F1α levels when compared with WT transfected cells. Under the hypoxic condition, the levels decreased by 33% (P < .001) and 34% (P < .001), respectively (Figure 3).
We compared PMEC viability caused by ectopic overexpression of WT PTGIS or mutants. Wild-type PTGIS was first overexpressed in PMECs and then cultured under hypoxic or normoxic conditions. As shown in eFigure 10 in the Supplement, cell viability increased by 20% and 30% when compared with empty vector-transfected cells under respective conditions. Although the exogenously expressed R252Q variant affected little of the cell viability, the A447T variant reduced cell viability at the baseline condition (9.6%; \( P < .001 \)) and under hypoxic conditions (12.0%; \( P = .01 \)) when compared with WT vector-transfected cells (eFigure 10 in the Supplement). Moreover, R252Q and A447T variants attenuated the antiapoptotic effect of WT PTGIS either at baseline or in response to the stimulation of tumor necrosis factor (TNF) and cycloheximide (CHX). \( P \) values were calculated using analysis of variance.

All experiments were repeated in 3 to 4 times. A, After plasmid transfection, the 6-keto-prostaglandin F\(_{1\alpha}\) (PGF\(_{1\alpha}\)) concentration was measured in pulmonary microvascular endothelial cells (PMECs) culture supernatant. B, The effect of the variants of PTGIS on apoptosis in PMECs was assessed by caspase 3/7 activity after stimulation with tumor necrosis factor (TNF) and cycloheximide (CHX). C, The effect of the variants of PTGIS on angiogenesis in PMECs was assessed by tube formation.

Discussion

Using data from WGS of patients with IPAH, we demonstrated that PTGIS might be a PAH susceptibility gene. Importantly, these patients lacked any BMPR2 variants, which suggests that the IPAH susceptibility of PTGIS variants is independent of BMPR2. The rare PTGIS variants identified in the discovery and replication cohorts conferred a greater odds ratio of 7.8-fold to develop IPAH. Furthermore, patients with PTGIS variants were more sensitive to iloprost stimulation. Functional studies revealed that the PTGIS splicing variant interfered with its transcription, and the 2 missense variants caused impaired enzyme activity, resulting in decreased viability of PMECs. These findings suggest that the rare loss-of-function variants of PTGIS may contribute to the genetic etiology of IPAH (eFigure 12 in the Supplement).

The advances in sequencing technology accelerate our understanding for human diseases. Recently, several novel PAH disease gene or susceptibility loci have been identified using WGS.\(^{21,22}\) However, identification of variants that are specifically associated with PAH is still difficult. To address this caveat, we first excluded the detrimental effect of BMPR2 variants. To minimize variability, we used the strategy of rare variant enrichment with stringent filtering criteria in the discovery cohort and found PTGIS as a candidate gene. We subsequently genotyped an additional 188 patients with IPAH and determined the association of the 3 risk alleles of PTGIS with IPAH.

Under physiological conditions, constitutive PTGIS couples with thromboxane-A synthase to regulate the prostacyclin-thromboxane A\(_{2}\) metabolism. The balance of prostacyclin-
thromboxane A₂ state is necessary for physiological homeostasis and functional endothelium. In contrast, under pathological conditions (eg, hypoxia, increased shear stress, and injury), the decreased expression of PTGIS in pulmonary endothelial cells leads to a deficiency in prostacyclin and a supraphysiological level of thromboxane A₂. Such a dysregulated prostacyclin axis would cause the apoptosis of lung endothelial cells and the remodeling of the pulmonary vasculature. In the present study, we identified 3 rare PTGIS variants that are overrepresented in patients with IPAH and without BMPR2 variants compared with healthy controls. Functional studies showed that all 3 rare variants exhibited an impaired function of PTGIS. Compared with R252Q, A447T is more proximal to the active site, which may explain why A477T was more deleterious than R252Q. Given the importance of PTGIS in the regulation of pulmonary vasculature tone, we reasoned that rare loss-of-function variants of PTGIS predispose to the risk of IPAH. Of note, the population minor allele frequency for A477T (0.31%) and R252 (0.16%) is higher in East Asian than in other populations (minor allele frequency, 0%-0.013% for R252Q, 0%-0.011% for A447T [gnomAD]). These findings may or may not only apply to individuals of East Asian ancestry, which needs further evaluation in future studies.

The inheritance of IPAH is autosomal dominant, whereas a female predominance is common. Compared to males, females with BMPR2 variants are about 2.5-fold more susceptible to familial PAH. Among ACVRL1 variant carriers who develop IPAH, the female-to-male ratio has been reported to be 3.5:1.0. In the present study, we also observed a sex bias in PTGIS rare variant carriers, with a female-to-male ratio of 6:1. The predominance of adult female carriers may be causative between sex hormones and PTGIS. Indeed, combined administration of estradiol-17β and progesterone to ovariectomized sheep increases levels of cytosolic phospholipase A₂ and cyclooxygenase-1 in uterine artery endothelial cells. Consistently, combined hormone administration also increases PTGIS levels in uterine artery vascular smooth muscle cells. Further studies are warranted to confirm the clinical characteristics of sex bias with respect to PTGIS rare variant carriers.

The previous study by Stearman et al has shown that functional PTGIS promoter polymorphisms exert a protective effect on the penetrance of BMPR2 variants. In the present study, our genetic data showed the significant deleterious effect of rare PTGIS variants on the development of IPAH independent of BMPR2. Given the strong association between rare PTGIS variants and IPAH, further studies are needed to assess whether these variants represent a true PAH disease state that is distinct from a grinder area or a potential contamination with secondary pulmonary hypertension. Moreover, our clinical data from acute pulmonary vasoreactivity testing demonstrate that rare PTGIS genetic variants are predictive of iloprost responsiveness, thereby providing a rationale for targeting the prostacyclin pathway as part of tailored therapies for managing patients with PTGIS variants.

Limitations
Our study has several limitations. First, because all the involved patients in the 2 cohorts had sporadic PAH and the DNA samples from the relatives were not available, familial segregation of rare PTGIS variants with PAH could not be demonstrated. Second, rare variant testing (eg, burden tests) was not performed owing to the small sample size, and future studies to validate these findings will be needed to explore results using contemporary rare variant statistical testing. Third, the pulmonary vascular response to iloprost was only measured at the acute phase for a relatively low number of patients with IPAH, with or without rare PTGIS variants. Future study among larger cohorts will be crucial to test the variability and prognostic value of rare PTGIS variants for long-term prostacyclin treatment or the relevance to different therapeutic strategy.

Conclusions
We have identified rare variants in the PTGIS gene that may represent a novel susceptibility factor for IPAH in Chinese patients. These variants may also modulate pulmonary vasodilator responsiveness to inhaled prostacyclin.

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Critical revision of the manuscript for important intellectual content: X-J Wang, Xu, Sun, K-Q Liu.

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