Critical Genomic Networks and Vasoreactive Variants in Idiopathic Pulmonary Arterial Hypertension

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Abstract

Rationale: Idiopathic pulmonary arterial hypertension (IPAH) is usually without an identified genetic cause, despite clinical and molecular similarity to bone morphogenetic protein receptor type 2 mutation-associated heritable pulmonary arterial hypertension (PAH). There is phenotypic heterogeneity in IPAH, with a minority of patients showing long-term improvement with calcium channel-blocker therapy.

Objectives: We sought to identify gene variants (GVs) underlying IPAH and determine whether GV differ in vasodilator-responsive IPAH (VR-PAH) versus vasodilator-nonresponsive IPAH (VN-PAH).

Methods: We performed whole-exome sequencing (WES) on 36 patients with IPAH: 17 with VR-PAH and 19 with VN-PAH. Wnt pathway differences were explored in human lung fibroblasts.

Measurements and Main Results: We identified 1,369 genes with 1,580 variants unique to IPAH. We used a gene ontology approach to analyze variants and identified overrepresentation of several

pathways, including cytoskeletal function and ion binding. By mapping WES data to prior genome-wide association study data, Wnt pathway genes were highlighted. Using the connectivity map to define genetic differences between VR-PAH and VN-PAH, we found enrichment in vascular smooth muscle cell contraction pathways and greater genetic variation in VR-PAH versus VN-PAH. Using human lung fibroblasts, we found increased stimulated Wnt activity in IPAH versus controls.

Conclusions: A pathway-based analysis of WES data in IPAH demonstrated multiple rare GV that converge on key biological pathways, such as cytoskeletal function and Wnt signaling pathway. Vascular smooth muscle contraction-related genes were enriched in VR-PAH, suggesting a potentially different genetic predisposition for VR-PAH. This pathway-based approach may be applied to next-generation sequencing data in other diseases to uncover the contribution of unexpected or multiple GV to a phenotype.

Keywords: whole-exome sequencing; pulmonary arterial hypertension; vasodilator responsive

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At a Glance Commentary

Scientific Knowledge on the

Subject: Although mutations in the bone morphogenetic protein receptor type 2 gene are known to be associated with heritable forms of pulmonary arterial hypertension and are occasionally found in patients with idiopathic pulmonary arterial hypertension (IPAH), little is known about the potential genetic underpinnings of IPAH. Moreover, there is phenotypic heterogeneity in IPAH, with a minority of patients showing long-term improvement with calcium channel-blocker therapy. We used whole-exome sequencing (WES) to identify gene variants that underlie IPAH and determine whether genetic variants differ in vasodilator-responsive IPAH (VR-PAH) versus nonresponsive IPAH (VN-PAH).

What This Study Adds to the

Field: Using a pathway-based analysis of WES data in 36 IPAH samples, we demonstrated multiple rare genetic variants that converge on a few key biological pathways, such as cytoskeletal function and Wnt signaling pathway. Vascular smooth muscle contraction-related genes were enriched in VR-PAH and suggest a potentially different genetic predisposition for VR-PAH versus VN-PAH. Differential Wnt pathway responses to stimulation were detected between IPAH and control lung fibroblasts, suggesting functional differences in this pathway in human IPAH. This pathway-based analytic approach may be applied to other diseases to uncover the role of multiple genes in a disease phenotype.

Pulmonary arterial hypertension (PAH) is a highly mortal disease with limited therapies characterized by progressive vascular obliteration and right heart failure (1). Although the association of rare variants with heritable forms of PAH (HPAH) has contributed substantially to the understanding of PAH pathogenesis, whether rare genetic variants (GVs) contribute to the development of idiopathic PAH (IPAH) is presently understudied

(2–4). Understanding the genetic determinants of IPAH may identify underlying disease mechanisms and new therapies. Moreover, there is substantial variability in responses to current PAH-directed therapy across all subtypes of PAH, and genetic predictors of response to therapy are presently unknown (5).

IPAH and HPAH share common pathology and are clinically indistinguishable (2). However, approximately 80% of patients with HPAH have mutations in the gene bone morphogenetic protein receptor type 2 (BMPR2) (6, 7), but only a minority of patients with IPAH have mutations in this receptor (8). Furthermore, in HPAH, pulmonary vascular resistance generally cannot be improved acutely by administration of vasodilators such as nitric oxide (9). However, in IPAH approximately 10% of patients normalize pulmonary artery pressure with administration of vasodilators, and these patients have markedly improved survival (2, 10, 11). We recently showed that patients with this vasodilator-responsive IPAH (VR-PAH) phenotype have different transcriptomic patterns in peripheral blood compared with those without a response (VN-PAH) (12), but whether GV drive these RNA expression patterns is presently unknown.

Whole-exome sequencing (WES) has been used to identify Mendelian genes in familial cardiopulmonary and other diseases (13–16). More recently this technique has been used to identify *de novo* GV associated with pulmonary vascular disease (15, 17–19). In addition, analytic techniques applied to WES data can identify the presence of polygenic disease (20, 21), which is particularly useful when conditions are not clinically known to be heritable. To date, there is a single report of WES in IPAH, which used a candidate gene approach for analysis (3). However, a discovery-based approach has not been used to analyze WES data in this disease, and, additionally, the overlay of WES data on prior genetic studies in IPAH may allow discovery of important, previously unrecognized GV. Furthermore, techniques such as these may uncover the role of multiple genes or point to new, unexpected variants within a pathway that contribute to disease risk or pathogenesis.

We hypothesized that GV may predispose to the development of IPAH and

that GV that underlie VR-PAH will differ from VN-PAH. We tested this hypothesis using WES in two cohorts of well-phenotyped patients with IPAH, both VR-PAH and VN-PAH, and further explored an identified pathway in human lung fibroblasts from IPAH and healthy control subjects.

Methods

Patients

Patients with IPAH were recruited from two centers: Vanderbilt University Center for Pulmonary Vascular Disease and the University of Chicago. All patients provided informed consent (institutional review board #9401 Vanderbilt University Medical Center) and peripheral blood was obtained for DNA. Patients were carefully phenotyped in accordance with published guidelines (2) as outlined in the METHODS section and Figure E1 in the online supplement.

WES

WES and genotyping were performed at the Center for Inherited Disease Research Core Facility, Johns Hopkins School of Medicine (Baltimore, MD). Please see the METHODS section of the online supplement for further details.

Variant Call and Assessment

We conducted two phases of sequencing analysis (Figure 1). Details of our variant calling and filtering strategy can be found in the METHODS section of the online supplement.

Functional Enrichment Analysis

To assess the function of the genes harboring variants detected from this WES project, we conducted functional enrichment tests using the online tool KOBAS (22). Those gene ontology (GO) terms or pathways were considered significantly enriched if their adjusted *P* value was <0.05, which was calculated by the hypergeometric test followed by the Benjamini-Hochberg multiple testing correction (23), as implemented in the KOBAS tool.

Connectivity Map Analysis of Drug Relatedness and Microarray Analysis

Drugs included in the connectivity map (cMAP) analysis were either known calcium channel blockers or known to elicit

Figure 1. Filtering strategy to identify rare genetic variants in whole-exome sequencing data. A total of 11,981 variants were filtered to 1,580 gene variants. CIDR = Center for Integrated Disease Research; IPF = idiopathic pulmonary fibrosis; PolyPhen2 = Polymorphism Phenotyping version 2; SIFT = Sorting Intolerant from Tolerant.

vasodilation in VR-PAH. Details of cMAP analysis can be found in the online supplement.

Network Analysis of Candidate IPAH Genes

To form a connected pathway for our potential gene set, we first constructed a nonredundant human pathway-based interactome based on the consecutive metabolic reactions and signaling transduction data from HumanCyc, NCI signaling pathway database, Reactome, and PANTHER pathway databases (downloaded from Pathway Commons database [24] on April 19, 2014 from <http://www.pathwaycommons.org/pc2/downloads.html>). Further details can be found in the online supplement.

Lung Fibroblast Isolation and Culture, Western Analysis, Wnt Activity Assay, Polymerase Chain Reaction

See the METHODS section of the online supplement.

Statistical Methods

See the METHODS section of the online supplement.

Results

Patient Characteristics

We selected 36 patients with IPAH for WES. Demographic data are presented in Table E1. Of these, 12 were from University of Chicago and 24 from Vanderbilt University Medical Center; 17 met criteria for VR-PAH and 19 met criteria for VN-PAH.

WES Results

In total, 215,406 variants were identified in 36 IPAH samples in the WES results. To narrow down the candidate variants, a strict filtering strategy was performed (Figure 1; see online supplement for details). After this initial variant filtering, we identified 1,580 variants in 1,369 genes in our 36 patients (Table E2). Among the detected 1,580 variants, 57 of these were small

insertions or deletions, and the remaining 1,523 were single-nucleotide polymorphisms (SNPs). In the VR-PAH group we identified 789 variants in 727 genes, and in the VN-PAH group we identified 797 variants in 722 genes (several variants are present in both groups and thus counted twice).

GO Term Enrichment Analysis

To obtain a functional overview for our identified mutated genes, we performed GO term enrichment analysis of the 1,369 mutated genes. Using all the human protein-coding genes as background, the statistically overrepresented pathways in descending order of strength of association were as follows: (1) microtubule motor activity, (2) ion binding, (3) carbohydrate derivative binding, (4) motor activity, (5) anion binding, (6) adenylyl ribonucleotide binding, (7) small molecule binding, (8) adenylyl nucleotide binding, (9) cell adhesion, (10) biological adhesion, (11) extracellular matrix, (12) extracellular matrix part, and (13) ATP binding (Table 1). Although overall these results suggest similar GO term groups as in prior publications on transcriptomic changes in PAH (6), particularly in the cytoskeletal findings, they also point to differences in ion and small molecule binding, on the basis of anionic binding, small molecule binding, ATP binding, and adenylyl ribonucleotide binding above, on a genomic level in this PAH population enriched for VR-PAH.

Overlay of WES-identified Variants with Genome-Wide Association Study of Common Variants

We next sought to explore the intersection of common variants previously identified through genome-wide association studies (GWASs) in IPAH with our own identified rare variants (25). We mapped the 1,580 novel mutations to the genome and compared this map with 318 SNP peaks identified from a prior GWAS in PAH (18), as these peaks represent moderately associated genomic regions in patients with IPAH (Figure 2) (26–29). In total, we found eight regions that are statistically significant ($P < 0.01$, hypergeometric test), including chr2:32100000–36600000, chr3:79800000–83500000, chr3:87200000–87900000, chr5:66700000–68400000, chr5:139500000–144500000, chr10:12200000–17300000, chr19:0–6900000, and chr17:75300000–81195210.

Table 1. Statistically Overrepresented Gene Ontology in 1,369 Genes with Identified Variants

GO Term	GO ID	P Value	Corrected P Value*
Microtubule motor activity	GO:0003777	9.94×10^{-7}	3.73×10^{-3}
Ion binding	GO:0043167	1.67×10^{-6}	3.73×10^{-3}
Carbohydrate derivative binding	GO:0097367	2.33×10^{-6}	3.73×10^{-3}
Motor activity	GO:0003774	2.84×10^{-6}	3.73×10^{-3}
Anion binding	GO:0043168	2.90×10^{-6}	3.73×10^{-3}
Adenyl ribonucleotide binding	GO:0032559	4.37×10^{-6}	3.91×10^{-3}
Small molecule binding	GO:0036094	4.46×10^{-6}	3.91×10^{-3}
Adenyl nucleotide binding	GO:0030554	4.56×10^{-6}	3.91×10^{-3}
Cell adhesion	GO:0007155	5.48×10^{-6}	3.95×10^{-3}
Biological adhesion	GO:0022610	6.03×10^{-6}	3.95×10^{-3}
Extracellular matrix organization	GO:0031012	6.14×10^{-6}	3.95×10^{-3}
Extracellular matrix part	GO:0044420	7.57×10^{-6}	4.36×10^{-3}
ATP binding	GO:0005524	7.89×10^{-6}	4.36×10^{-3}

Definition of abbreviation: GO = gene ontology.

*The P values are corrected using the Benjamini-Hochberg method (23).

In examining all the regions together, there were 346 WES-identified gene variants that overlapped to 318 GWAS peaks. We again performed a pathway analysis on these 346 genes using REACTOME and found that the five most overrepresented pathways in decreasing order were: (1) WNT5A-dependent internalization of FZD2, FZD5, and ROR2; (2) HSF1-dependent transactivation; (3) attenuation phase; (4) formyl peptide receptors; and (5) PKA activation in glucagon signaling (Table E3).

Multiple Analyses Suggested an Enrichment of Wnt Signaling Variants

Although our prior analyses were discovery based, we also sought to identify variants and pathways of interest in a hypothesis-driven approach. On the basis of prior publications suggesting a potential role for the Wnt pathway in PAH (30–32), we chose to focus on this pathway for more detailed study. First, we looked for Wnt variants in our WES variants that correlated to GWAS peaks as shown in Figure 2. Of the 436 genes, 20 were associated with Wnt signaling by REACTOME. In addition, we identified a region in chromosome 5 with a high degree of intersection between GWAS and WES that included a gene cluster with nine protocadherin genes related to Wnt signaling pathway (Figure 3), including *PCDHA12*, *PCDHAC2*, *PCDHB5*, *PCDHB6*, *PCDHB12*, *PCDHGA6*, *PCDHGB7*, *PCDHGA11*, and *PCDH12*.

In a second approach, we next sought to narrow the list of candidate variants by

examining GV shared by two or more patients (33, 34). There were 37 variants from 37 genes meeting this criterion (*ANKRD6*, *ANLN*, *AQPEP*, *CASP8AP2*, *CHD7*, *CRIPAK*, *CRYBB1*, *CSRP3*, *CTBP2*, *CUBN*, *DOPEY2*, *FCRL4*, *GNLY*, *GRID1*, *GTF2F1*, *GULP1*, *LRRC66*, *LTBP4*, *MROH2B*, *MTFR1*, *MUC5B*, *MYH13*, *NACC2*, *PRB3*, *REV1*, *RGMA*, *SNED1*, *SYTL5*, *TAS2R46*, *TPSG1*, *TTC31*, *TUBA3E*, *TYRP1*, *WIF1*, *ZAN*, *ZBTB41*, and *ZDHHC20*). In this smaller list of shared GVs, three Wnt signaling genes were found: *CTBP2*, *MYH13*, and *WIF1* (hypergeometric test, $P = 0.01$). Three of the Wnt genes were from regions other than the chromosome 5 variants identified above.

We next sought to define the frequency of Wnt variant genes in the 1,369 genes with identified variants in our cohort. There were 24 genes related to the Wnt signaling pathway detected in 24 patients (Table E4; hypergeometric test, $P = 3.05 \times 10^{-5}$). For example, different variants in *MYH13* were present in six patients with IPAH. One variant in *MYH13* (chr17:10216635, G->A) is shared between two samples. Taken together, these three separate hypothesis-driven analyses suggest a high degree of genetic variation in Wnt signaling pathway genes in the patients with IPAH studied.

Integration of WES Data to cMAP Expression Profiles Suggests Enrichment of Vascular Smooth Muscle Contraction Variants in VR-PAH

Recent data have suggested that there may be molecular differences between VR-PAH and VN-PAH (12, 35). We thus sought to

determine if different GVs or variant patterns underlie these two distinct clinical phenotypes. We tested the hypothesis that cMAP-derived expression patterns of vasodilator drugs could be used to prioritize the candidate variants in VR-PAH. Using our list of vasodilator medications (found in the METHODS section of the online supplement), we retrieved the cMAP Instance ID and found 56 expression profiles for 11 drugs. We next overlapped the cMAP top- and bottom-ranked probes with VR-PAH and VN-PAH gene variant lists (Figure 4A) to identify genes found in common (Table E5). In total, we found 26 cMAP up-regulated genes also shared by the VR-PAH group. Although 24 of the 26 genes represent distinct molecular activities, 2 of the 26 genes are involved in vascular smooth muscle contraction (hypergeometric test, $P = 3.90 \times 10^{-5}$). Another four genes related to vascular smooth muscle contraction were found in the gene list only from VR-PAH (hypergeometric test, $P = 0.018$).

We next tested the hypothesis that VR-PAH is associated with different pathway variants from VN-PAH and compared the most enriched by GO or cMap analysis or biologically relevant pathways in each phenotype (Table 2). There was overrepresentation of vascular smooth muscle contraction GVs in the VR-PAH cohort compared with the patients with VN-PAH (seven affected patients with VR-PAH with six genes vs. one affected patient with VN-PAH and one gene; Fisher exact test on the number of mutated samples, $P = 1.55 \times 10^{-2}$). Variants were identified in the following genes in VR-PAH: *ADCY4*, *ADCY8*, *GNAS*, *PLA2G4E*, *PPP1R12B*, and *RAF1*. More variants were also identified in cardiac muscle contraction in VR-PAH (four patients, four genes vs. one patient with one gene). These affected genes are *ATPIA4*, *CACNA2D3*, *RYR2*, and *UQCRI0*. In addition, although there were relatively similar numbers of individual variants presented in two or more patients in both VR-PAH and VN-PAH, the number of affected genes was different in each phenotype (Figure 4B, Table E5). There were 33 genes with more than one variant in VR-PAH and only 10 genes with more than one variant in VN-PAH (Fisher exact test on the mutated genes, two-tail $P = 5.00 \times 10^{-4}$). These data suggest a high frequency of GVs within both smooth muscle and cardiac contraction in

Figure 2. Circos plot to facilitate the identification and analysis of similarities and differences arising from comparisons of the 36 idiopathic pulmonary arterial hypertension exomes. Variation in genome structure between chromosomes (1–22, X, and Y) is demonstrated in this plot, which allows for demonstration of the frequency of variations according to phenotype. From outside to inside, the *outer rim* demonstrates the frequency of variants in the vasodilator-nonresponsive pulmonary arterial hypertension (VN-PAH) group according to chromosomal location, and the *second-most outer rim* demonstrates the frequency of variants in the vasodilator-responsive pulmonary arterial hypertension (VR-PAH) group according to chromosomal location. Next the frequency of genome-wide association study (GWAS) common variations (single-nucleotide polymorphisms) are sandwiched between the abundance of novel VN-PAH variants (*outer faint orange circle*) and the abundance of novel VR-PAH variants (*inner faint orange circle*). Relevant identified genes are shown in the *innermost circle* (see Figure 3).

VR-PAH and, more broadly, that VR-PAH may have a higher frequency of variants common in two or more patients, than does VN-PAH.

Network Analysis of Variants in Multiple Pathways

Because we did not identify a single variant that was uniformly associated with IPA, we

hypothesized that there might be pathways or biological networks of affected genes that were central to development of PAH. To test this hypothesis, we performed

Figure 3. Region of interest on chromosome 5. Magnification of a region of chromosome 5 on the Circos plot in Figure 2 for closer evaluation of a portion with a high degree of intersection between our whole-exome sequencing data and the published genome-wide association study (GWAS) dataset. This region included a gene cluster with nine protocadherin genes related to the Wnt signaling pathway. PAH = pulmonary arterial hypertension; VN-PAH = vasodilator-nonresponsive pulmonary arterial hypertension; VR-PAH = vasodilator-responsive pulmonary arterial hypertension.

a network analysis focusing on key pathways or gene ontology groups and high-variant gene clusters identified in our prior analyses (Table 2). We created a central mutation map for IPAH (Figure 5) in which there were 75 nodes and 107 links in total. Four of the five categories were fully connected. Only vascular smooth muscle contraction was disconnected from the other functional groups. This fully connected map showed the relevance of the Wnt signaling pathway, which potentially connected to the transforming growth factor (TGF)- β signaling pathway (of which *BMPR2* is a member) via four steps starting at *WIF1*, *CDC25C*, *SRC*, *PLA2G4A*, to TGF- β_1 . Although most of the 37 mutated genes (shared in at least two samples) are not linked to known biological processes, they may partly bridge the signaling events among Wnt signaling pathway, extracellular matrix, and TGF- β signaling pathway.

Sequence and Functional Validation

To confirm the accuracy of our WES data, we selected GV for validation using Sanger sequencing (Table 3). Rationale for selection of the variants and their frequency is in the online supplement (Table E6).

We further sought to determine if any of the variant pathways identified through WES have functional consequences using

skin fibroblasts cultured from patients with IPAH (32). Gene ontology analysis was performed via Webgestalt (36) using genes with altered regulation in skin fibroblasts in patients with IPAH compared with control subjects. The most highly significant gene ontology groups are listed in Table E7, and they include cell adhesion and developmental genes, including Wnt pathway members, similar to the patterns found in our WES analysis. Multiple Wnt-related genes have altered expression in sporadic PAH-derived skin fibroblasts, including *DKK2*, *FZD7*, *SFRP2*, *WIF1*, *WNT2*, and *WNT5A*, among others. These data demonstrate transcriptional patterns in human IPAH cells that reflect the pattern analysis of our WES data, suggesting biological function of the identified variant pathways.

To further validate the microarray and gene variant findings in the Wnt pathway, we undertook studies of lung fibroblasts isolated from patients with IPAH at the time of lung transplantation or healthy control failed lung donors (see Table E8 for demographic information). Fibroblasts have previously been shown to be important in PAH, and can be isolated from patients with IPAH (37, 38). We first tested the hypothesis that GVs in Wnt genes

may result in altered expression of Wnt genes. In our IPAH fibroblast lines, we found significantly increased expression of *SFRP1* and *SFRP2*, Wnt antagonists, and increased expression of *WISP1*, a downstream target of Wnt pathway activation ($P < 0.05$ for all; Figure 6). Examining protein expression, we found that *Wisp1* trended to be increased in IPAH compared with control fibroblasts but did not reach statistical significance ($P = 0.05$). Finally, we tested the hypothesis that Wnt pathway dysregulation via GVs could be detected in a functional assay using our previously described Wnt activity assay (32). We first measured baseline Wnt activity in our lung fibroblast lines indirectly via TCF/LEF luciferase activity and normalized to *Renilla* luciferase activity after transfection. We also stimulated our control and IPAH cell lines using Wnt3a and LiCl. We found no baseline differences in Wnt activity but significantly increased activity of this pathway when stimulated with LiCl. Our data suggest that there is broad dysregulation of the Wnt pathway in IPAH compared with control subjects and differential responses to stimulation of the Wnt pathway in IPAH compared with control subjects, suggesting a potential functional role for GVs in this pathway in IPAH pathogenesis.

Figure 4. Connectivity map (cMAP) analysis was used to define expression arrays associated with vasodilator drugs and prioritize genetic variants associated with vasodilator-nonresponsive pulmonary arterial hypertension (VN-PAH) and vasodilator-responsive pulmonary arterial hypertension (VR-PAH). (A) The areas of intersection are shown on this Venn diagram. There was overrepresentation of vascular smooth muscle genes in the VR-PAH cohort using this analysis ($P < 0.05$). (B) Heat map showing frequency of variants in specific pathway and number of affected pathways per patient. Rows represent individual patients and columns are affected pathways. Color key represents number of affected genes. down exp = decreased expression; IPAH = idiopathic pulmonary arterial hypertension; up exp = increased expression.

Discussion

We analyzed the genomic DNA of 36 subjects with IPAH using WES and enriched our cohort for the subphenotype of VR-PAH to identify genetic signatures of IPAH broadly and determine whether genetic

differences underlie clinical observations in VR-PAH compared with VN-PAH. Although no single rare variant was detected to explain all IPAH cases, multiple analytic approaches consistently highlighted the association of variants in several key pathways with IPAH of all types. In

particular, variation in the Wnt pathway was highly associated with IPAH. To demonstrate the capacity of our approach to understanding gene variant data, we performed functional studies of this pathway in human lung fibroblasts and were able to demonstrate differences in Wnt pathway stimulation in IPAH compared with control subjects. This validation enhances confidence in the analytic approach to identify other, potentially important and perhaps unexpected pathways or GVs. Furthermore, consistent with our hypothesis, we identified differences in variant frequency and affected pathways in the VR-PAH and VN-PAH, including enrichment of pathways relevant to pulmonary vascular smooth muscle contraction in the VR-PAH subphenotype. Our data support the concept that IPAH may be a disease with a genetic basis, or at least a genetic susceptibility; however, multiple variants within biologically important pathways may be required for the development of the IPAH phenotype.

Next-generation sequencing techniques such as WES are well known to identify hundreds of rare GVs in healthy individuals, complicating the identification

Table 2. Comparison of Pathways and Variant Frequency in Vasodilator-Responsive Pulmonary Arterial Hypertension versus Vasodilator-Nonresponsive Pulmonary Arterial Hypertension

	VR-PAH (n = 17)		VN-PAH (n = 19)	
	No. of Patients with Variant	No. of Affected Genes	No. of Patients with Variant	No. of Affected Genes
Wnt signaling	13	14	11	12
Vascular smooth muscle contraction	7	6*	1	1
Mutated variants with 2+ IPAH	12	33	10	10
Extracellular matrix organization	10	17	13	16
TGF- β signaling	5	7	3	3

Definition of abbreviations: IPAH = idiopathic pulmonary arterial hypertension; TGF = transforming growth factor; VN-PAH = vasodilator-nonresponsive pulmonary arterial hypertension; VR-PAH = vasodilator-responsive pulmonary arterial hypertension.

* $P = 0.0155$.

Figure 5. Central mutation map for idiopathic pulmonary arterial hypertension (IPAH). We performed a network analysis focusing on key pathways or gene ontology groups and high-variant gene clusters. The central mutation map for IPAH shows 75 nodes and 107 total links. Implicated linker genes were not identified in the variant analysis but are required to connect other identified gene variants in this analysis. At a minimum, four steps linked the candidate pathways of Wnt and transforming growth factor (TGF)- β ; starting from WIF1 to CDC25C to SRC to PLA2G4A to TGF- β_1 .

of variants that directly contribute to the disease of interest (39). This challenge may be overcome through the use of an appropriate filtering strategy that includes potentially important rare variants but appropriately excludes variants that are unrelated to disease (40). Our project used two techniques to address this issue: first,

we reduced variability by performing WES on a moderate number of well-phenotyped patients with IPAH, considering the frequency of the disease. Second, we used a filtering algorithm that used stringent criteria based on prediction of disease-causing variants. In particular we excluded GV's that have been identified in idiopathic pulmonary fibrosis (41), under the hypothesis that the two diseases have different molecular etiology. This strategy is grounded in basic science and clinical data, yet there remains a risk that pathogenic variants may be removed from the dataset and thus missed. Our strategy involved filtering common variants, which allowed focus but does inherently risk losing potentially important common SNPs. In addition, as with all WES, the data must be interpreted with some caution, as WES only evaluates coding regions; thus, potentially important variants in the noncoding genome will be excluded, epigenetic modifications are not assessed, and, moreover, not all variants identified were validated, although our validation rates are within published norms (42). Our initial filtering strategy did not show a single genetic variant, although we

Table 3. Results of Variant Validation through Resequencing

Gene Name	Gene ID	Variant Locus	Sequencing Results
ADCY8	114	chr8:131826409:131826409:A:G	Validated
COL15A1	1306	chr9:101748162:101748162:C:T	Validated
TGFB1	7040	chr19:41850733:41850733:G:A	Validated
LTBP4	8425	chr19:41119074:41119074:G:T	Failed
CTBP2	1488	chr10:126727616:126727616:A:-	Failed
MYH13	8735	chr17:10216635:10216635:G:A	Validated
MTFR1	9650	chr8:66620310:66620310:C:G	Validated
ANKRD6	22881	chr6:90337349:90337349:G:T	Validated
DNAH1	25981	chr3:52380743:52380743:G:A	Validated
FER1L5	90342	chr2:97335910:97335910:G:C	Validated
RGMA	56963	chr15:93616963:93616963:C:T	Failed
ZDHHC20	253832	chr13:21976996:21976996:T:C	Validated
CDH23	64072	chr10:73571088:73571088:G:A	Validated
		chr10:73563154:73563154:G:C	Validated
		chr10:73490244:73490244:G:T	Validated
		chr10:73468866:73468866:G:T	Validated
		chr10:73406479:73406479:G:C	Validated
PIM1	5292	chr6:37138142:37138142:G:T	Failed
CRCP	27297	chr7:65579834:65579834:G:T	Validated
HLA-DRB1	3123	chr6:32549501:32549501:A:T	Failed
WNT5A	7474	chr3:55513419:55513419:T:A	Validated
WIF1	11197	chr12:65445142:65445142:T:C	Validated
		chr12:65445142:65445142:T:C	Validated

Figure 6. Analysis of Wnt pathway in human lung fibroblasts. Lung fibroblasts were isolated from human patients with idiopathic pulmonary arterial hypertension and healthy, failed donor control subjects and used for these studies ($n = 3-4$ each). (A) Polymerase chain reaction of cell homogenates for SFRP1, SFRP2 (Wnt antagonists), and AXIN2 (canonical Wnt signaling) show increased expression of Wnt antagonists and the Wnt target gene, WISP1. (B) Western analysis for Wisp1 demonstrated a trend to increased Wisp1. (C) Wnt pathway activity was assessed using a dual luciferase assay at baseline and after 72-hour stimulation with Wnt3a and LiCl. Idiopathic pulmonary arterial hypertension cells demonstrated significantly greater stimulation of Wnt activity after LiCl stimulation. * $P < 0.05$. HPRT = hypoxanthine phosphoribosyltransferase; LiCl = lithium chloride; PAH = pulmonary arterial hypertension.

and other groups have identified single variants in other forms of heritable pulmonary vascular disease using advanced genetic analytic techniques (15, 18, 19, 43). In this context, given the possibility that multiple genes are required to develop some disease phenotypes, we hypothesize that IPAH may develop due to either (1) multiple GVs in certain key pathways, or (2) multiple affected key pathways within an individual patient. Using a gene ontology analysis, we were able to identify several pathways that were affected by GVs at a higher degree than expected in the general population. Several of these pathways could be combined broadly into cytoskeletal genes or ion-binding genes. Prior transcriptomic analyses in heritable PAH and IPAH and in other reports have pointed to cytoskeletal dysfunction in PAH

(6, 44, 45). Similarly, several animal models have found ion-channel dysfunction in pulmonary vascular disease (46–48), but the identification of these pathways as a potential genetic predisposition to IPAH is new. In addition, as our patient population was enriched in VR-PAH, the ion-binding genes were particularly promising as being important to this subset in particular.

Although just one of the many pathways highlighted by our variant analysis, we chose to focus on the Wnt pathway for further study as an example of further narrowing and understanding data from WES. Wnt pathway GVs were overrepresented within several chromosomal regions. Further strengthening this association are Wnt variants present in multiple patients. Finally,

to strengthen the gene variant findings, we tested the hypothesis that this pathway was of relevance to the IPAH (37, 38). Using human lung fibroblast lines, we found evidence of altered expression of Wnt pathway-modulating genes; borderline increased expression of Wisp1, a Wnt target regulating proliferation; and significantly increased Wnt activity in response to stimulation by LiCl. These functional studies in human cell lines from lung fibroblasts suggest functional alteration in a pathway identified through our gene variant analysis. In the future, transgenic rodent models querying all the identified variants may help to lend further specificity as to which variants in particular are important in PAH pathogenesis, and our analytic techniques to next-generation sequencing data may facilitate confident

identification of new GV and pathways involved in pathogenesis in IPAH or other diseases. Taken together, these analyses suggest a potential role for this pathway as a basis for a genetic predisposition in IPAH and are congruent with other reports demonstrating a role for Wnt signaling in pulmonary vascular remodeling (30, 32).

Our population was intentionally enriched in patients with VR-PAH, as usually this subphenotype accounts for less than 10% of patients with IPAH. We have shown differences in blood transcriptome in VR-PAH compared with VN-PAH, but whether GVs are associated with these different phenotypes was previously unknown. We used the cMAP as a tool to point to important pathways in VR-PAH compared with VN-PAH and identified two important features of VR-PAH, acknowledging the limitation that this tool is not specific to lung expression. First, vascular smooth muscle contraction genes were more enriched in the patients with VR-PAH than in those with VN-PAH. This may explain why many patients with VR-PAH reduce pulmonary arterial pressure when treated with several vasodilators with different mechanisms of action, as the defect may be a shared gene regulating smooth muscle cell contraction. In addition, the number of variants found in pathways per patient was higher in VR-PAH than in

VN-PAH, suggesting there may be either modification of important pathways in VN-PAH that result in the VR-PAH phenotype or, perhaps, a completely different genetic predisposition for VR-PAH than VN-PAH.

Our study has limitations including a relatively small patient population. Although our analysis did not identify a monogenic variant underlying all IPAH, the gene ontology approach was informative, showing that patients with IPAH have a high degree of GVs within certain pathways that have biological plausibility as contributing to PAH. In addition, although the analysis of common variations in small numbers of subjects is prone to false discovery, the overlay of WES data with common variant information previously published allowed for the reduction in genetic pathways of interest to focus our discoveries. The frequency of multiple variants in each pathway in individual patients suggests a “dose effect,” such that multiple variants within at least one pathway may be required to develop the IPAH phenotype. This observation suggests that IPAH may be a polygenic disease and reinforces the role of these key pathways in the molecular etiology of IPAH. Given the limits of WES for detection of mitochondrial genomic variants (49) and its inability to detect nonexomic variants or epigenetic (50) or downstream mediators,

these data do not exclude potentially important roles for these factors in PAH. The functional consequence of many variants identified in these analyses will need to be tested in animal models and in cell culture including pulmonary arterial and endothelial cells and also validated in other humans with IPAH. Finally, our samples did not all have matching expression data, which prevented quantitative trait locus analysis and analysis of genes previously associated with RNA expression differences in VR-PAH.

In conclusion, our systematic WES screening of GVs in 36 IPAH samples revealed that rare GVs may converge on a few key biological pathways, such as cytoskeletal function and Wnt signaling. Some of the mutated genes related to vascular smooth muscle contraction suggest a potentially different genetic predisposition for patients with VR-PAH and VN-PAH. The role of key gene nodes associated with IPAH needs to be confirmed in the future using patients with IPAH and preclinical models, but this pathway-based analytic approach that we have validated with functional studies may also be informative for other diseases to confidently uncover the role of multiple genes or unexpected GVs that may contribute to disease phenotype. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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