INTERSTITIAL LUNG DISEASE

Genetic mutations in surfactant protein C are a rare cause of sporadic cases of IPF


Background: While idiopathic pulmonary fibrosis (IPF) is one of the most common forms of interstitial lung disease, the aetiology of IPF is poorly understood. Familial cases of pulmonary fibrosis suggest a genetic basis for some forms of the disease. Recent reports have linked genetic mutations in surfactant protein C (SFTPC) with familial forms of pulmonary fibrosis, including one large family in which a number of family members were diagnosed with usual interstitial pneumonitis (UIP), the pathological correlate to IPF. Because of this finding in familial cases of pulmonary fibrosis, we searched for SFTPC mutations in a cohort of sporadic cases of UIP and non-specific interstitial pneumonitis (NSIP).

Methods: The gene for SFTPC was sequenced in 89 patients diagnosed with UIP, 46 patients with NSIP, and 104 normal controls.

Results: Ten single nucleotide polymorphisms in the SFTPC sequence were found in IPF patients and not in controls. Only one of these created an exonic change resulting in a change in amino acid sequence. In this case, a T to C substitution resulted in a change in amino acid 73 of the precursor protein from isoleucine to threonine. Of the remaining polymorphisms, one was in the 5′ UTR, two were exonic without predicted amino acid sequence changes, and six were intronic. One intronic mutation suggested a potential enhancement of a splicing site.

Conclusions: Mutations in SFTPC are identified infrequently in this patient population. These findings indicate that SFTPC mutations do not contribute to the pathogenesis of IPF in the majority of sporadic cases.

Despite many years of research into the cause of idiopathic pulmonary fibrosis (IPF) and its pathological correlate usual interstitial pneumonitis (UIP), the aetiology underlying most forms of the disease remains unknown. However, clustering of cases in families suggests that some forms may have a genetic basis. The incidence of IPF is estimated to be 10.7 cases per 100 000 per year in males and 7.4 cases per 100 000 per year in females. Of these, 0.5–3.7% are estimated to be familial. In these familial forms pulmonary fibrosis appears to be similar to sporadic forms, but diagnosis tends to occur at a younger age.

Over 68 kindreds with familial pulmonary fibrosis (FPF) have been reported with analysis suggesting inheritance in an autosomal dominant manner with reduced penetrance. Among sporadic cases of IPF, reports have suggested that polymorphisms in the genes encoding tumour necrosis factor-α, interleukin-1 receptor antagonist, and complement receptor 1 may be important. Another recent report suggested that polymorphisms in transforming growth factor β1 are not associated with development of the disease but are associated with progression of the disease in IPF.

Recent studies have revealed that some cases of IPF are associated with alterations in surfactant protein C (SFTPC). In 2001 Nogee et al reported a mother and infant child who had a mutation in the gene encoding for SFTPC. The mother had desquamative interstitial pneumonitis while the infant had a mutation in the SFTPC gene which resulted in the substitution of glutamine for leucine at amino acid position 188 of the mature SFTPC, but no genetic mutation in SFTPC was detected. Since these reports, Nogee et al have reported a number of childhood cases of interstitial lung disease associated with heterozygous mutations in SFTPC. In 2002 Thomas et al reported a large kindred in which 14 members had pulmonary fibrosis, some with UIP and others with cellular NSIP. Affected family members had a heterozygous mutation in the SFTPC gene which resulted in the substitution of glutamine for leucine at amino acid position 188 of the carboxy terminal region of pro-SFTPC. This report was the first to associate alterations in SFTPC with UIP.

These reports of associations of FPF with alterations in SFTPC raised questions regarding whether or not genetic mutations in SFTPC could be responsible for some cases of sporadic forms of IPF. With this possibility in mind, we evaluated a cohort of patients with sporadic IPF with genetic sequencing of the SFTPC gene.

METHODS

IPF patient cohort

This investigation was approved by the Vanderbilt University Institutional Review Board and by the Royal Brompton Harefield and NHLI ethics committee. Patients followed in the pulmonary clinic at Vanderbilt and at the interstitial lung disease unit at the Royal Brompton Hospital comprised the study cohort; 89 had UIP and 46 had NSIP. Diagnosis was made in accordance with ATS/ERS consensus statements. 11 12 14 Individuals with the known lung disease served as controls. Of the remaining 104 individuals with no known lung disease, 84 were drawn from the Vanderbilt interstitial lung disease patient population and 20 were randomly selected from the Vanderbilt School of Medicine database. Of these, 46 were male and 58 were female.

Abbreviations: ESE, exonic splicing enhancer; FPF, familial pulmonary fibrosis; IPF, idiopathic pulmonary fibrosis; NSIP, non-specific interstitial pneumonitis; SFTPC, surfactant protein C; SNP, single nucleotide polymorphism; UIP, usual interstitial pneumonitis
controls. The control population was drawn from the same local areas as the patient group and all patients and controls were white. Patient- and control groups included both men and women, but the groups were not necessarily age or sex matched. The demographic characteristics of the cohort are shown in table 1. Blood was collected from each patient and was used in the genetic sequence evaluation.

### SFTPC locus amplification and sequencing

Sequencing of the SFTPC locus was performed from genomic DNA isolated from peripheral blood leucocytes obtained from each patient. Sequencing was performed with the investigator blinded to the diagnosis. Primers were obtained from MWG-Biotech (Charlotte, NC, USA). 10 ng of human genomic DNA (Roche Diagnostics, Indianapolis, IN, USA) was combined with a PCR buffer containing 10 pmol each of forward and reverse primers (see table S1 on the website at www.thoraxjnl.com/supplemental), 10% 10× PCR buffer (PE Biosystems, Foster City, CA, USA), 2 mM MgCl₂ (PE Biosystems), 2% dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO, USA), 5 mM DTT (Bio-Rad Laboratories, Hercules, CA, USA), 200 μM of each dNTP (Promega Corp, Madison, WI, USA), and 0.625 units of TaqGold (PE Biosystems) with 1% by units Pfu Turbo Hotstart (Stratagene, La Jolla, CA, USA) in a total volume of 20 μl per reaction. The reaction tubes were cycled using the following protocol: 95°C for 12 minutes followed by 35 cycles consisting of 95°C for 30 seconds, 60°C for 20 seconds, 72°C for 1.5 minutes, with a final extension at 72°C for 6 minutes. Excess primers were removed using ExoSAP-IT (USB Corp, Cleveland, OH, USA) following the manufacturer’s protocol. Electrophoresis to assess quality of the amplicons was performed using 2× TBE (Invitrogen, Carlsbad, CA, USA) with 1% agarose gels containing ethidium bromide (BMA Biosciences, Madison, WI, USA), and 0.625 units of TaqGold (PE Biosystems) using dye set “H”, and the resulting data were analysed using PhredPhrap/Phrap/Consed Suite (Codon Code Corp, Rockland, MA, USA) for base calling and contig alignment.

Sequence reactions were performed using 2 μl of each amplicon, 1.4 pmol of primer, 1.5 μl 5× Big Dye dilution buffer, and 2 μl of BigDye terminator ready reactions mix version 3.0 (Applied Biosystems) per 10 μl reaction. Reaction components were assembled in a 96-well multiplate and briefly pulsed in a centrifuge to mix. The cycling protocol consisted of 95°C for 5 minutes followed by 35 cycles consisting of 95°C for 30 seconds, 55°C for 20 seconds, and 60°C for 4 minutes. Finished sequence reaction plates were pulsed in a centrifuge and 10 μl of 10% 1-butanol was added to each well. The plates were pulsed again to mix and samples were transferred to a Sephadex (Sigma Chemical Co, St Louis, MO, USA) matrix for dye removal. After the samples were transferred to the Sephadex matrix, a MicroAmp optical 96-well reaction plate (Applied Biosystems) was placed under the Sephadex plate and the cleaned samples were collected by spinning the two plates again at 900g for 5 minutes. The collected samples were dried under vacuum. Formamide (7.5 μl/sample) was added and the DNA was resuspended and denatured by heating to 95°C for 5 minutes, 80°C for 5 minutes, and 4°C for 5 minutes. The samples were sequenced on an ABI Prism 3700 DNA analyser (Applied Biosystems) using dye set “H”, and the resulting data were analysed using PhredPhrap/Phrap/Consed Suite (Codon Code Corp, Rockland, MA, USA) for base calling and contig alignment.

### Evaluation for possible exonic splicing enhancers (ESE)

Identified sequence variations were screened for the possibility that the new genetic sequence could affect exonic splicing. This was performed using ESEfinder 2.0 from the website http://exon.cshl.org/ESE/. Of the splicing enhancer proteins used in the ESEfinder program, the SRp55 protein is known to be present in mature epithelial cells so SRp55 results were analysed using the website pre-set threshold.

### Data analysis

Single sample proportion confidence intervals (CI) were calculated using Wilson’s method.

### RESULTS

We identified 10 sequence variations in the SFTPC gene which occurred in patients with pulmonary fibrosis but not in controls. Table 2 summarises the findings and the corresponding clinical diagnosis. One of these was in the 5′ UTR, three were exonic, and six were intronic. Of the three exonic sequence variations, all were heterozygous single nucleotide polymorphisms and only one predicted a change in the amino acid sequence of the translated protein. In this patient, a T6108C change in intron 4 was found to encode an Ile73Thr substitution. Considering information from FPF associated with heterozygous mutations in SFTPC including recent reports of this same mutation in an infant with lung disease, it is possible that this sequence alteration contributed to the lung disease in this patient. Of the intronic variations, a homozygous C6699T change in intron 4 was found in four patients (three UIP, one NSIP) and a heterozygous G5089A change in intron 1 was found in two patients (one NSIP, one UIP). One patient with UIP had two heterozygous intronic sequence variations in intron 1 (G5236A and G5574A). Each sequence alteration was screened for a potential ESE change as described in the methods section. One intronic sequence alteration (G5574A variation in intron 1) may have implications with regard to splicing enhancement as it predicted a possible SRp55 splice enhancement.

### DISCUSSION

These findings show that sequence variations in SFTPC are rarely found in DNA from subjects with sporadic pulmonary fibrosis. Thirteen of 135 patients (9.6% (95% CI 5.7 to 15.8)) had SFTPC genetic sequence variations that were not found in controls, but only one patient (0.7% (95% CI 0.1 to 4.1)) had a sequence variation (T6108C) that predicted a change in

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**Table 1** Demographic characteristics of the patient population

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No of patients*</th>
<th>Age†</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>UIP</td>
<td>89</td>
<td>63</td>
<td>74% male</td>
</tr>
<tr>
<td>NSIP</td>
<td>46</td>
<td>57</td>
<td>57% male</td>
</tr>
<tr>
<td>Controls</td>
<td>104</td>
<td>43</td>
<td>53% male</td>
</tr>
</tbody>
</table>

*81 of the 89 patients with UIP were from the Royal Brompton Hospital and eight were from Vanderbilt Hospital. All patients with NSIP were from the Royal Brompton Hospital. Diagnoses of UIP and NSIP were made in accordance with ATS/ERS consensus statements .11 18 of the UIP patients and 23 of the NSIP patients had surgical lung biopsies.

†Age for UIP and NSIP represents age at time of diagnosis. Age for controls represents age at which they donated blood for the study.
Amino acid sequence of the precursor protein (Ile73Thr). In one other patient the possibility exists that altered splicing proficiency due to genetic sequence alteration could play a role in the disease, although this has not yet been investigated.

Alternative splicing contributes significantly to the complexity of the human genome, with estimates suggesting that 60% of genes have alternative splice forms.14 Included in this growing field is the concept of splicing regulation by splicing ‘‘enhancers’’ and ‘‘silencers’’, in which sequence elements within exons or introns can promote or inhibit splicing at nearby splice sites.15 Thus, an intronic or exonic mutation could be postulated to affect transcription and thus protein translation. In this study SRp55 was examined in the ESE analysis since it is a splicing factor known to be present in mature epithelial cells.16 While it is not known if SRp55 splicing site alterations lead to non-neoplastic disease states, it has been found that the ratio of SRp55 to other splicing factors is altered in preneoplastic epithelium.17 Whether or not SRp55 has any effect on the expression of SFTPC will require further examination before it can be postulated to be a contributor to IPF in these patients. Nonetheless, it is an important concept to consider in future evaluations.

While SFTPC may not be the primary gene of interest in IPF, information from familial SFTPC cases18–20 may still help us to understand the pathogenesis of IPF, particularly the role of type II alveolar epithelial cells in its initiation and propagation. Currently, it is not known how changes in SFTPC lead to pulmonary fibrosis in affected families. SFTPC is encoded by a gene on chromosome 8p21 with transcription resulting in a 197 amino acid precursor protein. Subsequent processing within the type II alveolar cell results in the functional SFTPC protein, a highly hydrophobic 35 amino acid polypeptide that is secreted into the alveolar space. This possibility is supported by the observation that mice deficient in SFTPC develop interstitial pneumonitis and emphysema, although in a strain dependent manner.20

We have shown that genetic mutations in SFTPC are not commonly found in association with sporadic cases of IPF. Nonetheless, SFTPC mutations still have implications for familial forms of interstitial lung disease, especially in children. A clearly defined genetic association for most cases of pulmonary fibrosis currently remains elusive, but studies are ongoing in a number of centres to determine genetic links to this disease.

For sequences and amplicons of the primers used in the study see table S1 on the Thorax website at www.thoraxjnl.com/supplemental.

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## REFERENCES


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**Table 2** Summary of single nucleotide polymorphisms (SNPs) found by genetic sequencing of the SFTPC gene

<table>
<thead>
<tr>
<th>Site of SNP</th>
<th>BP* with change</th>
<th>Nucleotide change</th>
<th>Predicted amino acid change</th>
<th>Predicted ESE change</th>
<th>No of patients with SNP</th>
<th>UIP v NSIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′ UTR</td>
<td>4702</td>
<td>GG to GC</td>
<td></td>
<td></td>
<td>1</td>
<td>NSIP</td>
</tr>
<tr>
<td>Exon 1</td>
<td>4859</td>
<td>CC to CG</td>
<td>Val8Val</td>
<td></td>
<td>1</td>
<td>UIP</td>
</tr>
<tr>
<td>Exon 1</td>
<td>4877</td>
<td>GG to GA</td>
<td>Pro14Pro</td>
<td></td>
<td>1</td>
<td>UIP</td>
</tr>
<tr>
<td>Intron 1</td>
<td>5089</td>
<td>GG to GA</td>
<td></td>
<td></td>
<td>2</td>
<td>UIP (1), NSIP (1)</td>
</tr>
<tr>
<td>Intron 1</td>
<td>5210</td>
<td>CC to CA</td>
<td></td>
<td></td>
<td>1</td>
<td>UIP</td>
</tr>
<tr>
<td>Intron 1</td>
<td>5234±</td>
<td>GG to G4+</td>
<td></td>
<td></td>
<td>1</td>
<td>UIP</td>
</tr>
<tr>
<td>Intron 1</td>
<td>5574±</td>
<td>GG to GA</td>
<td></td>
<td></td>
<td>1</td>
<td>UIP</td>
</tr>
<tr>
<td>Intron 2</td>
<td>5786</td>
<td>AA to CC</td>
<td>V70L</td>
<td></td>
<td>2</td>
<td>UIP (1), NSIP (1)</td>
</tr>
<tr>
<td>Exon 3</td>
<td>6108</td>
<td>TT to TC</td>
<td>Ile73Thr</td>
<td></td>
<td>1</td>
<td>UIP</td>
</tr>
<tr>
<td>Intron 4</td>
<td>6699</td>
<td>CC to TT</td>
<td></td>
<td></td>
<td>1</td>
<td>UIP</td>
</tr>
<tr>
<td>Intron 5</td>
<td>5604</td>
<td>GG to GA</td>
<td></td>
<td></td>
<td>4</td>
<td>UIP (3), NSIP (1)</td>
</tr>
</tbody>
</table>

*Base pair (BP) numbering is directly from GenBank accession #AY337315.1.
†SNP previously reported as documented in GenBank accession #AY337315.1.
‡These two sequence variations were in the same patient.

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LUNG ALERT

A paradox of TB immunity

To address whether protective immunity induced by natural infection was any different from that induced by BCG, investigators infected mice—either BCG vaccinated or previously infected with M tuberculosis and then cured with chemotherapy—with a low dose of M tuberculosis H37Rv. Protection against post primary M tuberculosis infection did not differ significantly between the two groups even when adoptive transfer of interferon (IFN)-γ positive splenocytes or serum was performed. After challenge infection, the number of IFN-γ positive splenocytes did not differ significantly between the groups. The authors conclude that, in this murine model, natural infection with M tuberculosis and vaccination with BCG do not differ in their capacity to induce protective immunity against tuberculosis. Consequently, any novel vaccine against tuberculosis has to perform better than both vaccination with BCG and immunity evoked by natural infection.

This study highlights one of the great paradoxes of tuberculosis: natural infection does not confer protective immunity yet only 10% of those that are infected progress to active disease. Indeed, recent human molecular epidemiological studies show that previous infection does not protect against re-infection progressing to active disease and, moreover, at the site of human disease there are high IFN-γ levels. This study raises the question of whether the strategy to identify vaccine candidates by using the IFN-γ response as a surrogate marker of protective immunity is a valid one. The future challenge will be to identify other correlates of protective immunity or, alternatively, components of M tuberculosis that induce a subversive host response.

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