Endothelin-1 Pathway Polymorphisms and Outcomes in Pulmonary Arterial Hypertension

Raymond L. Benza¹, Mardi Gombert-Maitland², Teresa Demarco³, Adaani E. Frost⁴, Adam Torbicki⁵, David Langleben⁶, Tomas Pulido⁷, Priscilla Correa-Jaque¹, Michael J. Passineau¹, Howard W. Wiener⁸, Mayumi Tamari⁹, Tomomitsu Hirota⁹, Michiaki Kubo⁹, and Hemant K. Tiwari¹⁰

¹Division of Cardiovascular Disease, Department of Medicine, Allegheny General Hospital, Pittsburgh, Pennsylvania; ²Division of Cardiovascular Disease, Department of Medicine, University of Chicago, Chicago, Illinois; ³Division of Cardiovascular Disease, Department of Medicine, University of California San Francisco, San Francisco, California; ⁴Baylor College of Medicine, Houston, Texas; ⁵Department of Pulmonary Circulation and Thromboembolic Diseases, Centre of Postgraduate Medical Education, ECZ, Otwock, Poland; ⁶Department of Medicine, Sir Mortimer B. Davis Jewish General Hospital, McGill University, Montreal, Canada; ⁷Cardiopulmonary Department, National Heart Institute, Mexico City, Mexico; ⁸Department of Epidemiology and ¹⁰Department of Biostatistics, University of Alabama at Birmingham, Birmingham, Alabama; and ⁹Institute of Physical and Chemical Research (RIKEN), Center for Integrative Medical Sciences, Yokohama, Japan

Abstract

Rationale: Pulmonary arterial hypertension (PAH) is a progressive fatal disease. Variable response and tolerability to PAH therapeutics suggests that genetic differences may influence outcomes. The endothelin pathway is central to pulmonary vascular function, and several polymorphisms and/or mutations in the genes coding for endothelin (ET)-1 and its receptors correlate with the clinical manifestations of other diseases.

Objectives: To examine the interaction of ET-1 pathway polymorphisms and treatment responses of patients with PAH treated with ET receptor antagonists (ERAs).

Methods: A total of 1,198 patients with PAH were prospectively enrolled from 45 U.S. and Canadian pulmonary hypertension centers or retrospectively from global sites participating in the STRIDE (Sitaxsentan To Relieve Impaired Exercise) trials. Comprehensive objective measures including a 6-minute-walk test, Borg dyspnea score, functional class, and laboratory studies were completed at baseline, before the initiation of ERAs, and repeated serially. Single-

nucleotide polymorphisms from ET-1 pathway candidate genes were selected from a completed genome-wide association study performed on the study cohort.

Measurements and Main Results: Patient efficacy outcomes were analyzed for a relationship between ET-1 pathway polymorphisms and clinical efficacy using predefined, composite positive and negative outcome measures in 715 European descent samples. A single-nucleotide polymorphism (rs11157866) in the G-protein alpha and gamma subunits gene was significantly associated, accounting for multiple testing, with a combined improvement in functional class and 6-minute-walk distance at 12 and 18 months and marginally significant at 24 months.

Conclusions: ET-1 pathway associated polymorphisms may influence the clinical efficacy of ERA therapy for PAH. Further prospective studies are needed.

Keywords: pulmonary arterial hypertension; endothelin receptor antagonists; pharmacogenetics; polymorphisms

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Correspondence and requests for reprints should be addressed to Raymond L. Benza, M.D., Allegheny General Hospital, 320 East North Avenue, 16th Floor, South Tower, Pittsburgh, PA 15212. E-mail: rbenza@wpahs.org

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At a Glance Commentary

Scientific Knowledge on the

Subject: Genomic studies in pulmonary arterial hypertension have focused on associative analyses to determine at-risk individuals. There have been few studies, however, studying the genomic underpinnings of patient responses to commonly used therapeutics.

What This Study Adds to the

Field: This is the largest conducted genomic analysis in pulmonary arterial hypertension studying the relationship between genetic variants and responses to therapy.

Untreated pulmonary arterial hypertension (PAH) is a progressive fatal disease (1, 2). A common histopathology among patients with PAH suggests a consistent mechanism of disease progression likely involving similar endogenous regulatory substances (3–6). Endothelin (ET)-1 is central to the pathophysiology of PAH. ET-1 has potent mitogenic effects and is abundantly expressed in the pulmonary vasculature (7–13). ET-1 expression is increased in the lungs of patients with primary pulmonary hypertension (PH) (14), and plasma levels of ET-1 are elevated in all cases of PAH, regardless of cause (15–19). Clinically, ET receptor antagonists (ERAs) in patients with PAH improve functional capacity, New York Heart Association (NYHA) functional class, time to clinical worsening, and perhaps mortality (20–27). However, significant treatment variability still exists and patients still progress clinically.

The dramatic interpatient variability in treatment response and agent tolerability, even with consideration of known prognostic factors, suggests that genetic differences among patients may play a role in the severity of PAH and the efficacy of treatment. Specifically, women with PAH obtain greater response to ERAs than do men, and white persons have a greater treatment benefit compared with black persons (28). Likely candidates are polymorphisms in genes controlling the regulatory factors of PAH, including ET-1. In addition, because both the ET-1 A and B receptors are G-protein couple receptors, polymorphisms within G-protein subunits

may also be particularly important in the subcellular signaling sequences promoted by ET-1 and mitigated by ERAs. Multiple polymorphisms have been identified in the ET-1 pathway genes and corresponding receptors, including both the ETA and ETB receptor and associated G proteins (29–35). Bosentan, macitentan, ambrisentan, and sitaxsentan are orally available ERAs with the latter two inhibiting primarily the ETA receptor. Only bosentan, macitentan, and ambrisentan are clinically available and Food and Drug Administration approved for treatment of PAH. Large, randomized, placebo-controlled trials demonstrated significant improvements in functional capacity, hemodynamics, clinical worsening, and perhaps survival (20) for each of these therapeutics. Sitaxsentan is no longer available because of case-reported significant liver toxicity.

Based on the key function of the ET pathway in PAH and the effect of ET-1 polymorphisms on blood pressure (30), we hypothesized that a pharmacogenetic interaction may occur in patients treated with ERAs who have certain ET-1 pathway polymorphisms, and may affect patient outcome. This study specifically examined the interaction of certain ET-1 pathway polymorphisms and clinical outcomes of patients with PAH treated with ERAs.

Methods

Patient Enrollment and Data Acquisition

A total of 1,198 patients with PAH treated with ERAs were enrolled prospectively from 45 U.S. and Canadian PH centers or retrospectively from global sites participating in the STRIDE (Sitaxsentan To Relieve Impaired Exercise) trials (36–40). PAH was defined by predefined clinical characteristics in the STRIDE trials. Sites enrolling patients prospectively were instructed to conform to the standard definition of PAH described in the 2007 Venice classification clinical guidelines.

For the retrospective STRIDE data, patients were asked to consent to and provide a blood sample when they were seen for a follow-up visit at the appropriate clinic. The collection of blood samples from the prospective patients with PAH from various PH centers in the United States treated with bosentan and ambrisentan (only available ERAs at the time of the study enrollment)

were collected in a manner similar to that described previously.

Assessments including a 6-minute-walk distance (6MWD) test, Borg dyspnea score, functional class, and standard laboratory tests were completed at baseline, before the initiation of ERAs, and repeated serially at prespecified times according to protocol (STRIDE enrollees) or local clinical practice. Collection of clinical data from prospective patients required the completion of case report forms by the clinic investigators. To obtain proprietary data from Encysive and later Pfizer, the sponsors of the STRIDE trials, a confidentiality agreement was enacted. The final patient cohort for these analyses consisted of 914 patients for whom a DNA sample and matching clinical data were available.

Each participating site was either required to hold a Federal-wide Assurance Number or to sign an “Individual Investigator Agreement” verifying understanding of the International Conference on Harmonization Good Clinical Practice principles. Importation of biologic specimens to the United States from international sites required adherence to the policies, procedures, and regulations set forth by the Department of Health and Human Services Center for Disease Control, International Air Transportation Association, and Council on Foreign Relations.

Selection of ET-1 Pathway Candidate Genes

The genes chosen for the current study included ET-1 (*EDN1*), the A and B receptors of ET (*EDNRA* and *EDNRB*), and guanine nucleotide binding proteins (alpha, beta, and gamma subunits) that participate in the response to ET. The single-nucleotide polymorphisms (SNPs) within ET-1 pathway genes were selected from recently completed genome-wide association studies (GWAS) on PAH (M. Gombert-Maitland, unpublished results). We used UCSC Genome Browser to select SNPs within the gene region and SNPs flanking 500 bp of the gene boundary. The numbers of SNPs per gene in 29 genes are given in Table 1.

Selection of SNPs from Genome-Wide Association Study Data Set and Quality Control

The University of Chicago and the Allegheny Singer Research Institute of Allegheny General Hospital had contributed

Table 1. SNPs Available from GWAS within and Flanking 500 Base Pairs of the Gene

Gene	Chromosome	Number of SNPs	Total Excluded	Effective Number of SNPs
GNB1	1	15	3	7
GNG12	1	34	9	15
GNG5	1	2	0	2
GNG4	1	30	7	13
GNB4	3	17	6	7
EDNRA	4	21	6	8
GNB2L1	5	5	1	3
EDN1	6	16	10	5
GNA12	7	32	7	11
GNGT1	7	88	21	39
GNG11	7	9	4	3
GNB2	7	4	2	2
GNA14	9	106	18	45
GNAQ	9	44	6	14
GNG10	9	3	0	2
GNG3	11	2	2	0
GNB3	12	5	0	3
EDNRB	13	23	13	6
GNG2	14	80	24	28
GNB5	15	20	10	4
GNG13	16	3	3	0
GNGT2	17	8	3	4
GNA13	17	14	8	3
GNG7	19	49	7	31
GNA11	19	12	4	5
GNA15	19	18	3	12
GNG8	19	3	0	3
GNAS	20	43	17	15
GNB1L	22	43	21	13
Total		749	215	303

Definition of abbreviations: GWAS = genome-wide association study; SNP = single-nucleotide polymorphism.

1,198 patients with PH for a GWAS of PAH through the Pharmacogenomics Research Network grant (U01GM061393) (M. Gomberg-Maitland, unpublished results). Our Japanese collaborators at the RIKEN Center for Genome Sciences performed genotyping on the Illumina Omni Express platform (Illumina, Inc., San Diego, CA) with appropriate HapMap quality control samples (41, 42). The standard protocol for genome-wide analyses was used for quality control (43). We used PLINK version 1.07 to ascertain the amount of missing genotype data, and filtered SNPs with less than 98.5% call rate across individuals and samples (44). The KING program was used to identify any related or duplicated samples, which were verified against the list of duplicates. For each duplicate, we retained the sample with less missing data for analysis (45). EIGENSTRAT (version 4.2) was used to identify homogeneous samples for analysis, and establish principal components of ancestry. Proc Discrim in SAS version 9.3 (SAS Institute, Inc., Cary, NC) was used to classify genotyped individuals into ethnic

groups based on the principal components of ancestries, which was matched with self-identified ethnic groups. Individuals that were classified as European American by this clustering method were retained for analysis. PLINK was again used to test for departure from Hardy-Weinberg Equilibrium (HWE) within these defined ethnic groups, and SNPs that failed with HWE test *P* less than 0.001 in any of the ethnic groups were removed. We calculated the correlations among genotypes within each gene, and used the methods of Nyholt (46) and Li and Ji (47) to determine the effective number of SNPs within each gene to account for linkage disequilibrium within gene region. We kept track of general quality metrics, such as plate effects tests, to include in interpreting results of analyses.

Demographics and Clinical Outcome Assessments

We analyzed all clinical and demographic variables in the European descent sample of 715 individuals by cohort and sex using analysis of variance for continuous

variables, and contingency table chi-square tests for categorical variables in each category. We chose to analyze the European descent sample because the sample size of other ethnicities was very small. Predefined outcomes for the analysis were derived from two sources: composite clinical endpoints and efficacy measures (6MWD and NYHA/World Health Organization [WHO] functional class). Two composites for clinical worsening were used: a composite (WOR) of an increase in NYHA/WHO functional class by greater than or equal to one class or NYHA/WHO functional class III with a greater than or equal to 15% decline in 6MWD; and a composite (Comb1) of WOR, time to death, time to hospitalization, and time to transplant.

Two composites of clinical improvement were used based on NYHA/WHO functional class and 6MWD thresholds and defined in the following manner: attainment of NYHA/WHO functional class no higher than two at some time and was never classified as having a higher NYHA/WHO level at any later time; and attained a particular 6MWD at some time and never had a shorter 6MWD at any later time. The distances attained and that defined these two composites in this second group were 33 m (48) more than the baseline distance (Attain33), or 400 m total (Attain400) (49, 50).

Association Methods

Associations with composite event outcomes were analyzed using Cox proportional hazard models using Proc PHREG in version 9.3 of SAS. Genotypes were included in the models in all three standard codings (additive, dominant, and recessive), and the strongest association of the three was retained and appropriate statistic was used for significance testing (51). Covariates included in addition to genotypes were sex; age; type of PAH (idiopathic and hereditary vs. secondary to congenital heart disease or connective tissue disease); baseline 6MWD; baseline WHO score; cohort of the individual (STRIDE vs. Prospective); PAH treatment use before inclusion in the study; mean right arterial pressure greater than 20 mm Hg; pulmonary vascular resistance greater than 32 Wood units; baseline creatinine levels; and use of the treatments phosphodiesterase inhibitors, prostacyclins, warfarin, or oxygen.

For each of the clinical outcomes of interest, we were interested in occurrence of the outcome at one of three time points after initiation of ERA (12, 18, and 24 mo), so separate analyses were done for each. Subjects that experienced an outcome after the specific time of interest for an analysis were treated as not having experienced the outcome for that analysis (in the terminology of survival analysis, they were censored for the analysis at that time point). For example, if a subject experienced an outcome at 20 months, they were treated as not yet having experienced that outcome for the 12- and 18-month analyses, but would be classified as having the event for the 24-month analysis. There were 702 SNPs within 29 genes, so some of the SNPs are correlated (i.e., in linkage disequilibrium). Some of the SNPs were excluded because

SNPs were either duplicate of other SNPs, had more than 1.5% missing, or had a *P* value for HWE test less than 0.001 and minor allele frequencies less than 0.05. Accounting for linkage disequilibrium patterns, the effective number of SNPs was 303. Using Bonferroni correction for multiple testing the *P* value threshold for significance was 1.65×10^{-4} .

Results

Clinical Characterization of the PAH Cohort

A total of 1,198 patients with PAH treated with ERAs were enrolled prospectively from 45 U.S. and Canadian PH centers or retrospectively from global sites participating in the STRIDE trials. A total

of 914 patients (103 African Americans, 715 European American, and 96 Hispanics) with adequate DNA and matching clinical data were evaluated by SNPs from candidate genes in ET pathway extracted from an existing GWAS. We focused our evaluation on those 715 patients of European descent for the present analysis. Demographic information for these 715 European American patients displayed by enrollment cohort, sex, and ethnicity is given in Tables 2 and 3, respectively. The *P* values are depicted in the last column of the tables. There was significant difference between Prospective and STRIDE cohorts in several clinical and demographic variables with respect to cohort, age, and sex, respectively.

This sample was 78.6% female and predominantly idiopathic PAH (53.5%). A total of 46.5% had associated forms of PAH

Table 2. Description of Sample by Source Cohort (Prospective or STRIDE)

Variable	Prospective	STRIDE	Overall	Missing	<i>P</i> Value*
Sex					1.09×10^{-2}
Male	63 (17.4%)	90 (25.5%)	153 (21.4%)	0	
Female	299 (82.6%)	263 (74.5%)	562 (78.6%)		
Age, yr	54.54 (14.97)	49.12 (15.94)	51.84 (15.68)	6	3.67×10^{-6}
Type of PAH					6.06×10^{-1}
IPAH and FPAH	195 (54.6%)	185 (52.4%)	380 (53.5%)	5	
Associated PAH	162 (45.4%)	168 (47.6%)	330 (46.5%)		
Association					2.24×10^{-9}
CHD	42 (25.9%)	90 (53.6%)	132 (40.0%)	0	
ASD	25 (59.5%)	25 (27.8%)	50 (37.9%)		
CTD	94 (58.0%)	76 (45.2%)	170 (51.5%)		
Scleroderma	56 (59.6%)	37 (48.7%)	93 (54.7%)		
Other	26 (16.0%)	2 (1.2%)	28 (8.5%)		
6MWD, m	335.29 (135.56)	380.22 (114.02)	358.55 (126.79)	39	3.55×10^{-6}
WHO class					1.12×10^{-10}
I	7 (2.1%)	17 (4.8%)	24 (3.5%)	32	
II	77 (23.3%)	164 (46.6%)	241 (35.3%)		
III	228 (68.9%)	161 (45.7%)	389 (57.0%)		
IV	19 (5.7%)	10 (2.8%)	29 (4.2%)		
PAPM, mm Hg	48.13 (14.79)	53.93 (18.02)	51.20 (16.81)	52	8.37×10^{-6}
PCWP, mm Hg	11.14 (5.84)	9.68 (3.99)	10.36 (5.00)	67	1.96×10^{-4}
PVR, Wood units	9.72 (6.15)	12.79 (17.13)	10.95 (6.92)	107	8.93×10^{-5}
RAPM, mm Hg	9.31 (5.46)	7.79 (5.03)	8.52 (5.29)	89	3.10×10^{-4}
ALT, U/L	25.97 (18.29)	18.43 (13.36)	22.09 (16.37)	37	1.26×10^{-9}
AST, U/L	27.90 (16.80)	20.34 (8.17)	23.98 (13.59)	41	1.85×10^{-13}
Creatinine, mg/dl	1.02 (0.63)	1.07 (0.25)	1.04 (0.48)	41	1.41×10^{-1}
Hematocrit, %	40.28 (9.52)	43.42 (7.58)	41.65 (8.86)	124	1.66×10^{-5}
Hemoglobin, g/dl	13.93 (2.80)	14.52 (2.63)	14.20 (2.74)	100	7.96×10^{-3}
Sodium, mmol/L	138.88 (3.74)	139.80 (3.25)	139.36 (3.52)	48	6.80×10^{-4}
Total bilirubin, mg/dl	0.96 (0.94)	0.86 (0.62)	0.91 (0.79)	41	1.19×10^{-1}
Uric acid, mg/dl	6.51 (1.95)	6.73 (1.89)	6.68 (1.90)	249	2.58×10^{-11}
Previous PAH Tx					2.09×10^{-11}
No	283 (78.2%)	337 (95.5%)	620 (86.7%)	0	
Yes	79 (21.8%)	16 (4.5%)	95 (13.3%)		

Definition of abbreviations: 6MWD = 6-minute-walk distance; ALT = alanine aminotransferase; ASD = atrial septal defect; AST = aspartate aminotransferase; CHD = congenital heart disease; CTD = connective tissue disease; FPAH = familial pulmonary arterial hypertension; IPAH = idiopathic pulmonary arterial hypertension; PAH = pulmonary arterial hypertension; PAPM = mean pulmonary artery pressure; PCWP = pulmonary capillary wedge pressure; PVR = pulmonary vascular resistance; RAPM = mean right arterial pressure; STRIDE = Sitaxsentan To Relieve Impaired Exercise; Tx = treatment; WHO = World Health Organization.

**P* values are for the test of differences of means for continuous variables or proportions of categorical variables.

with most having either connective tissue or congenital heart diseases. The mean age of patients at time of initial ERA administration was 51.8 (± 15.68) years. A total of 51.1% were on the ERA sitaxsentan, 43.1% on bosentan, and 5.8% on ambrisentan. A total of 13.29% of patients were on another class of PAH medication (prostacyclin or phosphodiesterase-5 inhibitor) before starting ERA therapy. This rose to 26.57% by the end of 1 year of ERA therapy. A description of the efficacy scores over the first year for the entire sample is summarized in Table 4. Survival was 99% at 1 year. However, a clinical worsening event (death, hospitalization, initiation of intravenous prostacyclin, atrial septostomy, lung transplant, or combined decline in WHO functional class and 6MWD) occurred in 9.5% of patients at 1 year, 13.2% at 18 months,

and 14.9% at 2 years. Most of these (92.6, 93.6, and 91.5%, respectively, at 1 yr, 18 mo, and 2 yr) were related to either a hospitalization or combined decline in WHO functional class and 6MWD.

Genotyping

The genes of interest included genes in the ET-1 gene, the ET receptor group (A or B), and the G-coupled protein subunits (α , β , and γ). After excluding SNPs that were monomorphic for the entire sample, there were a total of 749 SNPs with data available. Of these, 14 were duplicates and 33 failed to meet the threshold for missing genotypes, or for the test of HWE. The proportion of SNPs with no missing data is similar by sex (58.82% in males, 53.38% in females) and by source cohort (54.97% in the Prospective cohort,

54.11% in the STRIDE cohort). In all groups, more than 90% of the individuals were missing no more than two SNP genotypes.

Association

A major determinant of the information available for the outcome analysis was the amount of information that was censored (i.e., the proportion of the sample that did not experience the event by the end of the period for the analysis [12, 18, or 24 mo]). The clinical worsening outcome WOR was censored for more than 90% of observations at all three time points across the sample. The amount of censoring overall was lower for the Comb1 outcome, only exceeding 90% at the first time point. There was more information available for the clinical improvement outcomes; slightly more than 79% of the data were censored for the Attain33 outcome at 12 months, but the censoring proportions were below 73% for this outcome at the other two time points. Censoring proportions for the Attain400 outcome remained below 69% at all three time points.

The details of censoring events with respect to sex and study cohort are given in Table E1 in the online supplement. Sex was never a significant predictor of the worsening outcomes (WOR and Comb1). It was only rarely significant for the attainment outcomes: less than 1% of the total models for Attain33 had a *P* value below 0.05 for sex, whereas almost 16% of the total models for Attain400 reached this threshold. In both cases, females attained the improvement more quickly. Similar to the effect of sex, type of PAH (idiopathic PAH vs. associated) was never a significant predictor of the worsening outcomes (WOR and Comb1). However, it was a significant predictor in almost every (>99%) model for both Attain33 and Attain400. In both cases, those with idiopathic PAH attained the improvement more quickly. The Table E2 gives details about each SNP's minor and major alleles, minor allele frequency, HWE test *P* value, function, chromosome, and chromosomal position in base pairs within selected genes. Table 1 gives the effective number of SNPs within each gene. Based on these effective numbers, the association results that reached significance within each gene are given in Tables E3A–E3C.

A variant in the gene *GNG2* (rs11157866) significantly increased the rate of developing the first improvement

Table 3. Description of Sample by Sex

Variable	Male	Female	Missing	<i>P</i> Value*
Age, yr	47.58 (17.48)	53.01 (14.96)	6	1.45×10^{-4}
Type of PAH				1.15×10^{-1}
IPAH and FPAH	91 (59.5%)	289 (51.9%)	5	
Associated PAH	62 (40.5%)	268 (48.1%)		
Association				4.10×10^{-6}
CHD	42 (67.7%)	90 (33.6%)	0	
ASD	10 (23.8%)	40 (44.4%)		
CTD	16 (25.8%)	154 (57.5%)		
Scleroderma	8 (50.0%)	85 (55.2%)		
Unknown	4 (6.5%)	24 (9.0%)		
6MWD, m	404.17 (115.65)	345.87 (126.93)	39	6.65×10^{-7}
WHO class				5.13×10^{-4}
I	13 (8.8%)	11 (2.1%)	32	
II	56 (37.8%)	185 (34.6%)		
III	72 (48.6%)	317 (59.3%)		
IV	7 (4.7%)	22 (4.1%)		
PAPM, mm Hg	53.97 (17.75)	50.46 (16.49)	52	2.79×10^{-2}
PCWP, mm Hg	10.65 (4.81)	10.28 (5.04)	67	4.38×10^{-1}
PVR, Wood units	10.95 (6.15)	10.94 (7.13)	107	9.88×10^{-1}
RAPM, mm Hg	8.74 (5.03)	8.46 (5.36)	89	5.94×10^{-1}
ALT, U/L	23.95 (14.72)	21.58 (16.77)	37	1.21×10^{-2}
AST, U/L	23.88 (10.21)	24.01 (14.39)	41	9.20×10^{-1}
Creatinine, mg/dl	1.23 (0.89)	0.99 (0.26)	41	8.23×10^{-8}
Hematocrit, %	44.30 (9.99)	41.00 (8.44)	124	2.92×10^{-4}
Hemoglobin, g/dl	15.03 (3.26)	13.98 (2.55)	100	1.15×10^{-4}
Sodium, mmol/L	139.44 (3.23)	139.33 (3.60)	48	7.49×10^{-1}
Total bilirubin, mg/dl	1.06 (0.80)	0.87 (0.79)	41	9.09×10^{-3}
Uric acid, mg/dl	7.48 (1.82)	6.45 (1.87)	249	8.13×10^{-7}
Previous PAH Tx				4.47×10^{-1}
No	136 (88.9%)	484 (86.1%)	0	
Yes	17 (11.1%)	78 (13.9%)		

Definition of abbreviations: 6MWD = 6-minute-walk distance; ALT = alanine aminotransferase; ASD = atrial septal defect; AST = aspartate aminotransferase; CHD = congenital heart disease; CTD = connective tissue disease; FPAH = familial pulmonary arterial hypertension; IPAH = idiopathic pulmonary arterial hypertension; PAH = pulmonary arterial hypertension; PAPM = mean pulmonary artery pressure; PCWP = pulmonary capillary wedge pressure; PVR = pulmonary vascular resistance; RAPM = mean right arterial pressure; Tx = treatment; WHO = World Health Organization.

**P* values are for the test of differences of means for continuous variables or proportions of categorical variables.

Table 4. Means and SEs of Efficacy Scores over the First Year

	Baseline	12 Weeks	52 Weeks
6MWD, m	358.01 (128.64)	379.41 (113.70)	392.13 (116.43)
Borg score	3.63 (2.23)	3.21 (2.16)	3.27 (2.28)
WHO score	2.61 (0.63)	2.40 (0.66)	2.30 (0.72)

Definition of abbreviations: 6MWD = 6-minute-walk distance; WHO = World Health Organization.

outcome (Attain33) at time points 12 months ($P = 1.36 \times 10^{-5}$) and 18 months ($P = 1.26 \times 10^{-4}$) and marginally significant at 24 months ($P = 1.69 \times 10^{-4}$) (Table 5). The associated minor allele (A) in rs11157866 has approximately 12.67% frequency. Figures 1A–1C are Kaplan-Meier plots for rs11157866 corresponding to 12, 18, and 24 months. The homozygous individuals with minor allele T/T for rs11157866 attained the improvement sooner than the individuals with other genotypes.

Discussion

The ability to predict clinical response to therapy would allow more efficient therapeutic decisions. Our study is one of the first and largest to assess potential genetic associations with clinical outcomes in patients with PAH treated with ERAs. We found several significant associations between clinical outcomes and SNPs within ET-1, ET receptors, and various subunits of the G-protein complex genes. The strongest associations, however, were noted with polymorphisms attributable to the G-protein complex. This later observation is important given the vital importance of these protein complexes and related genetic variations with human disease (34, 35).

The association between clinical responsiveness to ERAs and G-protein subunit polymorphisms is likely complex, mainly because of the enormous variation in the subunits themselves, but also because of the relative paucity of information regarding the

association of the various alpha/beta/gamma complexes with their cognate receptors, and their composition within various tissues. Nonetheless, several of the genomic associations in the GNG2 gene (Table 5) and others (see Tables E3A–E3C) tell a plausible biologic story. The GNG2 gene is located on chromosome 14, and the GNG2 protein is expressed in the adult adrenal gland, brain, white blood cells, and lung (52) and seems to be particularly important in angiogenesis and cellular migration and proliferation (53–57). There is also evidence that GNG2 is expressed in malignant tissues, particularly of the skin (53–55), and that GNG2 expression levels could be a novel biomarker for malignant melanoma (55). Indeed, increased protein expression levels of GNG2 alone inhibits proliferation of malignant melanoma cells *in vitro* and *in vivo* and overexpression of GNG2 alone inhibits metastasis in human malignant melanoma cells related to decreased focal adhesion kinase activity. GNG2 also mediates Akt signaling for nuclear factor- κ B activation (58, 59). Interestingly, SNPs located within or near GNG2 were associated with clinical response and measures of thiopurine metabolism (60). Thus, GNG2's activity and regulation may be particularly important in PAH given this particular factor's roles in apoptosis, cell proliferation, transcription, cell migration, inflammation, and drug responses. Whether this particular G-protein component is specifically regulated by activation or inhibition of the ET-1 receptor, by ET-1, or ERAs, respectively, is an area of open investigation.

The GNAS gene is located on chromosome 20 and target RNA interference of GNAS in porcine fibroblast cells leads to lower mRNA expression of Bcl-2, Fas, and Caspase-3, which are recognized as apoptosis-related markers (61). GNAS also couples receptors to the effector enzyme adenylyl cyclase and is required for receptor-stimulated intracellular cAMP generation. Generation of cAMP is critical to several key pathways involving cellular growth and apoptosis, particularly in several tumor types and in vascular tissue (62–67). These activities are particularly relevant to PAH both in terms of the pathophysiologic underpinnings of the disease and the beneficial effects on clinical outcome with prostacyclins, whose main activity is mediated via activation of cAMP (68–70). It is also well known that activation of the G-protein coupled, ETB receptor on endothelial cells is coupled to the stimulation of cyclooxygenases and increased endogenous prostacyclin and hence increase cAMP (71). Whether GNAS activity mediated by ETB stimulation is responsible for this aforementioned cascade is at present unknown. Mutations and sequence variations in this gene lead to a variety of tumors, fibrous dysplasia of bone, and McCune-Albright syndrome (72–76). In addition, SNPs in the GNAS gene have been shown to predict the postoperative course in solid tumors (77) and may predict benefit from platinum-based neoadjuvant chemoradiotherapy for esophageal cancer (78). Thus similar to GNG2, potential alterations in ET-mediated GNAS activity via SNPs may be particularly relevant to PAH, but a direct causal link has yet to be established in preclinical studies.

For other associations, the biologic story is more speculative or altogether unclear and the associations with clinical outcome in PAH less rigorous (see Tables E3A–E3C). However, because of their potential unique relationship to mechanisms underpinning PAH, a brief description is offered. GNAQ and GNA14

Table 5. Significant SNP Associations with Bonferroni Correction Accounting for Multiple Testing ($P < 1.65 \times 10^{-4}$)

Time	Outcome	Gene	Chr	Position	Locus	HWE	MAF	P Value	Log HR
12 mo	Attain33	GNG2	14	52,371,313	rs11157866	0.61	0.13	1.36×10^{-5}	2.09
18 mo	Attain33	GNG2	14	52,371,313	rs11157866	0.61	0.13	1.26×10^{-4}	1.76

Definition of abbreviations: Chr = chromosome; HR = hazard ratio; HWE = Hardy-Weinberg Equilibrium; MAF = minor allele frequency; SNP = single-nucleotide polymorphism.

Figure 1. (A–C) Kaplan-Meier plot for rs11157866 corresponding to 12, 18, and 24 months. The homozygous individuals with minor allele T/T for rs11157866 attained the improvement sooner than the individuals with other genotypes.

are known to bind to phospholipase C (79), which in turn mediates vascular smooth muscle cell contraction, whereas *GNA13* is known to be involved in cellular migration (80) and angiogenesis (81). Both of these signal-amplification enzymes are involved in some degree with the pathology of PAH and can be regulated by the ET-1 axis.

In summary, the overall findings of our study are encouraging because the most significant associations identified correlate with improved, rather than worsened, outcomes following an ERA-based treatment scheme. Thus, we have potentially defined a subgroup of “responder” patients, when treated with an ERA-based treatment regimen. If prospectively validated, these findings offer a first step toward a clinically practical genetic diagnostic tool to contend with one of the most vexing challenges in PAH pharmacotherapy, namely the choice of front-line therapeutic agents. Numerous studies have shown that survival is optimized if initial response to monotherapy is favorable, but the choice of the monotherapy agent remains the domain of physician preference. Our results offer hope that this paradigm may soon change, with genotyping providing a tool for guiding front-line treatment decisions. Although another GWAS in PAH is currently available, its focus was on association not outcome (82). In light of this, further studies, both to prospectively validate our findings in patients treated with ERAs and to expand this approach to patients treated with other PAH therapy, are warranted to advance treatment toward a personalized medicine approach.

Limitations

This study suffers from several limitations. Because we are investigating time to event outcomes there could have been misclassification caused by patients being on ERA therapy before they entered the study. However, we may have compromised the power of the association study because of misclassification. Clinical data collection was not complete for all patients and the results have not been validated in another clinical dataset. The missing data also leads to loss of power. However, the sample size used after removing the missing data is big enough (715) not to create bias unless missingness is not at random. This later shortcoming is the focus of our ongoing sample and data collection protocols. In addition, the biologic hypotheses and the

cellular processes regulated by these SNPs, as noted in our discussion, are speculative and need to be proved in appropriate preclinical models and studies.

A major limitation in any study in which the major analytic tool is survival analysis is the proportion of the sample that is censored (i.e., that does not experience the

outcome of interest by the end of the observation period). Censoring proportions are above 90% for outcome WOR, and above 85% for Comb1 at all time points. The improvement outcomes (Attain33 and Attain400) are better, but most of the individuals in this sample still do not experience the events by the end of the

observation periods. These large censoring proportions can severely impact the power of the study. This must be considered in the interpretation of the results, especially the results for worsening outcomes. ■

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

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