ORIGINAL ARTICLE

PCSK6 and Survival in Idiopathic Pulmonary Fibrosis

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Abstract

Rationale: Idiopathic pulmonary fibrosis (IPF) is a devastating disease characterized by limited treatment options and high mortality. A better understanding of the molecular drivers of IPF progression is needed.

Objectives: To identify and validate molecular determinants of IPF survival.

Methods: A staged genome-wide association study was performed using paired genomic and survival data. Stage I cases were drawn from centers across the United States and Europe and stage II cases from Vanderbilt University. Cox proportional hazards regression was used to identify gene variants associated with differential transplantation-free survival (TFS). Stage I variants with nominal significance ($P < 5 \times 10^{-5}$) were advanced for stage II testing and meta-analyzed to identify those reaching genome-wide significance ($P < 5 \times 10^{-8}$). Downstream analyses were performed for genes and proteins associated with variants reaching genome-wide significance.

Measurements and Main Results: After quality controls, 1,481 stage I cases and 397 stage II cases were included in the analysis. After filtering, 9,075,629 variants were tested in stage I, with 158 meeting advancement criteria. Four variants associated with TFS with consistent effect direction were identified in stage II, including one in an intron of *PCSK6* (proprotein convertase subtilisin/kexin type 6) reaching genome-wide significance (hazard ratio, 4.11 [95% confidence interval, 2.54–6.67]; $P = 9.45 \times 10^{-9}$). PCSK6 protein was highly expressed in IPF lung parenchyma. PCSK6 lung staining intensity, peripheral blood gene expression, and plasma concentration were associated with reduced TFS.

Conclusions: We identified four novel variants associated with IPF survival, including one in *PCSK6* that reached genome-wide significance. Downstream analyses suggested that PCSK6 protein plays a potentially important role in IPF progression.

Keywords: IPF; genome-wide association study; genomics; survival; PCSK6 protein

Idiopathic pulmonary fibrosis (IPF) is a devastating disease characterized by progressive lung scarring and poor survival (1, 2). Two antifibrotic therapies were approved for the treatment of individuals with IPF after randomized controlled trials demonstrated efficacy in slowing lung function decline (3, 4). Despite this advance, outcomes remain poor, and antifibrotic therapy appears to provide only modest survival benefit (5). To improve IPF outcomes, novel therapeutic targets are needed.

We and others have identified molecular IPF risk factors through unbiased investigation of the genome, transcriptome, and proteome (6–15). Among the strongest molecular determinants of IPF is a common variant in the promoter region of *MUC5B* (mucin 5B, oligomeric mucus/gel-forming), which increases the odds of developing IPF by nearly fivefold per risk allele (6–9). Despite this strong association with IPF onset, the MUC5B promoter was paradoxically associated with improved survival (16), though this association was potentially confounded by index event bias (17). Few other susceptibility-associated gene variants have been shown to reliably predict differential IPF survival, suggesting that

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molecular determinants of IPF susceptibility and progression may have limited overlap.

To better understand molecular drivers of IPF progression and identify new therapeutic targets, we conducted a two-stage, multicenter, international genome-wide association study (GWAS) of IPF survival, followed by downstream analysis of genes and proteins associated with top survival-associated variants to determine whether these circulating proteins also predicted differential survival. Some results of this study have been previously reported in the form of abstracts at the American Thoracic Society (18) and British Thoracic Society (19) national meetings and in preprint form (https://www.medrxiv.org/content/10.1101/2022.05.06.22274705v1).

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Data sharing statement: Genome-wide association study summary statistics for this study are available at https://github.com/genomicsITER/PFgenetics.

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This article has a related editorial.

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

At a Glance Commentary

Scientific Knowledge on the Subject: A host of gene variants that increase one's risk of developing idiopathic pulmonary fibrosis (IPF) have been identified through genomic analyses. Few of these variants have been associated with differential outcomes, however, suggesting that genomic determinants of IPF susceptibility and progression may have limited overlap. To identify genomic determinants of IPF outcomes, a genome-wide association study of IPF survival was conducted.

What This Study Adds to the Field: We identified four novel gene variants associated with differential IPF survival, including one in *PCSK6* (proprotein convertase subtilisin/kexin type 6) that reached genome-wide significance. Downstream analysis showed that PCSK6 was highly expressed in IPF lung parenchyma and that PCSK6 lung staining intensity, peripheral blood gene expression, and plasma concentration were associated with reduced transplantation-free survival. These findings suggest that PCSK6 may play a potentially important role in IPF progression.

Methods

Cohorts and Case Selection

All patients provided informed consent for research blood draws in accordance with protocols approved by the institutional review board at each participating institution. GWAS stage I cases consisted of unrelated patients with IPF of European ancestry from three previously described case-control GWAS data sets from the United States (9), the United Kingdom (7), and a combined cohort from the United States, United Kingdom, and Spain (UUS) (6). Available outcome data were gathered for all cases meeting international consensus criteria for IPF (20), and survival was plotted for individual cohorts within each data set. Patients without available outcome data were excluded, as were clinical trial cohorts because of short follow-up (*see* the online supplement). Stage II cases consisted of previously described, unrelated patients with IPF of European ancestry from Vanderbilt University (21). Vital status was assessed in U.S.- and Spain-based cohorts by chart review and contact with family members and in United Kingdom–based cohorts by review of national vital status databases.

Genotyping and Quality Control

Genotypes were generated for stage I cases using SNP genotyping arrays according to previously described methods (6, 7, 9). A summary of arrays used for each cohort is provided in the online supplement. Imputation for stage I cases was performed using the Michigan Imputation Server using the Haplotype Reference Consortium panel (version 1.1 2016). Genotypes for the stage II cases were determined by whole-genome sequencing, as previously described (21). Stringent quality control measures were applied, with a two-tier variant-filtering scheme in which variants with minor allele frequencies (MAFs) of 0.5–1% were retained when imputation R^2 was $\geqslant$0.8 and those with MAFs $\geqslant$1% were retained when imputation R^2 was $\geqslant$0.5. Variants deviating from Hardy-Weinberg equilibrium ($P < 1.0 \times 10^{-6}$) were removed.

Genome-wide Survival Analysis

The primary endpoint assessed was transplantation-free survival (TFS), defined as the time in months from the site-determined date of IPF diagnosis to event (death or lung transplantation) or censoring date. Variants associated with differential TFS were identified using a multivariable Cox proportional hazards regression model adjusted for age, sex, center, and first 10 genetic principal components, with principal components calculated separately for each cohort. Variant genotypes were treated as a continuous variable, with each patient having an imputed genotype dosage between zero and two risk alleles. To avoid considering results obtained for just one of the three individual studies (United States, United Kingdom, and UUS), only those variants with association results available for at least two data sets (United States, United Kingdom, and UUS) were meta-analyzed using a fixed-effect inverse variance–weighted meta-analysis (METAL [http://csg.sph.umich.edu/abecasis/metal/] version 2011-03-25) to generate stage I results.

Variants nominally associated with TFS in stage I were defined as those with Wald P values $<$0.05 in at least two data sets with the same direction of effect and $P < 5.0 \times 10^{-5}$ in stage I meta-analysis. Conditional analysis of these SNPs to deduce their independence was performed with GCTA-COJO version 1.26 (https://yanglab.westlake.edu.cn/software/gcta/#Overview). The proportional hazards assumption was then assessed for each independent variant meeting advancement criteria by testing whether Schoenfeld residual rank varied by genotype strata. Variants that satisfied the proportional hazards assumption were advanced for stage II testing. Stage I and II cases were then meta-analyzed using METAL with the genome-wide significance threshold set at $P < 5.0 \times 10^{-8}$. *In silico* assessments were used to infer the biological effect of variants associated with TFS after stage II testing.

PCSK6 Tissue Expression

Formalin-fixed paraffin-embedded human lung tissue sections obtained from patients with IPF undergoing surgical lung biopsy were compared with sections from control subjects undergoing lung resection for malignancy, with sections distal to areas of malignancy used. Immunohistochemistry was performed using standard methods (*see* the online supplement), and mean staining intensity of PCSK6 protein was compared between IPF cases and non-IPF control subjects using a Mann-Whitney U test. Single-cell RNA sequencing data from prior published data sets (11, 22, 23) were reanalyzed and jointly annotated using label transfer from an updated annotated version of GSE135893. Cell-type annotation was performed using the TransferAnchors function in Seurat version 4 (https://yanglab.westlake.edu.cn/software/gcta/#Overview) (24). Data visualization was performed using Scanpy version 1.7.2 (https://scanpy.readthedocs.io/en/stable/) (25). The code used for analysis and presentation is available at https://github.com/KropskiLab/ipf_survival_gwas.

PCSK6 Clinical Outcome Association

The association between circulating *PCSK6* gene expression and TFS was assessed using three previously published microarray data sets from the COMET (Correlating Outcomes with Biochemical Markers to Estimate Time-Progression in IPF) trial, Imperial College, and the University of Chicago (UChicago) (*see* the online supplement) (26), which were

analyzed separately, with results meta-analyzed and presented as a forest plot. Circulating plasma PCSK6 protein concentration was then determined in patients with IPF from the University of California, Davis (UC-Davis), and the University of Chicago (UChicago) (*see* the online supplement), $\log_2$ transformed, and tested for TFS association using Cox proportional hazards regression (27). The proportional hazards assumption was satisfied for all downstream survival analyses.

Results

Case Selection for Stage I

Patients constituting the U.S. cohort included those from UChicago ($n = 118$) and the University of Pittsburgh ($n = 200$) (*see* Figures E1 and E2). Those constituting the United Kingdom cohort included patients from the University of Edinburgh ($n = 119$), the Trent Lung Fibrosis Study ($n = 210$), a subset of those participating in the prospective, multicenter PROFILE (Prospective Study of Fibrosis In the Lung Endpoints) study (NCT 01134822) ($n = 175$), and aggregated patients from smaller United Kingdom centers (Hull and Papworth) ($n = 61$) (*see* Figures E1 and E3). Patients constituting the UUS cohort included those from UChicago (independent of those from the U.S. cohort; $n = 187$), the PROFILE study (independent of those in the United Kingdom data set; $n = 299$), the University of California (Davis and San Francisco) ($n = 84$), and aggregated patients from centers in Spain ($n = 28$) (*see* Figures E1 and E4).

Baseline characteristics and outcomes

After phenotypic exclusions, 1,481 patients constituting stage I were included in the analysis. These included 318 patients from the U.S. data set, 565 from the United Kingdom data set, and 598 from the UUS data set. Baseline characteristics for each data set are shown in Table 1. The mean age ranged from 67 to 72 years, and men constituted 71–75% of each data set. The mean percentage predicted FVC and diffusion capacity of the lung for carbon monoxide (D_{LCO}) was lowest in patients constituting the U.S. data set and highest in the United Kingdom data set. A majority of patients in each data set were classified as gender, age, and physiology stage I or II (28). Median survival was highest in the United Kingdom cohort (53.2 mo), followed by the UUS cohort (40.6 mo) and the U.S. cohort (39.3 mo) ($P = 0.001$) (*see* Figure E2). Survival also varied substantially across the centers constituting each cohort (*see* Figures E3–E5). Median survival was 48 months in the Vanderbilt University validation cohort (*see* Figure E6). Survival was similar between stage I and II cases through 24 months of follow-up but could not be compared thereafter because of violation of the proportional hazards assumption (*see* Figure E6).

Genome-Wide Survival Analysis

After filtering, 7,873,835 variants in the U.S. data set, 8,591,398 variants in the United Kingdom data set, and 8,620,496 variants in the UUS data set were tested for TFS association. Quantile-quantile plots for each stage I cohort suggested acceptable inflation (29) (*see* Figure E7). After stratifying stage I cohorts by MAF, inflation was higher for rare variants compared with low- and high-frequency variants, but within an acceptable range for each group ($\lambda < 1.1$) (*see* Figure E8). For meta-analysis, 9,075,629 variants were tested for TFS association in the aggregated stage I cohorts. One hundred sixty-one independent SNPs had Wald P values <0.05 in at least two data sets with the same direction of effect and $P < 5.0 \times 10^{-5}$ in stage I meta-analysis (Figure 1). Of those, 158 satisfied the proportional hazards assumption and advanced for stage II testing (*see* Table E1).

Genotype data were available in the Vanderbilt University cohort for 154 of the 158 variants advanced from stage I. Six variants were associated with TFS in the Vanderbilt University cohort at $P < 0.05$, including four with consistent effect direction that strengthened in TFS association after meta-analysis (Table 2; *see* Table E2). These four were rs184498750 near *SUCLG1* (succinate-CoA ligase

Table 1. Baseline Characteristics and Outcomes of Stage I and II Data Sets

Characteristic	Stage I ($n = 1,481$)			Stage II ($n = 397$)
	United States* ($n = 318$)	United Kingdom† ($n = 565$)	UUS‡ ($n = 598$)	Vanderbilt§
Age, yr, mean (SD)	67.3 (8.9)	72.1 (8.4)	69.8 (8.3)	65.7 (9.0)
Male sex, *n* (%)	234 (73.6)	403 (71.3)	449 (75.1)	295 (74.3)
FVC, % predicted, mean (SD)	62.7 (17.9)	77.7 (18.5)	70.2 (18.6)	65.0 (16.1)
D_{LCO}, % predicted, mean (SD)	36.7 (14.6)	43.3 (14.3)	39.9 (14.6)	39.1 (13.5)
GAP stage, *n* (%)				
I	72 (23.1)	106 (35.2)	135 (24.2)	115 (29.8)
II	149 (47.8)	144 (47.8)	286 (51.4)	201 (52.1)
III	91 (29.2)	51 (16.9)	136 (24.4)	70 (18.1)
Death, *n* (%)	189 (59.4)	366 (64.8)	257 (43.0)	202 (50.9)
Transplantation, *n* (%)	52 (16.4)	2 (0.4)	26 (4.4)	32 (8.1)
Death or transplantation, *n* (%)	241 (75.8)	366 (64.8)	283 (47.3)	234 (58.9)
Median survival, mo (IQR)	39.3 (13.4–70.6)	53.2 (24.8–92.5)	40.6 (19.9–75.5)	48 (15–105)

Definition of abbreviations: GAP = gender, age, and physiology; IQR = interquartile range; SD = standard deviation; UUS = United States, United Kingdom, and Spain.
*Missing data: FVC, $n = 6$; D_{LCO}, $n = 31$: GAP stage, $n = 6$.
†Missing data: FVC, $n = 241$; D_{LCO}, $n = 264$: GAP stage, $n = 241$.
‡Missing data: FVC, $n = 30$; D_{LCO}, $n = 41$; GAP stage, $n = 30$.
§Missing data: FVC, $n = 4$; D_{LCO}, $n = 9$; GAP stage, $n = 4$.

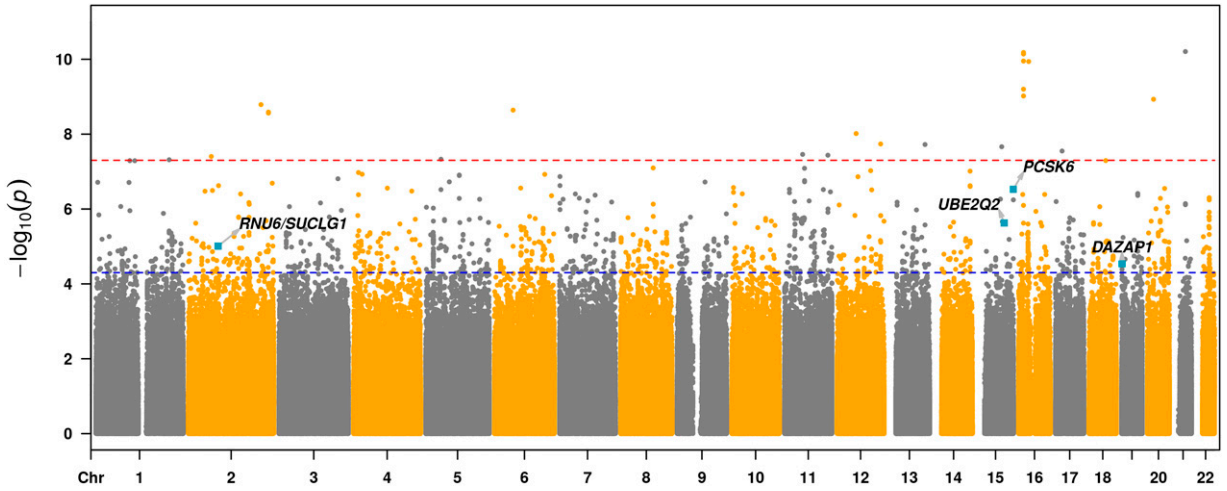

Figure 1. Manhattan plot of stage I gene variants associated with idiopathic pulmonary fibrosis survival. Each dot represents a gene variant, arranged on the *x*-axis by chromosome. Variants falling above the blue line, which corresponds to $P < 5 \times 10^{-5}$, are considered to reach nominal significance, whereas those falling above the red line, which corresponds to $P < 5 \times 10^{-8}$, are considered to reach genome-wide significance. All variants crossing the nominal significance threshold were advanced for stage II testing. Chr = chromosome; *DAZAP1* = deleted in azoospermia-associated protein 1; *PCSK6* = proprotein convertase subtilisin/kexin type 6; *RNU6* = U6 small nuclear RNA; *SUCLG1* = succinate-CoA ligase GDP/ADP-forming subunit α; *UBE2Q2* = ubiquitin-conjugating enzyme E2Q family member 2.

GDP/ADP-forming subunit α), rs60514164 near *UBE2Q2* (ubiquitin-conjugating enzyme E2Q family member 2), rs35647788 in an intron of *PCSK6* (proprotein convertase subtilisin/kexin type 6), and rs3893252 in an intron of *DAZAP1* (deleted in azoospermia-associated protein 1) (Table 2). Of these, rs35647788 (*PCSK6*) showed the strongest TFS association across stage I (hazard ratio [HR], 4.76 [95% confidence interval (CI), 2.62–8.64]; $P = 2.96 \times 10^{-7}$) and stage II (HR, 3.12 [95% CI, 1.37–7.11]; $P = 6.70 \times 10^{-3}$) cohorts and crossed the genome-wide significance threshold in meta-analysis (HR, 4.11 [95% CI, 2.54–6.67]; $P = 9.45 \times 10^{-9}$) (Table 2). With the exception of rs60514164 (*UBE2Q2*) (MAF = 8%), these SNPs were low frequency, with MAFs of ~1% in the study population. Regional association plots for each of the four variants are shown in Figure E9.

In multivariable analysis, each variant except rs3893252 (*DAZAP1*) maintained survival association after adjustment for relevant confounders of IPF survival (*see* Table E3). Among patients with the rs35647788 (*PCSK6*) variant, all were heterozygotes (*see* Table E4) and were evenly distributed across centers constituting the United Kingdom and UUS cohorts. No rs35647788 (*PCSK6*) variants were observed in the U.S. cohort despite good imputation quality ($r^2 = 0.74$). In sensitivity analysis of the *PCSK6* variant, results were consistent when censoring transplantations (*see* Table E5). *In silico* testing revealed functional effects for each of the four variants associated with TFS after stage II meta-analysis (*see* Table E6), none of which had a known association with fibrotic lung disease. Using Genotype-Tissue Expression, we found multiple common sentinel PCSK6 expression quantitative trait loci in high linkage disequilibrium ($D' = 1$) with rs35647788 (*see* Table E7).

PCSK6 Tissue Expression

Morphologic assessment of histological sections from lung tissue of patients with IPF and control subjects without fibrotic lung disease was performed. In IPF lung, cytoplasmic PCSK6 expression localized to ciliated epithelial cells and alveolar epithelial cells and was markedly higher than PCSK6 expression in non-IPF control sections (Figure 2). Western blot confirmed the presence of only a single PCSK6 band (*see* Figure E10). Relative staining intensity was twofold higher in IPF lung samples ($n = 86$) compared with non-IPF control samples ($n = 9$) ($P < 0.001$) (Figure 3A). Increased PCSK6 protein staining score was associated with reduced TFS in those with available survival data ($n = 71$), with staining scores above the median associated with greater than twofold increased risk of death or lung transplantation (HR, 2.41 [95% CI, 1.12–5.16]; $P = 0.024$) (Figure 3B).

Interrogating previously published single-cell RNA sequencing data from IPF and control lungs, PCSK6 expression was highest in lymphatic endothelial cells and adventitial fibroblasts, while broad expression in the airway epithelium was observed (*see* Figure E11).

PCSK6 Clinical Outcome Association

When assessing *PCSK6* gene expression in the COMET ($n = 75$), Imperial College ($n = 55$), and UChicago ($n = 45$) cohorts, increasing PCSK6 expression was associated with increased mortality risk in each cohort, with each one-unit increased associated with greater than threefold increased risk of death or lung transplantation in meta-analysis (HR, 3.43 [95% CI, 1.62–7.25]; $P = 0.0012$) (Figure 4A). When assessing PCSK6 plasma concentration in patients with IPF from UC-Davis ($n = 138$) and UChicago ($n = 181$), increasing plasma PCSK6 concentration was associated with reduced TFS, with each one-unit change in log-transformed plasma concentration associated with a nearly 50% increase in outcome risk (HR, 1.47 [95% CI, 1.14–1.89]; $P = 0.0031$). These results were consistent across UC-Davis (HR, 1.47 [95% CI, 1.14–1.89]; $P = 0.08$) and UChicago (HR, 1.34 [95% CI, 0.92–1.94]; $P = 0.12$) cohorts. After stratification of the combined cohort by tertiles, those with PCSK6 concentration in the highest tertile displayed significantly

Table 2. Results for the SNPs Nominally Associated across Stages and with Same Direction of Effects on Idiopathic Pulmonary Fibrosis Survival

Variant Location (hg19) and Nearest Gene							Stage I Results			Stage II Results			Meta-Analysis	
Chr	Position	SNP rsID	Gene	Ref	EA	R^2	EAF (%)	HR (95% CI)	*P* Value	EAF (%)	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
2	84291167	rs184498750	*SUCLG1*	G	T	0.86	1.1	3.11 (1.88–5.15)	9.83×10^{-6}	1.2	2.05 (1.79–4.18)	0.049	2.71 (1.79–4.08)	2.07×10^{-6}
15	76081200	rs60514164	*UBE2Q2*	C	T	0.77	7.0	1.60 (1.32–1.95)	2.35×10^{-6}	7.7	1.42 (1.04–1.93)	0.026	1.55 (1.31–1.83)	2.23×10^{-7}
15	101914234	rs35647788	*PCSK6*	C	T	0.93	0.8	4.76 (2.62–8.64)	2.96×10^{-7}	0.8	3.12 (1.37–7.11)	6.72×10^{-3}	4.11 (2.54–6.67)	9.45×10^{-9}
19	1412985	rs3893252	*DAZAP1*	C	T	0.82	0.6	3.57 (1.97–6.49)	2.91×10^{-5}	1.5	2.09 (1.05–4.15)	0.036	2.84 (1.81–4.45)	5.81×10^{-6}

Definition of abbreviations: CI = confidence interval; *DAZAP1* = deleted in azoospermia-associated protein 1; EA = effect allele; EAF = effect allele frequency; HR = hazard ratio; *PCSK6* = proprotein convertase subtilisin/kexin type 6; R^2 = lowest imputation quality value across studies; Ref = reference allele; *SUCLG1* = succinate-CoA ligase GDP/ADP-forming subunit α; *UBE2Q2* = ubiquitin-conjugating enzyme E2Q family member 2.

worse survival than those in the second and third tertiles ($P = 0.0018$) (Figure 4B).

Overlap between IPF Risk and TFS

Variants previously associated with IPF risk (6–10) were investigated for outcome association (*see* the online supplement). None of the 15 genetic variants previously associated with IPF risk (6–10) was associated with TFS after Bonferroni correction ($P = 0.0033$) (*see* Table E8). As previously reported (16, 17), individuals with the *MUC5B* promoter polymorphism (rs3570590) displayed better overall survival, though this did not reach significance after adjustment for multiple testing. None of the four validated survival variants was associated with differential IPF risk (*see* Table E9). When combing the effect of thousands of IPF risk variants in a polygenic risk score, this risk score was not significantly associated with TFS for any significance threshold used (*see* Figure E12), again suggesting that variants that affect disease risk may have little impact on survival times after diagnosis.

Discussion

In this investigation, we conducted the first GWAS of IPF survival, identifying a variant intronic to *PCSK6* that associated with differential TFS at genome-wide significance in two independent IPF cohorts totaling nearly 2,000 patients. We subsequently found that PCSK6 protein was highly expressed in IPF lung tissue, localizing to the airway epithelium, which plays a key role in IPF onset and progression (30). Finally, we found that PCSK6 lung staining, peripheral blood gene expression, and circulating plasma concentration were negatively correlated with TFS across independent IPF cohorts. To our knowledge, this study is the first to systematically identify gene variants associated with IPF survival and the first to identify PCSK6 as a potentially relevant mediator of IPF progression.

PCSK6, also called *PACE4*, encodes a widely expressed calcium-dependent serine endoprotease, which we demonstrate is expressed most highly in the airway epithelium, adventitial fibroblasts, and lymphatic endothelial cells in the lung. PCSK6 is a critical mediator of TGF-β (transforming growth factor-β) processing and is crucial for reproduction, embryological development, and blood pressure regulation (31–35). A *PCSK6* gene variant has been implicated in the development of hypertension (35), and dysregulated *PCSK6* gene expression has been linked to vascular disease (36–38), and cardiac remodeling after myocardial ischemia (39). These cardiovascular remodeling effects make PCSK6 of particular relevance to IPF, as *PCSK6* overexpression can lead to increased collagen I and III deposition, TGF-β activation, and extracellular matrix formation (39, 40), which are cardinal features of IPF pathogenesis (2). In addition, PCSK6 may bind tissue inhibitors of metalloproteinases (41), potentially counteracting the antimetalloproteinase activity of tissue inhibitors of metalloproteinases (42).

PCSK6 dysregulation has also been implicated in the development of cancer of the lung (43), breast (44), ovary (45), thyroid (46), and prostate (47). PCSK6 has been shown to regulate apoptosis in prostate cancer (48) and pancreatic cancer (49) and is also linked to increased cancer cell invasiveness by enhancing bioactivity of matrix metalloproteinases and cytokines (50). Accordingly, PCSK6 has been proposed as an antitumor therapeutic target (51, 52), and a bioavailable formulation of an anti-PCSK6 molecule is currently under investigation (53). *In vitro* PCSK6 inhibition has already been shown to reduce fibroblast proliferation, migration, and invasion in rheumatoid arthritis–associated synovitis (54). Given its relatively high expression in adventitial (including airway-associated) fibroblasts and the airway epithelium, PCSK6 could serve as a potential therapeutic in patients with pulmonary fibrosis. However, additional research is needed to better characterize the role PCSK6 may play in IPF progression before therapeutic blockade is considered.

Despite being the largest genomic analysis of IPF survival performed to date, the modest size of this cohort limited our ability to identify higher frequency SNPs with modest effect sizes. In addition, the rare nature of the *PCSK6* variant identified in this study suggests that it is unlikely to singularly explain subsequent gene expression and protein findings. *In silico* analyses identified several nearby regulatory elements that may include common functional variants, with smaller effects, in linkage disequilibrium with this *PCSK6* variant. Further research is needed to determine whether rs35647788 and other rare variants in *PCSK6* have additive effects on gene expression, together with investigation of regulatory elements,

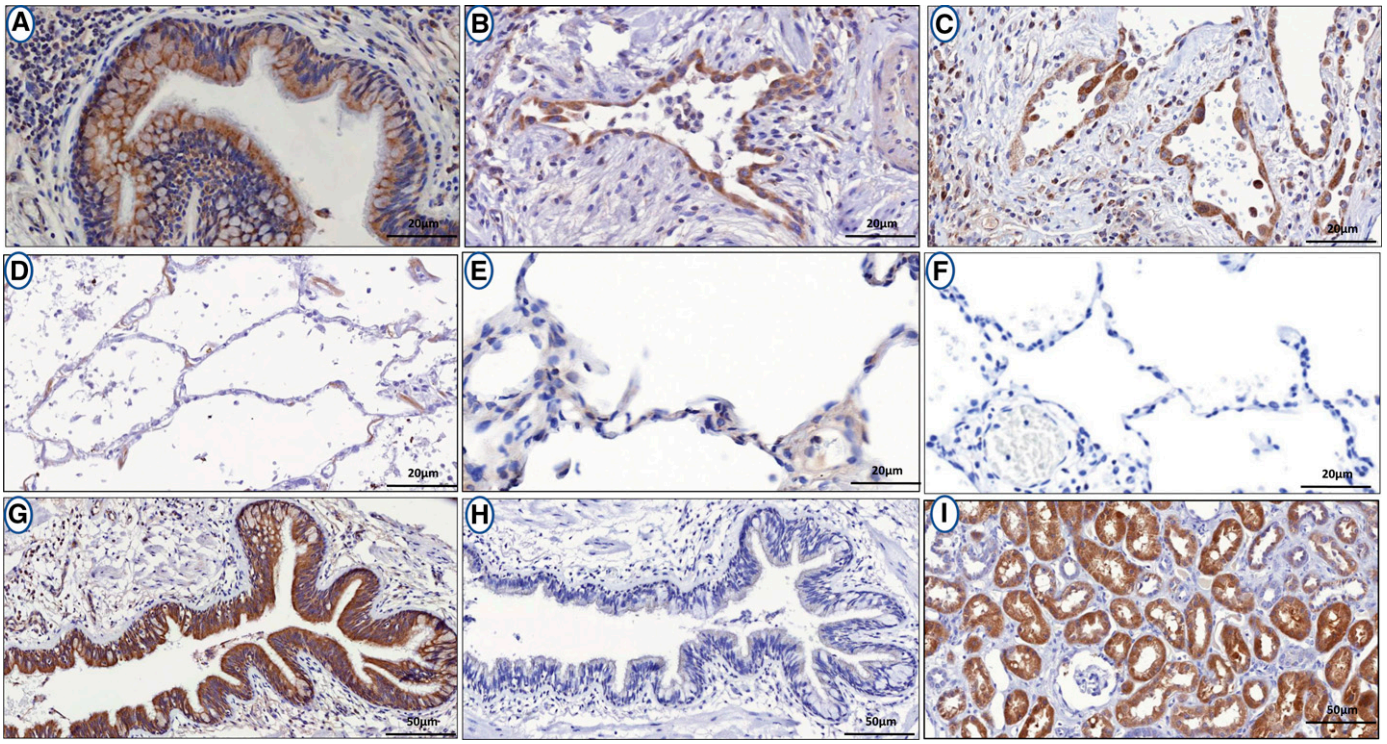

Figure 2. (*A–F*) PCSK6 immunohistochemistry showed increased cytoplasmic PCSK6 (proprotein convertase subtilisin/kexin type 6) expression in ciliated epithelial cells (*A*) and alveolar epithelial cells (*B* and *C*) compared with normal lung control sections (*D–F*). (*G* and *H*) Parallel idiopathic pulmonary fibrosis sections confirm increased PCSK6 staining (*G*) compared with control section (*H*). (*I*) Human kidney positive control is provided for reference. Scale bars, 20 μm (*A–F*) and 50 μm (*G–I*).

expression quantitative trait loci, and more complex structural variants that may be contributing to our findings.

These results, and others recently published by our group (55), demonstrate the value of case-only GWAS to identify genes associated with a relevant trait or outcome within a disease state. This approach is subject to temporal selection bias, however, as the timing of blood draw is likely associated with outcome when using the date of blood draw as the starting point for determining survival time (56). This approach was necessary given difficulties defining the date of diagnosis, together with variable periods of preclinical disease in patients with IPF, but variability in survival across cohorts suggests that temporal

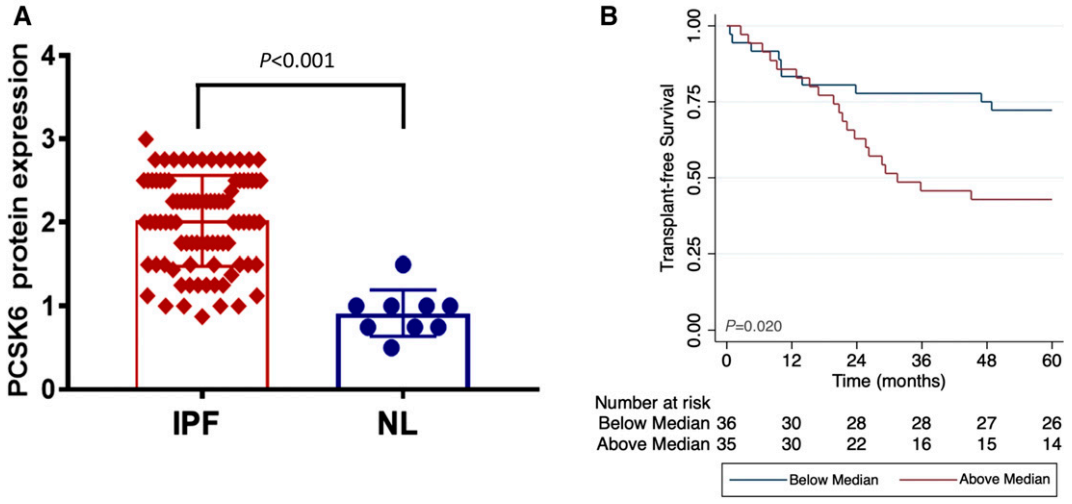

Figure 3. (*A*) Comparison of relative PCSK6 (proprotein convertase subtilisin/kexin type 6) staining intensity between idiopathic pulmonary fibrosis (IPF) cases and non-IPF control subjects demonstrated significantly higher median intensity in IPF lungs ($P < 0.001$). (*B*) Survival was lower among IPF cases with PCSK6 staining intensity above the median. NL = non-IPF control subjects.

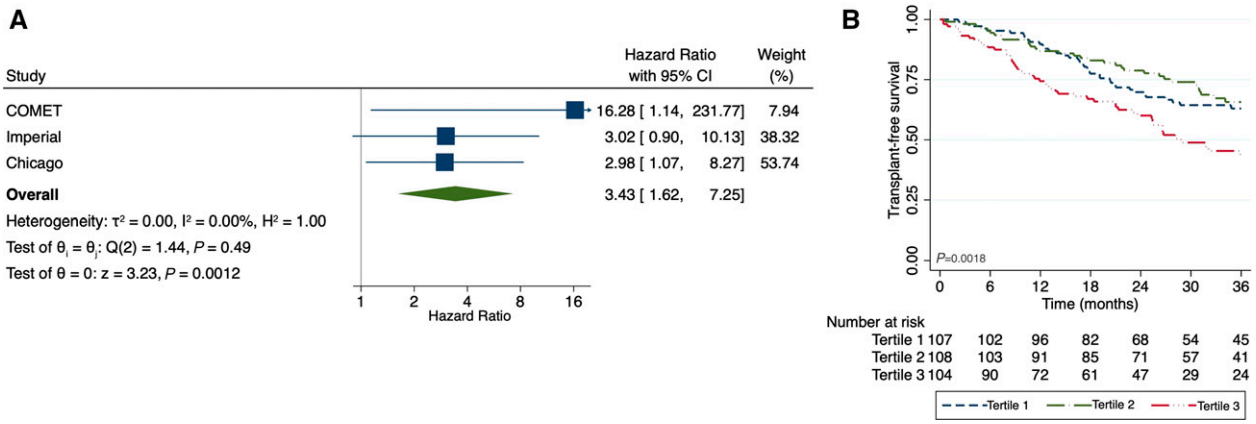

Figure 4. Relationship between proprotein convertase subtilisin/kexin type 6 and clinically relevant idiopathic pulmonary fibrosis endpoints. (*A* and *B*) Higher peripheral blood gene expression (*A*) and circulating plasma protein concentration (*B*) are associated with reduced transplantation-free survival. CI = confidence interval; COMET = Correlating Outcomes with Biochemical Markers to Estimate Time-Progression in IPF.

selection bias was likely present in our study. This study also suggests potentially important differences between genomic determinants of IPF susceptibility and survival, whereby those involved in disease onset may be independent of those driving disease progression. No survival-associated variant identified in this analysis was associated with IPF risk. Although we replicate prior IPF risk association for the *MUC5B* promoter polymorphism, we found only a weak association with favorable survival, an observation that may be influenced by index event bias (17). As none of the survival-associated variants showed an association with disease risk, it is unlikely that these survival results are affected by index event bias. These findings have potential implications for drug development, as genes associated with IPF survival may represent more effective therapeutic targets than those associated with IPF onset.

This study has several limitations. Given sample size constraints for this rare disease, we pursued a two-stage approach with meta-analysis of candidate variants rather than a discovery and replication approach, which would have required substantially higher sample sizes in each cohort. Furthermore, there was heterogeneity in the distribution of the *PCSK6* variant, as this variant was not detected in any individuals from the U.S. cohort. This was likely driven by the rare nature of this variant, but consistent effect association in the stage II cohort and genome-wide significance for the *PCSK6* variant after meta-analysis increase confidence that this represents a true association, as does the downstream clinical outcome analysis showing PCSK6 gene expression and protein concentration to be associated with differential TFS. Next, we relied on all-cause mortality when modeling these data, leaving it unclear what proportion of subjects died of IPF. This could have biased results to some extent if variants were associated with death events unrelated to IPF. There were also substantial differences in transplantation across cohorts, which could have biased results when modeling TFS. This did not appear to be an issue for the *PCSK6* variant, which maintained strong association with death when censoring transplantation events (*see* Table E5). In addition, a large proportion of patients constituting the UUS data set were recruited after the U.S. approval of pirfenidone and nintedanib, which may affect survival (57), and patients recruited before 2012 may have been exposed to potentially harmful immunosuppression (58). Finally, to facilitate accurate imputation, we focused on individuals of European ancestry, leaving it unclear whether these findings would extend to patients of non-European ancestry. The *PCSK6* variant was not present in any of the 123 patients excluded because of non-European ancestry, but dedicated analysis in this population is warranted. Additional variants of interest that failed to reach genome-wide significance were also identified, including one in DAZAP1, which lies close to PCSK4 on chromosome 19. Additional research is needed to ascertain the role these genes nearby these variants may play in IPF progression.

Conclusions

Here we present results from the first GWAS of IPF survival conducted to date. This study sheds important light on the genetics of IPF progression and identified novel variants that may contribute to this process, including rs35647788 in an intron of *PCSK6*. Downstream analysis demonstrated PCSK6 protein lung staining, peripheral blood gene expression, and circulating plasma concentration to be associated with reduced IPF survival, suggesting that PCSK6 may serve as a potential therapeutic target in patients with IPF. ■

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