

UNDERSTANDING THE BIOLOGY OF PULMONARY FIBROSIS: WHAT GENES TELL US FOR IDIOPATHIC INTERSTITIAL PNEUMONIA

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ABSTRACT

Pulmonary fibrosis is the formation of fibrous tissue as a reaction or a repair process in the lung. It is the main cause of severe morbidity and mortality in interstitial lung disease (ILD). Pathogenesis in most ILDs is triggered by exposition to exogenous factors, such as asbestos and radiation, or endogenous factors, such as auto-immune disease and drugs. However, in a subgroup of ILD, the idiopathic interstitial pneumonias (IIPs), causative environmental triggers have not been identified. Fibrogenesis in IIP seems to be an autonomous pulmonary process, making a strong genetic contribution to disease more plausible. In fact, in the last decade, several studies have shown a contribution of genetic variations to the development of IIP, from highly penetrant mutations to strongly predisposing common risk alleles. This review summarises the results of candidate gene analyses, family-based linkage analyses, and case control whole genome association studies that have identified germ-line gene variants that predispose to the development of IIP. Genetic analyses in both familial and sporadic diseases have shown congruent results and point to genes, their corresponding protein product, and effect on cell function in several pathways. Present data tell us that IIP result from a complex interaction involving the alveolar compartment, in particular alveolar epithelial Type 2 cells, but recent studies also emphasise a role for mucin and immune-regulatory genes.

Keywords: Pulmonary fibrosis, idiopathic interstitial pneumonias, alveolar epithelial cell, surfactant, telomeres, genetic variation, genetic predisposition, mutation.

IDIOPATHIC INTERSTITIAL PNEUMONIA (IIP)

IIPs are uncommon and difficult to diagnose. Diagnosis requires a multidisciplinary team of experts and, when clinical and radiological data are insufficiently informative, often includes histological evaluation.¹ For histological confirmation, surgical lung biopsy is the modality of choice, but due to increased surgery related mortality in IIP, this should be considered with care.^{2,3} Idiopathic pulmonary fibrosis (IPF) is the most common and severe form of IIP. Median survival in IPF is 2-3 years regardless of therapy.⁴ Clinically, distinction between the different entities within IIP is important with regard to prognosis, response to therapy, timing of lung transplantation, or palliative care. Genetically,

however, the question has arisen whether the different types of IIP have different pathogeneses. In a large cohort of families with two or more cases of IIP, also called familial interstitial pneumonia (FIP), it was found that a diagnosis of IPF was most frequent, but all subtypes of IIP were represented.⁵

Evidence for Genes

There are several lines of evidence suggesting that development of IIP has a genetic basis. Firstly, ethnic differences in the disease have been observed. IPF appears to be more common in Hispanics than in Whites, and least present in Blacks, Maori, and Pacific islanders.^{6,7} The most compelling evidence is based on statistics. IPF is an orphan disease with an annual incidence in the US and Europe ranging between 0.22-17.4 per 100,000,

depending on country and inclusion criteria.⁸ The probability of the presence of IPF in two or more first degree family members is therefore infinitely small, and can only be explained by the presence of a common environmental or genetic cause. An environmental cause would require the affected family members to be clustered in space and time. A genetic cause however, allows for differences in space and time that are often found in familial disease, such as sibling from different environments, and parent-offspring disease with an interval of decades.^{5,9-11} FIP families, like these, most commonly showed a dominant disease transmission pattern and have formed the starting point in the search for causative genetic variations.

Surfactant Metabolism

The first causative mutation for IIP was discovered by candidate gene sequencing of surfactant protein C (*SFTPC*). The family consisted of a baby girl with paediatric interstitial lung disease (ILD), a mother with desquamative interstitial pneumonitis, a type of IIP, and the infant's maternal grandfather who had died from lifelong lung disease of unknown cause.¹² This report already contained many features of disease associated with *SFTPC* mutations: familial ILD, dominant expression, variable penetrance, and expressivity resulting in acute and chronic lung disease in individuals ranging from newborn to adult.¹³⁻¹⁵ *SFTPC* is exclusively expressed in the Type 2 alveolar epithelial cells (AECs); surfactant protein-C is processed and stored in lamellar bodies, and secreted into the alveolar space. Pulmonary surfactant consists of a mixture of lipids and specific proteins that lower alveolar surface tension, thereby preventing alveolar collapse at the end of expiration.^{16,17}

Mutations in *SFTPC* can cause abnormal processing and protein misfolding, leading to endoplasmic reticulum (ER) stress and activation of unfolded protein response in alveolar epithelial cell lines of mouse, rat, and human origin.¹⁸⁻²⁰ In turn, ER stress can induce epithelial-to-mesenchymal transition in lung epithelial cells.¹⁹ The most common *SFTPC* mutation, I73T, does not cause substantial elevation of ER-stress,²¹ but is trafficked into early endosomes and suspected of clogging the surfactant recycling system,²² altering surfactant lipid composition, and activating immune cells.²³ Furthermore, the study of human lung tissue from patients with *SFTPC* BRICHOS mutations has shown the presence of amyloid, composed of mature SP-C, suggesting that these patients suffer from amyloid disease.²⁴

ER Stress

Lamellar bodies, the secretory organelles unique to Type 2 AEC, are crucial for biosynthetic processing and transportation of pulmonary surfactant. The limiting membrane of lamellar bodies is encoded by the gene *ABCA3*.¹⁶ The first mutations in *ABCA3* were found in newborns with respiratory distress syndrome.²⁵ Recessive mutations in *ABCA3* cause lethal surfactant deficiency in neonates to chronic ILD in children.^{26,27} The highest expression of *ABCA3* has been observed in Type 2 AEC.¹⁶ In these cells, *ABCA3* mutations cause abnormal processing, trafficking, and functionality of the *ABCA3* protein,^{28,29} resulting in impaired lipid transport³⁰ or retention in the ER compartment, elevated ER-stress, and apoptotic signalling.³¹ Two recent reports show that *ABCA3* mutations can also cause adult IIP.^{32,33} A French patient with *ABCA3* mutations presented with combined pulmonary fibrosis and emphysema (CPFE).³³ CPFE typically occurs in male smokers, but also has similarities with radiographs of IIP patients with *SFTPC* mutations.^{15,34,35}

Another surfactant associated gene was implicated when missense mutations in *SFTPA2* were identified in two families with adult early-onset pulmonary fibrosis (PF) or lung cancer, and autosomal dominant disease transmission.³⁶ The mutant protein, when expressed in AECs, was not excreted but retained in the ER and induced ER-stress.³⁷ Most importantly, different studies showed that AECs in fibrotic tissue from non-mutated familial PF and sporadic IPF patients were positive for ER-stress markers.^{18,38}

PF and Macrophages

Interestingly, further research in several types of lung epithelial cell lines, showed that expression of genetic variants in *SFTPA1*, *SFTPA2*, and *SFTPC*, lead to increased secretion of transforming growth factor beta 1 (TGF- β 1), a profibrotic cytokine.^{39,40} The significance of this finding is yet unclear, because highest TGF- β 1 secretion level was induced by a common *SFTPA1* polymorphism.⁴⁰ Raised TGF- β 1 levels are known as potent inducers of PF.⁴¹ Surfactant protein A2 is a C-type lectin important in the defense against respiratory pathogens and is found in AEC Type 2, clara cells, and alveolar macrophages.^{42,43} It has been shown that TGF- β 1 driven lung fibrosis is macrophage-dependent and that alternatively activated macrophages play a major role during disease development.^{44,45}

The role of macrophages was investigated in a mouse model of Hermansky Pudlak Syndrome (HPS). PF in HPS shares many similarities with that observed in IIP^{46,47} and mouse models of HPS1 and 2 have constitutive activation of lung macrophages.⁴⁸ In reciprocal bone marrow transplantation experiments between HPS and wild-type mice, bleomycin-induced fibrosis (BIF) was conferred by the genotype of the lung epithelium and not by the bone marrow-derived, cellular compartment. Furthermore, altered alveolar macrophage activation was only observed in HPS mice and not in wild type mice with transplanted HPS bone-marrow.⁴⁹

HPS is a rare autosomal recessive and genetically heterogeneous human disorder, characterised by reduced pigmentation of skin, hair, eyes, and bleeding diathesis. Mutations in *HPS1*⁵⁰ lead to giant lamellar bodies in Type 2 AECs, that can be recognised on biopsy as foamy Type 2 cells.^{51,52} Due to unfamiliarity with the disease and the variable degree of albinism and bleeding disorders, HPS could be misdiagnosed as IIP. In a cohort of patients ever having been diagnosed with IPF, four patients with two or more features consistent with HPS were identified. One out of four was compound heterozygous for mutations in *HPS1* and had received a diagnosis of HPS upon tertiary referral.⁵³

Telomere maintenance genes

Although very important in familial disease, and extremely informative on biological mechanisms that may lead to PF, mutations in surfactant associated genes play a minor role in sporadic IIP. However, the following genes might have broader significance. When a pedigree with autosomal dominant dyskeratosis congenita due to a mutation in the gene encoding telomerase reverse transcriptase (*TERT*) was identified, it was noted that the family lacked the typical mucocutaneous features, and one family member only suffered from PF.⁵⁴ The gene *TERT* encodes telomerase reverse transcriptase, which together with the transcript of the telomerase RNA-component (*TERC*), is required to maintain telomere length. Testing the hypothesis that mutations in these genes might cause FIP, resulted in the discovery of six mutations in *TERT* or *TERC* and corresponding short telomeres.⁵⁵ At the same time, a different group of researchers identified six *TERT* mutations in FIP, one in sporadic IIP, and one in *TERC*, after a family based genome-wide linkage scan had pointed out the gene region.⁵⁶

Later, a genome-wide single-nucleotide polymorphism (SNP) association study, identified a disease susceptibility SNP in intron 2 of *TERT* comparing Japanese sporadic IPF cases with healthy controls.⁵⁷ SNPs are common genetic variations that act as biological markers to locate genes that are associated with disease. SNPs do not necessarily directly affect a gene's function, but together, these three studies were the first to show that genetic variation in *TERT* predisposes to IIP in both familial and sporadic patients.

Telomere Length

Single codon substitutions in one *TERT* allele can cause haploinsufficiency with decreased telomerase activity, resulting in premature ageing disorders, such as IPF, bone marrow failure, and cryptogenic liver cirrhosis.⁵⁸ However, it is not the presence of the mutation per se, but the length of telomeres that confers the risk for disease. Measuring telomerase activity of 19 disease-associated mutant alleles in both rabbit reticulocyte lysates and in human cell lines showed that ten mutations cause <40% loss of function. In a heterozygous individual carrying one wild type and one mutant allele, this would result in >80% overall telomerase activity, which is considered normal.⁵⁹ However, through genetic anticipation, the continuous inheritance of ever-shorter telomeres over several generations, carriage of mutations that cause a minor decrease in overall telomerase activity, will eventually lead to telomere disorders.⁶⁰

Telomere length shortens with each cell division, and, when critically short, replicative senescence is induced. It was shown that telomere lengths of genomic DNA of circulating leukocytes in FIP and IIP patients, both with and without telomere mutations, were significantly shorter, compared to age-matched controls, suggesting that telomere attrition is key to IIP pathogenesis in at least a subgroup of patients.^{61,62} Furthermore, alveolar epithelial cells yielded shorter telomeres in IPF patients when compared with age-matched individuals.⁶²

AEC Type 2 Cells

Genetic evidence of the surfactant-related genes has pointed towards the AEC Type 2 cell. These cells are stem cells of adult lung, have self-renewing capacity, and are responsible for growth, differentiation, and repair in alveoli.⁶³ Alveolar Type 2 cells are progenitors of Type 1 cells, and, in case

of alveolar injury, react by immediate proliferation along the alveolar basement membrane.⁶⁴ Critically short telomeres cause cellular senescence which is mediated by cell cycle regulator p53. Increased levels of proapoptotic molecules, such as p53, have been observed in hyperplastic AECs in IPF patients.⁶⁵ IPF patients that carried the minor alleles of rs12951053 or rs12602273 of the gene encoding p53, were shown to have a significantly worse 4-year survival rate.⁶⁶

P53-induced growth arrest is mediated by cyclin-dependent kinase inhibitor 1A (*CDKN1A*), the gene encoding p21. P21 can delay cell cycle progression to allow optimal DNA repair and thereby reduction of apoptosis.⁶⁷ Genotypes rs2395655GG and rs733590CC in *CDKN1A* were associated with an increased risk of developing IPF and lower mRNA expression levels in healthy controls.⁶⁶ PF and liver cirrhosis can be the first manifestations of telomere disorders running in a family. It is postulated that telomere dysfunction serves as the first hit, however, a second hit is required that determines the phenotype.⁵⁸ In PF, smoking is highly suspected of causing a second hit, even in familial disease,^{5,68,69} but also dust, metal, or viruses may be involved.⁷⁰ In Finnish families, ELMO domain containing 2 (*ELMOD2*) was found to be associated with IPF. The risk allele caused significantly decreased *ELMOD2* messenger-RNA expression in IPF lung compared to a healthy lung.⁷¹ *ELMOD2* is expressed in lung epithelial cells and macrophages, and participates in antiviral responses. Viral stimulation of these cells can downregulate *ELMOD2* expression.⁷²

A COMMON VARIANT IN IDIOPATHIC DISEASE

Exposure to putative predisposing environmental factors, such as viruses, smoking, and fibrogenic dust, is common, but does seldom cause PF. This supports a prominent role for genetic factors. Those rare individuals that do develop IIP after exposure might have a unique pulmonary profibrotic genetic make-up. Linkage and fine-mapping analysis identified the minor allele of a common SNP, rs35705950, in the putative promoter region of *MUC5B*, to be associated with disease in both FIP and IPF.⁷³ Odds ratios (ORs) were above five, extremely high for a common risk allele.^{73,74} In a lung from unaffected subjects, the risk allele was associated with increased *MUC5B* expression.⁷³ Surprisingly, the risk allele significantly associates

with improved survival in IPF,⁷⁵ less severe pathological changes in FIP,⁷⁶ and slower decline in forced vital capacity for IPF,⁷⁷ but also with increased severity of cough.⁷⁸

Interestingly, the *MUC5B* risk allele is associated with the presence of interstitial lung abnormalities on high-resolution computed tomography in a general population, suggesting a role in induction of patterns of interstitial pneumonia.⁷⁹ Such patterns can also develop in systemic inflammatory diseases. Therefore, *MUC5B* was investigated in four independent Caucasian populations with systemic sclerosis^{77,80,81} and one British sarcoidosis cohort.⁷⁷ Allele frequencies in patients did not differ from controls, and suggest that PF in inflammatory disease is genetically distinct from IPF. In all Caucasian control panels, risk allele frequency of rs35705950 is approximately 10%. However, in African-Yoruban, African-American, and Asian populations, risk allele frequencies vary from 3 to 0% (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=35705950). This suggests that either the SNP in Caucasians is in linkage with a different, not yet identified, genetic variant, or that IPF is heterogeneous and Caucasian patients carrying the risk allele suffer from a specific type of disease.

Airway Involvement

MUC5B is not expressed by Type 2 AEC, but shows preferential expression in normal distal airway epithelium.⁸² In general, raised expression of gel-forming mucins are a major pathological feature of chronic airway diseases. Proinflammatory cytokines interleukin-1 beta (IL-1 β) and IL-17A are potent inducers of *MUC5B* mRNA expression in human bronchial cells. *MUC5B* induction by IL-1 β was both time and dose-dependent.⁸³ *MUC5B* protein was found in IPF lesions, and IPF patients had significantly increased expression of *MUC5B* in the lungs compared with controls.⁷³ Several exogenous, including cigarette smoke, and endogenous factors have been shown to increase *MUC5B* expression or decrease clearance in the lung.⁸⁴⁻⁸⁶ Many hypotheses have been generated regarding the mode of action of *MUC5B* in IPF, including degradation of mucosal host defense and interference with alveolar repair, but all need further investigation.⁷³ Studies in *MUC5B* deficient mice show that mucociliary clearance is dependent on *MUC5B*.⁸⁷ Absence of *MUC5B* caused accumulation of apoptotic macrophages, impaired phagocytosis, and chronic infection. Mouse studies investigating *MUC5B* dose-dependent reactions in relation

to fibrotic disease and predisposing factors are still lacking.

Genome-wide Association (GWA) Studies

Two GWA studies were published last year. Comparison of fibrotic IIP patients with controls confirmed associations with common genetic variants in *TERT*, *TERC*, and *MUC5B*, but also identified seven new loci, harbouring genes involved in host defense, cell-cell adhesion, and DNA repair.⁸⁸ The product of one of these genes, desmoplakin, an adhesive intercellular molecule that tightly links adjacent cells, was more highly expressed in cases compared to controls, and relative expression increased with the number of copies of the risk allele.⁸⁸ The other GWA study was conducted with IPF patients. It confirmed the *MUC5B* association and discovered new associations with toll interacting protein (*TOLLIP*) and signal peptide peptidase like 2C (*SPPL2C*).⁸⁹ *TOLLIP* encodes a protein that interacts with toll-like receptors (TLRs), regulates inflammatory signalling, IL-1 receptor trafficking, and TGF- β 1

signalling.⁸⁹ Furthermore, minor protective alleles in *TOLLIP* is associated with increased mortality and reduced expression in IPF.⁸⁹

TOLLIP is located at chromosome 11p15.5, right next to *MUC5B*. The GWAS study with IIP patients also identified *TOLLIP* SNPs, but the effect disappeared after correction for the effect of *MUC5B*.⁸⁸ In the IPF-study however, analysis was pursued because linkage disequilibrium between *TOLLIP* and *MUC5B* SNPs was found to be very low, $r^2 < 0.16$.⁸⁹ Linkage disequilibrium describes the non-random association of genetic markers, based on the frequencies of the marker alleles. It is often represented as the correlation coefficient r^2 between markers. Another measurement for linkage disequilibrium, D' , provides information about the recombination breakpoints of chromosomes. SNPs with low r^2 values can reside in a linkage disequilibrium block with a high level of D' between markers. In such a case disease associations are not independent.⁹⁰ Further studies are therefore needed to fully understand the contribution of this region to disease.

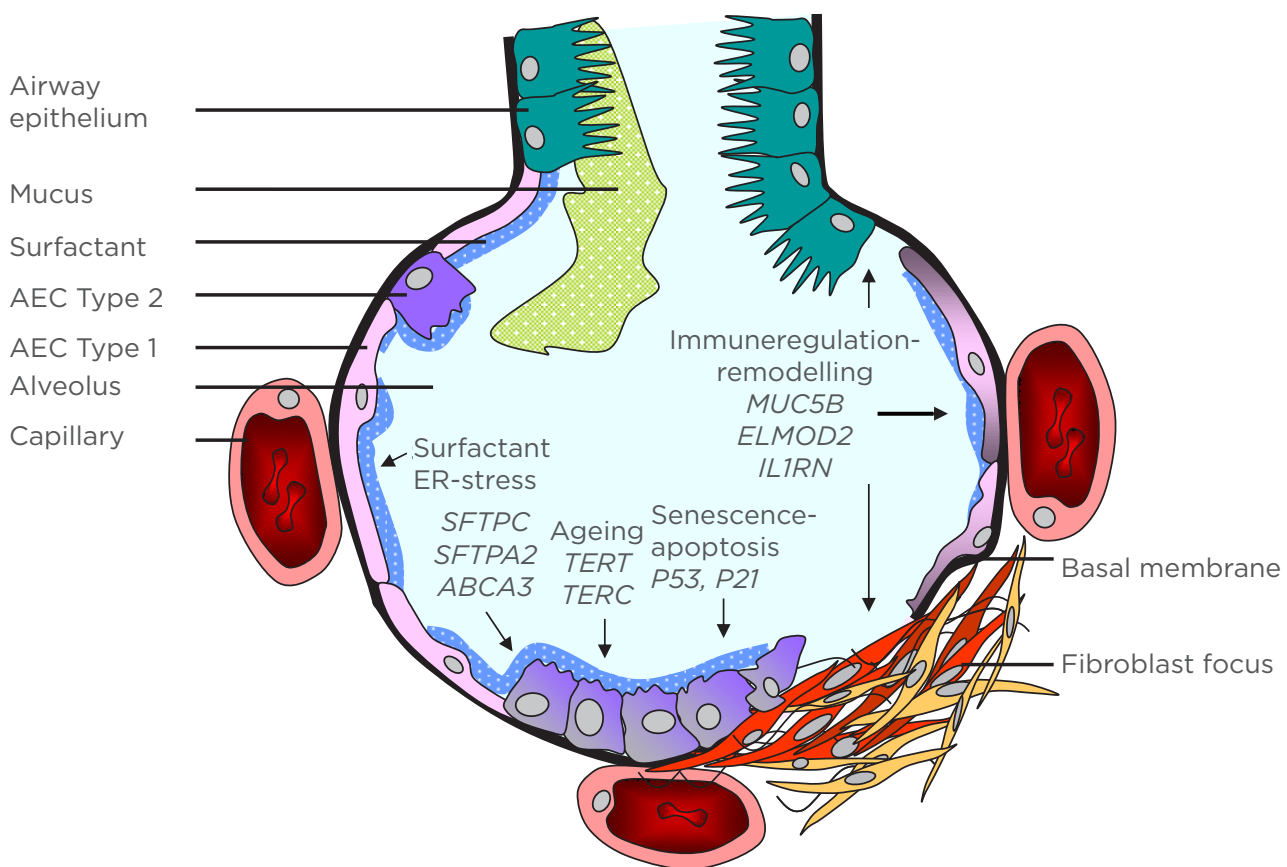


Figure 1: Genes involved in the pathogenesis of pulmonary fibrosis in idiopathic interstitial pneumonia. AEC: alveolar epithelial cell; ER: endoplasmic reticulum.

REVISITING OLD HYPOTHESES

Over a decade ago, pathogenesis of PF was thought to result from a chronic inflammatory process. Polymorphisms in cytokines were therefore among the first to be studied. Most study populations were small and heterogeneous, and results were either negative or depended on a single cohort.⁹¹ An exception was interleukin-1 receptor antagonist (*IL1RN*), the gene encoding the protein, interleukin-1 receptor antagonist (IL-1Ra). A recent meta-analysis of five independent cohorts revealed that carriage of an *IL1RN* haplotype was associated with IPF with an OR of 1.6.⁹² A risk allele influenced expression of *IL1RN* mRNA in healthy carriers and associated with IL-1Ra to IL-1 β ratio in bronchoalveolar lavage fluid in IPF.⁹³ Reduction of the IL-1Ra to IL-1 β ratio causes a profibrotic environment, while the addition of IL-1Ra can prevent fibrosis in mice with BIF.⁹⁴

Although drugs that directly interfere with IL-1 are currently used in inflammatory diseases, they have never been put to trial in IPF. Recently, there has been a flood of publications on genetic variation in immune regulatory genes, including *TGFB1* and TLRs⁹⁵⁻⁹⁹ (and many others). Many of these studies present positive results regarding genetic

predisposition to IPF or IPF disease characteristics, although most with relatively small ORs. In future, subsequent meta-analyses of these studies should provide more insight into the importance of these variations.

CONCLUSION

Genetic variations associated with IIP point towards an essential role for Type 2 AEC function and highlight that negative changes in its functional capacity have detrimental effects on alveolar integrity (Figure 1). On the other hand, *MUC5B* associations point towards the involvement of airway epithelium and mucus production in Caucasian IPF patients. Furthermore, both old and new SNP studies highlight the growing importance of immune regulatory genes. Further investigation is required in order to determine if these phenomena, Type 2 AEC dysfunction, altered mucin production, and immunoregulation act together or separately in fibrotic IIP. While so far none of the genes involved point towards the fibroblast, the genes involved in familial disease have definite consequences for Type 2 AEC, and tell us that therapies aimed at rescuing Type 2 cell function hold promise for an effective cure in, at least, a subgroup of IIP patients.

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