

**Genetics of Lung Fibrosis:
New Genes, New Mutations, Same Pathway**

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Internal Medicine Grand Rounds

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Biographical Information

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Purpose and Overview

To provide a relevant update of the genetics of interstitial lung disease

Educational Objectives

- Understand the current epidemiology, clinical features and FDA-approved treatments for idiopathic pulmonary fibrosis (IPF)
- Recognize the difference in effect size and frequency of rare and common variants associated with lung fibrosis
- Appreciate that rare mutations in six different genes are associated with familial interstitial pneumonia and a common pathway of telomere shortening
- Understand that telomere length may be a biomarker relevant to the underlying pathogenesis and survival characteristics of IPF

Introduction: Case Presentation

Mrs. M is a 68 year old white female who was referred by her pulmonologist from the University of Texas Health Science Center at Tyler in May of 2011 for a second opinion regarding her interstitial lung disease. She has had a cough and gradual worsening of shortness of breath with exertion over the last year. She partly attributes the cough productive of white sputum to existing sinus disease and allergic rhinitis. The dyspnea has been progressively worsening. Back in 2010 she notes that she was able to walk up two flights of stairs at church, but now she is unable to walk up these stairs without stopping. She denies any chest pain, recent respiratory infections, arthralgias, sicca symptoms, difficulty swallowing, muscle pains or skin rashes.

She has a family history of interstitial lung disease as both her father and uncle died from pulmonary fibrosis. Because of this, her primary care physician has been getting yearly chest x-rays. She says that there was first evidence of pulmonary fibrosis in 2006, five years ago. However, she was not symptomatic at that time. In 2010, the chest x-ray demonstrated worsening and so she was referred to Dr. Varsha Taskar at UTHSC Tyler. Dr. Taskar had started her on Imuran and N-acetyl cysteine. She has not taken any prednisone and no surgical lung biopsy or bronchoscopy has been performed.

Her past medical history is notable for treated hypertension, hypothyroidism, GERD, osteopenia, depression and a history of Graves' disease. She is a never smoker and denies drinking alcoholic beverages. She has been married for 49 years and has two sons, both without any medical problems. She is currently retired, but she previously worked raising ~250,000 chickens per year. The poultry farming was initially her father's business for about 25 years before he sold it in 1995. She denies any other fibrogenic exposures.

Her physical exam is normal except for mild bilateral proptosis, mild L eye esotropia and loud, dry inspiratory crackles over the posterior lung bases. Otherwise, she has a normal exam and a resting oxygen saturation of 95%.

Data collected prior to the evaluation included pulmonary function tests which were consistent with moderately severe restriction, no evidence of a bronchodilator response, and a severely decreased diffusion capacity. In comparison with testing from January 2011, there appears to be a drop in significant drop in lung volumes (FVC fall of 250 cc) and the diffusion capacity over the past 5 months.

Her high resolution chest computed tomography scan (**Figure 1**) was categorized as being Indeterminate for a usual interstitial pattern (UIP) as per diagnostic radiographic categories¹. Her

hemogram was notable for mild anemia (Hemoglobin of 11 g/dL, Hematocrit 32.6%) with a normal mean corpuscular volume. Her antinuclear antibodies were measured at a titer of 1:80 in a speckled pattern. All other autoantibodies (RF, CCP, ENA panel) were negative.

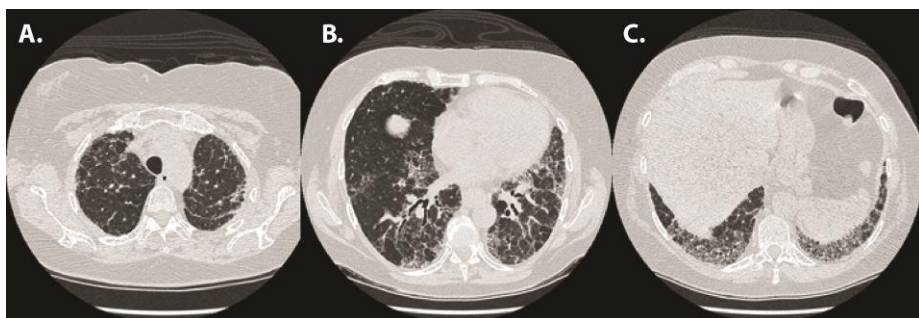


Figure 1. High resolution CT chest scan for patient. Axial images of 1 mm thickness seen at the level of the apices (A), the heart (B) and the bases (C). The tomography scan demonstrates an axial distribution of reticulations with associated ground glass opacities, with more involvement of the lower lobes than the upper lobes. There is not a very striking zonal distribution, as the degree of peripheral involvement is very similar to that seen in the central regions of the lung. There is traction bronchiectasis but evidence of honeycombing. One of the UTSW chest radiologists has considered this scan (Figure 1) to be indeterminate for a usual interstitial pattern (UIP) pattern.

A clinical diagnosis of probable idiopathic pulmonary fibrosis was made. However, this diagnosis could not be definitively classified as such in the absence of a surgical lung biopsy given the indeterminate radiographic diagnosis. She was advised to stop taking the Imuran and N-acetylcysteine.

This case brings up a number of pertinent questions:

1. What is the expected prognosis?
2. What is the best treatment?
3. What is the cause of her interstitial lung disease?

Interstitial Lung Disease (ILD)

The ILDs, also called the diffuse parenchymal lung diseases, are a heterogeneous collection of >100 different non-neoplastic, non-infectious chronic lung diseases with varying patterns of inflammation and fibrosis and similar clinical, radiographic and physiologic features^{2,3}. The interstitium is the space between the epithelial cells lining the alveoli and the endothelial basement membrane; it is thought to be one of the primary sites of injury. However, these disorders frequently affect many of the structures of the distal airways, including the alveoli, the blood vessels and the small peripheral airways (alveolar ducts, respiratory bronchioles and terminal bronchioles). Normally, the delicate interstitium contains very few numbers of resident macrophages and fibroblasts. After injury, there can be leakage of serum proteins across the alveolar capillary basement membrane, recruitment of inflammatory cells, fibroblasts and myofibroblasts into the interstitium and deposition of extracellular matrix proteins, including collagen, elastin, fibronectin and laminin. All of these changes have profound effects on the alveolar-capillary interface, resulting in impaired gas exchange.

The clinical classification of the diffuse parenchymal lung diseases are listed in **Table 1**. In broad terms, the sub-classifications consist of disorders of known causes (connective tissue diseases, drug related causes, primary systemic disorders and environmental causes) and disorders of unknown cause.

Table 1. Clinical Classification of the Diffuse Parenchymal Lung Diseases (abbreviated)

<u>Related to Known Causes:</u>		<u>Unknown Causes:</u>
Connective Tissue Disease-ILD	Primary Systemic Disorders	Major Idiopathic Interstitial Pneumonias
Scleroderma	Langerhans' cell histiocytosis	Idiopathic pulmonary fibrosis (IPF)
Rheumatoid arthritis	Gaucher's disease	Idiopathic nonspecific interstitial pneumonitis (NSIP)
Antisynthetase syndromes	Niemann-Pick disease	Respiratory bronchiolitis interstitial pneumonitis (RB-ILD)
Systemic lupus erythematosus	Hermansky-Pudlak syndrome	Desquamative interstitial pneumonia (DIP)
Mixed connective disease	Neurofibromatosis	Cryptogenic Organizing pneumonia (COP)
Pulmonary vasculitis	Tuberous sclerosis/LAM	Acute interstitial pneumonia (AIP)
Drug-Induced or Therapy-Related ILDs	Occupational/Environmental ILDs	Rare Idiopathic Interstitial Pneumonias:
Antiarrhythmics (amiodarone)	Asbestosis	Idiopathic lymphoid interstitial pneumonia
Antibiotics (nitrofurantoin, sulfasalazine)	Silicosis	Idiopathic pleuroparenchymal fibroelastosis
Chemotherapeutics	Berylliosis	Unclassifiable idiopathic interstitial pneumonia
Anti-inflammatory (gold, penicillamine)	Hard metal pneumoconiosis	
Anticonvulsants (Dilantin)	Bird fancier's lung disease	Characterized by Granulomas:
Therapeutic radiation	Farmer's lung disease	Sarcoidosis

Sources: Murray and Nadel, Textbook of Respiratory Medicine², ATS/ERS Consensus Classification AJRCCM 2002³ and Travis et al AJRCCM 2013⁴

The idiopathic interstitial pneumonias are a subset of the ILDs of unknown etiology whose classification has evolved over recent years. In the past, these disorders were based solely upon "gold standard" lung histopathology. But since 2002, these diagnoses have been made by considering the clinical features in the context of radiographic and/or pathologic patterns. A multidisciplinary clinical-radiologic-pathologic approach is used for cases that do not neatly fit one particular diagnosis³. A recent update to the classification of the idiopathic interstitial pneumonias was made in 2013⁴. The terminology used to describe the different disorders has been confusing, a veritable "alphabet soup" of abbreviations.

It is important to remember that the idiopathic interstitial pneumonias are diagnosed after excluding the diffuse parenchymal lung disease of known cause. Some patients are commonly misclassified as having an idiopathic interstitial pneumonia because of inadequate information. For this reason, the diagnostic process is dynamic and the diagnosis may need to be revised as more details of history are obtained, after results of biopsies (where appropriate) become available, or as the radiographic pattern evolves.

Idiopathic pulmonary fibrosis (IPF)

Idiopathic pulmonary fibrosis is the most common of all the different idiopathic interstitial pneumonias and the prototypic ILD. It is a distinct type of chronic fibrosing interstitial pneumonia of unknown cause limited to the lungs and associated with a surgical lung biopsy and/or a radiographic pattern of usual interstitial pneumonia (UIP)^{5,6}. Its diagnosis requires exclusion of other known causes of ILDs and abnormal pulmonary function studies showing restriction and/or impaired gas exchange. Definitive histologic diagnosis requires a surgical lung biopsy showing a UIP pattern. If a biopsy is obtained, sampling from more than one lobe of the lung and from areas of varying severity improves diagnostic accuracy. In some cases of severe end-stage lung disease (diffusion capacity of carbon monoxide (DLco) <35% predicted), a surgical lung biopsy cannot be obtained safely given the significant mortality and morbidity associated with this procedure^{7,8}. The diagnosis of IPF in the absence of a surgical lung biopsy requires a high resolution chest computed tomography (HRCT) scan with a UIP pattern or a possible UIP pattern and multidisciplinary discussion.

IPF is more common in Whites. In a large study on survival of IPF patients listed for lung transplantation (n=2635), 82% were White, 11% were Black and 7% were Hispanic⁹. More men are affected than women, and the majority of patients have a history of cigarette smoking. There are no large-scale studies of the incidence and prevalence of IPF on which to base formal estimates. However, there is a striking increased incidence with older age, with presentation typically occurring in the sixth or later decades. Patients with IPF less than 50 years of age are rare; for such patients, there should be a careful search for an alternate diagnosis. Disease incidence is estimated to range from 6.8 - 16.3 per 100,000 per year^{10,11}. In individuals older than 75 years of age, the prevalence may approach 150 per 100,000, or 1.5%, of this age group.

While IPF is, by definition, a disease of unknown etiology, a number of potential risk factors have been described⁶. Chief among these is cigarette smoking, especially smoking >20 pack-years. An increased risk for IPF has been associated with a variety of environmental factors. These include exposure to metal dust, wood dust, stone cutting, farming, birds, livestock, hair dressing and various vegetable and animal proteins. Several studies have noted an association between abnormal acid gastroesophageal reflux and presumed microaspiration. For these reasons, most IPF patients are advised to minimize all potential "fibrogenic" exposures and treat symptomatic GERD.

The natural history of IPF is generally characterized by progressive decline in pulmonary function until death from respiratory failure^{12,13} (which occurs in ~60% of cases) or an accompanying comorbidity, such as coronary artery disease, pulmonary embolism or lung cancer. Data from the placebo arms of a number of clinical trials have shown that the mean rate of decline in the forced vital capacity (FVC) is approximately 150 to 200 ml/year¹⁴. Although a general decline is seen across cohorts of IPF patients, the natural history for each individual patient is unpredictable and highly variable. There may be periods of a slow, gradual progression over many years which are punctuated by acute exacerbations of more rapid decline. Predictors of increased mortality include advanced age, reduced pulmonary function test FVC and diffusion capacity for carbon monoxide (DLco) measurements, oxygen desaturation on the 6-minute walk test, extent of fibrosis assessed radiographically, and specifically, honeycombing on the

HRCT, and presence of pulmonary hypertension. Overall, the morality burden attributable to IPF is higher than that of many different types of cancer.

Pharmacologic Therapies

Over the last 15 years, a large number of clinical trials have tested the use of therapies that had previously been considered by some clinicians to be efficacious based upon small observational studies or uncontrolled retrospective studies. These pharmacologic therapies include corticosteroids, immunomodulators (azathioprine, cyclophosphamide, cyclosporine A, etanercept), antioxidants (N-acetylcysteine), antifibrotics (colchicine, interferon gamma), anticoagulants (warfarin), a tyrosine kinase inhibitor (imatinib) and medications already approved for the treatment of pulmonary hypertension (sildenafil, bosentan). Please refer to the 2011 review of the clinical trials evaluating each pharmacologic agent and providing an overall quality of evidence rating for each study⁶. Only one of these negative clinical trials will be addressed in this presentation. Since surgical biopsies of IPF lungs demonstrate variable amounts of inflammatory cell infiltrates, it had been assumed that anti-inflammatory medication may assuage disease progression. The PANTHER trial evaluated the efficacy of so called “triple” therapy (a combination of prednisone and azathioprine in conjunction with the antioxidant N-acetylcysteine). After ~50% of the data was collected, an interim analysis showed an 8-fold increased rate of death ($p = 0.01$) and a 3-fold increased risk of hospitalization ($p < 0.001$). The data and safety monitoring board recommended early termination of the treatment group.

As of October 2014, there are now two FDA-approved pharmacological therapies for IPF. Neither medication has been found to cure IPF, but they do appear to slow disease progression for those with early disease. Pirfenidone is an antifibrotic agent whose mechanism of action involves inhibition of transforming growth factor beta (TGF- β)-stimulated collagen synthesis, fibroblast proliferation and deposition of extracellular matrix. Three different large clinical trials (CAPACITY 004, CAPACITY 006, ASCEND) demonstrated a benefit of slowing disease progression in patients with mild-to-moderate disease (defined as patients having a FVC $>50\%$ and DLco $>35\%$). There was a possible mortality benefit in the pooled analysis. The second agent is nintedanib, a receptor blocker for multiple tyrosine kinases that mediate cell signaling of fibrogenic growth factors (PDGF, VEGF, and FGF). It showed a reduction in the rate of decline in lung function in one phase 2 trial (BIBF 1120) and two phase 3 trials (INPULSIS-1 and INPULSIS-2). As with the pirfenidone clinical trials, this medication has only been studied in IPF patients with mild to moderate disease.

Choice between these two medications is generally guided by availability, patient preference and a consideration of potential adverse effects. Elevated liver function test abnormalities and diarrhea are the most common adverse effects associated with nintedanib vs. nausea, dyspepsia and photosensitivity for pirfenidone. They are not currently recommended for patients with an ILD diagnosis other than IPF. It is not currently known if these medications are effective for IPF patients with severe disease.

Lung Transplantation

Lung transplantation is the only treatment that offers a cure for patients with IPF. Lungs are allocated based upon age, geography, blood compatibility and the Lung Allocation Score (LAS), which reflects risk of mortality while on the wait-list and probability of survival. Since the implementation of the LAS system in May 2005, candidates on the lung transplant wait-list are more likely to be older, to have a diagnosis of a fibrosing restrictive lung disease (Group D), and to be sicker¹⁵. The proportion of Group D patients continues to rise every year and was at an all-time high in 2012, representing 49.5% of all patients. A recent study has shown a survival benefit of double-lung transplantation in patients with IPF than single-

lung transplantation (adjusted median survival of 65.2 months (IQR 21.4-91.3) vs. 50.4 months (17.0-87.5), $p < 0.001$)¹⁶.

While overall donation rates have increased over the last decade, there are still far fewer donors to meet the needs of the wait list. Overall, donors age 15 to 34 years of age, the age group with the highest donation rate for lung transplant, provided 12.2 donations per 1,000 deaths¹⁵. Lungs from donors aged 55 years and older were rarely used, with donor rates of 0.5 and 0.04 for donors 55-64 years and 65-74 years, respectively¹⁵. Compared with recipients of other solid organs, lung transplant recipients experienced the highest rates of re-hospitalization with 43.7 per 100 patients in year one and 36.0 per 100 patients in year two. Complications post-transplant are common and mostly result from the long-term use of immunosuppressive medications. Complications include drug-related hypertension (66.7%), drug-related hyperlipidemia (53.8%), renal dysfunction (49.8%), diabetes (42.5%), and malignancy (18.3%)¹⁵. Infection, not graft failure, is the most likely cause of death for all time points following the first month after transplantation. The National Foundation for Transplants estimates that the average patient care costs for a double lung transplant is \$800,000 during the first year alone¹⁷.

Despite the successes of transplantation, the limitation of donor lungs, complications post-transplant and the economic burden associated with this procedure does not allow for its widespread use for all patients with IPF. Thus, more effective medications that prevent its occurrence or slow its progression are desperately needed. Over the last ten years, I have used a genetic approach to determine the underpinnings of the disease with the hope that this information will inform rationale therapeutics based on underlying disease mechanism. Precedence for the success of therapies based upon the underlying genetic cause of lung disease include: (1) replacement therapy for alpha-1-antitrypsin deficiency associated emphysema^{18,19} and (2) ivacaftor (VX-770) for treatment of cystic fibrosis patients with the G551D CFTR mutation²⁰.

Genetics of Lung Fibrosis: Genetic-Allelic Spectrum

Genetic variants are distinguished by their overall frequency and their overall effect size. In general, common variants have low effect sizes and rare variants have larger effect sizes

Single nucleotide polymorphisms (SNPs) are common variants which are defined as variants that are found in the population at an allele frequency of at least 0.01 or 1%. Rare variants are much less common and can be so rare that they have never been found in any other individual. "Ultra" rare variants not found in existing genetic databases are also called novel variants or singletons. An example of a common variant with clinical significance is the Factor V Leiden polymorphism (R506Q, rs6025), which gives rise to an increased risk of thrombosis. This allele has an allele frequency of 0.022 (2.2%) across a sample size of >120,000 alleles²¹. In contrast, the CFTR G551D mutation (rs75527207), mentioned above, is rare, with an allele frequency of <0.001 across a large population of over 120,000 alleles²¹.

Common Variants

Using a genome-wide screen, a common variant located 3 kb upstream of the MUC5B transcription start site (rs35705950) was found to be present at a higher frequency in IPF patients. This variant is found in ~9% of controls (an overall allele frequency of 0.10) and 38% of patients with IPF, $p = 2.5 \times 10^{-37}$. The odds ratio for IPF disease among subjects who are heterozygous or homozygous for the minor allele of this SNP were 9.0 (CI 6.2-13.1) and 21.8 (5.1-93.5), respectively²². The variant allele was associated with up regulation of MUC5B expression (up to 37.4 times as high as compared with those homozygous for the wild type allele) in lung tissue of unaffected subjects²².

A genome wide association study (GWAS) was performed comparing the frequency of common SNPs in patients with fibrotic idiopathic interstitial pneumonia (n=1616) with controls (n=4,683). A replication analysis included 876 cases and 1,890 controls. A MUC5B promoter variant (rs868903) was found to be significantly associated with fibrotic lung disease, with a meta-analysis P value of 9.2×10^{-26} .²³ There was a broad region on 11p15 including the *MUC5B*, *MUC2* and *TOLLIP* genes, that were found to demonstrate genome-wide significance. After adjusting for the MUC5B promoter SNP found in the earlier study (rs35705950)²², most of the variants in this broad region were no longer significantly associated, suggesting that the associations seen for these other SNPs were due to weak linkage-disequilibrium. The GWAS study confirmed the association of IPF with variants in the *TERT*²⁴ and *TERC* genes, with meta-analysis p values of 1.7×10^{-19} and 4.5×10^{-8} , respectively. Overall, common variants in three telomere-related genes (*TERT*, *TERC*, and *OBFC1*)²⁵⁻²⁷ were found to be associated with lung fibrosis in this study. The GWAS study also found seven new loci located near genes involved with host defense, cell-cell adhesion and DNA repair.

The MUC5B promoter SNP is present in 5-10% of the general population. However, as discussed above, IPF is not a common disease as it affects 6.8 - 16.3 per 100,000 individuals per year. Many more individuals have subtle reticulations found on high resolution chest CT scans (termed interstitial lung abnormalities, ILAs) than are diagnosed with IPF. The clinical significance of these ILAs is currently unknown. The MUC5B promoter (rs35705950) has been found to be associated with ILAs found in the Framingham Heart Study²⁸, thus linking it to an early manifestation of IPF. Once patients develop clinical disease, the presence of this SNP appears to have a protective effect. Individuals who are heterozygous or homozygous for the MUC5B SNP risk allele appear to have a better survival among IPF patients enrolled in the interferon-gamma 1b trial²⁹.

Rare Mutations found in Rare Kindreds

A number of rare variants in a handful of genes have also been linked to pulmonary fibrosis. All of these have been discovered by sequencing the coding regions of the genes in kindreds with familial pulmonary fibrosis, as opposed to genotyping genetic variants across diseased cohorts or populations. Each of these variants is very rare and is generally only found in the family in which it was discovered. They are so rare that they cannot be found in population-based databases, such as dbSNP or the 1000 Genome project. However, when other similarly-affected individuals are analyzed in the family, the rare variant is found to co-segregate with disease. Based upon the penetrance of rare *TERT* mutations in kindreds, the odds ratio has been estimated to be ~1000.

Kindreds with familial pulmonary fibrosis (FPF) include those with 2 or more cases of an idiopathic interstitial pneumonia or unclassified pulmonary fibrosis. In nearly all cases the disease segregates with an autosomal dominant pattern of inheritance with incomplete penetrance³⁰⁻³³. The first population-based study of FPF in the United Kingdom suggested that 0.5-2.2% of IPF is genetic, with a prevalence of 1.3 cases per million³³. In cohorts of IPF patients with early-onset disease, such as those referred for lung transplantation, there is a higher prevalence of familial disease, up to 19%^{30,34}.

As of 2014, genetic mutations in four different genes had been linked to adult-onset FPF. These genes include two encoding surfactant proteins (*SFTPC*, *SFTPA2*) and two encoding telomerase (*TERT*, *TERC*)^{35,36}. Please refer to the referenced review detailing the history of the discovery of these mutations in FPF kindreds and their underlying mechanism of disease³⁷. Briefly, the expression of the surfactant proteins is largely restricted to the lung alveolar epithelial cells. Rare mutations in the genes encoding either surfactant protein C or surfactant protein A2 cause increased endoplasmic reticulum (ER) stress and lung fibrosis³⁸⁻⁴⁰.

Rare mutations in the genes encoding telomerase (*TERT* and *TERC*) in FPF kindreds were discovered in 2007 by our group and by the laboratory of Dr. Mary Armanios^{35,36}. All telomerase mutations are individually ultra-rare and are not found in the general population. However, collectively, *TERT* mutations are the most common genetic defect found in FPF as they are found in ~15% of all FPF kindreds and 2-3% of patients with sporadic IPF, or those with no known family history of disease³⁷. The mutations segregate with pulmonary fibrosis in the FPF kindreds and all lead to a reduction of *in vitro* telomerase activity^{35,36,41}. Patients and at-risk family members carrying rare heterozygous *TERT* mutations have telomere lengths that are shorter than age-matched controls and demonstrate progressive telomere shortening over successive generations⁴².

Telomerase (**Figure 2**) is a ribonucleoprotein enzyme composed of a protein component (*TERT*)⁴³ and a RNA component (hTR) (*TERC*)⁴⁴ that catalyzes the addition of hexameric G-rich nucleotide repeats to the ends of linear chromosomes^{45,46}. Mutations in the telomerase genes are found in patients with dyskeratosis congenita (DC). This is the most severe presentation of all genetic telomere diseases, with high penetrance, multi-organ dysfunction and a clinical manifestation during childhood. DC families with mutations in *TERT* or *TERC* display genetic anticipation, with a worsening of disease severity and an earlier onset of symptoms with successive generations correlating with progressive telomere shortening^{43,47}. All DC patients have short telomeres, regardless of specific genetic mutation, with age-adjusted telomere lengths <1st percentile.

Pulmonary Fibrosis due to Telomerase Mutations

To more clearly define the *TERT* specific ILD phenotype, we characterized 134 *TERT* mutation carriers ranging from 5 to 88 years of age from 21 unrelated families, including 53 individuals with pulmonary fibrosis⁴². We found that the development of lung fibrosis in *TERT*-mutation carriers was age-dependent and associated with environmental exposures. By age 60, 50% of female *TERT* mutation carriers and 60% of male mutation carriers have a self-reported diagnosis of pulmonary fibrosis. The penetrance of lung fibrosis was related to the subject's personal history of smoking [OR 4.0 (1.2-14.5), P-value=0.02] and/or other fibrogenic exposures [OR 13.6 (1.7-636.8), P-value=0.005]. We found that the mean life expectancy of *TERT* mutation carriers with a diagnosis of lung fibrosis was 3 years, regardless of the specific ILD diagnosis. Thus, the clinical outcome of *TERT* mutation-associated pulmonary fibrosis mirrors the clinical course of IPF.

Although most ILD patients with *TERT* mutations do not have the full spectrum of phenotypes found in DC patients, they can exhibit mild anemia⁴² or macrocytosis^{48,49}. It was initially reported that these patients can have severe hematologic complications after lung transplantation, leading to death or requiring multiple transfusions of blood products⁵⁰⁻⁵². Seven *TERT* mutation carriers with end stage ILD have been transplanted at UTSW. Together with cases collected by the lung transplant center UCSF, we described the post-lung transplantation complications for a cohort of a total of 14 subjects⁵³. The median post-lung transplantation observation time was 3.2 years, with only one death observed over this time period. None of the patients were found to be transfusion-dependent, although the degree of leukopenia post-transplant prompted cessation of mycophenolate mofetil in five patients. Similarly, another lung transplantation center has reported that about one-third of patients with end stage ILD and short telomere lengths who were prospectively evaluated by bone marrow biopsy had an occult hematologic disorder that affected their candidacy for transplantation⁵⁴. Thus, hematologic phenotypes seem to be the most

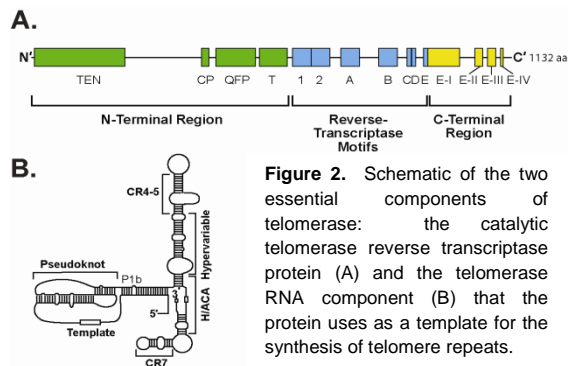


Figure 2. Schematic of the two essential components of telomerase: the catalytic telomerase reverse transcriptase protein (A) and the telomerase RNA component (B) that the protein uses as a template for the synthesis of telomere repeats.

clinically-relevant extrapulmonary manifestations of telomerase mutations carriers, often affecting a patient's candidacy for lung transplantation or leading to adjustment of immunosuppressant medications.

We recruited 20 *TERT*-mutations carriers and 20 family member controls to participate in a clinical observational study to determine if the manifestations of lung fibrosis occur before or concurrently with clinical diagnosis⁴⁹. We found that *TERT* mutation carriers exhibit preclinical signs of lung fibrosis. The preclinical pulmonary phenotypes include: reduced diffusion capacity of lung for carbon monoxide (DL_{CO}), reduced recruitment of DL_{CO} with exercise, increased subjective scores of lung peripheral reticulations on high resolution computed tomography (HRCT) scans of the chest and increased fractional tissue volume quantitative scores of the HRCT scans (**Figure 3**). The non-pulmonary phenotypes of *TERT*-mutation carriers include significantly lower RBC counts (but not hemoglobin or hematocrit levels), higher mean corpuscular volumes (MCV) and increased graying of hair.

New Genes and New Mutations in Familial Pulmonary Fibrosis

Overall, 40% kindreds with familial pulmonary fibrosis have short telomere lengths and only about half are explained by mutations in the *TERT* or *TERC* genes⁴¹. Therefore, we hypothesized a role for other genes involved with telomere maintenance. In 2010 we used a candidate gene approach to sequence the coding exons of 15 genes that had been linked to human diseases or the pathway of telomere shortening. We did not find any mutations in these genes by using this candidate gene approach.

Recently, we collaborated with Dr. Rick Lifton and the Yale Center for Genome Analysis to take an unbiased approach of sequencing the full exome (the coding sequences of ~21,000 genes) of 99 probands from FPF kindreds of unknown genetic cause⁵⁵. In these studies we used principal component analysis of the genotypes to demonstrate that 79 of the 99 probands clustered with HapMap subjects of European ancestry. The burden of variants found per gene in these 79 probands was compared to the number of variants found in 2,816 ethnicity-matched controls sequenced on the same platform.

Given that the population based frequency of FPF is ~1 per 1,000,000, we assumed that genetic mutations of large effect, like the ones previously found in the surfactant and telomerase genes, would be rare. Therefore, we limited the analysis to variants that were not found in the NIH dbSNP database, the 1000 Genomes project database or the NHLBI Exome Sequencing project. We found that there were 49.0 new variants per case and 50.3 new variants per control subject. We also assumed that dominant alleles of large effect would probably alter the function of the encoded protein and would include damaging variants (defined as premature termination, frameshift and splice site alleles) or highly conserved missense variants (defined as those found in at least 46 of 47 orthologs). We found that two genes surpassed the threshold for genome-wide significance (p -value $< 2.4 \times 10^{-6}$ accounting for the examination of 21,000 genes) when comparing the number of observed vs. expected novel mutations in cases and controls.

We found 5 new heterozygous variants in *RTEL1* in FPF probands (2 damaging variants and 3 conserved missense variants, **Figure 4**), whereas we observed 4 singletons among 2,816 controls ($p = 1.6 \times 10^{-6}$). The *RTEL1* gene encoding the regulator of telomere elongation helicase 1 has a known role in telomere

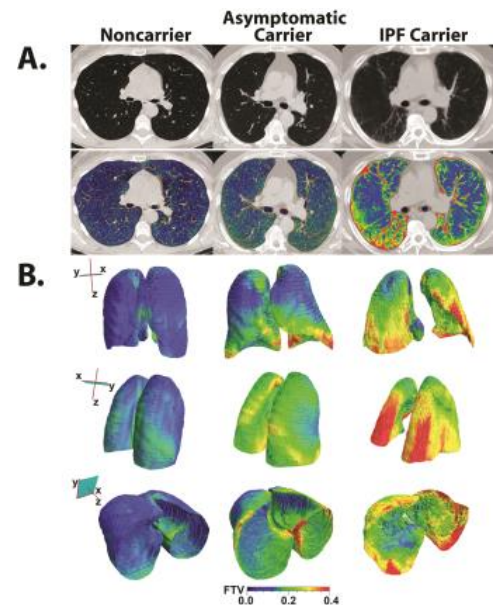
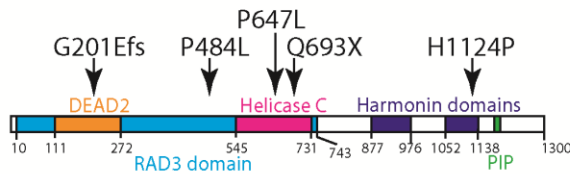


Figure 3. HRCT Analysis of a family member control (left), an asymptomatic *TERT* carrier (center) and an IPF patient with a rare *TERT* mutation (right).

maintenance. Mutations in this gene were recently shown to cause Hoyeraal-Hreidarsson syndrome, a severe variant of dyskeratosis congenita⁵⁶⁻⁵⁹. Affected individuals present in childhood, have very short telomere lengths, and generally

RTEL1 Heterozygous Mutations



PARN Heterozygous Mutations

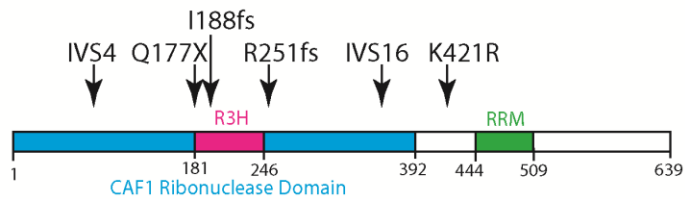


Figure 4. Schematic of the functional domains of the RTEL1 and PARN proteins with the positions of the mutations indicated by arrows. Conserved protein domains are indicated by colored bars.

exoribonuclease that had not been previously linked to any disease or the pathway of telomere maintenance. A new conserved missense variant (K421R) was also discovered in one proband.

It was curious that two of the probands had the identical loss of function *PARN* splice acceptor mutation. Both families lived in East Texas within 85 miles of each other and neither had any knowledge of the existence of the other family. One of the probands (Mrs. M) is the subject of the clinical case presented at the beginning of this protocol. We compared the family tree of Mrs. M with genealogic information for the other proband posted online and found that they shared the same great grandmother. Similarly, their kinship was confirmed by analyses that demonstrated that these two probands share ~6.4% of their overall genome. The *PARN* mutation is located on a genomic segment that is shared identical by descent between the two probands. As a further test for the relevance of the *PARN* mutations, we compared their co-segregation to pulmonary fibrosis in extended kindreds. We found that the all the affected individuals had inherited the same *PARN* mutation identified in the index case, an event that is highly unlikely to have occurred by chance. The overall backward LOD score across all informative *PARN* kindreds was 3.6, or an odds ratio of 4,096:1, in favor of linkage of the *PARN* variants in an affected-only analysis.

The telomere lengths of genomic DNA isolated from leukocytes were measured for the *PARN* and *RTEL1* mutation carriers and compared to those with *TERT* and *TERC* mutations, as well as controls. Telomere lengths were measured both by a Southern blot technique (telomere length restriction fragment analysis) and by a multiplexed quantitative PCR assay. By both assays, the mean age-adjusted telomere lengths of the *PARN* and *RTEL1* mutation carriers were significantly shorter than normal controls ($p < 0.001$). Altogether, new damaging and missense *PARN* and *RTEL1* mutations were found in 12% of the FPF probands sequenced. With extrapolation to the entire cohort, *PARN* and *RTEL1* mutations are found in 4% and 3% of kindreds, respectively. Thus, these frequencies are intermediate between those of *TERT* mutations (~15%) and *SFTPC* (2%) or *SFTPA2* (1%) mutations.

In addition to the study described above, two other recent papers have independently linked *RTEL1* mutations to FPF^{60,61}. All three of these studies utilized whole exome sequencing to identify the variants, all found variants that were identified as novel and very rare, and all found that the mutation carriers have

have biallelic mutations. In contrast, those affected with FPF in our study were heterozygous for a novel loss of function or missense mutation. All amino acid residues affected by the mutations are within known protein domains.

We found a total of five novel damaging variants in 79 cases and zero damaging variants in the controls, including 2,816 Yale controls and 4,300 NHLBI controls ($p = 1.33 \times 10^{-8}$ and 1.69×10^{-9} , respectively) **Figure 4.** *PARN* (polyadenylation-specific ribonuclease deadenylation nuclease) encodes a 3'-

short telomere lengths in comparison with age-matched controls. Different *RTEL1* mutations were described with no overlap across studies, underscoring the rarity of the individual mutations linked to this disease in patients from around the world.

Short Telomere Syndromes

The prototype of the short telomere syndromes is dyskeratosis congenita (DC, alternatively known as Zinsser-Cole-Engman syndrome), which was first characterized by the mucocutaneous triad of lacy reticulated skin pigmentation, nail dystrophy and oral leukoplakia⁶². An expanded clinical phenotype was later documented, including bone marrow failure and multi-system involvement⁶³. There is substantial variable expressivity, or a range of signs and symptoms, in individuals with this disease. Clinical diseases that have substantial overlap with DC include Hoyeraal-Hreidarsson syndrome, Revesz syndrome, Coat's retinopathy and cerebroretinal microangiopathy with calcification and cysts. It is now known that DC develops as a result of defective telomere maintenance, so this syndrome is known as a telomeropathy or a telomere biology disorder.

Table 3. Short Telomere Syndromes

	Dyskeratosis congenita (DC)	Aplastic anemia	Pulmonary fibrosis	Liver cirrhosis
Inheritance	XLR, AD, AR	AD, AR	AD	AD
Age of onset	1-30	All ages	>40 years	>40 years
Mucocutaneous sx	Yes	No	No	No
Bone marrow failure	Yes	Yes	Macrocytosis	?
Somatic phenotypes	Yes	Rare	Rare	?
Cancer (AML)	Yes	Yes	?	?
Genetic mutations	DKC1 (XLR) TERT (AD, AR) TERC (AD) TINF2 (AD) NOP10 (AR) NHP2 (AR) TCAB1 (AR) CTC1 (AR) RTEL1 (AD, AR) ACD (AR) PARN (AR)	TERC TERT	TERT TERC RTEL1 PARN DKC1* TINF2*	TERT TERC
Short telomeres	Yes (<1 st percentile)	Yes	Yes	Yes

Abbreviations: XLR, X-linked recessive; AD, autosomal dominant; AR, autosomal recessive; sx, symptoms; bone marrow failure syndromes include aplastic anemia and myelodysplastic syndrome; AML, acute myeloid leukemia; DKC1, Dyskerin; TERT, telomerase; TERC, telomerase RNA component; TINF2, TRF1-interacting nuclear factor 2; NOP10, NOP10 ribonucleoprotein; NHP2, NHP2 ribonucleoprotein; TCAB1, telomere Cajal body-associated protein 1; CTC1, CTS telomere maintenance complex component 1; RTEL1, regulator of telomere elongation helicase 1; ACD, telomere protection protein 1 or adrenocortical dysplasia homolog; PARN, polyadenylation-specific ribonuclease deadenylation nuclease.

*, Described in a single family

Dyskeratosis congenita was first thought to be solely an X-linked recessive disease because early publications described the disease in only males. Mutations in the dyskerin (*DKC1*) gene were identified as the cause of X-linked DC in 1998⁶⁴. Technical advances in genomic sequencing have led to the rapid discovery of new genes. We now know that autosomal dominant and recessive manifestations of the disease also exist and are explained molecularly by mutations in different genes: *TERC*, *TERT*, *NOP10*, *NHP2*, *TINF2*, *TCAB1*, *RTEL1*, *ACD*, and *CTC1*^{56-59,65-67}. To date, about 60-70% of DC patients have an identifiable germline mutation in genes that are responsible for the proper functioning and maintenance of telomeres. Mutations in *PARN* (the 11th gene) were described in a paper published this month⁶⁸.

Compared to DC and other disorders of telomere dysfunction caused by single gene defects, it has been estimated that IPF is the most common manifestation of telomere-related disease⁶⁹. The discovery of mutations in these two genes, *PARN* and *RTEL1*, further strengthens the link between telomere attrition

and lung fibrosis. Case reports of a mutation in the *DKC1* gene and the *TINF2* gene have been reported for single FPF kindreds^{70,71}. Altogether, mutations in six different genes have been found in FPF kindreds with short telomere lengths. Three of the genes, *TERT*, *RTEL1* and *PARN*, demonstrate a gene dosage effect with homozygous or compound heterozygous mutations found in pediatric DC patients and heterozygous mutations in adult ILD patients. All patients with a monogenic form of pulmonary fibrosis (**Table 3**) are now considered to have a telomere biology disorder and are being welcomed by the DC Outreach patient support organization.

Telomere length as a Biomarker

The finding of inherited rare telomerase mutations and short telomere lengths in non-familial, sporadic, IPF patients suggests that reduction in endogenous telomerase activity may be important for the pathogenesis of IPF. Peripheral blood telomere lengths of ~25% sporadic IPF patients are <10th percentile, suggesting that short peripheral blood telomeres are a risk factor for the development of IPF^{41,72}. The association of both rare and common telomerase variants with IPF underscores the importance of telomerase dysfunction in the pathogenesis of IPF. While telomere shortening may be related to underlying genetic mechanisms, environmental effects from cigarette smoking and/or oxidative damage may also play a role⁷³⁻⁷⁶.

To determine if telomere length relates to IPF disease progression or survival, we measured peripheral blood telomere lengths from 381 Interstitial Lung Disease (ILD) patients prospectively followed by the UTSW Advanced Lung Disease clinics. Telomere length was associated with transplant-free survival time for patients with IPF in unadjusted analysis (P-value = 0.004) and in multivariate analysis after adjustment for age, gender and pulmonary function (HR 0.17, 0.06-0.50, P-value = 0.001)⁷⁷. There is a step-wise decrease in transplant-free survival for patients stratified by telomere length quartiles (**Figure 5**, P-value = 0.027). Similar findings were observed in the two independent IPF replication cohorts. Since patients with IPF demonstrate a widely variable clinical course, telomere length may be a useful prognostic biomarker.

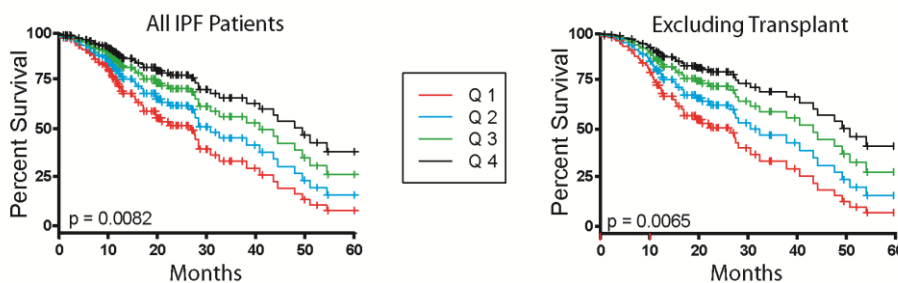


Figure 5. Shorter telomere lengths predict worse survival for IPF patients in the Dallas cohort. Estimated survival functions for transplant-free survival (A) and time to death (B) for IPF patients stratified by telomere length quartiles.

Molecular Pathogenesis

Telomeres, or the protective caps at the ends of linear chromosomes⁷⁸, are markers of human age. Their lengths are controlled by the opposing actions of the enzyme telomerase, which lengthens telomeres in certain proliferative or stem cell populations, and the end replication problem, which leads to telomere attrition⁷⁹. Telomeres acts as biologic "mitotic clocks," with age-dependent progressive shortening^{80,81}. Telomeres shorten in cells that do not express telomerase and in stem cells that have low but regulated telomerase activity. Exogenous expression of telomerase in human cells⁸² or upregulation of telomerase in cancer cells⁸³ leads to telomere elongation. Telomere length, therefore, reflects the integration of multiple effects: starting telomere length, telomerase activity and cellular replication.

Rare inherited mutations in the telomere related genes informed us of the underlying pathogenesis of IPF. It has been proposed that germline telomerase mutations cause telomere shortening, cell senescence, recruitment of quiescent stem cells with repetitive cycles of proliferation, further telomere shortening and cellular senescence, exhaustion of tissue-specific stem cells and clinical disease⁸⁴. Mouse models of telomerase (*mTert*, *mTerc*) dysfunction support this model as they have shortened telomere lengths and reduced proliferation of stem cells and shortened life spans⁸⁵⁻⁹⁰. However, these mice do not develop lung fibrosis, perhaps because of their inherently longer telomere lengths, their ubiquitous expression of telomerase or their relatively short life spans. In this regard, study of human IPF kindreds and patients has led to the identification of novel genes relevant to molecular pathogenesis of telomere attrition, aging and lung fibrosis. Future studies will be needed to determine whether the identification of this underlying molecular etiology of IPF will lead to effective targeted therapies.

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