Genetic variants associated with susceptibility to idiopathic pulmonary fibrosis in people of European ancestry

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Genetic variants associated with susceptibility to idiopathic pulmonary fibrosis in people of European ancestry: a genome-wide association study


Summary

Background Idiopathic pulmonary fibrosis (IPF) is a chronic progressive lung disease with high mortality, uncertain cause, and few treatment options. Studies have identified a significant genetic risk associated with the development of IPF; however, mechanisms by which genetic risk factors promote IPF remain unclear. We aimed to identify genetic variants associated with IPF susceptibility and provide mechanistic insight using gene and protein expression analyses.

Methods We used a two-stage approach: a genome-wide association study in patients with IPF of European ancestry recruited from nine different centres in the UK and controls selected from UK Biobank (stage 1) matched for age, sex, and smoking status; and a follow-up of associated genetic variants in independent datasets of patients with IPF and controls from two independent US samples from the Chicago consortium and the Colorado consortium (stage 2). We investigated the effect of novel signals on gene expression in large transcriptomic and genomic data resources, and examined expression using lung tissue samples from patients with IPF and controls.

Findings 602 patients with IPF and 3366 controls were selected for stage 1. For stage 2, 2158 patients with IPF and 5195 controls were selected. We identified a novel genome-wide significant signal of association with IPF susceptibility near A-kinase anchoring protein 13 (AKAP13; rs62025270, odds ratio [OR] 1·27 [95% CI 1·18–1·37], p=1·12×10⁻⁶⁶) and desmoplakin (DSP; rs2076295, OR 1·44 [1·35–1·54], p=7·81×10⁻²⁸). For rs62025270, the allele A associated with increased susceptibility to IPF was also associated with increased mRNA expression of AKAP13 mRNA in lung tissue from patients who had lung resection procedures (n=1111). We showed that AKAP13 is expressed in the alveolar epithelium and lymphoid follicles from patients with IPF, and AKAP13 mRNA expression was 1·42-times higher in lung tissue from patients with IPF (n=46) than that in lung tissue from controls (n=51).

Interpretation AKAP13 is a Rho guanine nucleotide exchange factor regulating activation of RhoA, which is involved in profibrotic signalling pathways. The identification of AKAP13 as a susceptibility gene for IPF increases the prospect of successfully targeting RhoA pathway inhibitors in patients with IPF.

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Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, fibrotic lung disease of unknown cause with a poor prognosis. The incidence of IPF in the UK is about 4·5–7·5 per 100 000 and is increasing,2 with a median survival of approximately 3 years.3–5 Since 2010, two new therapies, pirfenidone and nintedanib, have been approved for the treatment of IPF, but these treatments only slow disease progression and do not halt or reverse pulmonary fibrosis.6–9 Furthermore, these drugs are not universally effective and the mechanisms of their antifibrotic effects are unknown. Therefore, a detailed understanding of the genetic risk factors for IPF and their associated molecular pathways is urgently required.

The current theory suggests that IPF is characterised by initial damage to the alveolar epithelium, which then signals to various cell types, predominantly fibroblasts and macrophages, promoting tissue damage and remodeling.

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www.thelancet.com/respiratory Vol 5 November 2017 869
Evidence before this study
We searched Web of Science between Aug 21, 2015, and June 7, 2017, with the search terms “pulmonary fibrosis” and “genome wide” with no restrictions on publication date or language. Previous genome-wide association studies (GWAS) have reported 16 independent signals associated, at genome-wide significance, with susceptibility to idiopathic pulmonary fibrosis (IPF). Although previous studies have shown that epithelial cell function, lung defence, cell–cell adhesion, and telomere maintenance might play important roles in IPF, the precise mechanisms through which the genes identified in previous GWAS promote IPF are still poorly understood.

Added value of this study
To our knowledge, this study is the largest genetic study of IPF done to date, bringing together 2760 patients with IPF and 8561 controls. We present a novel genome-wide significant genetic association signal and novel gene expression data that implicate A-kinase anchoring protein 13 (AKAP13) as an IPF susceptibility gene. AKAP13 is a Rho guanine nucleotide exchange factor (RhoGEF) that regulates activation of Rho A. RhoA is a molecule with a known role in profibrotic signalling pathways; however, AKAP13 has not previously been implicated in the pathogenesis of IPF. These studies provide genetic veracity for targeting Rho signalling in IPF.

Methods
Study design
This GWAS used a two-stage approach to identify novel genome-wide significant signals associated with susceptibility to IPF. In stage 1, a GWAS was done using patients with IPF from nine different centres in the UK and controls from UK Biobank. Variants showing a significant association (p<5×10⁻⁶) in stage 1 alone were not reported as showing an association.

Stage 1 comprised patients with IPF selected from nine different centres across the UK (appendix p 1). All diagnoses were made in accordance with accepted international criteria.Controls were selected from UK Biobank and were matched for age, sex, and smoking distributions, showed no evidence of having any interstitial lung disease, and had genetic data available (appendix p 1). Stringent quality control testing of all samples was done, such as removing individuals with poor call rates, heterozygosity outliers, duplicates, related individuals, ancestry outliers, and sex mismatches (appendix p 1). All individuals were of European ancestry.

Stage 2 comprised two additional independent case-control studies that have been previously described by Noth and colleagues (Chicago consortium) and Fingerlin and colleagues (Colorado consortium). For both these studies, patients with IPF were diagnosed using American Thoracic Society and European Respiratory Society
Procedures and statistical analysis

For stage 1, patients with IPF and a third of the UK Biobank controls were genotyped using the Affymetrix Axiom UK BiLEVE array (Affymetrix, Santa Clara, CA, USA). The remainder of the controls were genotyped using the Affymetrix Axiom UK Biobank array (95% identical to the UK BiLEVE array). Genotyping and imputation procedures for studies contributing to stage 1 and 2 are described in the appendix p 1. Because the UK Biobank controls genotyped on the Axiom UK BiLEVE array had originally been selected on the basis of lung function and smoking behaviour, it was reasonable to assume that allele frequency differences between the controls genotyped on the two arrays could feasibly be driven by either technical array artefacts or genuine associations with lung function and smoking. To account for this in our analysis, and to avoid incorrectly reporting associations driven by array as associations with IPF susceptibility, we did additional sensitivity analyses (appendix p 2).

The genome-wide association analysis of IPF susceptibility was done assuming an additive genetic effect and conditioning on age, sex, and the first ten principal components to adjust for ancestry.23 The analysis was run using a score test because of its computational efficiency, using SNPTEST24 version 2.5.2. For variants with minor allele count less than 400 and score test p<5×10−6, the analysis was rerun using the Firth test using `EPACTS`25 version 3.2.4 (appendix p 2).

Independent variants reaching a threshold of p<5×10−6 in association testing in stage 1 were followed up in stage 2. Conditional analyses were used to identify additional independent signals in the same genomic region (appendix p 2).

In the Chicago consortium, analyses were done using the Firth test, adjusting for age and sex. In the Colorado consortium, analyses were done using the Firth test on a logistic regression model, adjusting for sex and the top three dimensions from a multidimensional scaling model.

For variants that were significantly associated with IPF susceptibility in stage 1 only after conditioning on another variant, the analysis was run in stage 2 conditioning on the same variant. The results from these stage 2 studies were meta-analysed using a fixed-effects model.

For variants that showed a significant (p<5×10−6) association with IPF risk in stage 1, available association test statistics from stages 1 and 2 were meta-analysed using a fixed-effects model. Signals that had genome-wide significance (p<5×10−8) when meta-analysing stages 1 and 2 were reported as significantly associated with susceptibility to IPF. A Bayesian approach was used to fine-map those signals to create 95% credible sets (a set of variants that was 95% likely to contain the causal variant; appendix p 3).

To investigate whether the variants showing an association with susceptibility to IPF were also associated with survival time in IPF, a Cox proportional hazards model was fitted in a subset of the stage 1 patients with IPF that had data for survival time. The model made adjustments for age, sex, the first ten principal components, and the recruiting study centre. Analysis was done using the Survival package in R version 2.2.3.

We searched for evidence that genetic variants associated with susceptibility to IPF were independently associated with expression of a particular gene, because an association could implicate a particular gene as the driver of the signal. Variants that were significantly associated with susceptibility to IPF and proxy variants (correlated with linkage disequilibrium r2>0.8) were investigated in three expression quantitative trait locus (eQTL) datasets: a lung eQTL database comprising individuals from three cohorts (University of British Columbia, Laval, and Groningen), a blood eQTL database, and in the Genotype-Tissue Expression project (GTeX) cohort (multiple tissues; appendix p 3).

RT-PCR gene expression analysis was done on RNA extracted from human lung tissues of 46 patients with IPF and 51 controls from the Lung Tissue Research Consortium using standard methods (appendix p 3). Initially, differential gene expression in the lung was compared between the patients with IPF and controls using two-tailed Student’s t test comparing the change in C values between groups. Linear regression analysis was used to compare A-kinase anchoring protein 13 (AKAP13) expression in controls and patients with IPF while also controlling for age, smoking (ever or never and pack-years), and sex.

Formalin-fixed paraffin-embedded human lung tissue sections were obtained from tissue distant from the tumour that was obtained during lung resection from controls and tissue from patients with IPF taken at either post-mortem examination or lung transplantation. Immunohistochemistry was done using standard methods (appendix p 3).

We performed in-silico analyses to establish whether known drugs target the proteins identified in the genome-wide and expression analyses or the proteins that interact with them (appendix p 4).

Role of the funding source

The funders had no role in the study design. GlaxoSmithKline Research and Design participated in collection of data and had access to the raw data from a subset of the UK IPF patient data. LVW and RG] were involved in all stages of study development and delivery, had full access to all data in the study, and had final responsibility for the decision to submit for publication.
A

677 patients with IPF

502 682 individuals in UK Biobank

65 excluded
8 failed Affymetrix quality control
6 heterozygosity outliers
33 duplicates
1 related
6 ancestry outliers
6 did not have IPF
5 sex mismatches

502 682 individuals with IPF

632 patients with IPF passed quality control

349 953 excluded because not genotyped

152 729 individuals with genotyped data

8 failed Affymetrix quality control
6 heterozygosity outliers
33 duplicates
1 related
6 ancestry outliers
6 did not have IPF
5 sex mismatches

632 patients with IPF passed quality control

112 190 individuals passed quality control

10 excluded for incomplete phenotype

3366 controls selected

602 patients with IPF selected

396B individuals included in discovery analysis

B

Chicago consortium

633 patients with IPF recruited

58 excluded
16 self-reported non-European ancestry
28 call rate <97%
10 sex mismatches
4 related

575 patients with IPF passing quality control

1545 controls recruited

118 excluded
26 self-reported non-European ancestry
69 call rate <97%
18 sex mismatches
5 related

1427 controls passing quality control

547 patients with IPF selected after genetic ancestry matching

1914 patients with IPF recruited

542 controls selected after genetic ancestry matching

542 controls after genetic ancestry matching

1054 individuals in analysis

7353 individuals in stage 2 meta-analysis

2138 patients with IPF

5195 controls

33 excluded because they could not be genetically matched to controls

885 excluded because they could not be genetically matched to patients

145 excluded†
41 sex mismatches
105 call rate <98%

6299 individuals in analysis

Colorado consortium

1914 patients with IPF recruited

4828 controls recruited

298 excluded
14 genetic outliers
126 related
8 high heterozygosity
150 call rate <98%

1616 patients with IPF passed quality control

6083 controls passing quality control

4828 controls selected after genetic ancestry matching

145 excluded†
41 sex mismatches
105 call rate <98%

6299 individuals in analysis

1054 individuals in analysis

30 excluded for incomplete phenotypes

7353 individuals in stage 2 meta-analysis

2138 patients with IPF

5195 controls
After sample quality control testing and genotype imputation, 602 patients with IPF and 3366 UK Biobank controls (selected on April 18, 2016) were included in the stage 1 analysis of 13076821 variants (figure 1A). The patients were diagnosed between June, 1996, and July, 2013, with the exception of 52 patients who were historical cases with unknown dates of diagnosis. In total, 44 independent signals were associated with susceptibility to IPF (p<5×10⁻⁶) and were followed up in stage 2 (figure 2).

A total of 2158 patients with IPF and 5195 controls were included in stage 2 (figure 1B, table 1). Of the 44 variants identified in stage 1, data were available for 27 variants in one or both of the stage 2 studies (appendix pp 8–9). Of these 27 variants, three were significant (p<1·85×10⁻³, after Bonferroni correction for 27 tests) in stage 2, and had genome-wide significance (p<5×10⁻⁸) in the meta-analysis of stages 1 and 2 together (table 2). A novel association for the single-nucleotide polymorphism (SNP) rs62025270, which was the most strongly associated SNP in a broad signal covering AKAP13 and kelch like family member 25 (KLHL25; figure 3), had genome-wide significance in the meta-analysis of stages 1 and 2 (minor allele A; odds ratio [OR] 1·27 [95% CI 1·18–1·37], p=1·32×10⁻⁹). This SNP was also significant in stage 2 alone after Bonferroni adjustment for 27 tests.
OR 1·22 [95% CI 1·11–1·33], p=9·96×10⁻⁶. The 95% credible set (a set of variants that was 95% likely to contain the causal variant) produced for this signal contained 113 variants (appendix pp 10–11). The other two genome-wide significant signals were previously reported variants in MUC5B (rs35705950) and DSP (rs2076295).10–12

We explored whether genotyping array differences could have influenced the association test result for SNP rs62025270. In the control–control comparison (appendix p 6), rs62025270 showed no association with the array (p=0·35, appendix p 12). We repeated the association analysis for rs62025270 restricted to controls who were genotyped on the same array as the patients (602 patients vs 1231 controls); the results were consistent with the original analysis (stage 1 OR 1·45, [95% CI 1·20–1·74], p=1·19×10⁻⁴; meta-analysis OR 1·25 [95% CI 1·17–1·33], p=1·86×10⁻⁸). We reimputed the region with only variants genotyped on both arrays using the new Haplotype Reference Consortium imputation reference panel;23 the results were again consistent with the original analysis (stage 1 OR 1·48 [95% CI 1·25–1·75], p=4·24×10⁻⁶; meta-analysis OR 1·27 [95% CI 1·18–1·37], p=1·32×10⁻⁹).

Previous GWAS reported 16 independent signals associated with susceptibility to IPF with genome-wide significance. In stage 1 of our study, which analysed previously unreported data only, nine of these signals had a p value less than 0·05 and a direction of effect consistent with the previously reported effects. Furthermore, seven signals met a Bonferroni adjusted significance threshold for 16 tests (p<3·13×10⁻³) with a
consistent direction of effect to previous GWAS; namely variants in MUC5B, DSP, family with sequence similarity 13 member A (FAM13A), TERT, mucin 2 (MUC2), DPP9, and TOLLIP (appendix p 13). Five of the remaining seven signals that were not significant had a consistent direction of effect with previous GWAS.

SNP rs62025270[A] was associated with increased expression of AKAP13 in the lung. To identify the functional consequence of the rs62025270 polymorphism, we assessed associations between rs62025270 and nearby variants and expression of genes (eQTLs). In non-diseased whole-lung tissue from the lung eQTL databases (University of British Columbia n=339, Laval n=409, and Groningen n=363), the minor allele A of rs62025270, associated with increased susceptibility to IPF, was associated with increased expression of AKAP13 (figure 4). SNP rs62025270 is in linkage disequilibrium (r²=0·60) with rs17636666, the variant that was most significantly associated with expression of AKAP13 in lung tissue. This co-localisation of IPF susceptibility association signal and strongest AKAP13 gene expression association signal (eQTL) in lung tissue (figure 3) further suggests that altered expression of AKAP13 has a role in IPF susceptibility. The minor allele A of rs62025270 (via proxy SNP rs2554, r²=0·93) was also associated with expression of AKAP13 in whole blood, which have different cell-type compositions. Furthermore, the minor allele A of rs62025270 was associated with decreased expression of AKAP13 in blood, rather than increased expression as seen in lung tissue. The SNP rs62025270 was also found to be associated with expression of RNA genes RP11–158M2.3, RP11–158M2.4, RP11–158M2.5, RP11–815J21.3, and RP11–815J21.4 in a range of tissues in GTEx (appendix p 14).

Having identified that the minor allele A of rs62025270 was associated with increased susceptibility to IPF and increased expression of AKAP13 in lung tissue, we did a further analysis in lung tissue to identify the cellular location of AKAP13 in the lung and compared levels of AKAP13 mRNA from patients with IPF versus controls. Morphological assessment of histological sections from 10 control and 10 patients with IPF showed that AKAP13 protein was expressed primarily in bronchial epithelium (figure 5A) and alveolar type 1 and 2 cells (figure 5B) in control lung samples. High AKAP13 expression was observed in fibrotic regions of lung tissue from patients with IPF, and the cells expressing AKAP13 were primarily epithelial cells, macrophages, and lymphoid aggregates (figure 5C–F). High AKAP13 expression was also seen in epithelial cells from less fibrotic alveolar regions of lungs of patients with IPF, with more AKAP13-expressing cells in alveoli of patients with IPF (figure 5G) than in alveoli of controls (figure 5H). Real-time PCR analysis of samples from 46 patients with IPF and 51 controls established that concentrations of AKAP13 mRNA were 1·42-times higher in whole lung tissue homogenates from patients with IPF.
than in lung tissue from controls (p=0·0011; figure 5I). Linear regression analysis confirmed that the significant increase in AKAP13 mRNA expression in lung tissue of patients with IPF was maintained after controlling for age, sex, and smoking history (p=2·03 × 10⁻⁴).

We tested the association of rs62025270 with survival time in all patients from stage 1 for whom survival data were available (n=565; 360 with recorded deaths and 205 with censored survival times, with a minimum follow-up time of 1112 days before censoring). The median survival time after diagnosis of IPF was 1621 days (4·4 years; IQR 764–2815 days). rs62025270 showed no association with survival time (hazard ratio 1·01 [95% CI 0·86–1·19], p=0·878).

11 proteins that interact with AKAP13 were identified as known targets for existing drugs or compounds in development, including aspirin, dextromethophan, and GSK-690693 (appendix pp 15–17).

Discussion
We studied 2760 patients with IPF and 8561 controls and identified a novel genome-wide significant association of variant rs62025270 that implicates AKAP13 as a potential driver of IPF pathogenesis. We showed that the minor allele (A) of rs62025270 (minor allele frequency [MAF]=25%), associated with increased susceptibility to IPF, was associated with increased expression of AKAP13 in lung tissue. Morphological analysis of control and diseased lung tissue identified AKAP13 expression primarily in epithelial cells, with some additional expression in lymphoid tissue in patients with IPF. Furthermore, we showed increased expression of AKAP13 in fibrotic lung tissue from patients with IPF compared with samples from healthy controls.

The high AKAP13 expression in epithelial cells further supports the hypothesis that IPF is a disease characterised by epithelial susceptibility to injury, consistent with findings of other genetic risk factors associated with epithelial cell function.13 AKAP13 is a RhoA guanine nucleotide exchange factor (RhoGEF) that is crucial for murine cardiac development. AKAP13-null mice are embryonic lethal because of arrested cardiac development at embryonic day 10.24 Conversely, enhanced AKAP13 signalling has been shown to promote profibrotic signals in cardiac fibroblasts25 and...
leads to cardiac hypertrophy in mice.\textsuperscript{20} Although AKAP13 has not previously been implicated in the pathogenesis of IPF, it plays a key role in profibrotic signalling pathways. Thrombin and lysosphatidic acid have been identified as key profibrotic mediators acting via a Gq and RhoA signalling pathway in epithelial cells to promote αvβ6 integrin-mediated transforming growth factor β (TGFβ) activation.\textsuperscript{21,22} AKAP13 coordinates thrombin and lysosphatidic acid receptor signalling through Ga12 to RhoA in fibroblasts\textsuperscript{31} and astrocytes,\textsuperscript{32} and has also been shown to functionally interact with Gq, promoting RhoA activation.\textsuperscript{31–33} Therefore, AKAP13 might promote epithelial αvβ6 integrin-mediated TGFβ activation in epithelial injury, thereby promoting IPF. A phase 2 clinical trial assessing the safety and tolerability of a humanised monoclonal antibody targeting AKAP13 binding partners, and the AKT pathway at genome-wide significance threshold for 16 tests (\(p<3\times10^{-5}\)), included five signals that reached a Bonferroni-adjusted significance threshold for 16 tests (\(p<3\times10^{-5}\)), and two additional signals that reached genome-wide significance (\(MUC5B\) \(p=1.86\times10^{-37}\) and \(DSP\) \(p=4.14\times10^{-12}\)). In addition, the diagnostic practices between the UK and USA might be different but the concordant results from the three case-control studies reassures us that these observations are robust.

A previous study of 119 patients with IPF and 50 donor lung controls from the Lung Tissue Resource Consortium reported a small decrease of AKAP13 expression in patients with IPF compared with controls using gene expression data from the Affymetrix Gene STI-0 array;\textsuperscript{23} whereas our data using RT-PCR showed a moderate increase of AKAP13 expression in patients with IPF compared with controls. The discordance
between these results could be due to a number of factors, including the difference in normalisation strategies between the two techniques and sample heterogeneity. The eQTL data that we present from control lung tissue shows that the minor allele A of rs62025270, associated with susceptibility to IPF, is also associated with increased expression of AKAP13 mRNA. Therefore, these data provide strong independent evidence that increased AKAP13 expression in alveolar cells has a role in IPF, and the cellular heterogeneity within the fibrotic lung samples used for RNA analysis is likely to explain this discordance.

In summary, we report a novel genome-wide significant association for IPF susceptibility with SNP rs62025270 and present evidence that this SNP might exert its effect via expression of the nearby gene AKAP13. AKAP13 is a RhoGEF that is known to interact with a central fibrogenic pathway—G protein-coupled receptor activation of RhoA—and expression is increased in fibrotic regions of lungs from patients with IPF. Expression of AKAP13 occurs primarily in the epithelium thereby reinforcing the idea that epithelial processes are central to IPF pathogenesis. The identification of a RhoGEF that regulates a pathway known to be involved in the pathogenesis of IPF, a pathway for which drugs are in development, could potentially provide a novel target for antifibrotic therapy for IPF.

Contributors
RGI, LVW, and MDT designed and supervised the study. RB, AH, RBH, VN, GS, NT, HLB, SPH, MRH, NH, TMM, RJM, ABM, PLM, HP, DMR, MKBW, WAF, RPM, IS, and IPH recruited and genotyped patients. RJA, JP, CF, TEF, IMO, BG-G, S-FM, TO, AES, MO, IVY, YB, DCN, DDS, WT, NS, IN, and DAS did analyses and experiments. RJA, LVW, and RGI wrote the manuscript with input from all authors.

Declaration of interests
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