ARTHRITIS & RHEUMATOLOGY
Vol. 68, No. 1, January 2016, pp 191–200
DOI 10.1002/art.39449


# Identification of Rare Variants in *ATP8B4* as a Risk Factor for Systemic Sclerosis by Whole-Exome Sequencing

Li Gao,[1] Mary J. Emond,[2] Tin Louie,[2] Chris Cheadle,[1] Alan E. Berger,[1] Nicholas Rafaels,[1] Candelaria Vergara,[1] Yoonhee Kim,[3] Margaret A. Taub,[1] Ingo Ruczinski,[1] Stephen C. Mathai,[1] Stephen S. Rich,[4] Deborah A. Nickerson,[2] Laura K. Hummers,[1] Michael J. Bamshad,[5] Paul M. Hassoun,[1] Rasika A. Mathias,[1] the National Heart, Lung, and Blood Institute GO Exome Sequencing Project, and Kathleen C. Barnes[1]

***Objective.*** **To determine the contribution of rare variants as genetic modifiers of the expressivity, penetrance, and severity of systemic sclerosis (SSc).**

***Methods.*** **We performed whole-exome sequencing of 78 European American patients with SSc, including 35 patients without pulmonary arterial hypertension (PAH) and 43 patients with PAH. Association testing of case–control probability for rare variants was performed using the unified sequence kernel association test with optimal kernel weighting and small sample adjustment by comparing all SSc patients with a reference population of 3,179 controls from the Exome Sequencing Project 5,500 exome data set. Replication genotyping was performed in an independent sample of 3,263 patients (415 patients with SSc and 2,848 controls). We conducted expression profiling of messenger RNA from 61 SSc patients (19 without PAH and 42 with PAH) and 41 corresponding controls.**

***Results.*** **The *ATP8B4* gene was associated with a significant increase in the risk of SSc ($P = 2.77 \times 10^{-7}$). Among the 64 *ATP8B4* variants tested, a single missense variant, c.1308C>G (F436L, rs55687265), provided the most compelling evidence of association ($P = 9.35 \times 10^{-10}$, odds ratio [OR] 6.11), which was confirmed in the replication cohort ($P = 0.012$, OR 1.86) and meta-analysis ($P = 1.92 \times 10^{-7}$, OR 2.5). Genes involved in E3 ubiquitin-protein ligase complex (*ASB10*) and cyclic nucleotide gated channelopathies (*CNGB3*) as well as *HLA–DRB5* and *HSPB2* (heat-shock protein 27) provided additional evidence of association ($P < 10^{-5}$). Differential *ATP8B4* expression was observed among the SSc patients compared to the controls ($P = 0.0005$).**

***Conclusion.*** ***ATP8B4*** **may represent a putative genetic risk factor for SSc and pulmonary vascular complications.**

Systemic sclerosis (SSc) is a rare multisystem autoimmune disease estimated to have a prevalence of 240 per million in the US population (based on a predominantly European American population) (1). SSc is characterized by small vessel vasculopathy, autoantibody production, excessive collagen deposition in the skin and internal organs, and abnormalities of the immune system. Whether they present with the limited or diffuse form, the survival rate of patients with SSc is negatively impacted by demographic characteristics such as older age at onset and male gender and by internal organ involvement of the lungs and kidney (2). In fact, the most common cause of death in SSc is pulmonary disease, manifesting as either interstitial lung disease (ILD) or pulmonary arterial

Supported in part by the NIH (National Heart, Lung, and Blood Institute grants R03-HL-114937 to Drs. Gao, Hassoun, and Barnes, HL-102923 to Drs. Gao and Barnes, and P50-HL-084946 to Dr. Hassoun). Dr. Gao's work was supported in part by the Gilead Sciences Research Scholars Program in Pulmonary Arterial Hypertension. Dr. Kim's work was supported in part by the Intramural Research Program of the National Human Genome Research Institute. Dr. Barnes' work was supported in part by the Mary Beryl Patch Turnbull Scholar Program.

[1]Li Gao, MD, PhD, Chris Cheadle, PhD, Alan E. Berger, PhD, Nicholas Rafaels, MS, Candelaria Vergara, MD, PhD, Margaret A. Taub, PhD, Ingo Ruczinski, PhD, Stephen C. Mathai, MD, MHS, Laura K. Hummers, MD, ScM, Paul M. Hassoun, MD, Rasika A. Mathias, ScD, Kathleen C. Barnes, PhD: Johns Hopkins University, Baltimore, Maryland; [2]Mary J. Emond, PhD, Tin Louie, MS, Deborah A. Nickerson, PhD: University of Washington, Seattle; [3]Yoonhee Kim, PhD: National Human Genome Research Institute, NIH, Baltimore, Maryland; [4]Stephen S. Rich, PhD: University of Virginia, Charlottesville; [5]Michael J. Bamshad, MD: University of Washington and Seattle Children's Hospital, Seattle.

Address correspondence to Kathleen C. Barnes, PhD, Johns Hopkins Asthma and Allergy Center, 5501 Hopkins Bayview Circle, Room 3A.62A, Baltimore, MD 21224. E-mail: kbarnes@jhmi.edu.

Submitted for publication July 17, 2014; accepted in revised form September 24, 2015.

hypertension (PAH) (3). PAH affects ~10% of patients with SSc, and these patients typically have a poorer response to PAH-specific medications and worse prognosis compared to patients with idiopathic PAH (4). Thus, SSc without PAH and SSc with associated PAH (or SSc patients with other common complications that are known to worsen outcomes such as ILD) are at opposite severity extremes within the spectrum of SSc diseases.

Genetic factors may play an essential role in SSc etiology. Multiple reports from candidate gene association and replication studies, together with genome-wide association studies (GWAS) in SSc have suggested that genes in the major histocompatibility complex (MHC) region, *STAT4*, *IRF5*, *BLK*, *BANK1*, *TNFSF4*, *CD247*, and *TNIP1* are SSc susceptibility genes (5–7). Additional loci in *IL12RB2* (8), *CSK* (9), and *PPARG* (10) were confirmed through GWAS followup. However, the contribution of rare variants (minor allele frequency [MAF] of <1–5%) to SSc susceptibility remains largely unexplored. Further, no genes directly involved in vascular or fibrotic pathways, the two critical mechanisms contributing to SSc pathogenesis, have been reproducibly identified. Whole-exome sequencing is a powerful and effective strategy for the discovery of rare coding variants that may be deleterious and relevant to disease risk (11). We hypothesized that rare variants of large effect influencing susceptibility to SSc are present at a much higher frequency in affected individuals than in the general population. Furthermore, multiple rare variants in the same gene could each have similar consequences on protein function and thus collectively identify an important SSc-associated gene. As part of the National Heart, Lung, and Blood Institute (NHLBI)–supported Exome Sequencing Project, we compared rare variants in whole-exome sequencing data from 78 individuals with SSc to those found in 3,172 exomes from unaffected controls using the Exome Sequencing Project 5,500 exome data set.

To our knowledge, this is the first report of whole-exome sequencing in SSc, a complex autoimmune disorder, with a novel gene identified for which we have demonstrated replication in an independent cohort and determined the messenger RNA (mRNA) expression levels in peripheral blood mononuclear cells (PBMCs) from patients.

## PATIENTS AND METHODS

**Study population.** Our study included 493 European American patients with SSc who were followed up at the Johns Hopkins Scleroderma Center and/or were referred to the Johns Hopkins Pulmonary Hypertension Program for the evaluation of pulmonary hypertension and performance of catheterization of the right side of the heart between October 2006 and June 2010. The study was approved by the Johns Hopkins University Institutional Review Board.

Each SSc patient satisfied 1 of the following criteria (12): the American College of Rheumatology preliminary criteria for SSc (13), 3 or more of the 5 features of CREST syndrome (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasias) (14,15), or the combination of definite Raynaud's phenomenon, abnormal nailfold capillaries, and an SSc-specific autoantibody (16). The onset of SSc was defined as the first non–Raynaud's phenomenon manifestation (2).

The SSc-only patients in our cohort did not have PAH when samples were obtained at diagnosis and remained free of PAH throughout the followup period. PAH was defined as a mean pulmonary artery pressure (PAP) of >25 mm Hg, pulmonary artery wedge pressure ≤15 mm Hg, and pulmonary vascular resistance (PVR) ≥3 Wood units by catheterization of the right side of the heart, in the absence of other known causes of pulmonary hypertension (17). Data on sex, race, disease severity, and duration of illness were available for all patients in both the discovery and replication cohorts. Data related to clinical, hemodynamic (right atrial pressure, mean PAP, cardiac index, and PVR), and echocardiographic parameters (e.g., tricuspid annular plane systolic excursion measurement) were available for the patients with SSc and PAH. Hemodynamic measurements obtained closest to the date of blood collection were used for genomic analyses.

A subset of unrelated European American individuals with SSc was selected for the discovery cohort as part of the Exome Sequencing Project. These included patients with SSc without PAH and patients with SSc-associated PAH (n = 78) matched for age (at blood draw), sex, and subtype of SSc (limited cutaneous SSc and diffuse cutaneous SSc [dcSSc]) (14). Briefly, in selecting patients for the study, we prioritized 1) patients with SSc and PAH for whom data on subtype of SSc and age at onset of PAH were available and 2) individuals who were male, had dcSSc, or both (Table 1). The 3,179 unaffected European American controls in the discovery cohort were drawn from the Exome Sequencing Project 5,500 exome data set. For unaffected controls in the replication cohort, we selected a subset of 2,848 European American subjects from the Lung Health Study (18) who participated in exome array genotyping but were not part of the Exome Sequencing Project (see Supplementary Methods, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39449/abstract).

A full list of the members of the NHLBI GO Exome Sequencing Project and their affiliations is provided in the Supplementary Appendix, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39449/abstract.

**Whole-exome sequencing.** DNA was extracted from whole blood samples collected from patients at the time of diagnosis. Details on quality control of sample DNA, library production and exome capture, clustering and sequencing, read mapping, and variant analysis have been described previously (19). Standard quality control approaches appropriate for exome data were used to assess both individual samples and variants (see Supplementary Methods, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39449/abstract). Originally, 80 subjects were

**Table 1.** Characteristics of the patients with SSc in the exome discovery and replication cohorts*

| | Exome discovery cohort | | | Replication cohort | | |
|---|---|---|---|---|---|---|
| | Patients with SSc without PAH (n = 35) | Patients with SSc with PAH (n = 43) | All patients with SSc (n = 78) | Patients with SSc without PAH (n = 323) | Patients with SSc with PAH (n = 92) | All patients with SSc (n = 415) |
| Age, mean ± SD years | 69.6 ± 5.4 | 61.9 ± 11.8 | 65.3 ± 10.2 | 56.2 ± 12.3 | 62.3 ± 10.3 | 57.5 ± 12.1† |
| Female, no. (%) | 28 (80.0) | 37 (86.0) | 65 (83.3) | 276 (85.4) | 79 (85.9) | 355 (85.5) |
| Age at onset of SSc, mean ± SD years (no. of patients) | 54.4 ± 10.8 (35) | 47.5 ± 13.5 (43) | 51.0 ± 12.7 (78) | 43.1 ± 12.5 (318) | 49.9 ± 13.5 (89) | 44.6 ± 13.0 (407)† |
| Disease onset at age ≥40 years, no./no. assessed (%) | 33/35 (94.3) | 30/43 (69.8) | 63/78 (80.8) | 194/318 (61.0) | 71/89 (79.8) | 265/407 (65.1)† |
| Type of SSc | | | | | | |
| No. of patients assessed | 35 | 41 | 76 | 311 | 72 | 383 |
| Diffuse cutaneous SSc, no. (%) | 6 (17.1) | 5 (12.2) | 11 (14.4) | 105 (33.2) | 8 (11.1) | 113 (29.5) |
| Limited cutaneous SSc, no. (%) | 29 (82.9) | 29 (70.7) | 58 (76.3) | 206 (66.2) | 50 (69.4) | 261 (68.1) |
| Unknown. no. (%) | 0 (0) | 7 (17.1) | 7 (9.2) | 0 (0) | 14 (19.4) | 9 (2.3) |

* SSc = systemic sclerosis; PAH = pulmonary arterial hypertension.
† $P < 2.2 \times 10^{-16}$ versus all patients with SSc in the exome discovery cohort, by *t*-test.

included in the exome sequencing. Two outliers, as indicated by the heterozygous-to-homozygous ratio, were discarded after applying quality control filters. Three *ATP8B4* sites were removed due to low quality (read depth <10).

**Genotyping and quantification of *ATP8B4* mRNA expression in PBMCs from patients.** Genomic DNA was extracted from peripheral blood samples. Validation genotyping for rs55687265 in *ATP8B4* was performed in an independent replication cohort of 415 European American patients (92 with SSc with PAH and 323 with SSc without PAH) by using TaqMan allelic discrimination assays on the 7900HT sequence detection system (Applied Biosystems). Positive controls representing 3 genotype clusters were run on every plate to ensure accurate clustering and allele calling. We replicated 10% of the samples for quality control. Additional genotyping for controls in the replication cohort was performed as part of the Illumina HumanExome-12v1_A BeadChip array (see Supplementary Methods, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39449/abstract).

We used existing data from high-throughput expression profiling using Illumina Sentrix Human BeadChips (HT12_v3) with mRNA from PBMCs of SSc patients without PAH (n = 19) and SSc patients with PAH (n = 42) and corresponding controls (n = 41). Control subjects were healthy individuals who had no known cardiovascular, pulmonary, or kidney disease. Details on isolation of PBMCs, purification of RNA, the microarray experiment, and analysis have been published previously (20). For comparison of gene expression between specified pairs of groups, fold changes, *P* values, and Benjamini and Hochberg false discovery rates (FDRs) (21) were obtained using custom software written in Interactive Data Language (Exelis Visual Information Solutions) (see Supplementary Methods, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39449/abstract) (20). For the validation analysis using quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR), we selected 24 samples (12 samples from the 61 cases and 12 from the controls) to represent the entire range of microarray expression values in the full data set, and the correlation between *ATP8B4* mRNA levels as detected by microarray and by RT-PCR was assessed (see Supplementary Methods, available on the *Arthritis & Rheumatology* web site at http:// online library.wiley.com/doi/10.1002/art.39449/abstract).

**Statistical analysis.** For the exome sequencing discovery cohort, we obtained *P* values for association by comparing all 78 patients with SSc to the reference population of 3,179 controls using the unified sequence kernel association test with optimal kernel weighting and small sample adjustment (aSKAT-O) (22). Testing was done collapsing by genes, and the model was adjusted for ancestry, using scores for 4 principal components (PC1, PC2, PC3, and PC16) from the principal-component decomposition of the exome data (see Supplementary Methods and Supplementary Figures 1 and 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary .wiley.com/doi/10.1002/art.39449/abstract). The significance level was defined as $3.2 \times 10^{-6}$ (*P* value of 0.05 divided by 15,625 genes tested). Analysis was restricted to the functional variants that are missense, nonsense, and splice sites. Only genes with at least 4 functional variants with a MAF of less than 0.05 were considered. We used a flexible beta weight that is the default for aSKAT-O, to upweight the influence of rarer variants. For both the discovery and replication cohorts, single-variant association testing between variant rs55687265 and disease status was done using Pearson's chi-square test with Yates' continuity correction, and we also performed a Fisher meta-analysis combining 2 Fisher's exact tests weighted by sample size with a Mantel-Haenszel odds ratio (OR) calculated.

## RESULTS

**Patient characteristics.** In the discovery cohort of 78 exomes from SSc patients (Table 1), affected individuals were predominantly women (83.3%) with a mean ± SD age of 65.3 ± 10.2 years at sample collection. Their mean ± SD age at SSc onset was 50.8 ± 12.7 years.

**Table 2.** Top 20 genes identified using the aSKAT-O method (sorted by *P* value from beta-weighted analysis) comparing the 78 patients with systemic sclerosis in the exome discovery cohort to the 3,179 controls

| Chromosome | Gene | Beta-weighted *P** | Logistic-weighted *P*† |
|---|---|---|---|
| 15 | *ATP8B4* | $2.77 \times 10^{-7}$ | $1.32 \times 10^{-7}$ |
| 7 | *ASB10* | $1.32 \times 10^{-5}$ | $1.18 \times 10^{-5}$ |
| 8 | *CNGB3* | $4.79 \times 10^{-5}$ | 0.015 |
| 11 | *HSPB2* | $5.93 \times 10^{-5}$ | $5.59 \times 10^{-5}$ |
| 6 | *HLA–DRB5* | $6.18 \times 10^{-5}$ | $6.18 \times 10^{-5}$ |
| 16 | *FAM195A* | $6.24 \times 10^{-5}$ | $6.17 \times 10^{-5}$ |
| 10 | *ADD3* | 0.00012886 | 0.00010348 |
| 14 | *SNX6* | 0.0002026 | 0.00020171 |
| 17 | *KRTAP17-1* | 0.00029338 | 0.00029338 |
| 10 | *GDF2* | 0.00029692 | 0.00029162 |
| 5 | *CTNND2* | 0.0002986 | 0.00028746 |
| 11 | *OR8B2* | 0.00032285 | $1.92 \times 10^{-6}$ |
| 16 | *LYRM1* | 0.00039352 | 0.00039535 |
| 17 | *TOM1L2* | 0.00042753 | 0.00040262 |
| 13 | *ZIC5* | 0.00048856 | 0.00046994 |
| 11 | *APIP* | 0.00060887 | 0.00061461 |
| 20 | *SDC4* | 0.00061663 | 0.00061266 |
| 17 | *NGFR* | 0.00063978 | 0.00042046 |
| 13 | *CCDC70* | 0.00065038 | 0.00061089 |
| 20 | *FKBP1A-SDCBP2* | 0.00066472 | 0.00066472 |

* *P* values from the beta-weighted sequence kernel association test with optimal kernel weighting and small sample adjustment (aSKAT-O) analyses.
† *P* values from the logistic-weighted aSKAT-O analyses.

The unaffected European American controls were 58.8% women and had a mean ± SD age of 55.6 ± 13.6 years. Although distributed among all 4 classes of the New York Heart Association functional classification of pulmonary hypertension (23), patients with moderate to severe PAH (class III and IV) accounted for 50% of the group of patients with SSc with PAH (see Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39449/abstract).

**Association of an *ATP8B4* deleterious variant with risk of SSc.** Testing for association between rare functional variants and risk of SSc was performed. The top 20 genes identified using aSKAT-O with $P < 10^{-3}$ are presented in Table 2. After Bonferroni adjustment, a single gene, *ATP8B4* on chromosome 15q21.2, was significantly associated with the risk of development of SSc (beta-weighted $P = 2.77 \times 10^{-7}$, Bonferroni-adjusted $P = 0.0043$) (*P* value from the beta-weighted analysis multiplied by 15,625 genes tested) (Figure 1). This result remained unchanged when the analysis was restricted to women only in both the discovery cohort (n = 65) and the control population (n = 1,682) (see Supplementary Figure 3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39449/abstract).

Among the 64 rare functional variants (missense, nonsense, or at/near a splice site) tested for *ATP8B4*, a single missense variant, F436L (rs55687265, chromosome 15: 50226359), provided the most compelling evidence of association ($P = 9.35 \times 10^{-10}$, OR 6.11), with an MAF of 8% among patients with SSc as compared to an MAF of 1.4% in the control population. Among the 3,179 controls (3,172 with nonmissing calls), 89 were heterozygous for the nonreference allele (C) at rs55687265. In contrast, among the 78 cases (75 with nonmissing calls), 1 was homozygous and 10 were heterozygous. A computational protein prediction program, Polymorphism Phenotyping version 2, predicted rs55687265 as a damaging variant with a score of 0.941 (under the HumDiv model) on a scale of 0–1, where 0 is benign (24). Notably, repeating the aSKAT-O analysis after removing variant rs55687265 eliminated the association with *ATP8B4* (beta-weighted $P = 0.5$), suggesting that rs55687265 was the main deleterious variant responsible for the association signal observed for *ATP8B4*. Indeed, rs55687265 had the highest MAF among the 64 rare variants tested for *ATP8B4*. Two additional intronic variants (rs17494791 and rs187777730) were in linkage disequilibrium with variant rs55687265 (an $r^2$ threshold of 0.8 in Europeans was used), according to HaploReg version 3 (http://www.broadinstitute.org/

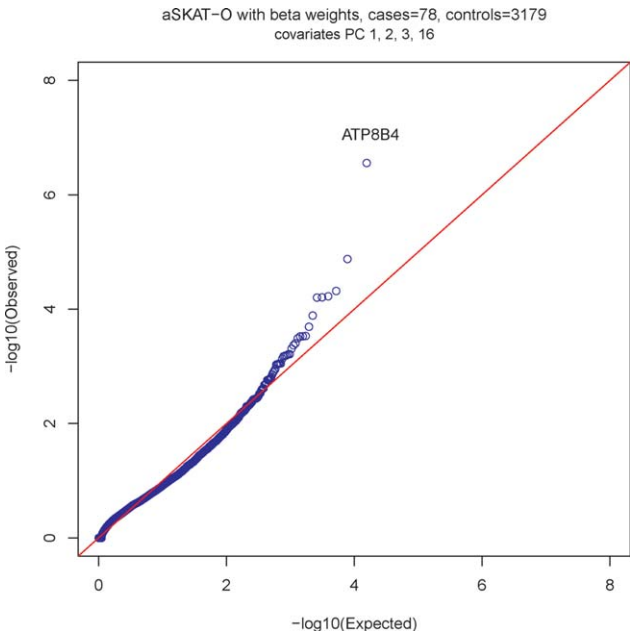


**Figure 1.** Quantile–quantile plot of *P* values for a rare variant test of association between each gene and the presence of the systemic sclerosis (SSc) phenotype. A total of 15,625 genes with at least 4 rare variants were tested for associations with a risk of SSc. The most significant association ($P = 2.77 \times 10^{-7}$) was with *ATP8B4*, with a collapsed score based on a single missense variant at c.1308C>G (rs55687265). aSKAT-O = sequence kernel association test with optimal kernel weighting and small sample adjustment; PC = principal component.

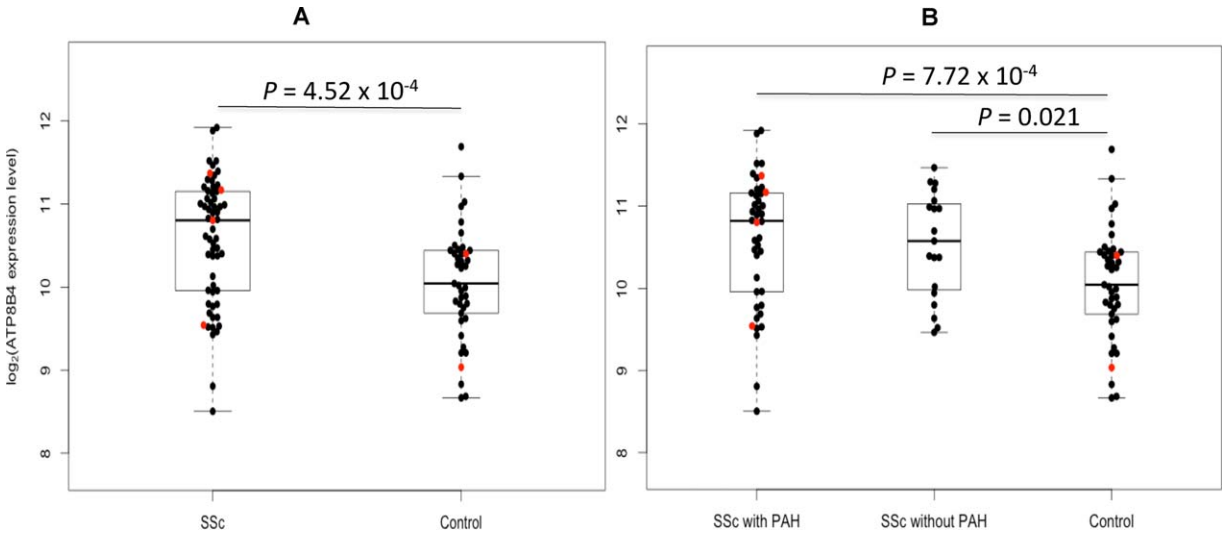


**Figure 2.** Overexpression of *ATP8B4* mRNA in peripheral blood mononuclear cells from patients with systemic sclerosis (SSc). *ATP8B4* (ILMN_1783956) expression was determined in **A,** the group of patients with SSc without pulmonary arterial hypertension (PAH; n = 19) and patients with SSc with PAH (n = 42) combined and the corresponding controls (n = 41) and **B,** patients with SSc without PAH, patients with SSc with PAH, and controls. Data are shown as box plots. Each box represents the interquartile range (IQR). Lines inside the boxes represent the median. Whiskers represent the closest values within 1.5 times the IQR. Circles represent individual subjects; red circles represent the 4 cases and 2 controls known to have the GC genotype.

mammals/haploreg/haploreg_v3.php). Of note, single-nucleotide polymorphism (SNP) rs55687265, but not the other 2 SNPs, overlaps with an enhancer in the fetal heart, suggesting the potential role of rs55687265 in influencing differential *ATP8B4* expression. However, neither of these variants was captured on the latest GWAS arrays (e.g., The Human Omni 2.5 BeadChip) or appeared in the GWAS Catalog (http://www.genome.gov/gwastudies/), a curated resource of SNP–trait associations.

Results from the subgroup analysis remained significant when we separately compared the patients with SSc without PAH and the patients with SSc with PAH to the controls ($P = 8.98 \times 10^{-6}$ and $P = 1.41 \times 10^{-4}$, respectively) (see Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39449/abstract).

**Validation in an independent cohort.** The association with variant rs55687265 was validated in an independent cohort of 415 European American patients with SSc (323 patients with SSc without PAH and 92 patients with SSc with PAH) (Table 1) and 2,848 control subjects (37.1% women; mean ± SD age 48.6 ± 6.8 years). Patients with SSc and PAH in the replication cohort had characteristics comparable to those of the discovery group, and there was no significant difference in hemodynamic measures among the patients with SSc and PAH (see Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39449/abstract). In the replication cohort, patients with SSc without PAH were also predominantly women (85.5%) but were younger, and there were fewer patients (61%) with onset of SSc as older adults (first SSc symptom after age 40 years) (see Supplementary Results, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39449/abstract) as compared to the discovery cohort (94.3%). Significant differences ($P < 2.2 \times 10^{-16}$) were observed for age, age at onset of SSc, and onset at older ages when the combined SSc group in the replication cohort was compared to the exome discovery cohort (Table 1).

Among the replication cohort, the frequency of carriers for the nonreference allele at rs55687265 among 415 SSc patients (with 21 carriers including 2 homozygous [MAF 2.77%]) was significantly higher ($P = 0.012$, OR 1.86 [95% confidence interval (95% CI) 1.134–3.025]) than that among the control group of 2,848 subjects (with 84 carriers including 2 homozygous [MAF 1.51%]). A meta-analysis of 493 cases (with an MAF of 3.57%) and 6,027 controls (MAF 1.45%) combining the discovery and replication cohorts provided an enhanced signal for the association with susceptibility to SSc ($P = 1.9 \times 10^{-7}$, OR 2.5 [95% CI 1.714–3.65]).

Similarly, we performed a subgroup analysis to test whether the variant was differentially associated with disease subtypes. Interestingly, we found that patients

with onset as older adults (61%) drove associations for the comparison between patients with SSc without PAH and the controls in the replication cohort ($P = 3.29 \times 10^{-3}$). In contrast, patients with disease onset as older adults made up 79.8% of the group with SSc with PAH in the replication cohort, and the difference between patients with SSc with PAH and the controls was slightly more significant ($P = 7.93 \times 10^{-4}$) (see Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39449/abstract).

Notably, carriers were significantly more likely to have onset of SSc at an older age (≥40 years) ($P = 0.005$) (see Supplementary Figure 4, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39449/abstract). Moreover, we recently reported that patients with onset of SSc at late ages (≥65 years) in this study population had a higher incidence of PAH at 5 years compared to those with disease onset at younger ages (12). Thus, our finding suggests that the rs55687265 variant may represent a genetic risk factor for greater severity as measured by PAH and thus poorer outcomes of SSc.

**Overexpression of *ATP8B4* in PBMCs of patients with scleroderma.** A summary of demographic and clinical descriptions of patients enrolled in the genomic analysis was presented previously (20). We observed significant overexpression of *ATP8B4* among 61 SSc patients (19 with SSc without PAH and 42 with SSc with PAH) as compared to 41 controls ($P = 4.52 \times 10^{-4}$, Benjamini and Hochberg FDR 0.003, fold change 1.43) (Figure 2A). We also performed a subgroup analysis comparing the expression levels of *ATP8B4* in the patients with SSc without PAH and the patients with SSc with PAH groups to controls. The same trend of differential expression of *ATP8B4* remained when we examined patients with SSc without PAH and patients with SSc with PAH separately ($P = 0.021$ and $P = 7.72 \times 10^{-4}$, respectively) (Figure 2B). The direct comparisons between patients with SSc without PAH and patients with SSc with PAH did not yield any significant differences, either at the genetic or the genomic level, suggesting that the subtype of SSc with PAH represents the extreme of SSc severity rather than being a distinct independent phenotype as compared to the subtype of SSc without PAH.

We further validated *ATP8B4* overexpression by qRT-PCR in the combined SSc case group compared to controls in 24 selected samples (12 samples from each group). The *ATP8B4* mRNA levels detected by microarray and RT-PCR were correlated (see Supplementary Figure 5, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39449/abstract) (Pearson's correlation coefficient 0.765, $P = 1.3 \times 10^{-5}$). However, we were unable to definitively determine the effect of the variant rs55687265 genotype on the expression level of *ATP8B4* due to limited sample size. Both the *ATP8B4* expression level and variant genotype were available for 56 patients (with either SSc without PAH or SSc with PAH) and 32 controls. Among the cases, 4 were carriers (all 4 of whom had SSc with PAH) who had the higher mean *ATP8B4* expression (presented as log base 2 of scaled expression values; mean ± SD 10.72 ± 0.82 [95% CI 9.42–12.03]) compared to the 2 carriers among the controls (mean ± SD 9.72 ± 0.97 [95% CI 1.05–18.39]). Cases who were noncarriers (n = 52) had significantly higher *ATP8B4* expression (mean ± SD 10.53 ± 0.78 [95% CI 10.31–10.75]) than the controls (n = 30; mean ± SD 10.12 ± 0.66 [95% CI 9.88–10.37]) who were also noncarriers ($P = 0.015$). Therefore, the tendency of *ATP8B4* overexpression in cases versus controls was preserved, albeit with a small number of subjects having the variant genotype. Thus, overexpression of the *ATP8B4* gene alone correlated with SSc status and is therefore a potential biomarker of SSc.

## DISCUSSION

In this study, we identified a novel gene and its variants as susceptibility loci for the development of SSc and for vascular complications within SSc. Using whole-exome sequencing and highly phenotyped subjects, we described a variant in the phospholipid transporter gene *ATP8B4* that is strongly associated with the risk of development of SSc, with or without PAH, a devastating complication with high morbidity and mortality. Further, we replicated these findings in a large, well-matched cohort, thereby strengthening their validity. Additionally, gene expression profiling data demonstrating overexpression of the *ATP8B4* gene in both the study and replication cohorts both in those with and in those without gene variants suggests a possible role in disease pathogenesis.

SSc is commonly complicated by PAH, a leading cause of mortality in the SSc spectrum of diseases. The main results from prior studies using both candidate gene and GWAS approaches have been the identification of genes belonging to immune pathways, which were mostly also associated with some other autoimmune genes ("shared autoimmunity"). Many of these studies have focused on the risk of developing SSc and not specifically on the risk of complications of SSc such as PAH. Interestingly, one study examining genetic associations with PAH in SSc also identified an

immune gene *TNFAIP3* (25), suggesting a possible role of immune system perturbations in the development of pulmonary vascular complications in SSc. As evidenced by recent efforts of whole-exome sequencing in successful identification of mutations in *CAV1* (26) and *KCNK3* (the gene encoding potassium channel subfamily K member 3) in familial and idiopathic PAH (27), it is highly suspected that rare variants with strong effects remain to be discovered for SSc and its associated pulmonary vascular complications.

In the present study, to strengthen the possibility of identification of genetic susceptibility factors for vascular complications, we included both subtypes of SSc (SSc without PAH and SSc with PAH) in a small, well-phenotyped cohort in the exome discovery phase, with targeted followup in an independent larger case–control cohort. Indeed, the identification of a novel phospholipid transporter gene *ATP8B4* and variants as susceptibility loci for SSc and pulmonary vascular complications (i.e., PAH) in our data suggest that this is a productive approach for identifying missing heritability in complex traits. Moreover, we identified rare coding-region variants in novel risk loci for SSc rather than those known loci (e.g., *STAT4* and *IRF5*) revealed by GWAS studies, supporting the notion that rare variants contribute to <3% of the heritability explained by common variants at known risk loci for autoimmune diseases (28).

*ATP8B4*, or probable phospholipid-transporting ATPase IM, is thought to participate in ATP biosynthesis and phospholipid transport via a variety of potential mechanisms. *ATP8B4* was recently identified as a risk factor for Alzheimer's disease via a GWAS study (29), and *ATP8B4* variants have also been associated with blood lipid phenotypes in the Framingham Heart Study (30) and risk of stroke in the SNP Typing for Association with Multiple Phenotypes from Existing Epidemiologic Data (STAMPEED) Cardiovascular Health Study (CHS) (dbGaP accession no. pha002887.1), which contains GWAS data for the subset of the CHS cohort selected for the NHLBI STAMPEED project. However, the function and mechanisms of *ATP8B4* remain largely unexplored given that only 10 related articles have been published to date. Human class I ATPase (ATP8A1, ATP8B1, ATP8B2, ATP8B3, and ATP8B4) is also called "P4-type ATPase," which functions as ATP-dependent aminophospholipid translocase and catalyzes phospholipid transport from the outer to the inner leaflet of membrane bilayers.

While no high-resolution structure for a P4 ATPase has been determined, the presence of well-conserved motifs found in all P-type ATPases suggests that they adopt the 4-domain structure observed with the $Ca^{++}$, $Na^{+}/K^{+}$, and $H^{+}$ ATPases that have been crystallized: the nucleotide-binding, phosphorylation, actuator, and membrane domains (31). All of these P-type ATPases are predicted to have 10 transmembrane segments composing the membrane domain. The actuator domain folds from cytosolic sequences preceding transmembrane segment 1 and in the loop between transmembrane segment 2 and transmembrane segment 3, while the phosphorylation and nucleotide-binding domains are formed from the cytosolic loop between transmembrane segment 4 and transmembrane segment 5. For cation transporters, binding of ATP to the nucleotide-binding domain and the subsequent phosphorylation of an Asp residue in the phosphorylation domain induces dramatic conformational transitions described as E1 to E2-P in the Post-Albers catalytic cycle (32–34). The deleterious *ATP8B4* variant F436L is located at amino acid position 436, which is between transmembrane segment 4 and transmembrane segment 5, where the phosphorylation and nucleotide-binding domains are formed from the cytosolic loop, thus possibly affecting the E1 to E2-P conformational transitions.

Because the molecular mechanisms by which P4-type ATPases relate to severe inherited human diseases remain largely unexplored, the study of these proteins presents a challenge. Mutations in *ATP8B1* cause progressive familial intrahepatic cholestasis type 1 or Byler disease (35) and are associated with an increased risk of pneumonia with markedly elevated amounts of cardiolipin (a major phospholipid of the mitochondrial inner membrane and a key factor for apoptosis [36]) in lung fluid (37). Defects in cardiolipin may also play an important role in the rapid development of right ventricular dysfunction and right-sided heart failure in persistent pulmonary hypertension of the newborn (38). Given the structural and functional similarities between *ATP8B1* and *ATP8B4*, we speculate that defects in *ATP8B4* may play important roles in regulating phospholipids in SSc-associated diseases as well as other organs in which it is a candidate for genetic and acquired diseases.

Despite the novelty of an exome-sequencing approach in SSc, the use of phenotypically well-matched extremes, and the efficient statistical approaches to the analysis of rare variants, our study has some limitations. More generally, our results suggest that rare variants, as risk factors for SSc, deserve further attention using a larger sample size of SSc cases, i.e., genotyping or targeted sequencing of thousands of SSc patients in order to validate our findings. Furthermore, the mechanisms by which variants in *ATP8B4* confer risk are unknown but clearly warrant further investigation. Expression modifiers are often located in promoters, distant promoters, or even intronic regions. Notably, *ATP8B4* gene

expression is possibly regulated by *HOXA9*, a DNA-binding transcription factor that binds in a distal promoter of *ATP8B4* and has been previously shown to be dysregulated in SSc (39). It is likely that genetic variants that act as "expression modifiers" affecting *ATP8B4* gene expression in *cis* and in *trans* are yet to be identified. In contrast, damaging variants in *ATP8B4* are likely to change the protein function such as the predicted E1 to E2-P conformational transition and subsequent phospholipid transport kinetic alteration. This may explain in part the weak correlation observed between the deleterious F436L variant and *ATP8B4* overexpression in SSc patients.

It is well established that the plasma membrane exhibits an asymmetric distribution of lipids between the inner and outer leaflets of the lipid bilayer. Recent studies suggest that this asymmetric distribution changes locally and temporarily, accompanied by cellular events (40,41). Future studies examining the function of the mutant *ATP8B4* might provide insights into the function of the protein, such as evaluating the mutant effects on lipid uptake by measuring the fluorescence of nitrobenzoxadiazole-labeled phosphatidylserine, a negatively charged phospholipid component of cell and blood platelet membranes typically located in the inner leaflet of the membrane.

A limitation of the gene array studies performed in our study includes a focus on PBMCs, which limits our ability to interrogate the impact on single cell populations and which may have caused us to miss important differences in gene expression in relevant, specific subsets of cells. By analyzing global RNA expression within individual tissues or cells and treating the expression levels of genes as quantitative traits, variations in gene expression that are highly correlated with genetic variation can be identified as expression quantitative trait loci (eQTLs). In future mechanistic studies, we anticipate using whole-transcriptome RNA sequencing analysis for identifying allele-specific expression and measuring gene expression variation derived from genetic variants as eQTLs. Single-cell whole-transcriptome RNA sequencing analysis facilitated by single-cell isolation methods, such as fluorescence-activated cell sorting or microfluidic analysis, is also promising and provides the expression profile of individual cells, especially for profiling rare or heterogeneous populations of cells (42,43). Thus, genome-scale studies using single-cell whole-transcriptome RNA sequencing and in-depth functional characterization of the target genetic variants are warranted.

To evaluate the vascular phenotype of SSc patients, peripheral vasculopathy and digital ulcers must be taken into consideration in addition to PAH. Our inability to show tissue specificity (i.e., in the lung) of the expression of *ATP8B4* gene and its protein products in our own data remains one of the limitations of our study. Correlations between genotype and tissue-specific gene expression levels will help identify regions of the genome that influence whether and how much a gene is indeed expressed. To explore the tissue-specific expression of the *ATP8B4* gene, we searched the Genotype-Tissue Expression (GTEx) Project database with newly released RNA sequencing data from 1,641 samples across 43 human tissues from 175 individuals (http://www.gtexportal.org/home/eqtls/byGene?geneId=ATP8B4&tissueName=Lung). Although rs55687265 was not found in the GTEx database, significant SNP–gene eQTL association was observed in lung tissue samples (n = 124) for 3 markers located directly upstream of the *ATP8B4* gene: an intronic marker in *SLC27A2* (rs112753726; $P = 2.6 \times 10^{-6}$) and 2 additional intronic markers in linkage disequilibrium in *HDC* (rs55828351 [$P = 8.5 \times 10^{-7}$] and rs72737086 [$P = 1.1 \times 10^{-6}$]).

This novel finding supports the lung as being the main target tissue where genetic variations in the genomic region of the *ATP8B4* gene is acting in *cis* (locally) and thus influencing its expression. Our research suggests that further investigation of whether overexpression of the *ATP8B4* gene by PBMCs can provide a noninvasive, cost-effective, easy-to-measure, and sufficiently sensitive and specific molecular biomarker is warranted. Such studies would include deeper phenotyping of patient cohorts, comparison with other known biomarkers, and characterization of signaling pathways that may support the notion of a role of *ATP8B4* in SSc pathogenesis.

It is noteworthy that evidence was also found for association of variants in several other genes with risk of SSc, with *P* values less than $10^{-5}$ (Table 2). These included *ASB10*, *CNGB3,* MHC class II gene *HLA–DRB5* and *HSPB2* (heat shock 27-kd protein 2, aka HSP27). *ASB10* is a component of the Skp, Cullin, F-box (SCF)–containing complex–like Elongin-Cullin-SOCS box protein (ECS) E3 ubiquitin-protein ligase complex, which mediates the ubiquitination and subsequent proteasomal degradation of target proteins (44); it is also related to the pathway MHC class I–mediated antigen processing and presentation and immune system. *CNGB3* is involved in several pathways, including activation of cAMP-dependent PKA, endothelial cell nitric oxide synthase signaling, and cellular effects of sildenafil (45,46). An important paralog of this gene is *KCNH6*. It is well known that vascular, hormonal, or neurologic irregularities can alter production of nitric oxide by NOS and disturb the balance between synthesis and degradation of cGMP while sildenafil, a drug used to treat erectile dysfunction and PAH, can inhibit the action of PDE5

to increase cGMP levels and, in turn, enhance prolonged smooth muscle relaxation/vasodilation. Thus, our evidence suggests that ASB10-mediated ubiquitin degradation pathways and cyclic nucleotide–gated channelopathies may play a role. Functional and pharmacogenomic studies are needed to determine the clinical and genetic significance of these genes in the molecular pathogenesis of SSc and pulmonary vascular complications.

Using optimized analytical approaches for rare variants and extensive studies of gene expression, we found compelling evidence that variants in *ATP8B4* are associated with the risk of SSc and pulmonary vascular complication. Dysfunction of *ATP8B4* may also have implications for other neurodegenerative, cardiovascular, respiratory, and autoimmune disorders, depending on its targeted tissue-specific expression and interaction with inflammation, angiogenesis, and vascular remodeling as well as immune–effector pathways.

## ACKNOWLEDGMENTS

We thank the patients for participating in this study. The authors would like to thank the NHLBI GO Exome Sequencing Project and its ongoing studies which produced and provided exome variant calls for comparison: the Lung GO Sequencing Project (HL-102923), the WHI Sequencing Project (HL-102924), the Broad GO Sequencing Project (HL-102925), the Seattle GO Sequencing Project (HL-102926), and the Heart GO Sequencing Project (HL-103010).

### AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Barnes had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study conception and design.** Gao, Emond, Vergara, Mathai, Rich, Nickerson, Bamshad, Hassoun, Barnes.

**Acquisition of data.** Gao, Cheadle, Rafaels, Vergara, Mathai, Nickerson, Hummers, Bamshad, Hassoun, Barnes.

**Analysis and interpretation of data.** Gao, Emond, Louie, Cheadle, Berger, Rafaels, Vergara, Kim, Taub, Ruczinski, Nickerson, Bamshad, Hassoun, Mathias, Barnes.

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