Genetic Risk Factors for Portopulmonary Hypertension in Patients with Advanced Liver Disease

Kari E. Roberts[1], Michael B. Fallon[2], Michael J. Krowka[3], Robert S. Brown[4], James F. Trotter[5], Inga Peter[6], Hocine Tighiouart[7], James A. Knowles[8], Daniel Rabinowitz[9], Raymond L. Benza[2], David B. Badesch[5], Darren B. Taichman[10], Evelyn M. Horn[4], Steven Zacks[11], Neil Kaplowitz[12], and Steven M. Kawut[10,13], for the Pulmonary Vascular Complications of Liver Disease Study Group*

[1]Department of Medicine, and [7]Institute for Clinical Research and Health Policy Studies, Tufts Medical Center, Boston, Massachusetts; [2]Department of Medicine, University of Alabama, Birmingham, Alabama; [3]Department of Medicine, Mayo Clinic, Rochester, Minnesota; [4]Department of Medicine, College of Physicians and Surgeons, and [9]Department of Statistics, Columbia University, New York, New York; [5]Department of Medicine, University of Colorado, Denver, Colorado; [6]Department of Genetic and Genomic Sciences, Mount Sinai School of Medicine, New York, New York; [8]Department of Psychiatry and [12]Department of Medicine, University of Southern California, Los Angeles, California; [10]Department of Medicine and [13]Center for Clinical Epidemiology and Biostatistics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania; [11]Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

***Rationale*: Portopulmonary hypertension (PPHTN) occurs in 6% of liver transplant candidates. The pathogenesis of this complication of portal hypertension is poorly understood.**

***Objectives*: To identify genetic risk factors for PPHTN in patients with advanced liver disease.**

***Methods*: We performed a multicenter case-control study of patients with portal hypertension. Cases had a mean pulmonary artery pressure >25 mm Hg, pulmonary vascular resistance >240 dynes·s^{-1}·cm^{-5}, and pulmonary capillary wedge pressure ≤15 mm Hg. Controls had a right ventricular systolic pressure < 40 mm Hg (if estimated) and normal right-sided cardiac morphology by transthoracic echocardiography. We genotyped 1,079 common single nucleotide polymorphisms (SNPs) in 93 candidate genes in each patient.**

***Measurements and Main Results*: The study sample included 31 cases and 104 controls. Twenty-nine SNPs in 15 candidate genes were associated with the risk of PPHTN ($P < 0.05$). Multiple SNPs in the genes coding for estrogen receptor 1, aromatase, phosphodiesterase 5, angiopoietin 1, and calcium binding protein A4 were associated with the risk of PPHTN. The biological relevance of one of the aromatase SNPs was supported by an association with plasma estradiol levels.**

***Conclusions*: Genetic variation in estrogen signaling and cell growth regulators is associated with the risk of PPHTN. These biologic pathways may elucidate the mechanism for the development of PPHTN in certain patients with severe liver disease.**

Keywords: genetic polymorphism; portal hypertension; hypertension, pulmonary

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

It is believed that inherited factors contribute to the development of certain forms of pulmonary arterial hypertension, such as that associated with portal hypertension.

What This Study Adds to the Field

Genetic polymorphisms in estrogen and other pathways are associated with the risk of portopulmonary hypertension in patients with advanced liver disease.

Pulmonary arterial hypertension (PAH) is characterized by elevated pulmonary artery pressure and pulmonary vascular resistance, right heart failure, exercise limitation, and an increased risk of death. Histopathologic examination reveals intimal proliferation, medial hypertrophy, and adventitial fibrosis in the small muscular pulmonary arteries. Plexiform lesions and *in situ* thrombosis are also seen. Most commonly idiopathic, PAH may also be associated with portal hypertension, termed portopulmonary hypertension (PPHTN). McDonnell and colleagues showed a prevalence of histopathologic changes of PAH of 0.61% in autopsies of patients with cirrhosis, and PPHTN was the third most common form of PAH in a population-based epidemiologic study in France (1, 2). Recent cohort studies showed that the prevalence of PPHTN in patients presenting for liver transplant evaluation is between 5 and 6% (3–5). Patients with PPHTN have an increased risk of death, even with specific PAH treatment (4, 6–8). In many cases, PPHTN greatly complicates or precludes liver transplantation, significantly affecting the course of hepatic failure in these patients (6,9,10).

The etiology of PAH in patients with portal hypertension (characterized by systemic vasodilatation) is unclear. We have shown that female sex and type of liver disease are associated with the risk of PPHTN (11). Although germline mutations in the gene that codes for bone morphogenetic protein receptor type II (BMPR2) have been associated with idiopathic and familial forms of PAH, they have not been found in patients with PPHTN (12). Genetic variation in the serotonin transporter (SERT) has been associated with the risk of PAH in some studies (13) but not in others (14, 15). We did not find an association between genetic variation at SERT loci and the risk of PPHTN (16).

We therefore hypothesized that variation in genes other than BMPR2 and SERT contribute to the risk of developing PPHTN. We performed a high-throughput candidate gene study in an attempt to identify common genetic variation associated with the risk of PPHTN in a group of patients undergoing liver transplantation evaluation. This work has been previously published in abstract form (17).

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*A listing of other members may be found before the beginning of the REFERENCES.

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Correspondence and requests for reprints should be addressed to Kari E. Roberts, M.D., Division of Pulmonary, Critical Care and Sleep Medicine, Tufts Medical Center, 800 Washington Street #257, Boston, MA 02111. E-mail:kroberts@tuftsmedicalcenter.org

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METHODS

Study Cohort and Study Sample

The Pulmonary Vascular Complications of Liver Disease (PVCLD) Study enrolled a cohort of 536 patients evaluated for liver transplantation or pulmonary hypertension at seven centers in the United States between 2003 and 2006. The only inclusion criterion was the presence of chronic portal hypertension with or without intrinsic liver disease. We excluded patients with evidence of active infection, recent (<2 wk) gastrointestinal bleeding, or who had undergone liver or lung transplantation. The institutional review boards at each of the participating centers approved this study, and informed consent was obtained.

We performed a case-control study. The study sample included newly referred patients who were evaluated with transthoracic echocardiography (routinely performed for pretransplant evaluation) during the study period. "Prevalent" patients who met the case definition (*see* below) were also included. We excluded patients with pulmonary function testing showing a significant obstructive or restrictive ventilatory defect (*see* online supplement). We also excluded patients with HIV infection or the presence of more than moderate aortic or mitral valvular disease or significant left ventricular dysfunction by transthoracic echocardiography.

Case and Control Definitions

Cases with PPHTN met the following criteria at initial evaluation: (*1*) mean pulmonary artery pressure $>$ 25 mm Hg, pulmonary capillary wedge pressure (or left ventricular end-diastolic pressure) $\leqslant$ 15 mm Hg, and pulmonary vascular resistance $>$240 dynes$\cdot$s$^{-1}\cdot$cm^{-5} measured by right heart catheterization; and (*2*) no other etiology for pulmonary hypertension. Controls met the following echocardiographic criteria at entry into the cohort: (*1*) right ventricular systolic pressure $<$ 40 mm Hg (if estimable), and (*2*) absence of right atrial or ventricular dilation, hypertrophy, or dysfunction. Prevalent cases who had previously undergone evaluation and were subsequently being treated were also included.

Clinical Variables and Blood Sampling

Data were collected from subjects and the medical record. The Model for End-stage Liver Disease (MELD) score was calculated (18). Phlebotomy was performed and blood was collected into EDTA-containing tubes. Plasma and buffy coat layers were stored at −80°C.

Candidate Genes and Single Nucleotide Polymorphism Selection

Ninety-three genes affecting vascular function were selected by the investigators (Table 1). For this study, 1,079 single nucleotide polymorphisms (SNPs) in the 93 candidate genes were genotyped (*see* Table E1 in the online supplement). We genotyped an additional set of 60 SNPs (null loci) from a validated list of Ancestry Informative Markers (19) to detect potential population stratification. (*See* online supplement for details of gene and SNP selection.)

Genotyping

Genomic DNA was isolated from peripheral leukocytes using standard procedures (Gentra Puregene; Qiagen, Valencia, CA). SNP genotyping was performed using the GoldenGate Assay (Illumina, Inc., San Diego, CA).

Statistical Analysis

Continuous data were summarized using mean ± standard deviation or median (interquartile range), as appropriate. Categorical variables were summarized using n (%). Unpaired Student *t* tests, Wilcoxon rank sum tests, chi-square tests, and Fisher exact tests were used, as appropriate.

Hardy-Weinberg equilibrium (HWE) was assessed for genetic alleles using Fisher exact tests in controls. The association of genotype with case/control status was assessed using additive models in multivariate logistic regression and expressed with odds ratios (ORs). We adjusted for sex and autoimmune liver disease (previously associated with case status [11]) in the final multivariate logistic regression models. Because the main goal of this study was hypothesis generation, adjustment for multiple comparisons was not performed. Single locus association analyses were performed using SAS/STAT (SAS Institute, Cary, NC).

For genes in which more than one SNP was associated with PPHTN, we determined haplotype structure and pairwise linkage disequilibrium between SNPs using Haploview 4.0 (20). The presence or absence of population stratification was assessed by comparing allele frequencies of the 60 null loci between cases and controls using chi-square tests (21). Sensitivity analyses assessed the potential impact of racial differences or cryptic subpopulations. $P < 0.05$ was considered significant for all analyses.

There was 80% power to detect odds ratios of $\geqslant$2.4 to 4.0 (or $\leqslant$0.25 to 0.42), depending on the minor allele frequency of the SNP (range, 0.45–0.05). Power analysis was performed using *QUANTO 1.2* (22).

RESULTS

There were 31 cases and 104 controls. The mean age of the subjects was 52 ± 10 years, and 60 (44%) were female. One hundred and twenty-one (90%) were white and seven (5%) were black. Sixteen (13%) of the white subjects were of Hispanic ethnicity (12% of the study sample). Subjects with PPHTN had a mean right atrial pressure of 10 ± 6 mm Hg (n = 30), a mean pulmonary artery pressure of 50 ± 9 mm Hg, and pulmonary capillary wedge pressure (or left-ventricular end-diastolic pressure) of 10 ± 4 mm Hg. The cardiac output was 5.5 ± 1.8 L/min, the cardiac index was 2.9 ± 0.9 L/min/m^2, and the pulmonary vascular resistance was 672 ± 374 dynes$\cdot$s$^{-1}\cdot$cm^{-5}.

Bivariate Analyses

Age, race, and severity of liver disease were similar between the groups (Table 2). Female sex and autoimmune hepatitis were associated with an increased risk for PPHTN, as previously reported in this population (11). One case and one control had α-1 antitrypsin deficiency, one control had biliary atresia, one case had sarcoid, and one case had portal vein thrombosis.

Genetic Analyses

Nine hundred and ninety-three (of the 1,079) SNPs conformed to HWE in controls ($P > 0.05$) and were included in the analysis. Twenty-nine SNPs in 15 genes were significantly associated with PPHTN after adjustment for sex and liver disease etiology (autoimmune hepatitis) (Table 3). In the gene coding for estrogen receptor 1 (ESR1), 7 of 36 SNPs were associated with either a significantly decreased (OR = 0.39–0.18) or increased (OR = 2.56–2.70) risk of PPHTN. The five protective SNPs included four intronic and one synonymous Exon 4 SNP (rs1801132, P324P). Pairwise linkage disequilibrium analyses demonstrated that these five loci represented a single haplotype block (D′ = 0.71–0.84) (Table E2 and Figure E1). Distal to these protective loci, two SNPs (rs7757956 and rs3020368, D′ = 0.89) were associated with an increased risk of PPHTN. Two promoter SNPs in the gene coding for aromatase (CYP19A1), the rate-limiting enzyme in the conversion of the androgens testosterone and androstenedione to estradiol, were associated with an increased risk of PPHTN (Tables 3 and E2, Figure E2).

Four of nine SNPs genotyped in phosphodiesterase 5 (PDE5A) were in tight linkage disequilibrium (r^2 = 0.95–1.00) (Table E2) and all were associated with an increased risk of PPHTN (all OR = 2.11; 95%CI, 1.05–4.22; P = 0.03) (Table 3). Two tightly linked SMAD3 intron 1 SNPs (rs4776881 and rs12324036, $r^2 > 0.97$) were associated with a decreased risk of PPHTN (both OR = 0.50; 95%CI, 0.26–0.95; P = 0.035). Two SNPs in each of three genes—calcium binding protein A4 (S100A4), angiopoietin 1 (ANGPT1), and retinoic acid receptor, β (RARB)—were associated with case status and were not in linkage disequilibrium (Tables 3 and E2, Figure E3). Of note, polymorphisms in BMPR2 or genes coding for bone morphogenetic protein receptor Type Ia (BMPR1A), activin A receptor

TABLE 1. CANDIDATE GENES (GENE ONTOLOGY ANNOTATION)

Pathway	Gene	RefSeq	Chr	SNPs
Control of blood	Angiotensin I converting enzyme (ACE)	NM_152831	17q23	15
circulation	Elastin (ELN)	NM_000501	7q11	5
GO:0008015	Endothelin 1 (EDN1)	NM_001955	6p24	7
	Endothelin converting enzyme 1 (ECE1)	NM_001397	1p36	10
	Endothelin receptor, nonselective type (EDNRB)	NM_000115	13q22	13
	Endothelin receptor, type A (EDNRA)	NM_001957	4q31	11
	Heme oxygenase 1 (HMOX1)	NM_002133	22q13	8
	Natriuretic peptide precursor A (NPPA)	NM_006172	1p36	13*
	Natriuretic peptide precursor B (NPPB)	NM_002521	1p36	13*
	Nitric oxide synthase 2 (NOS2A)	NM_000625	17q11	15
	Phosphodiesterase 5 (PDE5A)	NM_001083	4q26	9
	Potassium channel, voltage-gated, shaker, member 5 (KCNA5)	NM_002234	12p13	9
	Rho-associated protein kinase 2 (ROCK2)	NM_004850	2p24	15
	Transient receptor potential cation channel, subfamily C, 6 (TRPC6)	NM_004621	11q21	18
Cell growth apoptosis	Activin A receptor, type II-like kinase (ACVRL1)	NM_000020	12q11	6
GO:0008283	Apolipoprotein E (APOE)	NM_000041	19q13	4
GO:0006915	BCL2-associated X protein (BAX)	NM_138764	19q13	6
	Bone morphogenetic protein receptor type 1a (BMPR1A)	NM_004329	10q22	20
	Bone morphogenetic protein receptor type 2 (BMPR2)	NM_001204	2q33	12
	Caveolin 1 (CAV1)	NM_001753	7q31	20*
	Caveolin 2 (CAV2)	NM_001233	7q31	20*
	Caveolin 3 (CAV3)	NM_033337	3p25	19
	CD14 molecule (CD14)	NM_000591	5q22	3
	Cyclin-dependent kinase inhibitor 2A (CDKN2A)	NM_000077	9p21	13
	Growth differentiation factor 2 (GDF2)	NM_016204	10q11	5
	Homolog of *Drosophila* mothers against dpp 3 (SMAD3)	NM_005902	15q21	34
	Homolog of *Drosophila* mothers against dpp 4 (SMAD4)	NM_005359	18q21	5
	Nitric oxide synthase 3 (NOS3)	NM_000603	7q36	10
	Nuclear factor kappa B p100 subunit (NFKB2)	NM_001077493	10q24	5
	Nuclear factor kappa B p105 subunit (NFKB1)	NM_003998	4q23	13
	Nuclear factor kappa B p65 subunit (RELA)	NM_021975	11q13	4
	Prostaglandin I2 synthase (PTGIS)	NM_000961	20q13	13
	Protein kinase C, alpha (PRKCA)	NM_002737	17q22	33
	Protein kinase C, beta 1 (PRKCB1)	NM_002738	16p11	13
	Protein kinase C, gamma (PRKCG)	NM_002739	19q13	5
	Transforming growth factor, Beta-1 (TGFB1)	NM_000660	19q13	5
	V-AKT murine thymoma viral oncogene homolog 1 (AKT1)	NM_005163	14q32	7
Blood vessel growth and	Angiopoietin 1 (ANGPT1)	NM_001146	8q22	37
development	Calcium-binding protein A4 (S100A4)	NM_019554	1q21	6
GO:0001568	Endoglin (ENG)	NM_000118	9q34	15
	Hypoxia-inducible factor 1, alpha subunit (HIF1A)	NM_001530	14q21	8
	Plasminogen (PLG)	NM_000301	6q26	21
	Runt-related transcription factor 1 (RUNX1)	NM_001754	21q22	58
	Thrombospondin-1 (THBS1)	NM_003246	15q15	5
	Tyrosine kinase with Ig and EGF factor homology domains (TIE1)	NM_005424	1p34	8
	Vascular endothelial growth factor (VEGF)	NM_00125366	6p12	7
Inflammation	Complement component 4A (C4A)	NM_007293	6p21	4
GO:0006954	C-reactive protein (CRP)	NM_000567	1q21	8
	Cytochrome b-245, NADPH oxidase 2, NOX2 (CYBB)	NM_000397	Xp21	6
	Lipopolysaccharide binding protein (LBP)	NM_004139	20q11	7
	Tumor necrosis factor (TNF)	NM_000594	6p21	5
Oxidation reduction	Dual oxidase 1 (DUOX1)	NM_017434	15q15	15*
GO:0006979	Dual oxidase 2 (DUOX2)	NM_014080	15q15	15*
	NADPH oxidase 1 (NOX1)	NM_007052	Xq22	7
	NADPH oxidase 4 (NOX4)	NM_016931	11q14	19
	Superoxide dismutase 1, soluble (SOD1)	NM_000454	21q22	3
	Superoxide dismutase 2, mitochondrial (SOD2)	NM_00636	6q25	3
	Xanthine dehydrogenase (XDH)	NM_00379	2p23	24
Tissue development	Homolog of *Drosophila* mothers against dpp 2 (SMAD2)	NM_005901	18q21	10
GO:0009888	Ikaros (IKZF1)	NM_006060	7p12	7
	Peroxisome proliferator activated receptor, gamma (PPARG)	NM_005037	3p25	13
	Recombination signal-binding protein 1 for J-kappa (RBPSUH)	NM_005349	4p15	13
Steroid hormone	Aromatase (CYP19A1)	NM_000103	15q21	24
GO:0008202	Estrogen receptor 1 (ESR1)	NM_000125	6q25	36
GO:0030518	Estrogen receptor 2 (ESR2)	NM_001437	14q24	14
	Farnesoid X receptor (NR1H4)	NM_005123	12q	7
	Pregnane X receptor (NR1I2)	NM_003889	3q13	13
	Sex hormone binding globulin (SHBG)	NM_001040	17p13	6
	Small heterodimer partner (NR0B2)	NM_021969	1p36	5

(*Continued*)

TABLE 1. (CONTINUED)

Pathway	Gene	RefSeq	Chr	SNPs
Extracellular matrix structure and regulation	Collagen, type XVIII, alpha-1 (COL18A1)	NM_130445	21q22	29
GO:0043062	Elastase 1 (ELA1)	NM_001971	12q13	8
GO:0006508	Elastase 2 (ELA2)	NM_001972	19p13	4
	Matrix metalloproteinase 2 (MMP2)	NM_004530	16q13	11
	Matrix metalloproteinase 3 (MMP3)	NM_002422	11q23	6
	Matrix metalloproteinase 9 (MMP9)	NM_004994	20q11	6
	Proteinase inhibitor 3; elafin (PI3)	NM_002638	20q12	4
	Tenascin C (TNC)	NM_002160	9q33	16
Coagulation	Plasminogen activator inhibitor 1 (SERPINE1)	NM_000602	7q21	9
GO:0050817	Thrombomodulin (THBD)	NM_000361	20p11	4
	Thromboplastin (HEMB)	NM_000133	Xq27	11
	Von Willebrand factor (VWF)	NM_000552	12p13	39
Serotonin	Serotonin 2B receptor (HTR2B)	NM_000867	2q36	8
GO:0006587	Tryptophan hydroxylase 1 (TPH1)	NM_004179	11p15	8
GO:0007210	Tryptophan hydroxylase 2 (TPH2)	NM_173353	12q21	16
Na/bile acid transporter	Solute carrier family 10, member 1 (SLC10A1)	NM_003049.1	14q24	5
GO:0008508	Solute carrier family 10, member 2 (SLC10A2)	NM_000452.1	13q33	12
Metabolism	5,10-methylenetetrahydrofolate reductase (MTHFR)	NM_005957	1p36	7
GO:0008152	Betaine-homocysteine methyltransferase (BHMT)	NM_001713	5q13	4
	Cystathionine-beta-synthase (CBS)	NM_000071	21q22	6
	Peroxisome proliferator activated receptor, alpha (PPARA)	NM_005036	22q12	9
Retinoic acid signaling	Retinoic acid receptor, alpha (RARA)	NM_000964	17q21	4
GO:0048384	Retinoic acid receptor, beta (RARB)	NM_016152	3p24	29
	Retinoic acid receptor, gamma (RARG)	NM_000966	12q13	6

Definition of abbreviations: Chr = chromosome; RefSeq = Reference Sequence; SNP = single-nucleotide polymorphism.

* Indicates adjacent genes that were defined by a single genomic region and tagging SNPs. Thus the number of SNPs indicated refers to the total number of SNPs assayed in the region containing both genes.

type II-like 1 (ACVRL1), or endoglin (ENG) were not associated with PPHTN.

There were no significant differences in allele frequencies of the 60 null alleles between cases and controls (all $P > 0.05$), lessening the chance of population stratification. We assessed for potential confounding by liver disease etiology (other than autoimmune hepatitis). Eight SNPs associated with PPHTN were also independently associated with various liver disease etiologies; in all cases, adjustment for liver disease etiology resulted in either no significant change or strengthening of the association between the SNP and case status (Table E3).

There were no significant differences in the results from the main analyses and results of analyses performed in females only (n = 60), self-identified whites (n = 121), and subjects with white genetic ancestry (n = 124) (data not shown), indicating that neither sex nor race (nor genetic ancestry-based) differences accounted for our results.

TABLE 2. DEMOGRAPHIC AND CLINICAL DATA FOR CASES AND CONTROLS

Variable	Cases (*N = 31*)	Controls (*N = 104*)	*P* Value
Age, years	54 ± 10	52 ± 10	0.41
Female sex	20 (65%)	40 (39%)	0.01
Race			0.37
White	29 (94%)	92 (88%)	
Black	0	7 (7%)	
Other	2 (6%)	5 (5%)	
Etiology of portal hypertension			
Alcohol	14 (45%)	45 (43%)	0.85
Hepatitis C	6 (19%)	51 (49%)	0.003
Autoimmune hepatitis	7 (23%)	4 (4%)	0.003
Nonalcoholic fatty liver disease	1 (3%)	8 (75%)	0.68
Hepatitis B	1 (3%)	6 (6%)	1.0
Primary sclerosing cholangitis	1 (3%)	9 (9%)	0.45
Primary biliary cirrhosis	3 (10%)	3 (3%)	0.13
Cryptogenic	2 (6%)	8 (8%)	1.0
Model for End-stage Liver Disease score	12 ± 4 (N = 29)	12 ± 5 (N = 103)	0.77

Plasma Estradiol

Estradiol levels were measured in 28 cases and 98 controls with available plasma (*see* online supplement for assay details). Estradiol levels increased in a dose-dependent fashion with the A allele of the aromatase rs7175922 SNP (the allele associated with an increased risk of PPHTN), even after adjustment for sex (Figure 1). Estradiol levels were not associated with genotypes of the other aromatase SNP associated with case status (data not shown).

DISCUSSION

This is the first study to document genetic risk factors for PPHTN. Using a high-throughput candidate gene approach, we found SNPs in a variety of genes that were associated with the development of PAH in patients with advanced liver disease. Pathways with multiple gene "hits" included estrogen signaling, cellular growth/apoptosis, and oxidative stress. Other SNPs associated with case status included those in genes coding for recombination signal-binding protein 1 for J-kappa (RBPSUH), inducible nitric oxide synthase (NOS2A), and plasminogen activator inhibitor-1 (SERPINE1 or PAI-1). A number of the genes and signaling pathways found here have also been implicated in human or experimental PAH, supporting the concept that there may be shared pathogenetic mechanisms. In addition, several novel associations have been shown that may provide important mechanistic and therapeutic insights.

The role of estrogen signaling and increased estradiol levels in the pathogenesis of PAH and PPHTN has not been defined. PPHTN (like idiopathic and familial PAH) affects females more commonly than males (11), an association that may be related to a high estrogen state. However, estrogen has traditionally been

TABLE 3. ADDITIVE MULTIVARIATE LOGISTIC REGRESSION MODELS FOR SINGLE NUCLEOTIDE POLYMORPHISMS AND THE RISK OF PORTOPULMONARY HYPERTENSION (ADJUSTED FOR SEX AND THE PRESENCE OF AUTOIMMUNE HEPATITIS)

		SNP			Risk Allele Frequency			
Chr	Gene	Identification	Location	Risk Allele	Cases	Controls	OR (95% CI)	*P* Value
6	Estrogen receptor 1 (ESR1)	rs1913474	Intron 3	A	0.13	0.26	0.33 (0.13–0.85)	0.022
		rs1801132	P324P	C	0.13	0.27	0.39 (0.12–0.76)	0.011
		rs3020317	Intron 4	G	0.13	0.26	0.18 (0.06–0.55)	0.003
		rs985694	Intron 4	A	0.11	0.23	0.19 (0.05–0.67)	0.010
		rs932477	Intron 4	A	0.07	0.16	0.25 (0.08–0.87)	0.030
		rs7757956	Intron 4	A	0.24	0.15	2.70 (1.19–5.88)	0.017
		rs3020368	Intron 5	A	0.19	0.12	2.56 (1.09–5.88)	0.031
15	Aromatase (CYP19A1)	rs7175922	5′	A	0.26	0.13	2.17 (1.00–4.55)	0.050
		rs1902584	Intron 1	A	0.15	0.04	3.85 (1.33–11.1)	0.014
4	Phosphodiesterase 5 (PDE5A)	rs11731756	Intron 7	C	0.39	0.24	2.11 (1.05–4.22)	0.036
		rs10034450	Intron 11	G	0.39	0.24	2.11 (1.05–4.22)	0.036
		rs1155576	Intron 11	C	0.40	0.25	2.11 (1.06–4.20)	0.033
		rs3775843	Intron 16	G	0.39	0.24	2.11 (1.05–4.23)	0.036
8	Angiopoietin 1 (ANGPT1)	rs4324901	Intron 1	A	0.26	0.38	0.48 (0.24–0.97)	0.041
		rs4268102	Intron 6	C	0.34	0.19	2.30 (1.16–4.56)	0.017
1	Calcium binding protein A4 (S100A4)	rs743687	3′utr	G	0.18	0.07	3.82 (1.53–9.53)	0.004
		rs1810765	3′utr	G	0.19	0.11	2.38 (1.09–5.20)	0.030
3	Retinoic acid receptor, beta (RARB)	rs871963	Intron 2	T	0.63	0.46	1.92 (1.05–3.54)	0.035
		rs1153584	Intron 3	A	0.35	0.49	0.44 (0.23–0.88)	0.019
7	Caveolin 1 (CAV1)	rs926198	Intron 2	G	0.23	0.38	0.40 (0.19–0.84)	0.016
15	Homolog of Drosophila mothers against dpp 3 (Smad3)	rs12324036	Intron 1	A	0.34	0.48	0.50 (0.26–0.95)	0.035
		rs4776881	Intron 1	G	0.34	0.48	0.49 (0.26–0.95)	0.035
21	Runt-related transcription factor 1 (RUNX1)	rs2294163	Intron 1	A	0.29	0.17	1.96 (1.00–3.85)	0.049
4	Recombining binding protein 1 for J-kappa (RBPSUH)	rs2077777	Intron 2	G	0.11	0.05	3.47 (1.15–10.45)	0.027
2	Xanthine dehydrogenase (XDH)	rs1896846	Intron 24	C	0.39	0.23	1.96 (1.06–3.70)	0.031
6	Superoxide dismutase 2 (SOD2)	rs5746136	3′utr	A	0.37	0.23	2.00 (1.02–4.00)	0.043
11	NADPH oxidase 4 (NOX4)	rs3017887	5′utr	A	0.05	0.14	3.88 (1.05–14.29)	0.042
7	Plasminogen activator inhibitor 1 (SERPINE1)	rs2227714	3′utr	A	0.06	0.02	7.14 (1.47–33.33)	0.014
17	Nitric oxide synthase 2A (NOS2A)	rs1137933	D384D	A	0.13	0.28	0.39 (0.17–0.91)	0.030

Definition of abbreviations: Chr = chromosome; CI = confidence interval; OR=odds ratio; SNP=single-nucleotide polymorphism; utr=untranslated region.

believed to play a protective role in the systemic and pulmonary vasculature, modulating proliferative and vasoactive signaling by direct and receptor-mediated mechanisms (23, 24). In animal models of pulmonary hypertension, estrogen increases nitric oxide and prostacyclin production and decreases endothelin-1 (25–27), resulting in beneficial vascular effects. Such data are seemingly difficult to reconcile with studies showing adverse cardiovascular effects of estrogen. For instance, the Women's Health Initiative revealed that (despite many observational studies suggesting otherwise) hormone replacement therapy actually increased the risk for adverse cardiovascular events (28).

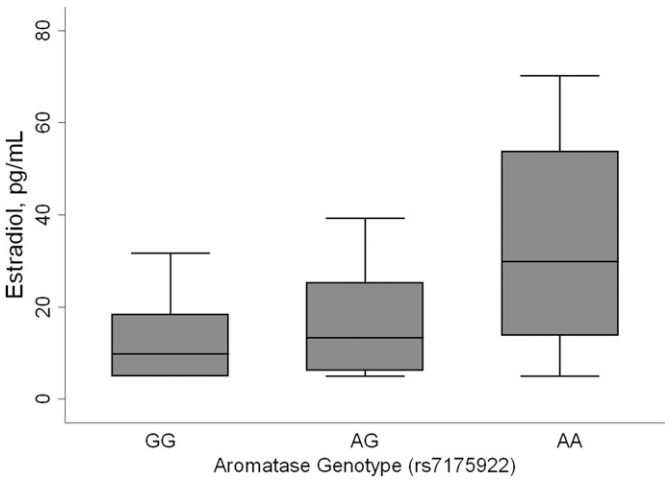

Figure 1. Estradiol levels and aromatase genotype adjusted for sex (test for trend, $P = 0.03$; n = 126). Median, interquartile range (*box*), and adjacent values (*whiskers*) are shown. Aromatase genotype distribution: GG (n = 88), AG (n = 34), AA (n = 4).

These apparent paradoxes may be explained by the complexity of the influence of estrogen on vascular homeostasis, resulting from variable expression of estrogen receptors 1 and 2 (α and β), cell and tissue specificity, and the influential balance between estrogen and other steroid hormones, such as testosterone and progesterone (29–31). We found that genetic variation in both the estrogen receptor 1 and aromatase (the rate-limiting enzyme in the conversion of androgens to estrogens) was associated with the risk of PPHTN, independent of sex. The two aromatase SNPs (rs1902584 and rs7175922) are located in the 93-kb region upstream (5′) of exon 2, where numerous tissue-specific promoters reside and thus could differentially influence aromatase expression in tissues (32). Furthermore, the association between the high-risk aromatase allele (rs7175922) and increased estradiol levels supports a functional effect of this SNP. Together, these findings strongly implicate estrogen signaling in the pathogenesis of PPHTN and define specific putative genetic factors that may contribute.

Several of the genes identified in our study participate in the regulation of cellular growth and apoptosis and have been implicated in human PAH and/or animal models of PAH. For example, we found that variation in PDE5A, which codes for a key enzyme in cyclic guanine monophosphate (cGMP) metabolism, was associated with PPHTN. Phosphodiesterase 5 inhibitors, such as sildenafil, potentiate the antiproliferative and vasodilatory effects of cGMP and improve hemodynamic features in PPHTN and other forms of PAH (33–35). Our

finding supports a role for altered cGMP production in causing disease in these patients and introduces PDE5A genotype as a potential pharmacogenomic target.

We also found a relationship between genetic variability in ANGPT1 and risk of PPHTN. ANGPT1 plays a pivotal role in angiogenesis, and enhanced ANGPT1 expression or signaling has been reported to have beneficial effects in several experimental models of PAH (36–38). Although the exact role of ANGPT1 in pulmonary hypertension remains obscure, our findings support a role for this molecule in human disease. Finally, we found an association between PPHTN and genetic variation in a SNP in S100A4, a member of a family of calcium-binding proteins involved in regulation of endothelial proliferation and adhesion (39). S100A4 is expressed in the plexiform lesions of individuals with certain types of PAH (40). In a murine model, overexpression of S100A4 results in increased arteriolar remodeling, plexiform lesions (41), and pulmonary hypertension in response to hypoxia (42). We have now found a potential causal link between S100A4 and human disease.

An additional 10 genes had SNPs associated with PPHTN. Six of these—SERPINE1, RARB (43), caveolin 1 (CAV1) (44), SMAD3, runt-related transcription factor 1 (RUNX), and RBPSUH (45)—play a significant role in angiogenesis. SERPINE1 codes for PAI-1, which modulates the proliferative and migratory properties of pulmonary artery smooth muscle cells (PASMC) and has been shown to be down-regulated in individuals with IPAH (46). We found that genetic variation in NADPH oxidase 4 (NOX4), xanthine dehydrogenase, and superoxide dismutase 2 was associated with PPHTN. Redox signaling has recently been implicated as a potential node of control for pulmonary vascular response (47). NOX4 localizes to the media of small pulmonary arteries and, in a hypoxic mouse model, contributes to PASMC proliferation. Pulmonary arterioles from IPAH patients demonstrate a significantly increased level of NOX4 protein, confirming a potentially important role of NOX4 overexpression in PAH (48). Last, genetic variation in NOS2A may contribute the hypercoagulability and vasoconstriction characteristic of PPHTN. By the nature of their roles in angiogenesis, control of coagulation, and vascular tone pathways, these 10 genes offer plausible candidates for determining the risk of PPHTN.

Disruption in bone morphogenetic protein/transforming growth factor β signaling has been demonstrated in familial and idiopathic forms of PAH (49, 50). Although we cannot rule out the possibility of a rare coding mutation in our subjects, use of regional linkage disequilibrium and haplotype-tagging SNPs makes a contribution of common genetic variation in BMPR2, BMPR1A, ACVRL1, or ENG to portopulmonary hypertension unlikely.

There are several limitations to this study. First, the sample size was small, limiting our ability to find genetic alleles associated with PPHTN that are rare, have small effect sizes, or whose effect depends on gene–gene or gene–environment interaction. However, this is one of the largest epidemiologic studies of PPHTN with very strict case and control phenotypes ever performed and the first in PAH to use high-throughput genotyping.

A fundamental challenge in high-throughput genetic analyses is the control of type I error. Given that we analyzed multiple SNPs for each of more than 90 genes, we can reasonably expect a certain number of statistically significant associations due to chance alone. We attempted to minimize the chance of false positives by using a curated candidate gene list, thus increasing the prior probability that one or more of these genes has mechanistic importance in PPHTN. There are commonly used frequentist methods to adjust for multiple comparisons in high-throughput studies, such as the Bonferroni correction and false discovery rate (51). Both methodologies assume that the association of each individual SNP with case status is entirely independent of those of the other SNPs. We have documented patterns of linkage disequilibrium between genotyped SNPs. Because most accepted methods to account for multiple comparisons do not consider such relatedness, they are overly conservative for this purpose. We have therefore presented the results without adjustment and consider these results to be hypothesis-generating. Although replication would be important, the biologic plausibility of our findings, the multiple gene "hits" in certain pathways (estrogen signaling and oxidative stress), and the demonstration of functionality (aromatase genotype and plasma estradiol levels) are reassuring in terms of the validity of the findings (52).

Our results implicate common genetic variation in the pathogenesis of PPHTN. Future studies should focus on replication in other populations and the mechanisms that explain the associations between the SNPs of interest and PPHTN.

***Conflict of Interest Statement*:** K.E.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.B.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.J.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.S.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.F.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. I.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. H.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.A.K. is on a scientific advisory board with Invitrogen, Inc., and received $12,500 in 2008. J.A.K. also holds a patent on the involvement of BMPRII in PAH and congenital heart disease. D.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.L.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.B.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.B.T. receives research support for participation in the REVEAL registry ($175,000, Actelion). E.M.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.Z. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. N.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.M.K. has received lecture fees, consultancy fees, funding for a CME course, and/or other support from Actelion, Pfizer, Gilead, Encysive, Lilly, INO Therapeutics, United Therapeutics, Gerson Lehrman, and Clinical Advisors. S.M.K. has received a $50,000 grant from Pfizer for an investigator-initiated clinical trial in chronic obstructive pulmonary disease.

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