The Genetic Approach in Pulmonary Fibrosis

Can It Provide Clues to This Complex Disease?

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Multiple investigators have undertaken genetic studies in idiopathic pulmonary fibrosis populations in attempts to define genetic links to disease in hopes that this would improve understanding of disease pathogenesis and target pathways for therapy. Multiple genes have been evaluated using a candidate gene approach with limited success, with results suggesting a disease modifier effect rather than a disease causing effect. Using this approach, associations have been observed between idiopathic pulmonary fibrosis and specific polymorphisms in genes encoding interleukin-1 receptor antagonist, tumor necrosis factor- α , and complement receptor 1. Recently investigators have used familial pulmonary fibrosis cohorts to evaluate for genetic mutations associated with idiopathic pulmonary fibrosis. Using one pulmonary fibrosis kindred, a mutation in the gene encoding surfactant protein C was identified as the cause of pulmonary fibrosis in this family. Subsequently, another individual with idiopathic pulmonary fibrosis was identified with a different mutation in surfactant protein C. Though rarely found in patients with idiopathic pulmonary fibrosis, these surfactant protein C mutations highlight the importance of the alveolar epithelium in disease pathogenesis. A recent collaboration between investigators at three major centers has resulted in the largest collection of families with pulmonary fibrosis to date, with hopes that this effort will identify genetic mutations associated with idiopathic pulmonary fibrosis. If genetic links to idiopathic pulmonary fibrosis are defined in this study, then the pathways involved with these genes and gene products can be targeted by investigators to help identify potential treatment options for this disease.

Keywords: familial pulmonary fibrosis; idiopathic pulmonary fibrosis; surfactant protein C

IDIOPATHIC PULMONARY FIBROSIS AND FAMILIAL PULMONARY FIBROSIS

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive lung disease characterized by the insidious onset of interstitial infiltrates in the lung parenchyma associated with progressive dyspnea and impaired pulmonary function (1). Estimates for prevalence suggest that approximately 20 per 100,000 males and 13 per 100,000 females have the disease (2). The outcome in most patients with IPF includes gradual progression to respiratory failure within 3 to 5 yr (2). Unfortunately, no clearly effective therapies are available for IPF, short of lung transplantation.

While the medical and scientific community has made significant improvements in understanding the pathogenesis of lung fibrosis, the etiology of IPF remains undefined, a fact that limits our ability to optimally prevent or treat this disease. As with other diseases, there has been great anticipation that identifying genetic links to IPF may help define etiologic factors, improve understanding of disease pathogenesis, and suggest potential treatment options. However, to date, a clear genetic link to most forms of IPF remains undefined.

Multiple lines of evidence suggest that genetic factors could impact the development of lung fibrosis (3). First, considerable variability exists in relationship to the development of lung fibrosis in individuals exposed to fibrogenic dusts, such as silica and asbestos. Second, inbred mouse strains have differential responses to experimentally induced fibrosis. Third, multiple defined genetic disorders have been described in which pulmonary fibrosis may be a manifestation of disease, including tuberous sclerosis, neurofibromatosis, Niemann-Pick disease, Gaucher disease, Hermansky-Pudlak syndrome, and familial hypocalciuric hypercalcemia. Fourth, and perhaps most important to defining the genetics of IPF, familial clustering of individuals with pulmonary fibrosis, including individuals raised in different environments, supports a genetic cause or predisposition to disease (3). It is this line of evidence that is most convincing of a genetic link to IPF.

Familial pulmonary fibrosis (FPF), also referred to as familial interstitial pneumonia (3) and familial idiopathic pulmonary fibrosis (4), is identified when two or more members of a family have an idiopathic interstitial pneumonia (IIP) (3–5). First described five decades ago (6–8), interest in FPF has recently undergone a resurgence because of the possibility that understanding familial forms could provide significant insight into the genetics and pathogenesis of the more common sporadic form of IPF. One of the earliest reports in 1950 described twin sisters raised in different geographic regions, both of whom died of IPF at age 46 (6). A year later, another report described deaths from IPF in a mother and daughter in another branch of this family (7). Since then, a total of 16 cases of IPF have been identified in this family, making it the largest reported (9, 10). Since these initial descriptions, well over 100 families with FPF have been reported (3, 8, 11, 12). Vertical transmission in families, including father-to-son transmission, suggests that FPF is inherited in an autosomal dominant fashion, although with reduced penetrance (3, 5, 10).

One of the earliest reviews of IPF suggested that approximately 25% of patients with the disease reported a positive family history (13), but more recent studies (11, 12) suggest that 0.5 to 3.7% of IPF is familial. However, the true percentage of IPF that is familial may be underestimated, as evidenced by the Vanderbilt Lung Transplant Program, in which 9 of 47 patients (19%) transplanted for IPF have a family history significant for interstitial lung disease (ILD) (5). In 2000, Marshall and colleagues reported 25 families with FPF that were identified from a survey of adult pulmonary physicians in the United Kingdom (11). From this study, they estimated that 0.5 to 2.2% of all IPF cases had a familial basis. This study was followed

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by a 2002 report by Hodgson and colleagues who reported 17 Finnish families with FPF (12). From this study they estimated a prevalence of IPF in Finland of 16 to 18 per 100,000 population with a prevalence of FPF of 5.9 per million, with the familial form of disease explaining 3.3 to 3.7% of IPF cases. Furthermore, they identified a clustering of cases in eastern Finland with analysis suggesting a founder effect among multiplex families.

In 2005, Lee and colleagues reported the clinical features and outcome of 27 patients from 15 FPF families whose complete clinical course had been followed at the Mayo Clinic in Rochester, Minnesota (4). Clinical, pathologic, and radiologic data were reviewed on these subjects from 1992–2002 and compared with nonfamilial cases of IPF. Overall, clinical findings were similar between familial and nonfamilial IPF, including symptoms, physical exam, imaging including high-resolution computed tomography (CT), pulmonary function, pathologic features, and survival. These studies reflected earlier observations by Marshall and colleagues that also suggested that the clinical, pathologic, and radiologic findings were indistinguishable between familial and nonfamilial cases, except that familial cases appeared to be diagnosed at a younger age (11). Nishiyama and colleagues (14) described nine patients with biopsy-proven familial IPF and reviewed serial CT scan findings. Disease progression was observed in most, and high-resolution CT findings on the familial IPF patients were similar to those of nonfamilial IPF patients except that familial IPF patients had less honeycombing and less predominance of lower lung zone disease distribution.

Recently, investigators at Duke University, National Jewish Medical Center/University of Colorado, and Vanderbilt University assembled a large cohort of patients with FPF in an attempt to identify genetic mutations responsible for IPF in these families. This collaborative effort resulted in a report describing the largest collection of FPF families to date (3). In this report, the investigators described 111 families with FPF, including 309 affected and 360 unaffected individuals. From this study, older age (68.3 vs. 53.1 yr, $p < 0.0001$), male sex (55.7 vs. 37.2%, $p < 0.0001$), and history of cigarette smoking (67.3 vs. 34.1%, $p < 0.0001$) were identified as risk factors for FPF. The effect of cigarette smoking in the setting of a FPF history suggests that these individuals may have a genetic predisposition to disease that is accentuated by an environmental exposure such as cigarette smoking. Another interesting finding in this study was the number of families that had more than one type of IIP. A total of 54.9% of the families had a uniform diagnosis of IPF/usual interstitial pneumonitis (UIP) among affected family members. The remaining 45.1% had radiographic and/or pathologic findings consistent with more than one type of IIP, including families in whom findings were pathologically confirmed. Within families that had heterogeneity of disease, 58.3% had IPF/UIP and unclassified ILD, whereas 35.4% included both UIP and nonspecific interstitial pneumonitis (NSIP). Other combinations of UIP with other IIPs were noted as well. These findings confirm previous reports of disease heterogeneity in FPF within individual families (15). This finding that different pathologic forms of IIP occur in different members within the same family, where the genetic transmission pattern suggests mutation in a single dominant gene, suggests that a common central mechanism is operative within each family, and that the pleiotropic phenotypes are influenced by its clinical expression in the background of specific individuals.

SURFACTANT PROTEIN C MUTATIONS AND LUNG FIBROSIS

Recent reports have linked some familial cases of pulmonary fibrosis to mutations in the gene encoding surfactant protein

C (SP-C). In 2001, Nogee and colleagues first reported that a mutation in the gene *SFTPC* for SP-C was associated with NSIP in an infant whose mother had desquamative interstitial pneumonitis (16). They described a heterozygous G to A transition of the first base of intron 4 (IVS4+1 G to A), which caused skipping of exon 4 with deletion of its 37 amino acids. This mutation was identified on only one allele of both individuals, consistent with an autosomal dominant pattern, and resulted in an abnormal pro-SP-C protein product. Subsequently, multiple other mutations in *SFTPC* have been found in infants with ILD (17–21), most of which are in the carboxy-terminal region of pro-SP-C.

When these reports demonstrated a link between *SFTPC* mutations and ILD, it raised questions about whether disease manifestations are due to altered SP-C expression or to epithelial dysfunction caused by the mutation (22). In addition to cases of ILD associated with mutations in *SFTPC*, one family was reported that had SP-C deficiency without a mutation in the gene. In this report, a mother and two daughters were diagnosed with chronic ILD (23). All three individuals were found to have an absence of SP-C on bronchoalveolar lavage and a marked decrease in immunostaining for pro-SP-C on lung tissue sections. No deviations from published *SFTPB* or *SFTPC* coding sequences were identified. Animal studies have supported the possibility that SP-C deficiency may play a role in lung fibrosis, with SP-C-deficient mice having a greater propensity to develop lung fibrosis in experimental models (24, 25).

Although *SFTPC* mutations may affect the level of SP-C expression, many lines of evidence suggest that they can cause disease through an abnormal protein product with aberrant intracellular processing with toxicity to the type II alveolar epithelial cell (AEC) (26, 27). Transcription of *SFTPC* and translation of the mRNA results in a 197-amino acid precursor protein (pro-SP-C). Pro-SP-C undergoes folding of the carboxy-terminal region in the endoplasmic reticulum (ER), sorting in the Golgi, and further processing in the secretory pathway. This results in the functional 35-amino acid SP-C protein that is secreted into the alveolar space. With carboxy-terminal mutations in *SFTPC*, translation results in a pro-SP-C protein that cannot be folded and processed correctly in the ER, leading to accumulation of the abnormal protein in the type II AEC and resulting in ER stress (28, 29).

The initial report by Nogee and colleagues, in 2001 (16), led to our selection of *SFTPC* as a candidate gene for IPF, and to discovery of a different *SFTPC* mutation in a large family with IPF (15). This multiplex kindred spans five generations, contains 97 total members, including 6 adults with IPF/UIP (confirmed by histopathology), 5 adults with probable IPF/UIP (ILD without biopsy), and 3 children with cellular NSIP. The *SFTPC* mutation in this family is a heterozygous exon 5+128 T to A transversion that substitutes glutamine for leucine at the highly conserved amino acid position 188 (L188Q) of the carboxy-terminal region of pro-SP-C. Lung tissue from affected family members revealed an abnormal distribution of staining when immunohistochemistry for pro-SP-C was performed as well as accumulation of atypical intracytoplasmic vesicles in type II AECs at the electron microscopy level (15). When the mutant L188Q *SFTPC* was transfected into mouse lung epithelial cells, greater cytotoxicity was noted as well as an accumulation of intracytoplasmic vesicles detected by electron microscopy when compared to mouse lung epithelial cells transfected with wild-type *SFTPC* (15), findings that are consistent with the purported action of carboxy-terminal *SFTPC* mutations described above.

After identifying the L188Q mutation in one family, we analyzed a cohort of sporadic IPF patients by sequencing the *SFTPC* gene in 89 patients with UIP and 46 patients with NSIP and comparing sequence results to those of 104 normal subjects (30).

In this study, we identified only one individual with a mutation in *SFTPC* that predicted an amino acid change in the pro-SP-C protein sequence. In this patient with UIP, a genetic mutation in exon 3 resulted in a substitution of threonine for isoleucine at amino acid position 73 (I73T). Considering that this mutation has been reported in multiple children with ILD (17–21), it is likely that this individual's IPF is related to this mutation. The I73T mutation was recently described in a family in which nine members had chronic lung disease, most with pulmonary symptoms since childhood (18).

Although rarely the cause of IPF, genetic mutations in *SFTPC* have been noted to cause ILD, with both the L188Q mutation and the I73T mutation having association with biopsy-proven UIP. Although *SFTPC* mutations are not the causative factors in the majority of IPF cases, these findings nonetheless highlight the importance of the type II AEC in disease pathogenesis and suggest that this cell population should be the target of future investigations into understanding the pathogenesis of IPF.

OTHER POTENTIAL GENETIC ASSOCIATIONS WITH IPF

In addition to using FPF families as a means of identifying potential genetic links to disease, multiple investigators have used cohorts of sporadic IPF cases to search for gene associations using a candidate-gene approach in which specific gene polymorphisms are analyzed in subjects with IPF and compared with a population of control subjects. Studies that have shown positive signals are described briefly below.

Interleukin-1 Receptor Antagonist and Tumor Necrosis Factor- α

In 2000, Whyte and colleagues reported association of interleukin-1 (IL-1) receptor antagonist and tumor necrosis factor- α (TNF- α) gene polymorphisms with IPF (31). IL-1 α and IL-1 β are proinflammatory cytokines that have fibrogenic properties. The IL-1 receptor antagonist (IL-1RN) is a protein that binds the type 1 IL-1 receptor but not does not elicit a response. A polymorphism at position +2018 has been implicated in inflammatory diseases, so these investigators chose to evaluate it in IPF. TNF- α , another proinflammatory cytokine, has been shown to be up-regulated in pulmonary fibrosis and its overexpression has resulted in lung fibrosis in murine models. A polymorphism in the promoter region of TNF- α , at position -308, has been implicated in inflammatory diseases, and thus this polymorphism was chosen for evaluation as well (31). In this study, the authors studied both an English cohort and an Italian cohort. In the English group, subjects homozygous for the IL-1RN +2018C>T polymorphism had an increased risk of IPF with an odds ratio (OR) of 10.2 (95% confidence interval [CI], 1.26–81.4; $p = 0.03$), whereas heterozygous subjects had an OR of 1.85 (95% CI, 0.94–3.63; $p = 0.075$). In the Italian group, subjects homozygous for the IL-1RN polymorphism had an increased risk of IPF with an OR of 2.54 (95% CI, 0.68–9.50; $p = 0.2$), whereas heterozygous subjects had an OR of 2.40 (95% CI, 1.26–4.60; $p = 0.008$). In the English cohort, carriage of the TNF- α -308G>A allele was associated with an increased risk of IPF with an OR of 1.85 (95% CI, 0.94–3.63; $p = 0.075$), whereas in the Italian cohort, carriage of the TNF- α allele was associated with an increased risk of IPF with an OR of 2.50 (95% CI, 1.14–5.47; $p = 0.022$).

Two other groups (Hutyrova and colleagues, Riha and colleagues) later evaluated an intron 2 variable number tandem repeat in the IL-1 receptor antagonist that is in strong linkage disequilibrium with the IL-1RN +2018C>T polymorphism and did not notice an association with IPF (32, 33). In the study by Riha and colleagues, the TNF- α -308G>A polymorphism

association with IPF was confirmed in a cohort of Australian patients (heterozygotes OR, 2.9; 95% CI, 1.2–7.2; $p = 0.02$; homozygotes OR, 13.9; 95% CI, 1.2–160; $p = 0.04$) (33). However, this association was not confirmed in a study by Pantelidis and colleagues in a group of IPF patients from the United Kingdom (34).

Complement Receptor 1

Erythrocyte complement receptor 1 (CR1) mediates the transport of immune complexes through the blood to phagocytes in the spleen and liver for clearance. A polymorphism in exon 33 (C5507G) has been found to correlate with the ratio of CR1 to erythrocytes (CR1/E), which may have implications for immune complex clearance. Individuals who are CC homozygous have a CR1/E ratio 10 times that of GG homozygotes. Individuals with the CG genotype have an intermediate CR1/E ratio. In 2002, Zorzetto and colleagues reported an association between sarcoidosis and the GG genotype for the C5507G polymorphism (35) and subsequently analyzed this polymorphism in a cohort of Italian patients with IPF (36). In their IPF study, they found that the GG genotype for the C5507G polymorphism was more common in patients with IPF compared with controls with an OR = 6.232 (95% CI, 2.198–18.419; $p = 0.00023$). Although it is not known how this polymorphism mechanistically affects IPF, these studies raise questions on how immune complex clearance may impact lung fibrosis and targets an area that warrants further investigation.

Surfactant Proteins A and B

Selman and colleagues evaluated specific polymorphisms in the genes (*SFTPA1*, *SFTPA2*, *SFTPB*, *SFTPC*, *SFTPD*) encoding the four surfactant proteins for associations with IPF in a group of subjects in Mexico (37). Evaluating the surfactant protein genes for genetic associations with IPF is an attractive approach because of the greater specificity for lung expression that these genes have compared with other candidates. In these studies, when both smokers and nonsmokers were considered together, no association between the studied polymorphisms and IPF was observed. However, when subjects were stratified according to smoking status, two associations were noted. On analysis of the *SFTPA1* and *SFTPA2* polymorphisms, the SP-A1_6A4 allele was associated with IPF in nonsmoking subjects compared with control subjects with an OR = 3.67 (95% CI, 1.34–10.07; $p = 0.01$). Regarding *SFTPB*, the B1580_C allele was associated with IPF in smokers compared with control subjects with an OR = 7.63 (95% CI, 1.64–35.4; $p = 0.01$). This particular *SFTPB* polymorphism has been described in association with chronic obstructive pulmonary disease (38), and thus this allele may be associated with smoking-related lung diseases rather than specifically associated with IPF. These investigators also evaluated two polymorphisms in *SFTPC* and two polymorphisms in *SFTPD* but did not find any associations between these polymorphisms and IPF.

Angiotensin Converting Enzyme

Angiotensin converting enzyme (ACE) converts angiotensin I to angiotensin II, which acts on the angiotensin II type 1 receptor. This may have implications for lung fibrosis because administration of ACE inhibitors or angiotensin II type 1 receptor antagonists can inhibit lung fibrosis in animal models. An insertion/deletion polymorphism in intron 16 of the ACE gene has been described that impacts the level of ACE expression in the serum. In 2001, Morrison and colleagues reported a study of a small group ($n = 24$) of subjects with UIP or fibrosing NSIP and noted an increased D allele frequency in these subjects (69 vs. 54% in control subjects; $p = 0.04$) (39), a finding that would benefit from further studies.

Transforming Growth Factor- β 1

Transforming growth factor- β 1 is a principal profibrotic cytokine and has been implicated in disease pathogenesis in multiple IPF studies and animal models. Thus, it is attractive to consider for possible genetic associations with IPF. Polymorphisms at codons 10 and 25 may affect production of transforming growth factor- β 1. The G915C polymorphism, predicting a change from arginine to proline at codon 25 (R25P), has been associated with post-lung-transplantation allograft fibrosis (40). This polymorphism has now been studied by two groups (Riha and colleagues, Xaubet and colleagues) without demonstration of an association with IPF (33, 41). In the study by Xaubet and colleagues, the T869C polymorphism, predicting a change from leucine to proline at codon 10 (L10P), was not associated with the development of IPF but was associated with its progression in gas-exchange abnormalities during follow-up (coefficient, 0.59; 95% CI, 0.23–0.96; $p = 0.002$) (41). This T869C polymorphism was recently shown to have implications in cystic fibrosis with the homozygous CC genotype having association with greater severity of lung disease (42).

Other Candidate Genes Evaluated

Polymorphisms in other genes have been evaluated using a candidate-gene approach in IPF (43). In 2003, Kim and colleagues reported an evaluation of the plasminogen activator inhibitor-1 promoter polymorphism 4G/5G in 88 patients with IIP (62 with UIP, 26 with NSIP) (44). No association was found between the plasminogen activator inhibitor-1 polymorphism and UIP, but the study did reveal an increased frequency of the 4G allele relative to the 5G allele in subjects with NSIP compared with control subjects ($p = 0.04$). In 2001, Pantelidis evaluated a cohort of patients from the United Kingdom for associations between IPF and polymorphisms in IL-6 and TNF receptor II (34). IPF was not associated with either the IL-6 (intron 4G) allele or the TNF receptor II (1690C) allele when each was considered alone. However, a strong association was noted between IPF and cocarriage of these two alleles ($p = 0.00093$) (34).

Other candidate genes with specific polymorphisms evaluated to date, but without association with IPF identified, include the following: IL-1 α (32), IL-1 β (32), lymphotoxin- α (34), IL-10 (45), IL-8 (46), CXC receptors 1 and 2 (46), IL-12p40 subunit (47), and IFN- γ (47). It should be noted that these studies have evaluated specific polymorphisms for each of the genes listed, and thus these evaluations do not necessarily rule out potential associations with other polymorphisms in these genes.

IMPLICATIONS FOR THE FUTURE

Data regarding the incidence and prevalence of FPF relative to total IPF cases is limited. Studies in the future should be designed to correct this deficiency. Our experience suggests that FPF may represent 20% of all cases of IPF. To date, the only clearly defined genetic mutations associated with IPF/UIP remain the L188Q and I73T genetic mutations in *SFTPC*. Though rare, these findings have the potential to guide future investigations into the pathogenesis of IPF. Specifically, these mutations highlight the importance of the alveolar epithelium in pulmonary fibrosis and support the possibility that AEC injury and the subsequent response may be critical components in the early pathogenesis of pulmonary fibrosis. From this, other candidate genes may be selected for evaluation of potential genetic links to disease. The effect of such an approach can be illustrated in the case of pediatric ILD. *SFTPC* mutations were first described as associated with pediatric ILD, and subsequently mutations in another gene in the SP-C processing pathway, ATP binding

cassette3 (*ABCA3*), were associated with pediatric ILD (48, 49). Many other candidate genes for future evaluation can be envisioned among the known components of the complex pathways in the pathogenesis of lung fibrosis. However, such an approach has limited potential to clarify genetic associations because each gene must be evaluated on an individual basis.

Unlike candidate-gene approaches for specific polymorphisms, utilizing FPF cohorts provides an opportunity to perform linkage analysis with a large number of DNA markers spaced throughout the entire genome. With the large number of families that have been assembled through the collaborative effort of Duke University, National Jewish Hospital/University of Colorado, and Vanderbilt University, it is hoped that a genomewide search will identify specific mutations responsible for pulmonary fibrosis in these families. These efforts, directed by Dr. David Schwartz, have shown some early promise, and further work is ongoing (50).

When considering the complexity of the pathogenesis of IPF, the fact that different polymorphisms are associated with disease in different populations, the difficulty with identifying clear genetic links to most cases of IPF, and the potential impact of environmental contributions on disease, it seems unlikely that a single aberrant gene is responsible for most cases of IPF. Specifically, in regards to the FPF families, it is likely that different genes are responsible for disease or predisposition to disease across families, albeit that vertical transmission in most families suggests a single dominant gene will be found responsible within each family. Nevertheless, a genetic approach to IPF is an important component of improving understanding of IPF and potentially identifying future therapeutic or preventive targets for disease management. If, for example, the Familial Pulmonary Fibrosis Study described above identifies genetic causes of disease in specific families, then the pathways involved with those genes and gene products become the targets of intensive investigation in relation to their role in the pathogenesis of IPF, allowing the medical and scientific communities to focus resources in a fashion that will increase ability to treat or prevent this devastating disease.

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