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Genetic Insights into Pulmonary Arterial Hypertension Application of Whole-Exome Sequencing to the Study of Pathogenic Mechanisms

Pulmonary arterial hypertension (PAH) is a rare, progressive, and fatal disease that predominantly affects women. Sustained pulmonary vasoconstriction and excessive pulmonary vascular remodeling are two major causes for the elevation of pulmonary vascular resistance (PVR) in patients with PAH. Without treatment,

the elevated PVR and pulmonary arterial pressure (PAP) increase the afterload to the right ventricle, which can progress to right heart failure and death. Despite significant progress in research, PAH remains a devastating disease with a poor long-term prognosis. The estimated median survival of patients with PAH is only 2.8 years, with 1-, 3-, and 5-year survival rates of 68, 48, and 34%, respectively (1). Current therapies remain insufficient with the absence of effective disease-modifying or preventive interventions. Investigations integrating novel and robust technologies and methods are

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urgently needed to help shed further insight into the molecular underpinnings of PAH and close these therapeutic gaps.

To date, the exact pathogenic mechanisms responsible for PAH are not fully understood; however, a number of potentially causative mutations in genes primarily related to PAH have been discovered via conventional linkage analysis and, more recently, next-generation sequencing (NGS) technologies. Rare variants in genes related to transforming growth factor β signaling, membrane receptors and ion channels, integral membrane proteins, serine-threonine kinases, and DNA-binding proteins have been reported in familial and sporadic cases of PAH (2–7). Most of these reports reveal gain-of-function or loss-of-function mutations, contributing to the development of PAH or leading to the increased risk of developing PAH. The first reports of genetic contributions to PAH were identified by gene linkage studies in which mutations in the gene encoding bone morphogenetic protein receptor type 2 (*BMPR2*) were found in approximately 75% of cases of heritable PAH and in ~20% of patients with idiopathic PAH (IPAH) (5, 6). Later, mutations in the genes encoding activin receptor-like kinase 1 (*ALK1*, also called *ACVRL1*) and endoglin (*ENG*), which are associated with hereditary hemorrhagic telangiectasia, were also reported to be associated with PAH (3, 8, 9). In rare cases, mutations in the genes encoding mothers against decapentaplegic homologs (*SMAD*), which are also involved in the transforming growth factor β /BMP signaling, have been identified as underlying causative factors in patients with PAH (10, 11). Furthermore, genetic variants in genes encoding canonical transient receptor potential 6 (*TRPC6*) and Kv1.5 (*KCNA5*) channels have been demonstrated to be associated with patients with IPAH, using a candidate gene sequencing approach (12, 13). Chida and colleagues screened for mutations in genes related to Notch3 signaling in 41 patients with PAH who had no other identifiable mutations of the genes known to be associated with PAH, and identified two novel missense mutations in *NOTCH3* in two patients (14).

Although genome-wide association studies have successfully reported numerous PAH susceptibility variants, in general, the risk alleles account for only a small proportion of estimated heritability (15, 16). Recently, Germain and colleagues first conducted a two-stage genome-wide association study of patients with IPAH and heritable PAH without detectable *BMPR2* mutations and identified the *CBLN2* locus associated with IPAH and heritable PAH (17). As with many other complex diseases, there are likely rare (<5% of the population) variants that exist in the pathogenesis of PAH that are not captured by genome-wide association studies, but could have a larger effect size while not being causative. With the decreasing cost of NGS technologies, whole-genome sequencing and whole-exome sequencing (WES) have enabled the rapid identification of many rare variants, some with a large effect size, that have facilitated the understanding of both Mendelian and complex traits. WES defines the protein-coding sequences of the human genome and has been a powerful tool for the detection of Mendelian variants. More recently, as the tool becomes cost-effective, there is a growing interest in the detection of potential variants in complex traits as well. In one of the earliest studies to apply WES to PAH, Austin and colleagues identified novel mutations in the gene encoding caveolin 1 (*CAV1*) in patients with either familial or idiopathic PAH (2). Using WES to study a family in which multiple members had PAH without mutations in *BMPR2*, *ALK1*, *ENG*, *SMAD*, and *CAV1*, Ma and

colleagues identified association of *KCNK3* with familial and idiopathic PAH (7). In another study using WES in PAH, Eyries and colleagues linked recessive variants of a single gene, *EIF2AK4* (also called *GCN2*), to pulmonary veno-occlusive disease development in all 13 families (18). More recently, by analyzing exome sequence data from 12 unrelated patients with IPAH lacking *BMPR2* mutations, de Jesus Perez and colleagues identified the DNA topoisomerase II-binding protein 1 (*TOPBP1*) gene, which is involved in the response to DNA damage and replication stress, as a risk factor for IPAH (4).

In this issue of the *Journal*, Hemnes and colleagues (pp. 464–475) have used WES in a small cohort of patients with IPAH that have been well-phenotyped with and without vasodilator responsiveness (19). The authors demonstrated multiple rare genetic variants that converge on cytoskeletal functions and the Wnt signaling pathway in patients with PAH. Importantly, using stringent filtering criteria and comparing it with idiopathic pulmonary fibrosis, the authors highlighted the potential for missed variants of significance. The current article opens up a new realm of possibilities in future PAH research and clinical practice as WES becomes more affordable. With WES, the potential to analyze known mutations (in *BMPR2*, *ALK1*, *KCNK3*, and *CAV1*, for example), as well as evaluate novel disease-modifying variants in patients with and without a family history of PAH, is now feasible with one approach. Although the authors did not find a variant across all forms of PAH, including vasodilator responsive and nonresponsive PAH, the technique also allows the search for these variants in other tightly phenotyped subgroups of PAH, such as scleroderma-PAH or HIV-associated PAH, in a research and clinical setting. Although no single SNP was significant across all patients with PAH in the current study, the use of pathway-based analyses, composed of several variants across multiple genes, highlighted WNT pathway-modifying genes associated with PAH. This observed molecular pathway was then validated in *in vitro* studies confirming elevated WNT activity in fibroblasts from patients with PAH. In addition, the authors identified (while low in sample size) a high frequency of genetic variations in smooth muscle cell (SMC) contraction genes and greater overall genetic variation per pathway in patients with vascular-responsive PAH than in patients with nonvascular response PAH; these novel observations strongly suggest genetic predisposition to the development of PAH, as well as the role of WNT pathway and SMC contraction genes in the development of PAH. Given that an increase in cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) is one of the major contributors to the development of PAH by inducing pulmonary arterial SMC (PASMC) contraction and pulmonary vasoconstriction, and by stimulating pulmonary vascular cell migration and proliferation, causing concentric pulmonary vascular wall thickening (20), Figure 1 shows a proposed mechanism by which the previously established PAH genes with observed rare variants, including those from the current study, link with the Ca^{2+} signaling cascade to induce pulmonary vasoconstriction and vascular remodeling.

In concert with previous findings in the genetics of PAH with identification of a number of causative genes, this study will undoubtedly open up new pipelines of research on PAH. However, these enlightening studies using NGS technologies raise several important issues. First, the role of the inherited variants remains difficult to ascertain, despite evidence to

Figure 1. Proposed mechanisms by which the genes associated with pulmonary arterial hypertension (PAH; based on emerging genetic sequencing studies) and the Wnt signaling cascade (based on the current study by Hemnes and colleagues) functionally interact with the Ca²⁺ signaling pathway to cause pulmonary arterial smooth muscle cell (PASMC) contraction and pulmonary vasoconstriction, PASMC proliferation/migration and pulmonary vascular wall thickening, and inhibition of PASMC apoptosis resulting in pulmonary vascular remodeling (19). On the basis of case-control studies, the current work by Hemnes and colleagues has found Wnt pathway and smooth muscle contraction pathway variants to be significantly overrepresented in PAH and in vasodilator-responsive PAH, respectively (19). Activation of Wnt signaling has been shown to induce Ca²⁺ release from the sarcoplasmic (SR) or endoplasmic (ER) reticulum by inositol 1,4,5-trisphosphate (IP₃)-mediated activation of IP₃ receptor, a Ca²⁺ release channel expressed in the ER/SR membrane. The Wnt-mediated Ca²⁺ mobilization from the SR/ER to the cytosol causes Ca²⁺ store depletion that subsequently opens the store-operated Ca²⁺ channels (SOC), formed by Orai1/2 and TRPC6 channel subunits, to induce store-operated Ca²⁺ entry (SOCE). Mutations in genes encoding KCNK3, a two-pore domain K⁺ channel, and KCNA5, a voltage-gated K⁺ channel, may lead to transcriptional down-regulation and/or functional inhibition of KCNK3 and KCNA5, causing membrane depolarization that opens voltage-dependent Ca²⁺ channels (VDCC) and induces voltage-dependent Ca²⁺ entry. Furthermore, up-regulated Notch3 and enhanced Notch signaling have been demonstrated to functionally activate receptor-operated Ca²⁺ channels (ROC) and induce receptor-operated Ca²⁺ entry (ROCE) via Notch intracellular domain (NICD) or other unknown mechanisms (22). Caveolin-1 (CAV1) has been shown to inhibit various K⁺ channels, cause membrane depolarization, and induce Ca²⁺ influx through VDCC. The increased cytosolic Ca²⁺ concentration ([Ca²⁺]_{cyt}) causes PASMC contraction, leading to pulmonary vasoconstriction, and stimulates PASMC migration and proliferation, leading to pulmonary vascular remodeling (or concentric pulmonary arterial wall thickening). Bone morphogenetic protein (BMP)/bone morphogenetic protein receptor type 2 (BMPRII)/Smad signaling has been demonstrated to induce PASMC apoptosis and inhibit PASMC and pulmonary arterial endothelial cell (PAEC) proliferation (23, 24). Mutations in the BMPRII gene identified in patients with idiopathic and heritable PAH result in the inhibition of PASMC apoptosis and enhancement of PASMC/PAEC proliferation, contributing to the development and progression of pulmonary vascular remodeling. Calmodulin (CaM) is a Ca²⁺-sensitive protein that physically interacts with Smad and induces Ca²⁺-dependent inhibition of transforming growth factor β /activin signaling (25, 26); this suggests that elevated [Ca²⁺]_{cyt} and activated CaM can lead to inhibition of BMP/BMPRII/Smad signaling, and ultimately to inhibited PASMC apoptosis and enhanced PASMC proliferation. DSH = dishevelled; ENG = endoglin; LRP = low-density lipoprotein receptor-related protein; Smad = mothers against decapentaplegic homologs.

support their genetic association with PAH susceptibility. How the causative genes associate with the PAH phenotype, and how several variations may interact with each other, remain unanswered. Clinical outcomes associated with the causative genes (and variants) may also exceed the boundaries of PAH because the same genes associated with PAH may also be associated with other cardiovascular disorders. Another current roadblock is the lack of knowledge in all of the pertinent

mechanisms through which these genes contribute to the development and progression of PAH. Further studies testing whether these variants lead to gain or loss of gene expression and activity, as well as their influence on PASMC contraction and pulmonary vascular cell proliferation, migration, and apoptosis, are required to validate their roles in the development and progression of PAH. In addition, although the technological sequencing methods have evolved and become more cost-

effective, a major bottleneck remains in the computational and bioinformatics methodology to analyze NGS data. We need improved tools in statistical and bioinformatics methods for reducing the rate of false-positive and false-negative variant calls, calling insertion/deletion, prioritizing candidate causal variants, and predicting and annotating the potential functional effect for disease gene discovery or molecular diagnostics (21). Although these issues appear daunting to tackle, leveraging these emerging genome-editing technologies in combination with the development of animal models that recapitulate the mutations of causative genes should lead to a more comprehensive understanding of the genetic and molecular causes of PAH.

In summary, WES and whole-genome sequencing have revealed a number of rare variants of genes involved in the development and progression of PAH and will continue to be an important tool for future research on PAH genetics. Larger cohorts of samples will be required in genetic studies, and therefore large-scale collaborative efforts involving international and national consortia, as well as physicians, pathophysiologists, geneticists, bioinformaticians, epidemiologists, and statisticians, are necessary to capitalize on these emerging technologies and their ability to dissect the underlying genetic mechanisms of PAH. Ultimately, these efforts and technologies can pave the way for personalized diagnostics and therapeutics in the clinical care for PAH. ■

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Continuous Positive Airway Pressure for Improving Glycemic Control in Type 2 Diabetes: Where Do We Stand?

Research over the course of the last decade has consistently shown that beyond the effects of obesity, the presence of obstructive sleep apnea (OSA) is associated with impaired glucose tolerance, insulin resistance, and increased risk of developing type 2 diabetes (1–5). Furthermore, both animal and human models of intermittent hypoxia and sleep fragmentation have revealed mechanistic links between OSA and alterations in glucose metabolism (6–9). Notably, among patients with type 2 diabetes, the prevalence of OSA appears to be exceedingly high, and increasing severity of untreated OSA has been associated with worsening glycemic control (10–13). Despite this overwhelming evidence for a strong link between OSA and type 2 diabetes, a clear benefit of treatment of OSA on glycemic control in patients with diabetes has yet to be demonstrated. Until recently, only one randomized controlled trial examined the effect of treatment of OSA on glycemic control in type 2 diabetes, and it reported no benefit after 3 months of continuous positive airway pressure (CPAP) in patients with poorly controlled diabetes (14). Now, almost a decade later, in two randomized controlled trials published in this issue of the *Journal*, Shaw and colleagues (pp. 486–492) (15) and Martínez-Cerón and colleagues (pp. 476–485) (16) report on the effects of CPAP treatment on glycemic control in patients with type 2 diabetes (Table 1).

In both trials, the intervention duration was 6 months, the control group received usual care, and HbA1c (a clinical marker of glycemic control) was the primary outcome. Shaw and colleagues conducted a relatively large multicenter trial and found no effect of CPAP treatment on HbA1c (15). In contrast, in the smaller trial by Martínez-Cerón and colleagues, CPAP resulted in a clinically meaningful improvement in glycemic control as compared with usual care after 6 months of intervention (16). It is noteworthy that the baseline demographics, including age, sex, and body mass index, were fairly similar between the two trials. Likewise, the severity of OSA was comparable despite separate criteria used for OSA diagnosis. So, what could be the potential explanations for the conflicting findings reported here?

An important aspect to consider is that the diabetes treatment regimens and degree of glycemic control at baseline varied between the two trials. In the study by Shaw and colleagues, about half of the patients were not taking any antidiabetes medications, and those receiving insulin therapy were excluded (15), whereas in the study by Martínez-Cerón and colleagues, almost all patients were treated with oral agents, and ~42% were receiving insulin therapy (16).

In addition, baseline HbA1c was lower by ~0.3% in the study by Shaw and colleagues (15). These observations are consistent with better glycemic control and milder disease at baseline in the study by Shaw and colleagues, which perhaps contributed to the failure of CPAP to further improve HbA1c (15). However, in post hoc analyses restricted to patients with poorer glycemic control at baseline (i.e., HbA1c >7.5%), Shaw and colleagues did still not find an effect of CPAP on glycemic control (15). Interestingly, in the study by Martínez-Cerón and colleagues, HbA1c levels did not change after CPAP treatment in the subgroup of patients who were receiving oral agents plus insulin therapy, whereas CPAP resulted in significant improvements of ~0.6% in HbA1c in those who were receiving oral agents alone (16). Overall, Martínez-Cerón and colleagues found no measurable change in antidiabetes medications between the groups at the end of the treatment period (16). Although these findings are intriguing, the question as to what extent antidiabetes regimens influence the ability of CPAP to improve glycemic control in patients with diabetes remains to be determined.

It is also worth noting that the mode of positive airway pressure was different between the two trials. The negative study by Shaw and colleagues used CPAP in auto-adjusting mode (i.e., auto-CPAP), whereas in the positive study by Martínez-Cerón and colleagues, fixed CPAP pressure was applied during the treatment period (15, 16). Interestingly, the prior negative study by West and colleagues (14) also used an auto-CPAP intervention. A few studies suggest that auto-CPAP may not be as effective as fixed CPAP in improving cardiometabolic outcomes (17, 18), perhaps as a result of greater sympathetic activation (19, 20). Although the precise mechanisms for differential effects of auto versus fixed CPAP are not known, it is possible that sleep fragmentation and mild obstructive respiratory events may persist with auto-CPAP, particularly at lower pressure settings. In addition, Shaw and colleagues reported an overall residual apnea–hypopnea index of ~6.2 from CPAP usage data, raising the possibility that a number of patients did not achieve optimal control of OSA, which thus may have attenuated the efficacy of CPAP to improve glycemic control (15). In support of this notion, studies have shown that mild OSA characterized mostly by arousals (21) or experimental sleep fragmentation (8, 9) adversely affects glucose metabolism in young, lean individuals.

Shaw and colleagues reported slightly lower CPAP adherence rates compared with Martínez-Cerón and colleagues, yet the authors found no effect of CPAP on glycemic control in post hoc