Genetic Analysis of Sporadic and Familial Interstitial Pneumonia

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Although much progress has been made in understanding the biology and clinical course of interstitial pneumonia, the etiology of this disease remains elusive. Epidemiologic studies have consistently identified cigarette smoke as an important exposure; however, most smokers do not develop interstitial pneumonia and many individuals with interstitial pneumonia do not smoke cigarettes. Moreover, interstitial pneumonias have been reported to cluster in families. Thus, a more thorough understanding of the genetic etiology of interstitial pneumonia may prove critically important in defining the biology and clinical course of this complex human disease.

Keywords: genetics; familial interstitial pneumonia; pulmonary fibrosis

Four lines of evidence suggest that the development of pulmonary fibrosis is, at least in part, determined by genetic factors. First, the familial clustering of pulmonary fibrosis, an uncommon disease, has been reported in monozygotic twins raised in different environments (1–3), in genetically related members of several families (3–6), in consecutive generations in the same families (3, 7, 8), and in family members separated at an early age (5). While a single report suggests that familial interstitial pneumonia is inherited as an autosomal recessive trait (9), other pedigrees demonstrate an autosomal dominant pattern of inheritance (5, 10, 11), perhaps with reduced penetrance (1, 2, 4–6, 10, 12, 13). Second, pulmonary fibrosis is observed in genetic disorders with pleiotropic presentation including Hermansky-Pudlak syndrome (14), neurofibromatosis (15), tuberous sclerosis (16, 17), Neimann-Pick disease (18), Gaucher disease (19), familial hypocalciuric hypercalcemia (20), and familial surfactant protein (SP)-C mutation (21). Third, considerable variability exists in the development of pulmonary fibrosis among workers exposed to similar concentrations of fibrogenic dusts or organic antigens. For instance, after exposure to asbestos, similarly exposed individuals may experience different outcomes (22, 23). Fourth, inbred strains of mice differ in their susceptibility to fibrogenic agents. In comparison with BALB/c or 129 mice, C57BL/6 mice develop more lung fibrosis when challenged with either bleomycin (24, 25) or asbestos (26, 27).

THE SEARCH FOR THE PULMONARY FIBROSIS GENE

Substantial advances made in technical and strategic approaches to the molecular identification of genes and loci have been

successfully applied to the investigation of human genetic disorders.

Some of the advances include sequencing of the human genome, improvement in processing and genotyping of DNA samples, continuous characterization of countless human genes, and technological achievements that permit us to sequence and genotype vast regions of DNA. Two general strategies have been adopted to take advantage of these advances: the candidate gene approach and genomewide linkage studies. In the candidate gene approach, specific genes are identified and studied on the basis of prior knowledge of their biological function and possible pathogenic role in the disease under study. In a linkage study, a genome-wide search using polymorphic genetic markers is performed and candidate genetic loci are identified on the basis of cosegregation between genetic markers and a disease trait.

CANDIDATE GENE STUDIES IN PULMONARY FIBROSIS

On the basis of our limited knowledge of the pathogenesis of pulmonary fibrosis, a number of candidate genes that may predispose individuals to develop this disease can be identified (Table 1). While activated alveolar macrophages and epithelial cells may release polypeptide growth factors (transforming growth factor [TGF]- β_1 , platelet-derived growth factor [PDGF]-B, insulin-like growth factor [IGF]-1, and fibronectin) that modulate the growth of mesenchymal cells, cytokines, such as tumor necrosis factor (TNF)- α , IL-1, and IFN- γ , may serve to amplify the local inflammatory response.

TGF- β_1 is a protein, formed by two identical chains, that has been implicated in pulmonary fibrosis through its promotion of fibroblast proliferation and collagen synthesis. TGF- β_1 has been found in excessive amounts in pulmonary epithelial cells and alveolar macrophages in areas of lung regeneration and remodeling in patients with idiopathic pulmonary fibrosis (IPF) (28, 29). Multiple studies have addressed the genetic variation in the TGF- β_1 gene in patients with IPF. Two related studies found that homozygosity for arginine at codon 25 of the leader sequences of TGF- β_1 , which correlates with higher production of TGF- β_1 *in vitro*, is more common in patients undergoing lung transplantation for fibrotic lung diseases as compared with control subjects and with patients undergoing lung transplantation for conditions other than lung fibrosis (30, 31). In addition, the majority of lung transplantation patients who developed lung allograft fibrosis were codon 25 homozygotes (31). Using *in situ* hybridization and immunohistochemistry, TGF- β_1 was found in abundance in foci containing activated fibroblasts in lung biopsies obtained from patients with IPF, in association with marked expression of TGF- β_1 mRNA in alveolar macrophages surrounding these sites (32).

PDGF is another potent mesenchymal cell growth factor that has been shown to be released in excess amounts by alveolar macrophages in patients with IPF (33, 34). Nagaoka and co-

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TABLE 1. GENETIC POLYMORPHISMS ASSOCIATED WITH INTERSTITIAL PNEUMONIA

Mechanism	Gene	Reference
Matrix turnover	TGF- β_1	30, 31
	Fibronectin	37
	IGF-1	38
Inflammation	TNF- α	39–41
	IL-4	43
	IL-6	40
	Complement receptor-1	44
	IFN- γ	45
	HLA genotypes	56–60
Oxidative stress	α_1 -Antitrypsin	54, 55
	p53	53
Phagocytosis	ELMOD2	69
Proteolysis	Angiotensin-converting enzyme	51
Surface tension	SP-C	21, 62, 63
Telomere length	TERT and TERC	65, 66

Definition of abbreviations: ELMOD2 = engulfment and cell motility-2; IGF-1 = insulin-like growth factor-1; SP-C = surfactant protein C; TERC = telomerase RNA component; TERT = telomerase reverse transcriptase; TGF- β_1 = transforming growth factor- β_1 ; TNF- α = tumor necrosis factor- α .

workers studied the transcription rates and mRNA levels for both PDGF-A and PDGF-B genes in alveolar macrophages from normal subjects and individuals with IPF (35). In IPF, both PDGF-A and PDGF-B mRNA levels were markedly increased, but with preservation of the 10-fold ratio of PDGF-B over PDGF-A found in normal subjects.

Fibronectin is an extracellular matrix glycoprotein that functions as an adhesive substrate and plays a role in cell proliferation, especially during tissue repair. Lung tissues from patients with IPF were found to have exaggerated expression of the fibronectin gene both in areas of early inflammation and areas of mature fibrosis, rich with fibroblasts (36). Avila and coworkers studied polymorphisms in the fibronectin gene in patients with pulmonary fibrosis associated with systemic sclerosis (37). Using restriction enzymes to assess the restriction fragment length polymorphisms, genotypes AB and CD (associated with restriction enzymes *Hae*III and *Msp*I, respectively) were found to be more frequent in patients with pulmonary fibrosis and systemic sclerosis as compared with healthy control subjects (45 vs. 29%; $P = 0.006$) with an increase in relative risk of developing pulmonary fibrosis of 1.99.

IGF-1 is a growth factor that induces fibroblast proliferation and stimulates their synthesis of collagen. Bloor and coworkers examined expression of four IGF-1 transcripts in bronchoalveolar lavage fluid cells from normal subjects, patients with IPF, and patients with sarcoidosis (38). There was differential expression of IGF-1 splice variants, in which patients with IPF had an increase in class 1 transcripts and failed to express class 2 IGF-1Eb forms.

TNF- α and IL-1 are proinflammatory cytokines that have been implicated in the pathogenesis of IPF. Numerous studies have investigated polymorphisms in their genes. Whyte and coworkers conducted a case-control study of two populations: English and Italian (39). For the rarer allele of the TNF- α gene (TNF-A) the relative odds of having pulmonary fibrosis were increased in homozygous subjects by an odds ratio of 1.85 in the English, and 2.50 in the Italian population. Similarly, for the less common allele of the interleukin-1 receptor antagonist (IL-1RA) gene (IL-1RN) the odds ratio was 10.95 in the English, and 2.54 in the Italian population. However, in a similar study by Pantelidis and coworkers, comparison of 74 patients with IPF with 100 control subjects found no significant deviations in genotypes or in allele or haplotype frequencies in 4 candidate

genes: TNF- α , lymphotoxin- α , TNF receptor II, and IL-6 (40). Freeburn and coworkers hypothesized that polymorphisms in the 3' untranslated region of the TNF- α gene would explain the previously observed TNF- α resistance to IL-10-mediated suppression in IPF (41). A single-strand confirmation polymorphism analysis was unable to detect such polymorphisms in 96 patients with IPF. In a more recent case-control study in a western Slavonic population by Hutyrová and coworkers, no polymorphisms in the IL-1 α , IL-1 β , and IL-1RA genes were found in 54 patients with IPF (42). Polymorphisms in helper T-cell type 2 cytokines (43) and complement receptor-1 (44) have also been reported more frequently in patients with IPF.

IFN- γ is an inflammatory cytokine that has drawn attention as a promising therapeutic agent for IPF. Earlier studies implicated IFN- γ in the pathogenesis of lung fibrosis, as demonstrated in one study by a significant increase in the frequency of allele 2 of the IFN- γ gene in a group of lung transplant recipients who developed fibrosis after transplantation (45). However, subsequent *in vitro* (46–48) and *in vivo* (49) data demonstrated a favorable effect of IFN- γ on fibrosis, with inhibition of the proliferation of lung fibroblasts and down-regulation of the transcription of the genes for TGF- β_1 .

The role of angiotensin-converting enzyme in renal and cardiovascular diseases has been well established. Angiotensin II has been shown to augment the *in vitro* proliferation of human lung fibroblasts by means of activating the angiotensin type 1 receptors (50). The angiotensin-converting enzyme gene has an insertion/deletion polymorphism in exon 16 on chromosome 17q23, resulting in three distinct genotypes: insertion/insertion (I/I), insertion/deletion (I/D), and deletion/deletion (D/D). Morrison and coworkers examined this polymorphism in 24 patients with usual interstitial pneumonitis or nonspecific interstitial pneumonitis and found a higher incidence of the D allele in this population compared with control subjects, as well as a higher incidence of the genotype D/D (51).

The tumor suppression factor p53 protein is a transcription factor that mediates the cellular response to DNA damage by irradiation or carcinogens. The chronic DNA damage in bronchial and alveolar cells and the higher incidence of lung cancer in patients with IPF have led to postulations that p53 may have a pathogenic role in IPF. Hojo and coworkers illustrated frequent heterogeneous point mutations of the p53 gene in patients with IPF (52). In the same line of investigation, Vassilakis and coworkers showed frequent genetic alterations in sputum samples of patients with IPF (53); namely, loss of heterozygosity and microsatellite instability, which are commonly detected in malignancies of various origin, including lung carcinomas.

α_1 -Antitrypsin (α_1 -AT) is a serum glycoprotein that exists in the bronchoalveolar lavage fluid of normal humans and plays a crucial role in the inhibition of several proteolytic enzymes, particularly neutrophil elastase. The most common phenotype in the PI loci encoding the α_1 -AT gene is MM, which results in normal serum levels of α_1 -AT, whereas the PI ZZ phenotype results in low serum levels of α_1 -AT. A highly significant increase in the frequency of a non-MM (MZ) phenotype was found among patients with pulmonary fibrosis with and without rheumatoid arthritis (RA), as compared with control subjects and patients with RA with no pulmonary fibrosis (54). These findings were replicated in patients with RA in a later study (55).

Investigation of the human leukocyte antigen (HLA) system, which is located on chromosome 6, has been inconclusive. IPF has been found at a higher frequency in individuals with HLA-B15 (56), HLA-Dw6 (57), and HLA-DR2 (58). Moreover, among patients with RA, the development of interstitial lung disease was associated with HLA-B8 (57), HLA-Dw3 (58),

HLA-DQw (59), and several specific HLA haplotypes (60). On the other hand, several studies did not identify a relationship between specific HLA alleles and pulmonary fibrosis among patients with IPF (12, 61). These inconsistencies may result from ethnic differences in the distribution of HLA alleles.

Mutation in the gene for SP-C was reported in two family members who developed interstitial lung disease (desquamative interstitial pneumonitis and nonspecific interstitial pneumonitis) in childhood (62). Lloyd and colleagues (21) have reported a family with a polymorphism in the fifth exon of the SP-C gene that was associated with the development of pulmonary fibrosis. Interestingly, this family had both childhood- and adult-onset cases of pulmonary fibrosis. However, mutations in SP-C are infrequently observed in patients with IPF (63). SP-A and SP-B genetic variants have also been reported more frequently in patients with IPF (64).

Armanios and coworkers (65) reported that 8% of families ($n = 73$) with familial idiopathic pulmonary fibrosis had heterozygous mutations in telomerase reverse transcriptase (TERT). They hypothesized that the "fibrotic lesion in patients with short telomeres due to TERT mutations is provoked by a loss of alveolar cells rather than by a primary fibrogenic process, such as one that would seem to occur in autoimmune disease associated with lung fibrosis." This hypothesis suggests a novel mechanism for the development of pulmonary fibrosis. To our knowledge, none of our families have evidence of an autoimmune component to disease. Specific evaluation of markers flanking TERT in our families failed to identify families that are conclusively linked to this gene, although some families did show slightly positive LOD (logarithm of the odds of linkage) scores. Subsequent to the initial findings, a missense mutation and a frameshift mutation in TERT were found to cosegregate with pulmonary fibrosis in two additional families, and a mutation in TERC (the RNA component of telomerase) was found in one family (66).

In summary, candidate gene studies of human genetic diseases, including pulmonary fibrosis, tend to point toward promising genes, but in general suffer from their small size and lack of consistency. Replication of such discoveries is paramount for further focused research striving to find specific genes involved in pulmonary fibrosis.

ROLE OF LINKAGE ANALYSIS IN THE SEARCH FOR THE PULMONARY FIBROSIS GENE

It is now feasible to identify the specific genes involved in human disorders by using strategies that involve "positional cloning," which aims to locate the gene on the basis of its map position with no functional information. Advances in molecular biology techniques and statistical genetic analysis have made genomewide linkage possible and has led to accelerated localization of genes for human genetic disorders such as cystic fibrosis (67) and Huntington's disease (68).

The first step in a genomewide linkage or positional cloning study involves the collection of families with two or more members affected with the trait (the disease of interest). The process of linking the disease trait to a genetic marker and, hence, a chromosomal location is termed "linkage analysis." DNA specimens from family members are genotyped for a series of genetic markers, spanning the entire genome ("genomic screen"). The segregation of alleles of two different genes is related to the distance separating them on the chromosome. If a disease trait segregates with a marker, it is likely that their genes reside in close physical proximity to each other. This relationship is tested using quantitative statistical tests, by comparing it with known genetic models and by obtaining an LOD

score. Genetic loci with an LOD score of 3 and above are considered favorable for linkage (69). These identified linked loci provide the impetus to search that region in more detail for specific genes, using the rapidly evolving knowledge of mapped human genes. These genes that map to the linked subchromosomal loci can then be prioritized on the basis of functional criteria (i.e., known oxidants, polypeptide growth factors, cytokines, etc.).

The positional candidate approach is advantageous over the candidate gene approach in pulmonary fibrosis, simply because of our lack of knowledge of the pathogenesis of pulmonary fibrosis. A focused examination of genes identified on the basis of polymorphisms in families with two or more cases of pulmonary fibrosis avoids laborious and expensive exploration of genes that may theoretically have a scientific basis but are ultimately far from the truth. However, given the late age of onset and poor prognosis of the disease, a linkage analysis is difficult in pulmonary fibrosis. Thus far, only one linkage analysis in pulmonary fibrosis has been published (70), showing that a region of chromosome 4 (4q31) had a shared haplotype among eight families. Moreover, the investigators found that *ELMOD2*, a gene involved in apoptosis, was differentially expressed in this region among patients with pulmonary fibrosis (Table 1). However, no mutations were identified in *ELMOD2* and no functional studies were presented.

CONCLUSIONS

In summary, the genetics of pulmonary fibrosis represent an exciting area for the clinician and the investigator. Although presentations of this disease are rare and account for a subset of a low-prevalence condition—interstitial lung disease—cases of familial interstitial pneumonia are recurrently observed in the practice of pulmonary medicine. Furthermore, pulmonary fibrosis is observed in several complex genetic disorders, and workers, as well as experimental animals, appear to respond differently to chronic inhalation of fibrogenic dusts. In aggregate, these findings strongly suggest that genetic factors predispose individuals to develop pulmonary fibrosis. Given this hypothesis, it is logical to speculate that specific gene products modulate the inflammatory/fibrotic response to fibrogenic agents and provide the pathogenic basis for enhanced genetic risk. Moreover, it is likely that the gene or genes that prove to be important in the development of the familial form of this disease will also be relevant in the pathogenesis of other forms of interstitial lung disease.

Conflict of Interest Statement: D.A.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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