Telomere Shortening in Familial and Sporadic Pulmonary Fibrosis

Jennifer T. Cronkhite[1], Chao Xing[1], Ganesh Raghu[2], Kelly M. Chin[3], Fernando Torres[3], Randall L. Rosenblatt[3], and Christine Kim Garcia[1,3]

[1]Eugene McDermott Center for Human Growth and Development, University of Texas Southwestern Medical Center, Dallas, Texas; [2]Division of Pulmonary and Critical Care Medicine, University of Washington Medical Center, Seattle, Washington; and [3]Division of Pulmonary and Critical Care Medicine, University of Texas Southwestern Medical Center, Dallas, Texas

***Rationale*: Heterozygous mutations in the coding regions of the telomerase genes, *TERT* and *TERC*, have been found in familial and sporadic cases of idiopathic interstitial pneumonia. All affected patients with mutations have short telomeres.**

***Objectives*: To test whether telomere shortening is a frequent mechanism underlying pulmonary fibrosis, we have characterized telomere lengths in subjects with familial or sporadic disease who do not have coding mutations in *TERT* or *TERC*.**

***Methods*: Using a modified Southern blot assay, the telomerase restriction fragment length method, and a quantitative polymerase chain reaction assay we have measured telomere lengths of genomic DNA isolated from circulating leukocytes from normal control subjects and subjects with pulmonary fibrosis.**

***Measurements and Main Results*: All affected patients with telomerase mutations, including case subjects heterozygous for newly reported mutations in *TERT*, have short telomere lengths. A significantly higher proportion of probands with familial pulmonary fibrosis (24%) and sporadic case subjects (23%) in which no coding mutation in *TERT* or *TERC* was found had telomere lengths less than the 10th percentile when compared with control subjects ($P = 2.6 \times 10^{-8}$). Pulmonary fibrosis affectation status was significantly associated with telomerase restriction fragment lengths, even after controlling for age, sex, and ethnicity ($P = 6.1 \times 10^{-11}$). Overall, 25% of sporadic cases and 37% of familial cases of pulmonary fibrosis had telomere lengths less than the 10th percentile.**

***Conclusions*: A significant fraction of individuals with pulmonary fibrosis have short telomere lengths that cannot be explained by coding mutations in telomerase. Telomere shortening of circulating leukocytes may be a marker for an increased predisposition toward the development of this age-associated disease.**

Keywords: idiopathic pulmonary fibrosis; pulmonary fibrosis; telomere length; aging; interstitial lung disease

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Rare mutations in the genes encoding telomerase are found in patients with familial and sporadic idiopathic interstitial pneumonia and are associated with short telomere lengths.

What This Study Adds to the Field

Short telomere lengths (<10th percentile) are commonly found in both the familial and sporadic forms of adult-onset pulmonary fibrosis.

The idiopathic interstitial pneumonias are characterized by damage to the lung parenchyma by a combination of fibrosis and inflammation. The prototype of these diseases is idiopathic pulmonary fibrosis (IPF), the prevalence and annual incidence of which increase dramatically with age (1). A clue to the genetic underpinnings of the familial subtype of this disorder emerged from the discovery that a subset (~15%) of patients with IPF is heterozygous for mutations in the genes encoding the protein component (*TERT*) and the RNA component (*TERC*) of telomerase, a ribonucleoprotein enzyme that catalyzes the addition of hexameric nucleotide repeats to the ends of linear chromosomes (2, 3). One mutation in *TERT* was found in a subject with the sporadic form of the disease who had no family history of IPF (2). The mutations in *TERT* segregate not only with individuals that met the strict clinical diagnosis of IPF (4), but also with several with pulmonary fibrosis favoring the upper lobes of the lung and others with unclassified pulmonary disease (2).

The missense, frameshift, and splice site mutations in *TERT* found in the familial and sporadic cases of pulmonary fibrosis span the entire coding regions of the gene but cluster in conserved domains. One frameshift mutation, V747fs, which is predicted to be missing half the reverse transcriptase domain, has little enzymatic activity in a recombinant *in vitro* assay; cotranslation of various ratios of plasmids encoding this mutation and the wild-type TERT protein suggests a mechanism of haploinsufficiency (2). The missense mutations in *TERT* that have been identified in patients with IPF have between 30 and 100% wild-type telomerase activity in a rabbit reticulocyte *in vitro* assay (2). The telomere lengths of circulating leukocytes of individuals with these mutations and pulmonary fibrosis are reproducibly shorter than those of age-matched control subjects (2, 3).

Telomere shortening is a common feature of dyskeratosis congenita and bone marrow failure syndromes, two diseases previously associated with mutations in *DKC1*, *TERT*, *TERC*, or *TINF2* (5) (reviewed in Garcia and coworkers [6]). Irrespective of the gene in which a mutation is found, patients with dyskeratosis congenita have short telomeres in their circulating leukocytes (7). Mutations in *TERT* are present in up to 4% of individuals with acquired aplastic anemia (8), and yet short telomere lengths are found in 34% of patients with this disease (9). In the patients with aplastic anemia, the severity of disease is directly related to the degree of telomere shortening; moreover, a lack of response to immunosuppressive agents is related to shorter telomere length (10, 11).

In this study, we have sequenced the genes encoding telomerase, *TERT* and *TERC*, in patients with the familial and sporadic forms of idiopathic interstitial pneumonias. We have compared

(*Received in original form April 11, 2008; accepted in final form July 17, 2008*)

Supported by National Institutes of Health grant K23-RR020632.

Correspondence and requests for reprints should be addressed to Christine Kim Garcia, M.D., Ph.D., University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-8591. E-mail: christine.garcia@utsouthwestern.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 178. pp 729–737, 2008
Originally Published in Press as DOI: 10.1164/rccm.200804-550OC on July 17, 2008
Internet address: www.atsjournals.org

telomere lengths in circulating leukocytes of both familial and sporadic case subjects with those of normal control subjects. All patients with pulmonary fibrosis in whom mutations were identified had telomere lengths that were less than the 10th percentile when compared with age-matched control subjects. In addition, we found that 20–25% of subjects with familial or sporadic pulmonary fibrosis who did not have any detectable mutations in telomerase also had telomere lengths less than the 10th percentile. These findings suggest that telomere shortening is a more generalized feature of IPF.

METHODS

Human Subjects

This study was approved by the University of Texas Southwestern Medical Center Institutional Review Board (Dallas, TX). Written informed consent was obtained from all subjects. Each participant completed a medical questionnaire. Medical records were obtained when available. Each sporadic case and at least one member of each kindred with familial pulmonary fibrosis carried a diagnosis of idiopathic interstitial pneumonia or unclassifiable interstitial pneumonia in concordance with established criteria (4). Kindreds with familial pulmonary fibrosis were defined as those in which there was at least one other relative of the proband who was affected with an interstitial lung disease; all sporadic case subjects had no affected first- or second-degree family members. Sporadic cases of pulmonary fibrosis due to known causes, associated with collagen vascular disease, sarcoidosis, and other diffuse parenchymal lung disease were excluded from study. All case subjects and affected family members were diagnosed at an age of 21 years or more. A subset of the probands (n = 46) and sporadic case subjects (n = 42) included in this study were also included in a previous study (2). Patients with idiopathic, familial, or anorexigen exposure associated pulmonary arterial hypertension and who fulfilled the following criteria were included: (*1*) right heart catheterization measurement of mean pulmonary artery pressure greater than 25 mm Hg, pulmonary capillary wedge pressure less than 15 mm Hg, and pulmonary vascular resistance greater than 3 Wood units; (*2*) a ratio of total lung capacity to forced vital capacity exceeding 70% plus a normal chest X-ray or a ratio of total lung capacity to forced vital capacity exceeding 50% plus a normal computed tomography scan of the chest; and (*3*) exclusion of pulmonary arterial hypertension associated with collagen vascular disease, congenital shunts, HIV infection, portal hypertension, and all other forms of pulmonary hypertension including pulmonary venous hypertension or that associated with lung disease, chronic thromboembolism, or disorders of the pulmonary vasculature. Genomic DNA samples for the population of normal control subjects (n = 201; age, 19–89 yr) were obtained from a sample of unrelated, multiethnic individuals from Dallas, Texas from H. Hobbs. The ethnicity of each subject was self-assigned. Genomic DNA was isolated from circulating leukocytes with an Autopure LS (Qiagen, Valencia, CA). Buccal swabs were obtained with sterile BD Falcon SWUBE applicators (BD Biosciences, San Jose, CA); DNA was isolated with Gentra (Qiagen) reagents.

Sequencing and Mutation Analysis

Sequencing of both *TERT* and *TERC* was performed as described (2). Sequences used in the comparative alignment were obtained from the NCBI website (www.ncbi.nlm.nih.gov) and aligned by ClustalW (www.ebi.ac.uk/clustalw), using the default settings. No new common single-nucleotide polymorphisms (frequency > 0.05) were found in this study; all variants were previously reported (2).

Telomerase Repeat Amplification Protocol Assays

Missense mutations in *TERT* were introduced into the parental plasmid pGRN125, using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The complete coding sequences for all the mutants were verified by sequencing. The activity of *in vitro* coexpressed recombinant telomerase protein and RNA (encoded by plasmid pKT26) was determined by telomerase repeat amplification protocol (TRAP) assay as previously described (2). The plasmids encoding the V747fs mutation and its wild-type control were previously described (2).

Terminal Restriction Fragment Length Analysis of Telomere Length

Terminal restriction fragment length (TRFL) analysis of genomic DNA isolated from leukocytes was performed in duplicate as described (2, 12). The observed mean coefficient of variation for the assay was 3.7% for 472 independent samples. Samples were assayed in duplicate again until their mean coefficient of variation less than 9%. The percentage of short telomeres for each sample was determined from the Southern blot as described (2).

Quantitative Polymerase Chain Reaction Determination of Telomere Length

Quantitative polymerase chain reaction (PCR) determination of telomere lengths was performed in a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA) as described previously (13, 14). *See* the online supplement for additional details about this assay. The ratio of the copy number of telomere DNA to a single-copy gene (T/S ratio) was normalized to a reference sample, MCF7 cells, which have short telomeres. Each relative T/S ratio represents the average of three independent experiments. The observed mean coefficient of variation for $Ct^{telomere}$ and $Ct^{\beta 2\text{-globin}}$ and the relative T/S ratio were 1.16, 0.58, and 13.6%, respectively, for 353 samples.

Statistical Methods

Linear regression analysis was performed with the R software package, version 2.4.1 (www.r-project.org) to assess the relationship between telomere length and age. We analyzed telomere length on the basis of three different parameters: (*1*) the mean TRFL, which was normally distributed; (*2*) the percentage of short telomeres (p, as defined in Reference 2), which had a skewed distribution and so was first logit-transformed; and (*3*) the relative T/S ratio, which was log-transformed. The 10th and 90th percentile prediction bands of the TRFLs, the logit(p), and the ln(relative T/S) for the normal subjects (n = 201) were determined from the linear regression model. Case and control subjects stratified by either the 10th or 90th percentile prediction lines were compared by Fisher exact test. Multiple regression was used to adjust for covariates when necessary.

RESULTS

Six New Mutations in *TERT* in Subjects with Adult-Onset Pulmonary Fibrosis

We sequenced the coding and flanking intronic sequences of *TERT* and *TERC* in 25 probands and 34 sporadic subjects with pulmonary fibrosis and identified six new mutations in *TERT*—five in probands with familial pulmonary fibrosis (Figure 1A) and one in a patient with sporadic pulmonary fibrosis (the lung phenotype is defined in METHODS). Additional clinical findings of the subjects and their relevant family members are provided in Table E1 (*see* the online supplement). Missense mutations were found in the probands of family F55 (K1050E), F80 (P702L), F106 (L1019F), F107 (V694M), and F119 (P704S). All three members of family F80 with IPF were heterozygous for the same missense mutation as the proband (P702L). One of the patients with sporadic disease had a missense mutation in the C-terminal region of TERT (S957R). All of these missense mutations involve highly conserved residues in regions of the protein (Figures 1B and 1C) that have postulated roles in enzymatic activity, processivity, and cellular location of the protein (15–17). None of the mutations were detected in a multiethnic panel of 528 individuals sequenced as control subjects (8). All of the mutations had not been identified previously except V694M, which was found in a 34-year-old subject with moderate aplastic anemia (8). The 60-year-old proband of family F107 with this mutation has a normal

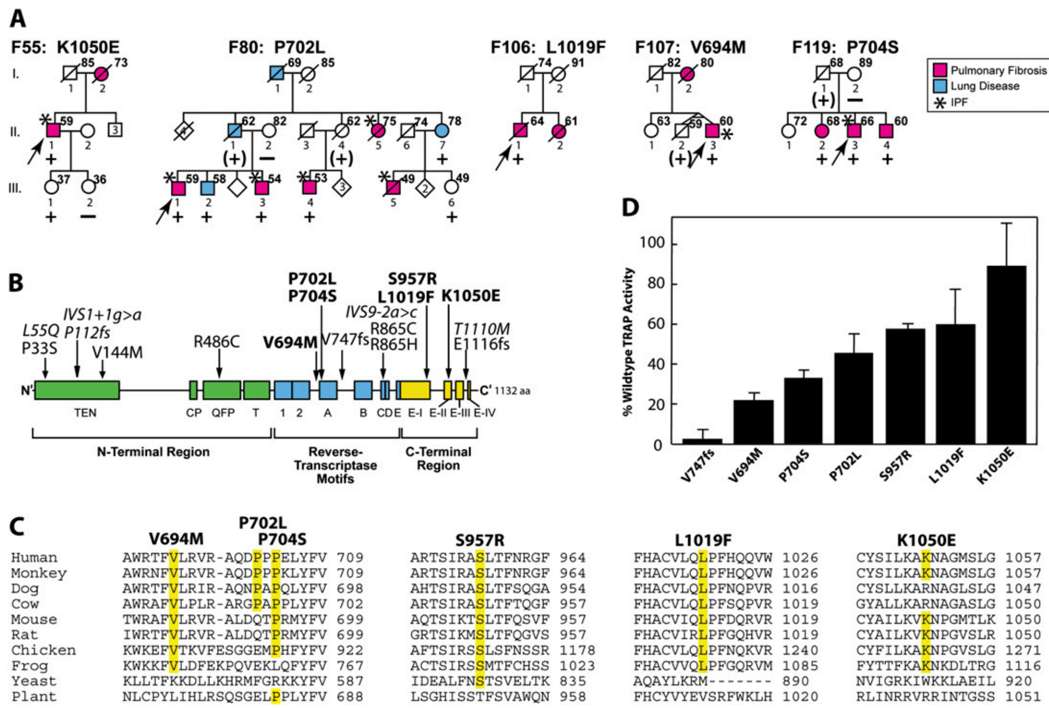

Figure 1. (*A*) Abridged pedigrees of kindreds F55, F80, F106, F107, and F119 with familial pulmonary fibrosis and *TERT* mutations; (*B*) schematic representation of the functional domains of TERT with the position of the mutations found in pulmonary fibrosis subjects relative to the domains; (*C*) alignment of the *TERT* sequences of human, *Macaca mulatta* (monkey), *Canis familiaris* (dog), *Bos taurus* (cow), *Mus musculus* (mouse), *Rattus norvegicus* (rat), *Gallus gallus* (chicken), *Xenopus laevis* (frog), *Schizosaccharomyces pombe* (yeast), and *Arabidopsis thaliana* (plant); and (*D*) relative telomerase activity of TERT mutants as measured by the telomere repeat amplification protocol (TRAP) assay. In (*A*), *shaded symbols* indicate individuals with pulmonary fibrosis (*pink*) or lung disease (*blue*); the presence or absence of the mutation is indicated by plus or minus signs, respectively. The current age or the age at death is listed to the right of each symbol. Mutations in the DNA and protein sequence are abbreviated by convention. Amino acids are listed as single letters. Additional details of the clinical features of individuals are listed in Table E1 in the online supplement. In (*B*), the N-terminal region domains (*green*), the reverse transcriptase motifs (*blue*), and C-terminal region domains (*yellow*) are shown. New mutations described in this article are indicated in boldface type; mutations previously described in Reference 3 (*italics*) and Reference 2 (*roman*) are shown for comparison. In (*D*), relative amounts of telomerase activity for seven different TERT mutants were calculated as a ratio of the intensity of the sample's telomerase products to that of an internal control band and normalized to wild-type activity in one representative experiment. *Error bars* represent the SD. Parallel reactions using [^{35}S]methionine were run on a sodium dodecyl sulfate–polyacrylamide gel to confirm equal expression of the TERT wild-type and mutant proteins.

complete blood count, which does not suggest any bone marrow dysfunction (*see* Table E1). Electropherograms of all the sequence mutations are shown in Figure E2.

To determine the effect of these amino acid substitutions in TERT on enzymatic activity, each mutation was reproduced by site-directed mutagenesis and tested *in vitro* after coexpressing the recombinant telomerase protein and RNA. For these studies we used the telomere repeat amplification protocol (TRAP) assay. As seen in Figure 1D, the missense mutations had between 23 and 88% of the telomerase activity of the wild-type protein.

Comparison of Telomere Lengths to Those of a Normal Population

To establish the normal range of telomere lengths, we studied 201 asymptomatic subjects ranging from 19 to 89 years of age. Table 1 lists demographic information for this control multiethnic group in comparison with subjects with pulmonary fibrosis. The rate of TRFL shortening in normal samples was 17 bp/year (Figure 2A), which is consistent with prior estimates that leukocyte telomere shorten by 15–40 bp/year within this age range (18–22). A small difference in mean TRFLs between the men (5.84 kb) and women (6.03 kb) was found ($P = 0.02$), which is consistent with other studies (21, 23). The 10th and 90th percentile predicted bands were determined from the linear regression model.

Next, we examined the telomere lengths of individuals from the families in Figure 1 and described by Tsakiri and coworkers (2), in which a *TERT* or *TERC* mutation was not identified (noncarriers). Six of 76 noncarriers fell below the 10th percentile predicted line. In contrast, 52 of the 61 *TERT/TERC* mutation carriers fell below this threshold. All the family members who had been diagnosed with pulmonary fibrosis were mutation carriers who were more than 48 years of age and their telomere lengths were uniformly less than the 10th percentile when compared with the control subjects (Figure 2B).

Telomere Shortening in Pulmonary Fibrosis Subjects without Mutations in *TERT* or *TERC*

To determine whether short telomeres are a more common feature of pulmonary fibrosis we assayed telomere lengths in

TABLE 1. DEMOGRAPHICS OF NORMAL CONTROL SUBJECTS, SUBJECTS WITH IDIOPATHIC INTERSTITIAL LUNG DISEASE AND *TERT* OR *TERC* MUTATIONS, PROBANDS OF KINDREDS WITH FAMILIAL PULMONARY FIBROSIS, AND SPORADIC CASES WITHOUT TELOMERASE MUTATIONS

		TERT/TERC Mutations		
		Present:	Absent	
	Control Subjects (*n* = 201)	PF Case Subjects (*n* = 20)	Familial PF Probands* (*n* = 59)	Sporadic PF Case Subjects* (*n* = 73)
Age, yr (mean)	55.0	58.6	61.6	59.1
Sex, %				
Male	49	85	58	56
Female	51	15	42	44
Ethnicity,%				
White	67	80	73	81
Black	20	0	3	11
Hispanic	12	10	24	8
Other	1	10	0	0

Definition of abbreviation: PF = pulmonary fibrosis.

* Subjects in this group do not have any detectable mutations in either *TERT* or *TERC*.

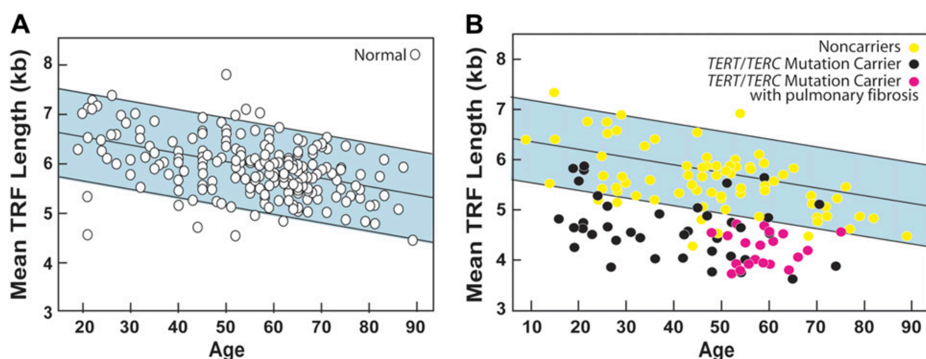

Figure 2. Mean terminal restriction fragment lengths (TRFLs) for (*A*) normal control subjects and (*B*) individuals from families with *TERT* or *TERC* mutations plotted against age. *Open circles* represent unrelated normal control subjects in (*A*). Symbols in (*B*) represent those without heterozygous *TERT* or *TERC* mutations (*yellow circles*) and those with *TERT* or *TERC* mutations either with (*pink circles*) or without (*solid circles*) a diagnosis of pulmonary fibrosis. The *blue region* delineates the 10th to 90th percentile predicted bands of the mean TRFLs for the normal control subjects. Linear regression was used to draw a best-fit line through the normal samples.

leukocytes from all those individuals in our collection of patients with familial or sporadic pulmonary fibrosis who did not have a mutation in the coding region of *TERT* or *TERC*. Figure 3A shows the telomere lengths in three unrelated kindreds with familial pulmonary fibrosis. The affected siblings in family F3 have telomeres that are of similar size as an unaffected older sister (data not shown) and an unrelated age- and ethnicity-matched control subject. In contrast, the 48-year-old proband of family F4 has markedly short telomeres as compared with his unaffected older sibling. His mean telomere length (4.1 kb) was shorter than that of the proband of family F55 (4.9 kb), who is heterozygous for a *TERT* missense mutation (K1050E).

We also analyzed the telomere lengths in our sporadic case subjects with pulmonary fibrosis who did not have a mutation in the coding region of telomerase. Figure 3B shows that some subjects, such as CKG059 and CKG973, have telomere lengths similar to those of age- and ethnicity-matched control subjects. Others, such as CKG030 and CKG545, have telomere lengths that are shorter than those of matched control subjects and are similar in length to the sporadic case who is heterozygous for the *TERT* S957R mutation (4.5, 4.4, and 4.1 kb, respectively).

We determined the distribution of telomere lengths in familial (n = 59) and sporadic (n = 73) case subjects with pulmonary fibrosis who did not have a detectable mutation in the telomerase genes. The majority of case subjects were male and white (Table 1), reflecting the demographics of this disease (1, 24). In general, affected individuals with telomerase mutations were similar to the familial and sporadic case subjects without telomerase mutations regarding smoking status, age at diagnosis, pathologic subtype of idiopathic interstitial pneumonia, and other comorbidities (Table 2). A comparison of the telomere lengths, as determined by the TRFL assay, of the probands and sporadic cases with normal subjects is provided in Figures 4B and 4C, respectively. As expected, all probands with mutations in *TERT* or *TERC* had mean TRFLs below the 10th percentile prediction line. In addition, 14 of the 59 probands (24%) with familial pulmonary fibrosis who did not have a mutation in telomerase had mean TRFLs below the 10th percentile prediction line; this is more than was observed in control subjects ($P = 8.0 \times 10^{-6}$). Some of these subjects had TRFLs that are just as short as those with mutations in *TERT* or *TERC*. If a more stringent cutoff was used, such as the 1st or 5th percentile, there were still significantly more subjects without telomerase mutations below these thresholds than control subjects (data not shown). Similarly, there were more sporadic case subjects without coding mutations in *TERT* or *TERC* who had telomere lengths that were below the 10th percentile prediction line than would be expected by chance (17 of 73 sporadic case subjects without telomerase mutations vs. 8 of 201 control subjects; $P = 2.6 \times 10^{-6}$) (Figure 4C). No consistent distinguishing phenotype was seen for familial and sporadic case subjects with short telomere lengths versus those whose telomere lengths were greater than the 10th percentile.

Telomere length of genomic DNA was also determined using an independent quantitative PCR method (*see* METHODS). The average telomere length was determined by assessing the ratio of telomere copy number repeats to a single copy gene, β_2-globin (T/S ratio) in experimental samples relative to a reference

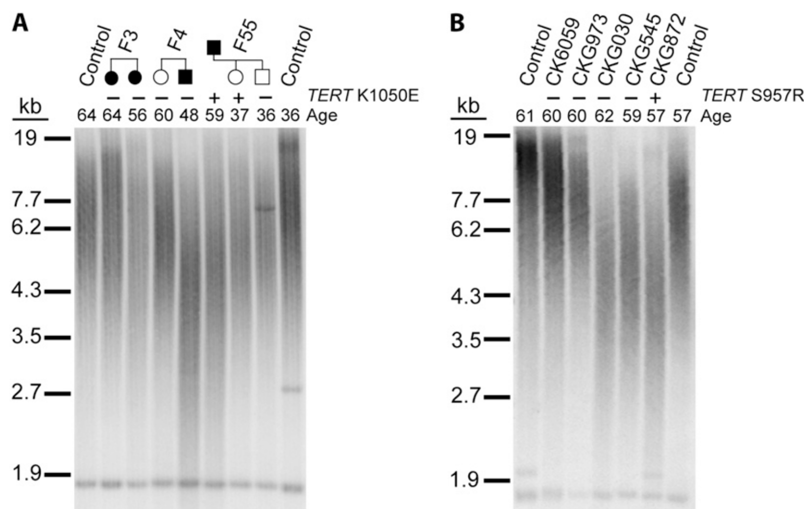

Figure 3. Telomere length determined by the terminal restriction fragment length (TRFL) assay of (*A*) subjects in kindreds with familial pulmonary fibrosis and (*B*) sporadic cases of idiopathic interstitial lung disease. Abridged pedigrees, the age of each individual, and the presence (+) or absence (–) of the *TERT* mutation are indicated above each Southern blot in (*A*) and (*B*). *Open symbols* represent normal individuals; *solid symbols* indicate individuals with pulmonary fibrosis.

TABLE 2. CHARACTERISTICS OF SUBJECTS WITH IDIOPATHIC INTERSTITIAL LUNG DISEASE AND *TERT* OR *TERC* MUTATIONS, PROBANDS OF KINDREDS WITH FAMILIAL PULMONARY FIBROSIS, AND SPORADIC CASES WITHOUT TELOMERASE MUTATIONS

	TERT/*TERC* Mutations		
	Present:	Absent	
	PF Case Subjects (*n* = 20)	Familial PF Probands* (*n* = 59)	Sporadic PF Case Subjects* (*n* = 73)
Smokers, %	65	70	63
Mean pack-years	12.5	17.2	20.0
Age at diagnosis			
Mean, yr	55.7	59.4	55.9
Range, yr	37–74	31–87	25–77
Deceased or transplanted, %	50	32	31
Diagnosis, %			
IPF	65	73	63
Open lung biopsy	60	59	81
UIP	67	69	63
Nonspecific fibrosis	25	20	15
Discordant pathology†	8	0	7
NSIP	0	6	7
COP	0	0	5
Other	0	6	3
Other diagnoses, %			
Cancer‡	5	8	14
Osteoporosis/osteopenia	50	37	42
GERD	57	68	59
Hypothyroidism	5	24	15
Anemia before lung transplantation	5	15	10
Telomere length			
Mean TRFL, kb	4.34§	5.25	5.39
Mean percent short telomeres‖	44.1%§	26.6%	23.0%
Mean relative T/S ratio¶	1.33§	1.74	1.84

Definition of abbreviations: COP = cryptogenic organizing pneumonia; DIP = desquamative interstitial pneumonia; GERD = gastroesophageal reflux disease; NSIP = nonspecific interstitial pneumonia; TRFL = terminal restriction fragment length; T/S/ ratio = ratio of the copy number of telomere DNA to a single-copy gene; UIP = usual interstitial pneumonia.

* Subjects in this group do not have any detectable mutations in either *TERT* or *TERC*.

† Pathologic diagnosis of the open lung biopsy and the explanted lung were discordant.

‡ Excludes completely localized and resectable epithelial cancers, that is, squamous cell carcinoma or basal cell carcinoma of the skin.

§ Indicates a statistically significant deviation from case subjects, either familial or sporadic, without telomerase mutations, using the Student's *t* test ($P < 0.05$).

‖ As defined in Reference 2.

¶ Relative to MCF7 cells as described in METHODS.

sample. We observed a good correlation between this method and the TRFL method for determining telomere length ($P < 2.2 \times 10^{-16}$; and *see* Figure E3). For control subjects, the relative T/S ratio decreased with age by 0.0096 unit/year, generally consistent with other reports of rates of attrition (14, 25, 26). The 10th and 90th percentile predicted bands were determined from the linear regression model (Figure 4D). The distributions for the probands of kindreds with familial pulmonary fibrosis and for the sporadic pulmonary fibrosis case subjects are shown in Figures 4E and 4F, respectively. As expected, all the probands of the familial cases with mutations in *TERT* or *TERC* fell below the 10th percentile prediction line (Figure 4E). When this band was used as an arbitrary cutoff, we again observed a significant excess of subjects with familial or sporadic pulmonary fibrosis with telomere lengths below this boundary (Table 3).

Consistent with telomere shortening with age, the percentage of short telomeres (determined from the TRFL Southern blots) also increased with age. All probands with telomerase mutations had logit(p) scores greater than the 90th percentile (Figure 5B), and again we found a significantly greater than expected number of probands and sporadic case subjects without telomerase mutations with logit(p) scores above the 90th percentile predicted band ($P = 9.5 \times 10^{-9}$) (Figures 5B and 5C; and *see* Table 3). All the probands of the familial cases with mean telomere lengths less than the10th percentile had logit(p) values greater than the 90th percentile.

Multiple regression analysis showed a correlation between telomere length and sex after controlling for age and ethnicity ($P = 4.9 \times 10^{-4}$). There was a small difference in mean TRFLs between men (5.25 kb) and women (5.45 kb) with pulmonary fibrosis without telomerase mutations ($P = 0.004$) (Table 4). Smoking has been associated with short telomere lengths (27, 28). In contrast with sex, we did not find a correlation between telomere length and smoking by multivariate analysis in the cases after controlling for sex and ethnicity. Because ethnicity was previously reported to be associated with telomere length (29), we analyzed white male and female case and control subjects separately and found similar results in these smaller subgroups when using all three methods for assessing telomere length (*see* Table E2).

To determine the specificity of the short telomere phenotype we measured telomere length by the quantitative PCR method for a cohort of patients with idiopathic, familial, or anorexigen-associated pulmonary arterial hypertension (*see* METHODS for a description of this patient population) (Figure 6). There was not

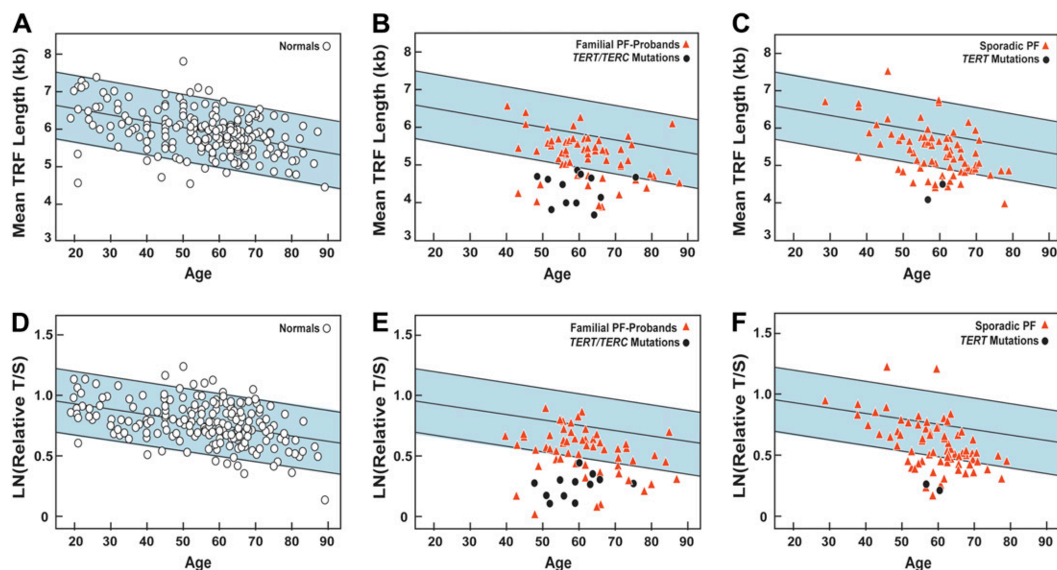

Figure 4. Telomere length as determined by the (*A–C*) terminal restriction fragment length (TRFL) assay and by the (*D–F*) quantitative polymerase chain reaction (PCR) assay for normal control subjects (*A* and *D*), probands of kindreds with familial pulmonary fibrosis (*B* and *E*), and sporadic case subjects with idiopathic interstitial lung disease (*C* and *F*) plotted against age. *Open circles* represent normal control subjects, *red triangles* represent unrelated probands and sporadic case subjects with pulmonary fibrosis, and *solid circles* represent probands and sporadic case subjects with pulmonary fibrosis and *TERT* or *TERC* mutations. The *blue region* delineates the 10th to 90th percentile predicted bands of the mean TRFLs or ln(relative T/S ratio) for the normal control subjects (T/S ratio, ratio of the copy number of telomere DNA to a single-copy gene). Linear regression analysis of the TRFL data of normal control subjects (*A*) established a linear relationship between telomere length and age by the following equation: TRFL = 6.87 – 0.0169 × age ($P = 6.1 \times 10^{-12}$). Linear regression analysis of the normal subjects (*D*) established a linear relationship between the logarithm of the relative T/S ratio and age by the following equation: ln(relative T/S ratio) = 1.02 – 0.00451 × age ($P = 2.67 \times 10^{-10}$).

a significant excess of subjects in this patient population below the 10th percentile predicted line when compared with control subjects, either for the entire group or sex- and ethnicity-matched case and control subjects.

DISCUSSION

In this article, we show short telomere lengths of circulating leukocytes in 25% or more of all subjects with familial or sporadic idiopathic interstitial pneumonia. The findings of the inherited mutations in telomerase in families and individuals with IPF initially suggested that telomerase dysfunction is important for the molecular pathogenesis of this disease. Here we find that of 71 unrelated probands, 12 (17%) with heterozygous mutations in *TERT* or *TERC* and an additional 14 (20%) probands without telomerase mutations have mean telomere lengths of circulating leukocytes that are shorter than the 10th percentile by the TRFL assay. Therefore, 37% of probands with the familial form of the disease have evidence of short telomeres. Similarly, of 75 unrelated sporadic cases, 2 (3%) have mutations in *TERT* and 17 have TRFLs less than the 10th percentile; so 25% of sporadic cases have short telomeres. These findings suggest that short telomere lengths are commonly associated with both the familial and sporadic forms of pulmonary fibrosis and can be only partially explained at the molecular level by coding mutations in *TERT* and *TERC*. The sequencing of both genes has been limited to the coding regions and their surrounding intronic splice sites. We

TABLE 3. DISTRIBUTION OF CASE SUBECTS AND CONTROL SUBJECTS STRATIFIED BY MEAN TERMINAL RESTRICTION FRAGMENT LENGTH, ln(RELATIVE T/S RATIO), OR LOGIT(p)

		TERT/TERC Mutations			
		Present:	Absent		
	Control Subjects (*n* = 201)	PF Case Subjects (*n* = 20)	Familial PF Probands* (*n* = 59)	Sporadic PF Case Subjects* (*n* = 73)	All Case Subjects* (*n* = 132)
Mean TRFL					
Upper (>10%)	193	0	45	56	101
Lower (<10%)	8	20	14	17	31
P Value†	—	$<2.2 \times 10^{-16}$	8.0×10^{-6}	2.6×10^{-6}	2.6×10^{-8}
ln(relative T/S ratio)					
Upper (>10%)	191	1	42	53	95
Lower (<10%)	10	19	17	20	37
P Value†	—	$<2.2 \times 10^{-16}$	2.0×10^{-6}	1.2×10^{-6}	8.2×10^{-9}
Logit(p)					
Upper (>90%)	8	20	18	17	35
Lower (<90%)	193	0	41	56	97
P Value†	—	$<2.2 \times 10^{-16}$	2.4×10^{-7}	1.4×10^{-5}	9.5×10^{-9}

Definition of abbreviation: PF = pulmonary fibrosis.

* Subjects in this group do not have any detectable mutations in either *TERT* or *TERC*.

† *P* Values were calculated comparing the number of case subjects and control subjects stratified by the indicated threshold, using the Fisher exact test.

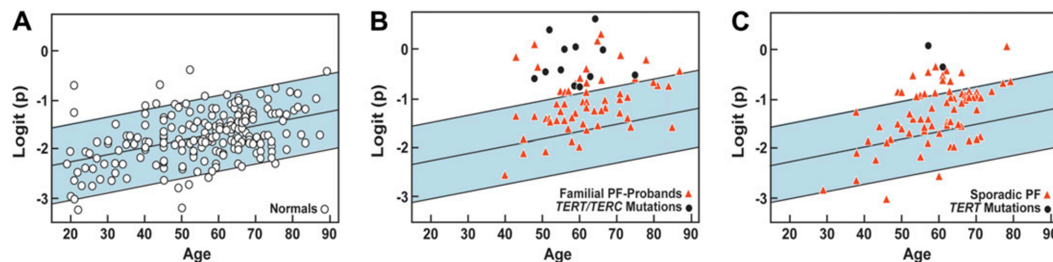

Figure 5. A measure of the percent short telomeres [logit(p)] for (*A*) normal control subjects, (*B*) probands of kindreds with familial pulmonary fibrosis, and (*C*) sporadic cases with idiopathic interstitial lung disease plotted against age. *Open circles* represent normal control subjects, *red triangles* represent unrelated probands and sporadic cases with pulmonary fibrosis, *solid circles* represent probands and sporadic cases with pulmonary fibrosis and *TERT* or *TERC* mutations. The *blue region* delineates the 10th to 90th percentile predicted bands of the logit(p) for the normal control subjects. Linear regression analysis of the logit(p) established a linear relationship for normal subjects between this measure and age by the following equation (where p is the percentage of short telomeres): $\text{logit}(p) = \ln[p/(1-p)] = -2.56 + 0.0148 \times \text{age}$ ($P = 3.4 \times 10^{-12}$).

cannot rule out noncoding mutations or small deletions in either gene. In one expanded family, the short telomere phenotype segregates with pulmonary fibrosis as an autosomal dominant trait but does not cosegregate with haplotypes about *TERT* and *TERC*, suggesting that other genetic loci may contribute to this trait (data not shown).

All families collected with familial pulmonary fibrosis have at least one affected member carrying a diagnosis of an idiopathic interstitial pneumonia or unclassifiable interstitial pneumonia; other affected family members have interstitial lung disease. Although IPF is the most common diagnosis among the affecteds, it is not the only diagnosis. In fact, only 65% of case subjects with coding mutations in *TERT* or *TERC* meet the clinical definition of IPF. Other mutation carriers within the same family have been diagnosed with nonspecific interstitial pneumonitis, granulomatous lung disease, and coal worker pneumoconiosis. Similarly, the occurrence of pathologic findings of diverse subtypes of non–usual interstitial pneumonitis in the same family has been reported for other cohorts of familial pulmonary fibrosis kindreds (30, 31). Granulomatous lung disease, as seen in the proband of family F106, has been associated with telomerase mutations earlier; we previously described one of the affected individuals in family F71 with chronic hypersensitivity whose open lung biopsy showed usual interstitial pneumonitis with features of noncaseating granulomas (2). The mutations in telomerase appear to increase the susceptibility to interstitial lung disease in general and are not associated with one particular clinicopathologic subtype. This raises the possibility that although the diagnosis of a specific interstitial lung disease may differ for individual family members with telomerase mutations due to different environmental or occupational exposures, the identified genetic predisposition leads to a tissue repair response of fibrosis in reaction to injury.

We also found that IPF is the most common, but not the only, pulmonary diagnosis seen in those familial and sporadic case subjects without telomerase mutations and whose telomere lengths are less than the 10th percentile. A diagnosis of IPF was found in 50–85% of these groups; other diagnoses such as nonspecific interstitial pneumonitis and cryptogenic organizing pneumonia were made by open lung biopsy. Patients in this study were collected on the basis of a diagnosis of idiopathic interstitial pneumonia, but we again found that telomere shortening was not associated with one particular clinicopathologic subtype. A collected cohort of patients with idiopathic, familial, or anorexigen-associated pulmonary arterial hypertension did not demonstrate an excess of individuals with short telomere lengths, suggesting some specificity of the association between pulmonary fibrosis and short telomeres. Additional cohorts of patients with different pulmonary phenotypes will be needed to delineate the range of pulmonary diagnoses associated with telomere shortening.

Although most individuals who carry a heterozygous mutation in *TERT* or *TERC* have short telomeres, 15% do not fall below the 10th percentile by the TRFL assay. Analysis of these individuals by the quantitative PCR method also demonstrates that they fall within the normal range (data not shown). In contrast, all of the individuals with diagnoses of pulmonary fibrosis and who carry a heterozygous mutation in *TERT* or *TERC* are more than 48 years of age and have telomere lengths below the 10th percentile predicted line. This strongly suggests that the pulmonary fibrosis phenotype is related to older age and short telomere lengths in this molecularly defined group of patients. Leukocyte telomere length may be influenced by other genetic, intrinsic, tissue-specific, or environmental effects. It is currently unknown whether the other mutation carriers have subclinical manifestations of the disease or whether they demonstrate incomplete penetrance. It is also not known whether the nature and degree of telomere shortening seen in circulating leukocytes is representative of the lung cells within these subjects. For eight control subjects and four subjects heterozygous for the *TERT* P702L mutation, we see a correlation between telomere lengths of DNA isolated from circulating leukocytes and oral buccal epithelial

TABLE 4. MEAN TERMINAL RESTRICTION FRAGMENT LENGTHS IN CASE SUBJECTS AND CONTROL SUBJECTS

		TERT/*TERC* Mutations			
		Present:	Absent		
	Control Subjects	PF Case Subjects	Familial PF Probands*	Sporadic PF Case Subjects*	All Case Subjects*
Male	5.84 kb (n = 99)	4.30 kb (n = 17)	5.14 kb (n = 34)	5.25 kb (n = 41)	5.25 kb (n = 75)
Female	6.03 kb (n = 102)	4.57 kb (n = 3)	5.40 kb (n = 25)	5.58 kb (n = 32)	5.45 kb (n = 57)
P Value†	0.02	0.20	0.04	0.08	0.004

Definition of abbreviation: PF = pulmonary fibrosis.

* Subjects in this group do not have any detectable mutations in either *TERT* or *TERC*.

† *P* Values were calculated by multiple regression with age included as a covariate.

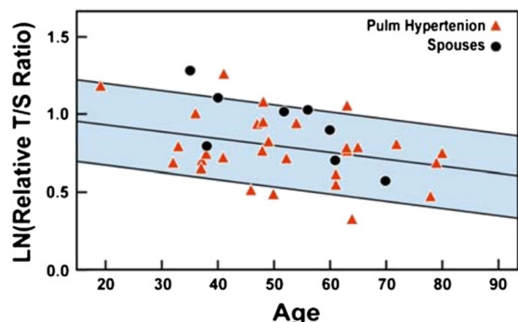

Figure 6. Telomere length as determined by the quantitative polymerase chain reaction assay for patients with pulmonary arterial hypertension and control subjects plotted against age. *Red triangles* represent unrelated cases of idiopathic, familial, or anorexigen-associated pulmonary arterial hypertension; *solid circles* represent available spouse control subjects. The mean age of both the case subjects and spouses is 52 years. This cohort of pulmonary hypertension case subjects includes 83% female and 17% male patients of the following ethnicities: white (75%), black (7%), Hispanic (10%), and other (7%).

cells (*see* Figure E4), suggesting that the germline mutations have a global effect on telomere shortening.

Because most of the case subjects in this study have been collected or referred from lung transplantation centers, many are severely affected, having demonstrated progressive worsening of disease despite treatment or withdrawal from presumed culprit exposures. An important question concerns whether mutations in telomerase or telomere length offer any prognostic information regarding the natural history of the disease. Although we have found that more subjects with the telomerase mutation had died or undergone transplantation over the course of this study than those without mutations in *TERT* or *TERC* (50 vs. 32%), these results are not statistically significant. Similarly, there is a trend in that more familial and sporadic case subjects without telomerase mutations below the 10th percentile had died or undergone lung transplantation than those with telomere lengths greater than the 10th percentile (43–53% vs. 25–31%), but these trends are not significant in both groups. We did not find a significant correlation between telomere length and diffusion capacity measurements for these individuals. Prospective studies and/or analysis of larger cohorts will be needed to determine whether telomerase mutations or telomere shortening are associated with rate of progression or severity of the pulmonary fibrosis phenotype.

Smoking is a known risk factor for IPF (32) and for the development of interstitial lung disease in at-risk individuals in kindreds with the familial form of the disease (30, 31). Those with mutations in *TERT* or *TERC* had a lower cumulative amount of cigarette smoking than those without mutations, 12.5 versus 19.2 pack-years. It may be that smoking of any level (even small amounts) may increase the risk of developing pulmonary fibrosis in those with an inherited predisposition. Similar results have been found in studies of carriers of a major lung cancer susceptibility locus identified by linkage analysis (33). Cigarette smoking and other environmental effects are likely important modifiers for the development of organ-specific disease in subjects with a global risk of telomerase dysfunction.

One of the mutations reported in this study, V694M, was identified in a 60-year-old smoker with no evidence of bone marrow dysfunction and whose 80-year-old mother had a history of pulmonary fibrosis. This same mutation has been reported in a 34-year-old man with moderate aplastic anemia that did not respond to immunosuppression and has short telomere lengths as measured by flow–fluorescence *in situ* hybridization (8). The development of pulmonary fibrosis versus bone marrow dysfunction in telomerase mutation carriers may be related to secondary "hits," such as environmental toxins (cigarette smoking), fibrosis-prone intrinsic host mesenchymal responses to injury (34, 35), or other susceptibilities. Understanding the influences on the development of lung disease in telomerase mutation carriers will be important to delineate.

In epidemiologic studies, IPF is diagnosed more commonly in males than females (1). Of the 20 case subjects with pulmonary fibrosis and telomerase mutations for which we had available DNA, 17 are male. The male-to-female ratio of 5.7:1 in this group suggests a lower penetrance of the pulmonary fibrosis phenotype in females with these dominantly inherited telomerase mutations. Women with *TERT* mutations and pulmonary fibrosis tend to be, on average, 11.9 years older than their male counterparts (66.0 vs. 54.1 yr for women and men, respectively) and many do not fit the narrow clinical diagnosis of IPF, having apical lung-predominant pulmonary fibrosis. The male-to-female ratio is 1.4:1 and 1.3:1 for the familial and sporadic case subjects without telomerase mutations, respectively. We did observe shorter mean age-adjusted telomere lengths for the men in all groups (Table 4) and found a significant correlation between telomere length and sex after controlling for age and ethnicity by multiple regression. These findings suggest that some of the observed excess of male cases may be explained by their shorter telomere lengths.

Both assays used in this study are straightforward and can measure telomere lengths of genomic DNA samples. We included only case subjects for whom we have good-quality DNA isolated from circulating leukocytes. The TRFL assay provides an indirect, rather than a direct, measure of telomere length because the undigested lengths of DNA contain telomeres and subtelomeric segments. To minimize the size of subtelomeric sequences, we digested the genomic DNA with six different 4-bp restriction enzymes. We observed a comparable rate of telomere length attrition with age (17 bp/yr) in normal individuals of this age range, as have other investigators who have used this same method (18–22). The quantitative PCR measurement of telomere length offers an independent method for estimating telomere length from genomic DNA and we found a good correlation between these two methods. It had been previously shown that a quantitative PCR method for estimating telomere length is fast, inexpensive, and requires significantly less DNA than measurement by Southern blotting (13). From the TRFL assay, but not the quantitative PCR assay, we can estimate the percentage of short telomeres in each sample which is the most biologically relevant measure (36).

Telomere length is known to be a heritable trait with parental effects (37). Twin studies suggest that telomere size, as measured by the TRFL assay, displays a heritability of 36–78% (22, 38). The quantitative trait of TRFLs has been mapped to various loci in normal subjects (38) or in small families with heart disease (19), but the causative genetic variants within these genomic intervals have not been pinpointed. Understanding the genetic underpinnings of telomere shortening in pulmonary fibrosis may lead to a more complete understanding of how this process contributes to the risk of developing irreversible lung scarring. In addition, such studies may more clearly define the pulmonary as well as other organ phenotypes associated with telomere shortening in aging humans.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors thank the affected individuals and their families for their participation in this study; Holly Brookman, Erica Solis, and especially Melissa Nolasco for excellent technical assistance; Dr. Helen Hobbs for the

control DNA samples; David Leonard for assistance with the statistical analysis; Dr. Yolanda Mageto, Dr. Craig Glazer, Dr. Borna Mehrad, Dr. Hal Collard, Dr. Edward Garrity, Dr. Keith Meyer, Dr. Varsha Taskar, Dr. Christopher Blewett, Dr. Carlos Girod, Dr. John Fitzgerald, Martha Kingman, and Barbi Estes for patient referrals; and Helen Hobbs, Jonathan Cohen, Jerry Shay, and Woody Wright for helpful discussions.

References

1. Raghu G, Weycker D, Edelsberg J, Bradford WZ, Oster G. Incidence and prevalence of idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2006;174:810–816.
2. Tsakiri KD, Cronkhite JT, Kuan PJ, Xing C, Raghu G, Weissler JC, Rosenblatt RL, Shay JW, Garcia CK. Adult-onset pulmonary fibrosis caused by mutations in telomerase. *Proc Natl Acad Sci USA* 2007;104: 7552–7557.
3. Armanios MY, Chen JJ, Cogan JD, Alder JK, Ingersoll RG, Markin C, Lawson WE, Xie M, Vulto I, Phillips JA III, *et al.* Telomerase mutations in families with idiopathic pulmonary fibrosis. *N Engl J Med* 2007;356:1317–1326.
4. American Thoracic Society; European Respiratory Society. American Thoracic Society/European Respiratory Society international multidisciplinary consensus classification of the idiopathic interstitial pneumonias. *Am J Respir Crit Care Med* 2002;165:277–304.
5. Savage SA, Giri N, Baerlocher GM, Orr N, Lansdorp PM, Alter BP. Tinf2, a component of the shelterin telomere protection complex, is mutated in dyskeratosis congenita. *Am J Hum Genet* 2008;82:501–509.
6. Garcia CK, Wright WE, Shay JW. Human diseases of telomerase dysfunction: insights into tissue aging. *Nucleic Acids Res* 2007;35:7406–7416.
7. Vulliamy TJ, Knight SW, Mason PJ, Dokal I. Very short telomeres in the peripheral blood of patients with X-linked and autosomal dyskeratosis congenita. *Blood Cells Mol Dis* 2001;27:353–357.
8. Yamaguchi H, Calado RT, Ly H, Kajigaya S, Baerlocher GM, Chanock SJ, Lansdorp PM, Young NS. Mutations in *tert*, the gene for telomerase reverse transcriptase, in aplastic anemia. *N Engl J Med* 2005;352:1413–1424.
9. Ball SE, Gibson FM, Rizzo S, Tooze JA, Marsh JC, Gordon-Smith EC. Progressive telomere shortening in aplastic anemia. *Blood* 1998;91: 3582–3592.
10. Brummendorf TH, Maciejewski JP, Mak J, Young NS, Lansdorp PM. Telomere length in leukocyte subpopulations of patients with aplastic anemia. *Blood* 2001;97:895–900.
11. Lee JJ, Kook H, Chung IJ, Na JA, Park MR, Hwang TJ, Kwak JY, Sohn SK, Kim HJ. Telomere length changes in patients with aplastic anaemia. *Br J Haematol* 2001;112:1025–1030.
12. Herbert B-S, Shay JW, Wright WE. Analysis of telomeres and telomerase. In: Bonifacino JS, Dasso M, Harford JB, Lippincott-Schwartz J, Yamada KM, editors. Current protocols in cell biology. New York: John Wiley & Sons; 2003.18.6.1–18.6.20.
13. Cawthon RM. Telomere measurement by quantitative PCR. *Nucleic Acids Res* 2002;30:e47.
14. Nordfjall K, Larefalk A, Lindgren P, Holmberg D, Roos G. Telomere length and heredity: indications of paternal inheritance. *Proc Natl Acad Sci USA* 2005;102:16374–16378.
15. Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, Harley CB, Cech TR. Telomerase catalytic subunit homologs from fission yeast and human. *Science* 1997;277:955–959.
16. Autexier C, Lue NF. The structure and function of telomerase reverse transcriptase. *Annu Rev Biochem* 2006;75:493–517.
17. Banik SS, Guo C, Smith AC, Margolis SS, Richardson DA, Tirado CA, Counter CM. C-terminal regions of the human telomerase catalytic subunit essential for *in vivo* enzyme activity. *Mol Cell Biol* 2002;22: 6234–6246.
18. Valdes AM, Richards JB, Gardner JP, Swaminathan R, Kimura M, Xiaobin L, Aviv A, Spector TD. Telomere length in leukocytes correlates with bone mineral density and is shorter in women with osteoporosis. *Osteoporos Int* 2007;18:1203–1210.
19. Vasa-Nicotera M, Brouilette S, Mangino M, Thompson JR, Braund P, Clemitson JR, Mason A, Bodycote CL, Raleigh SM, Louis E, *et al.* Mapping of a major locus that determines telomere length in humans. *Am J Hum Genet* 2005;76:147–151.
20. Brouilette S, Singh RK, Thompson JR, Goodall AH, Samani NJ. White cell telomere length and risk of premature myocardial infarction. *Arterioscler Thromb Vasc Biol* 2003;23:842–846.
21. Benetos A, Okuda K, Lajemi M, Kimura M, Thomas F, Skurnick J, Labat C, Bean K, Aviv A. Telomere length as an indicator of biological aging: the gender effect and relation with pulse pressure and pulse wave velocity. *Hypertension* 2001;37:381–385.
22. Slagboom PE, Droog S, Boomsma DI. Genetic determination of telomere size in humans: a twin study of three age groups. *Am J Hum Genet* 1994;55:876–882.
23. Nawrot TS, Staessen JA, Gardner JP, Aviv A. Telomere length and possible link to X chromosome. *Lancet* 2004;363:507–510.
24. Lederer DJ, Arcasoy SM, Barr RG, Wilt JS, Bagiella E, D'Ovidio F, Sonett JR, Kawut SM. Racial and ethnic disparities in idiopathic pulmonary fibrosis: a UNOS/OPTN database analysis. *Am J Transplant* 2006;6:2436–2442.
25. Cawthon RM, Smith KR, O'Brien E, Sivatchenko A, Kerber RA. Association between telomere length in blood and mortality in people aged 60 years or older. *Lancet* 2003;361:393–395.
26. van der Harst P, van der Steege G, de Boer RA, Voors AA, Hall AS, Mulder MJ, van Gilst WH, van Veldhuisen DJ. Telomere length of circulating leukocytes is decreased in patients with chronic heart failure. *J Am Coll Cardiol* 2007;49:1459–1464.
27. Valdes AM, Andrew T, Gardner JP, Kimura M, Oelsner E, Cherkas LF, Aviv A, Spector TD. Obesity, cigarette smoking, and telomere length in women. *Lancet* 2005;366:662–664.
28. Morla M, Busquets X, Pons J, Sauleda J, MacNee W, Agusti AG. Telomere shortening in smokers with and without COPD. *Eur Respir J* 2006;27:525–528.
29. Canela A, Vera E, Klatt P, Blasco MA. High-throughput telomere length quantification by fish and its application to human population studies. *Proc Natl Acad Sci USA* 2007;104:5300–5305.
30. Rosas IO, Ren P, Avila NA, Chow CK, Franks TJ, Travis WD, McCoy JP Jr, May RM, Wu HP, Nguyen DM, *et al.* Early interstitial lung disease in familial pulmonary fibrosis. *Am J Respir Crit Care Med* 2007;176:698–705.
31. Steele MP, Speer MC, Loyd JE, Brown KK, Herron A, Slifer SH, Burch LH, Wahidi MM, Phillips JA III, Sporn TA, *et al.* Clinical and pathologic features of familial interstitial pneumonia. *Am J Respir Crit Care Med* 2005;172:1146–1152.
32. Baumgartner KB, Samet JM, Stidley CA, Colby TV, Waldron JA. Cigarette smoking: a risk factor for idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 1997;155:242–248.
33. Bailey-Wilson JE, Amos CI, Pinney SM, Petersen GM, de Andrade M, Wiest JS, Fain P, Schwartz AG, You M, Franklin W, *et al.* A major lung cancer susceptibility locus maps to chromosome 6q23-25. *Am J Hum Genet* 2004;75:460–474.
34. Waghray M, Cui Z, Horowitz JC, Subramanian IM, Martinez FJ, Toews GB, Thannickal VJ. Hydrogen peroxide is a diffusible paracrine signal for the induction of epithelial cell death by activated myofibroblasts. *FASEB J* 2005;19:854–856.
35. Muro AF, Moretti FA, Moore BB, Yan M, Atrasz RG, Wilke CA, Flaherty KR, Martinez FJ, Tsui JL, Sheppard D, *et al.* An essential role for fibronectin extra type III domain A in pulmonary fibrosis. *Am J Respir Crit Care Med* 2008;177:638–645.
36. Hemann MT, Strong MA, Hao LY, Greider CW. The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. *Cell* 2001;107:67–77.
37. Njajou OT, Cawthon RM, Damcott CM, Wu SH, Ott S, Garant MJ, Blackburn EH, Mitchell BD, Shuldiner AR, Hsueh WC. Telom ere length is paternally inherited and is associated with parental lifespan. *Proc Natl Acad Sci USA* 2007;104:12135–12139.
38. Andrew T, Aviv A, Falchi M, Surdulescu GL, Gardner JP, Lu X, Kimura M, Kato BS, Valdes AM, Spector TD. Mapping genetic loci that determine leukocyte telomere length in a large sample of unselected female sibling pairs. *Am J Hum Genet* 2006;78:480–486.