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Research Article **Pulmonology**

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Telomerase mutations in smokers with severe emphysema

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Introduction

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death in the United States (1). Aside from cigarette smoke, age is the major risk factor. However, only a subset of smokers, approximately 10%, develops COPD, and the clustering of emphysema in families has suggested that genetic factors explain a significant portion of this susceptibility (2). Alpha-1 antitrypsin deficiency is the known Mendelian cause for emphysema (2). It manifests autosomal recessive inheritance because of biallelic mutations in the *SERPINA1* gene and accounts for young-onset, severe COPD in 0.5%–1% of smokers of European descent (3). The other monogenic factors that underlie COPD susceptibility are not fully known (2, 4).

Telomeres are the DNA-protein structures that protect chromosome ends. Telomeres shorten with cell division and advancing age, and short dysfunctional telomeres signal a DNA damage response that provokes cellular senescence and apoptosis (5, 6). Telomerase is the specialized polymerase that synthesizes new telomere repeats (7–9). It has two core components: TERT, the telomerase reverse transcriptase, and TR, the telomerase RNA that provides the template for telomere repeat addition (9, 10). Mutations in *TERT* and *TR* cause telomerase haploinsufficiency, and the consequent short telomere defect is most frequently recognized in clinical settings as autosomal dominant pulmonary fibrosis (6). Even though lung disease is the primary life-threatening presentation in these patients, the telomere defect is systemic and can manifest concurrently in a predictable, syndromic pattern that includes bone marrow failure, liver disease, and osteoporosis (11, 12). Because extrapulmonary telomere-mediated disease can cause significant morbidity, recognizing this subset of pulmonary fibrosis patients has been shown to be relevant for treatment decisions in several settings (6, 12, 13).

We recently sought to understand the mechanisms by which telomere dysfunction causes pulmonary fibrosis by studying telomerase-null mice with short telomeres. Although these mice have no obvious de novo lung defects, they develop emphysema after chronic cigarette smoke exposure (14). Coincident with these findings, we identified a family with a deleterious *TR* mutation that included two female siblings who developed premature emphysema, and emphysema combined with fibrosis, after modest smoking histories (14). Another family was recently reported that had a similar clustering of emphysema with fibrosis in association with a *TERT* mutation (15). The observations in mice, in light of these anecdotal cases, led us to systematically test the hypothesis that mutations in telomerase may be a risk factor for emphysema in unselected populations. Using a candidate gene approach,

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Table 1. Clinical characteristics of subjects analyzed for telomerase gene sequences

	COPDGene		Lung Health Study
Subjects	Controls (*n* = 206)	Cases (*n* = 209)	(*n* = 83)
Age at FEV_1 assessment, mean yr (range)	70.4 (65–81)	57.4 (46–63)	56.8 (41–64)
Sex			
Male	92	109	45
Female	114	100	38
Ethnicity (self-reported)	European American	European American	European American
Smoking history, mean pack-years (range)	50.0 (25–120)	50.1 (10–117)	47.4 (10–129)
Smoking status[A]			
Current	41	50	72
Former	165	159	11
FEV_1 baseline, l/s (range)	2.60 (1.41–4.27)	0.93 (0.30–1.98)	2.10 (1.20–3.06)
FEV_1 5-year visit, l/s (range)	–	–	1.38 (0.69–2.01)
GOLD stage[A]			
0	206	0	0
1	0	0	0
2	0	0	0
3	0	104	82
4	0	105	1

GOLD, Global Initiative for Chronic Obstructive Lung Disease (a scale used to measure chronic obstructive lung disease severity). [A]Smoking status and GOLD stage are from year 5 data for the Lung Health Study subjects. FEV_1, forced expiratory volume in 1 second.

in two cohorts of smokers with severe emphysema/COPD, along with pedigree data from a Johns Hopkins–based study, we show that deleterious telomerase mutations are a risk factor for COPD and may occur at a frequency that is similar to alpha-1 antitrypsin deficiency in individuals with severe, early-onset disease.

Results

Rare TERT variants cluster with the severe COPD phenotype. To test whether telomerase mutations are a risk factor for emphysema, we examined the telomerase gene sequences in exome data from the COPDGene cohort, a study designed to understand the genetic determinants of COPD susceptibility. These data have been recently made accessible through the National Heart Lung and Blood Institute (NHLBI) Exome Sequencing Project (Lung GO) (16). The clinical characteristics of the control group (*n* = 206) and emphysema subjects (*n* = 209) are summarized in Table 1. We designed a filtering strategy to identify novel or rare variants that could then be functionally examined (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI78554DS1). In the control group, one rare *TERT* variant, Arg653Cys, was identified; however, this missense substitution did not affect telomerase catalytic activity (97% ± 5% of wild-type telomerase activity ± SEM, *P* = 0.49, Student's *t* test, Supplemental Figure 2). In contrast, among the smokers with emphysema, there were two heterozygous missense variants in *TERT*: Arg599Gln and Thr726Met (2 of 209, 1%) that we subsequently found to be functionally deleterious, as shown below. The Arg599Gln variant was, to our knowledge, novel, and Thr726Met was previously reported in a child with bone marrow failure (17). We obtained archived DNA from the COPDGene parent study and confirmed the presence of these *TERT* variants by Sanger sequencing (Figure 1A).

We next tested the frequency of telomerase mutations in a second cohort of smokers with severe COPD. We selected cases from the Lung Health Study (18) that matched the criteria of age and forced expiratory volume in 1 second (FEV_1) used in the COPDGene exome sequencing study and examined the *TERT* and *TR* sequences along with the severe alpha-1 antitrypsin deficiency *SERPINA1* alleles (Table 1). Among 83 cases that fulfilled these criteria, we found no homozygous *SERPINA1* mutations, but we identified an individual with a heterozygous missense *TERT* mutation: His925Gln (Figure 1A). This mutation was previously reported in several members of a family with pulmonary fibrosis and liver disease (19). In total, across the two COPD cohorts, there were three *TERT* variants among the severe COPD cases (3 of 292, 1%), and all of them fell in conserved motifs within the telomerase reverse transcriptase domain (Supplemental Figure 3). The *TERT* gene contains few deviations from reference sequence (20, 21), and none of these variants were found in 2,020 controls, including 1,092 from the 1000 Genomes Project (http://www.1000genomes.org/), 528 published controls (21), and 400 individuals of similar European ancestry whom we additionally sequenced (Supplemental Table 1). These data suggested a clustering of rare telomerase variants in smokers with severe emphysema.

TERT variants compromise telomerase function and telomere length. To test the functional significance of the rare variants, we reconstituted each of the TERTs in cells. We measured telomerase enzyme activity by quantifying the telomere products using the direct primer-extension assay. The three emphysema-associated *TERT* variants substantially compromised enzyme activity compared with wild-type telomerase as evidenced by the decreased intensity of the telomere repeat ladder ($P < 0.001$, Student's *t* test, Figure 1, B–D). The reduction in activity was similar to that resulting from pathogenic *TERT* and *TR* mutations documented in pulmonary fibrosis (12, 22). In contrast, the enzyme activity of the *TERT* variant from the control group was comparable to that of wild-type telomerase (Supplemental Figure 2). These data indicated that the clinical phenotype of severe emphysema enriched for individuals with rare, deleterious *TERT* mutations and that this clustering was statistically significant (3 of 292 COPD subjects [1%] vs. 0 of 2,226 controls [2,020 healthy controls and 206 control smokers], *P* = 0.002, Fisher's exact test).

We examined the functional impact of the variants in vivo by measuring telomere length. In two deceased subjects we measured telomere length using archived DNA by quantitative PCR and found it was short relative to that in age-matched healthy controls (*P* = 0.018, Student's *t* test, Figure 1E) and comparable to that in *TERT* and *TR* mutation carriers with idiopathic pulmonary

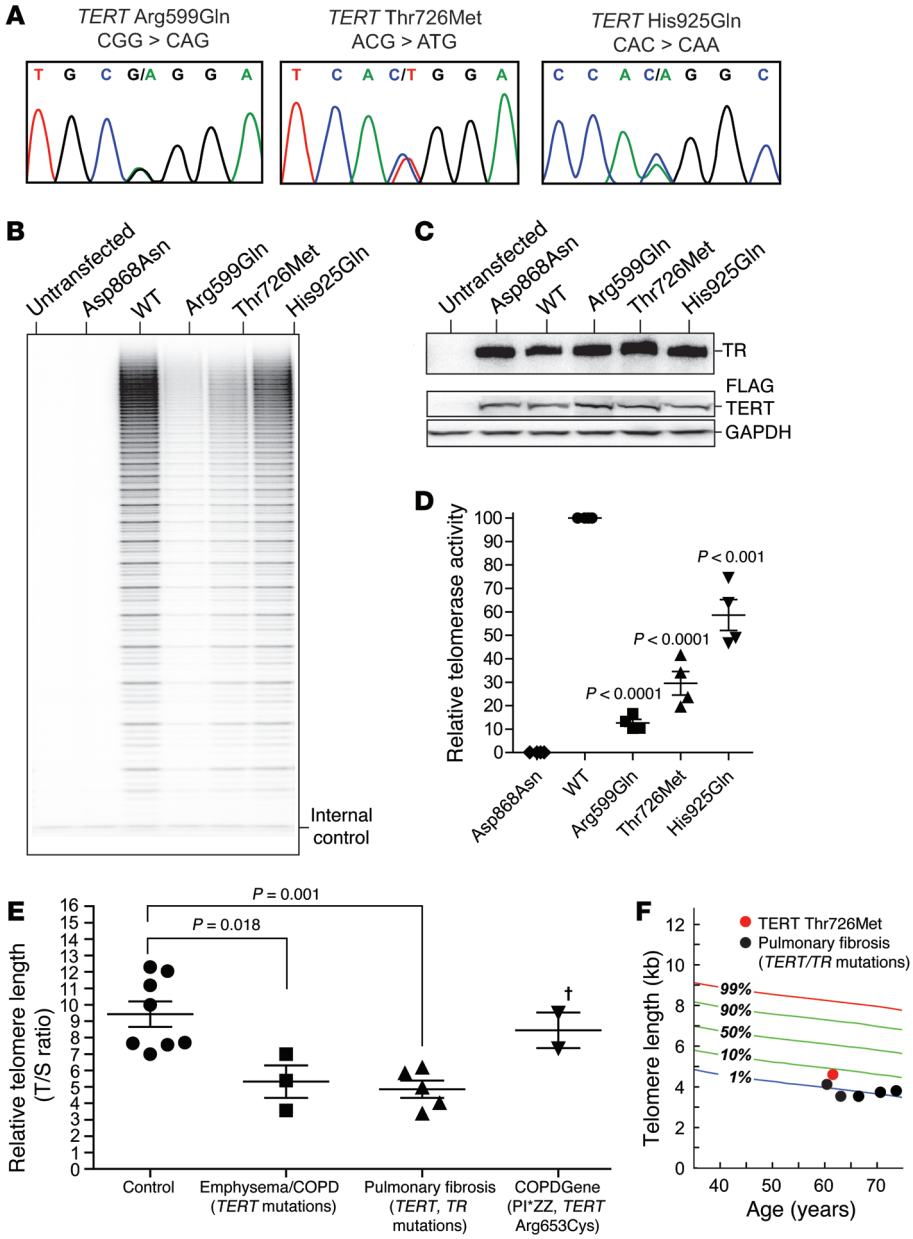


Figure 1. Functional consequences of telomerase variants identified by chronic obstructive pulmonary disease subjects. (**A**) Chromatograms of PCR-amplified products of variants identified by next-generation sequencing. (**B**) Gel image of telomere repeat ladder generated from wild-type and mutant telomerases reconstituted in vivo and immunopurified. The decreased intensity of the DNA repeat products reflects impaired enzymatic activity of TERT Arg599Gln, Thr726Met, and His925Gln. TERT Asp868Asn is a negative control, catalytically defective in one of the aspartic acid residues essential for reverse transcription. ^{32}P end-labeled 18-mer oligonucleotide was included as an internal control for the recovery of DNA products. (**C**) Northern blot for TR levels from immunopurified telomerases (top). Western blot for TERT expression in cells (bottom) was performed with anti-FLAG and anti-GAPDH antibodies for ectopically expressed FLAG-tagged TERT and endogenous GAPDH, respectively. (**D**) Mean telomerase activity was derived from 4 activity assays from cell lysates prepared from two separate transfections. (**E**) Relative telomere length as measured by quantitative PCR in age-matched controls (ages 37–64, n = 8), *TERT* mutation carriers (ages 46–57, n = 3) from the COPDGene Study and the Lung Health Study (LHS), telomerase mutation carriers with pulmonary fibrosis (ages 45–63, *TERT* n = 2, *TR* n = 3), and COPDGene controls: a homozygous *SERPINA1* Glu366Lys mutation carrier (formerly coded Glu342Lys, rs28929474, PI*ZZ genotype, age 46) and the control *TERT* Arg653Cys variant (age 68). T/S ratio, ratio of telomere repeat number to single gene copy number. †PI*ZZ mutation carrier. (**F**) Lymphocyte telomere length by flow cytometry and FISH of a *TERT* mutation carrier and telomerase mutation carriers with pulmonary fibrosis relative to a nomogram of 400 controls. Error bars represent SEM, and 2-sided P values were calculated using Student's t test.

fibrosis (P = 0.66). Telomere length in the telomerase-associated emphysema cases was also short compared with that in the control with the functionally intact *TERT* variant as well as the *SERPINA1* mutation carrier with alpha-1 antitrypsin deficiency from the COPDGene Study (Figure 1E). We additionally measured lymphocyte telomere length by flow cytometry and FISH in the one living subject we could contact and found it fell near the 10th age-adjusted percentile, similar to that in mutation carriers with idiopathic pulmonary fibrosis who were also included in the quantitative PCR telomere length analysis (Figure 1, E and F). These data supported the observation that the emphysema-associated *TERT* variants we identified were functionally deleterious.

Telomerase mutations may be associated with a more severe emphysema phenotype. We examined the clinical data available from COPDGene and the Lung Health Study records and found all three mutation carriers were female with a mean age of 48 years at diagnosis (Supplemental Table 2). Chest CT scans in the COPDGene subjects showed apical airspace destruction, and one subject had additional interstitial lung abnormalities and bronchiectasis (Figure 2, A–D). The dyspnea was severe, requiring supplemental oxygen support, and one subject died within 5 years of enrollment (Supplemental Table 2). Lung Health Study subjects had spirometry documented over 5 years, and, in the subject with the *TERT* His925Gln mutation, lung function declined faster than the lowest quartile of this 5,887-person cohort of smokers (18), suggesting an accelerated disease course (Figure 2E). Family history was not detailed in study records, but we learned that the only living subject reported her mother, who was a smoker, was oxygen-dependent for the diagnosis of COPD.

Female gender and telomerase-associated emphysema. Mutations in telomerase and short telomeres are a risk factor for pulmonary fibrosis (20), and we sought to understand the predictors of emphysema onset compared with fibrosis. We examined the clinical features of 50 telomere syndrome cases with lung disease that

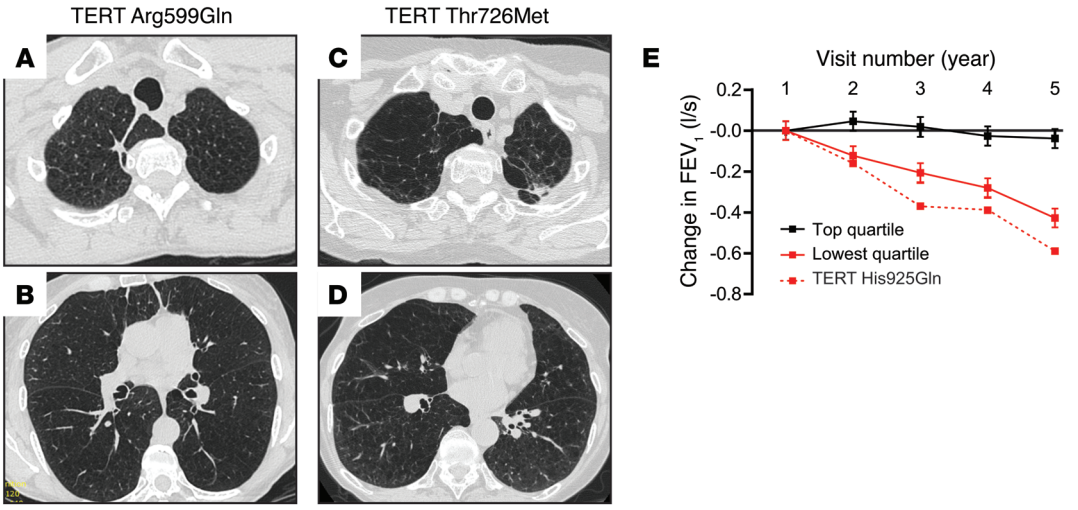


Figure 2. Radiographic and pulmonary function studies of telomerase mutation carriers with COPD. (**A**–**D**) High-resolution inspiratory CT images from COPDGene subjects with telomerase mutations. The panels are labeled with the subject's mutation. Images from *TERT* mutation carriers show apical centrilobular emphysema (**A**–**D**). In the subject with *TERT* Arg599Gln, bronchiectasis and a reticular, subpleural interstitial lung abnormality could also be appreciated (**B**). (**E**) Rate of change in forced expiratory volume in 1 second (FEV_1) from baseline across the 5 years of the Lung Health Study in the subject with a *TERT* His925Gln mutation. The rate of change is graphed relative to the highest and lowest quartiles of the 5,887 study population and based on longitudinal data available through dbGaP from 163 and 174 participants, respectively. Error bars represent SEM.

were consecutively recruited as part of a Johns Hopkins–based study (Table 2). Among never-smokers, there were no cases of emphysema (0 of 39, 0%). However, among 11 smokers, we identified 7 emphysema cases (64%). Notably, the emphysema cases were predominantly female, with all the female smokers (6 of 6, 100%) developing either emphysema alone (n = 2) or combined with fibrosis (n = 4). In contrast, only 1 in 5 male smokers had radiographic evidence of emphysema (P = 0.015, Fisher's exact test for enrichment of the emphysema phenotype in female smokers). These data suggested that short telomeres mediate a unique genetic-environmental interaction that predisposes to emphysema, but only in smokers; this interaction seems to manifest predominantly in females.

We analyzed the clinical features of telomere-associated emphysema phenotype by combining the Johns Hopkins cases with those we identified in the COPD cohorts (Table 3). In aggregate, 9 of the 10 cases were female. The average smoking history was 30 pack-years (range, 15–48), and of those who died, the mean age at the time of death from lung disease was 62 years (range, 46–68, n = 7, Table 3). Notably, 3 of 9 subjects had spontaneous pneumothorax (33%), a rare, life-threatening complication of COPD that normally affects 5% of cases (23). Relative to historic estimates, the likelihood of recurrent spontaneous pneumothorax in this genetically uniform subset occurring by chance alone is low (P = 0.016, Fisher's exact test). These clinical observations, albeit in a relatively small number, suggested that the telomere-associated COPD phenotype is associated with an increased risk for spontaneous air leaks.

Emphysema shows autosomal dominant inheritance with pulmonary fibrosis. We examined the pattern of inheritance in the 5 pedigrees of the Johns Hopkins emphysema cases. Four families carried known deleterious mutations in telomerase (12, 14, 22), including our originally reported family with the mutant *TR*, and one had classic features of a telomere syndrome (11). The emphysema phenotype in these pedigrees showed an autosomal dominant inheritance pattern with pulmonary fibrosis and other telomere phenotypes, including bone marrow failure and liver disease (Figure 3A). The severity of the telomere defect did not predict the lung disease phenotype, as both emphysema and fibrosis patients had equally short telomere length (Figure 3B). Notably, even within a single family that shared a telomerase mutation, emphysema appeared in female smokers who had an apical bleb distribution, while never-smokers developed pulmonary fibrosis (Figure 3, A–C). These data indicated that telomere-mediated emphysema

Table 2. Characteristics of consecutive telomere syndrome cases with parenchymal lung disease (n = 50)[A]

	Male	Female
Number of subjects	25	25
Age at diagnosis, mean yr (range)	53.1 (32–77)	54.2 (34–68)
Smoking status		
Never-smokers	20	19
Smokers	5	6
Lung disease type to smoking history		
Smokers with emphysema	1	6[B]
Smokers with fibrosis	4	0
Never-smokers with emphysema	0	0
Never-smokers with pulmonary fibrosis	20	19
Smoking history, mean pack-years (range)	24 (10–41)	30 (25–37)

[A]Subjects from 31 unrelated families (*TERT*, n = 15; *TR*, n = 6, other telomere gene mutation, n = 5; clinical telomere syndrome or dyskeratosis congenita, n = 5). [B]P = 0.015 for proportion of female smokers with emphysematous changes relative to males (Fisher's exact test, 2-sided).

Table 3. Clinical characteristics of telomerase mutation carriers with emphysema alone or combined with fibrosis (*n* = 10)

Age at diagnosis (yr)	Sex	Smoking history (pack-years)	Genetic diagnosis	Interstitial lung disease	Pneumothorax	Reference
34 (d.46)	Female	15	*TR* del375–377	Severe UIP	Yes	Family 2
44	Female	29	*TR* del375–377	None	Yes	Family 2
45	Female	18	*TERT* His925Gln	Imaging not collected	History not collected	Lung Health Study
48 (d.49)	Female	32	*TERT* IVS9-2 A⟶C	Severe	No	Family 4
44 (d.54)	Female	25	Telomere syndrome	Minimal UIP	No	Family 5
49 (d.62)	Female	43	*TERT* Arg599Gln	None	Yes	COPDGene
53	Female	48	*TERT* Thr726Met	None	No	COPDGene
60 (d.67)	Female	30	Telomere syndrome	None	No	Family 5
62 (d.63)	Female	37	*TR* 98G⟶A	Moderate	No	Family 1
63 (d.66)	Male	20	*TERT* Val747fsX20	Moderate UIP	No	Family 3

d., age at the time of death from lung disease; UIP, usual interstitial pneumonia, the hallmark of idiopathic pulmonary fibrosis.

manifests as an autosomal dominant trait, along with pulmonary fibrosis, but only appears in smokers.

Discussion

We report here that germline mutations in telomerase are a risk factor for severe emphysema in smokers. Because telomere dysfunction lowers the threshold to emphysema in animal models, we tested whether telomerase mutations predispose to human emphysema and found, in two independent cohorts, 1% of cases carried deleterious mutations in *TERT*. This frequency, although it constitutes a relatively small subset, is similar to that reported for alpha-1 antitrypsin deficiency in matched COPD populations (3). The emphysema-associated *TERT* variants compromised telomerase catalytic activity, and mutation carriers had abnormally short telomeres. Although a family history of pulmonary fibrosis was not detailed in the COPD cohorts we studied, in the families we fully characterized, emphysema showed autosomal dominant inheritance with pulmonary fibrosis and other telomere phenotypes. The familial clustering of emphysema with fibrosis suggests that these two lung phenotypes, heretofore considered distinct pathologies, may in some cases represent a continuum of degenerative lung disease that shares telomere dysfunction as a genetic susceptibility. For emphysema, in contrast to fibrosis, cigarette smoke exposure is a necessary second hit.

The evidence we document in human emphysema is compelling because short telomeres are a determinant of emphysema susceptibility in telomerase-null mice (14). In these animals, short telomeres lower the threshold to damage caused by cigarette smoke in epithelial cells (14). The additive damage of these "two hits" provokes a DNA damage response that causes epithelial senescence (6, 14). Senescence and the resultant loss of regenerative capacity may thus be critical events that drive the airspace destruction in telomere-mediated emphysema (J.K. Alder and M. Armanios, unpublished observations). Telomere length is normal in emphysema patients with alpha-1 antitrypsin deficiency (24). Our data, in light of these observations, indicate that telomere dysfunction may be a second, independent mechanism of emphysema susceptibility that is distinct from the protease imbalance characteristic of alpha-1 antitrypsin deficiency.

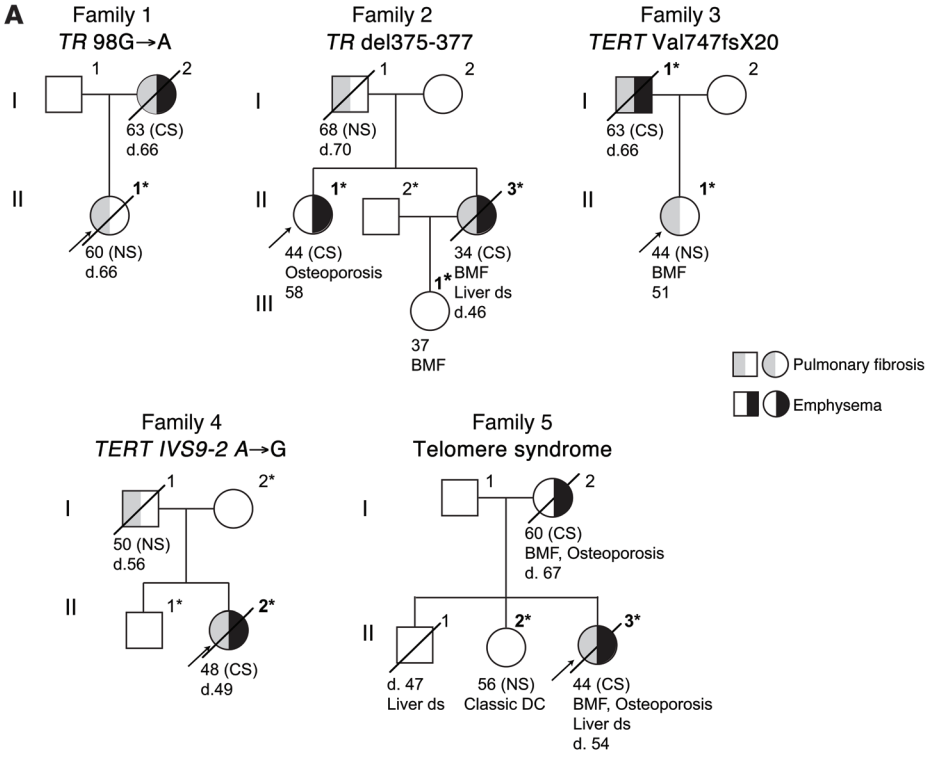
Several pieces of evidence suggest that short telomeres may play a broader role in emphysema susceptibility beyond the small subset of cases we identified. First, the coverage for *TERT* in COPDGene included only 75% of the coding sequence, similar to what has been seen previously (25). Moreover, in addition to *TERT* and *TR*, a number of other telomerase and telomere genes have been implicated in the monogenic telomere disorders (26, 27). It is therefore possible that although the telomerase genes may account for a small subset, other mutant telomere pathway genes will collectively explain a larger proportion of susceptibility. Even when telomerase is wild-type, telomere length is genetically determined, and short telomeres are sufficient to predispose to degenerative disease in the lung and elsewhere (28, 29). The prevalence of telomerase and telomere gene mutations in smokers with emphysema will require future confirmation in other cohorts.

Our findings are relevant for patient care because individuals with telomerase mutations are at risk for recurrent syndromic features including liver disease, osteoporosis, and certain malignancies (6). Some of these same telomere syndrome morbidities are known to occur at higher frequency in patients with severe emphysema (30). Our data suggest the inherited telomere defect may play a role in simultaneously predisposing to these systemic comorbidities along with the lung disease. A telomere-mediated sub-phenotype of COPD may thus require individualized clinical care algorithms. Identifying telomere syndrome patients at the bedside is particularly important in the setting of lung transplant, since some of these patients may be at increased risk for serious toxicities of immunosuppressive medications because of limited reserves in the bone marrow, gastrointestinal tract, and elsewhere (13). Relatives of telomerase mutation carriers may also be at risk for telomere syndrome complications, which may occur at an earlier age in successive generations because of genetic anticipation (26). Given the public health burden of COPD, our report suggests that emphysema may be a recurrent manifestation of telomere syndromes in populations where smoking remains prevalent.

Methods

Subjects

COPDGene study. We accessed the Database of Genotypes and Phenotypes (dbGaP; http://www.ncbi.nlm.nih.gov/gap) on March 1, 2013, after review and approval from the Johns Hopkins Medicine Insti-



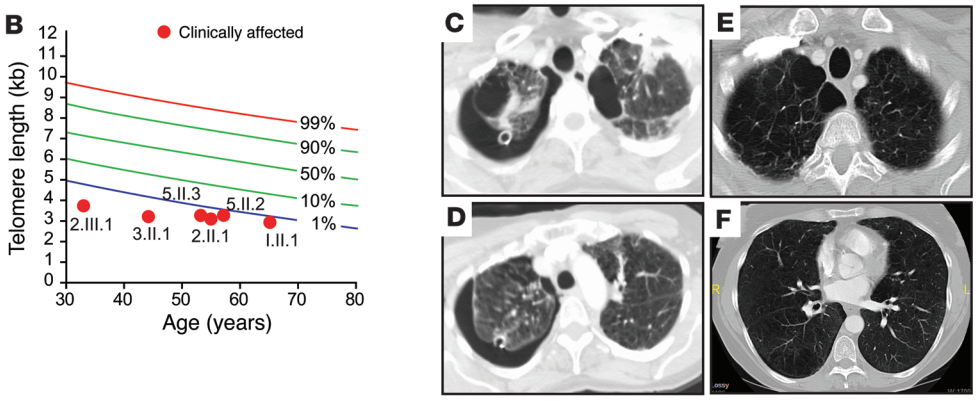


Figure 3. Pedigrees of telomere syndrome cases with emphysema. (**A**) Pedigrees of emphysema cases with telomere defects and their relatives' clinical history. The asterisk denotes individuals with DNA sequence data available and/or telomere length measurement performed. Boldface indicates individuals who carried the mutant gene and/or had very short telomeres (shown in **B**). DC, dyskeratosis congenita, a telomere syndrome defined by mucocutaneous features; CS, positive smoking history; NS, never-smoker; BMF, bone marrow failure; d., age at death from lung disease; ds, disease. (**B**) Measurement of lymphocyte telomere length by flow cytometry and FISH shows the short telomere defect in affected members relative to age-matched controls. The nomogram was based on data from 400 controls. (**C**–**F**) Apical and mid-lung chest CT cuts from two female cases (2.II.1 [**C** and **D**] and 5.II.3 [**E** and **F**]) show severe apical emphysema with blebs. In addition, 2.II.1 has a right-sided pneumothorax that arose spontaneously.

tutional Review Board and the NHLBI Data Access Committee. We analyzed the dbGaP clinical and exome data for the COPDGene study (32) and examined the *TERT*, *TR*, and *SERPINA1* gene sequences (phs000179.v3.p2 and pht002239.v2.p2.c1). Supplemental Figure 4 summarizes the depth of coverage for the coding and exon-flanking sequences of the candidate genes.

The COPDGene subjects who underwent exome sequencing (total $n = 415$) were smokers who were selected for extreme phenotypes (33). Case subjects were selected to enrich for a severe, early-onset phenotype: younger than 63 years, with severe or very severe obstruction (FEV_1 less than 50% of predicted values), and greater than 15% emphysema on CT scan ($n = 209$) (32). Controls were older than 65 years, had FEV_1 greater than 80%, and showed less than 5% emphysema on CT ($n = 206$). Quantitative chest CT scan assessment was based on the percentage of the lung with low attenuation areas below –950 Hounsfield units. Although subjects with known severe alpha-1 antitrypsin deficiency were excluded from COPDGene based on protein phenotyping, one PI*ZZ subject was inadvertently included. To verify exome variant calls, we obtained and sequenced archived DNA after review and approval of the COPDGene Ancillary Study Committee.

Lung Health Study. To test the hypothesis in a second cohort, we selected Lung Health Study subjects who fulfilled criteria similar to those in the COPDGene study for candidate gene sequencing. Smokers had FEV_1 less than 50% of predicted values and were younger than 65 years ($n = 83$). Lung Health Study participants had mild or moderate obstruction at the time of study entry (33), so we used selection criteria to identify subjects who developed severe obstruction at 5 years, the last time point at which pulmonary function was documented. The Lung Health Study longitudinal pulmonary function data were generated from dbGAP files (phs000291.v2.p1 and pht002273.v1.p1.c1) accessed on March 1, 2013.

Johns Hopkins registry. Families were recruited through the Johns Hopkins Telomere Syndrome Registry from July 1, 2005, to June 30, 2014. This study aims at understanding the genetics and natural history of telomere-mediated disease (34). The study was approved by the Johns Hopkins Medicine Institutional Review Board, and all sub-

jects gave written informed consent. Lung disease type was assessed for each of the subjects by CT imaging and review of the medical records, including pulmonary function studies and death certificates. Chest CT images were available for review in 90% of the subjects, and, in the remaining cases, chest X-rays and death certificate information were ascertained to determine the diagnosis.

Exome sequence analysis

Sequence files were annotated using publicly available software (35). Variants were prioritized for functional analysis if they were absent in 1000 Genomes (36) as well as dbSNP build 130 (http://www.ncbi.nlm.nih.gov/SNP/), an uncontaminated earlier version of variants, and additionally had less than 0.001 minor allele frequency in the Exome Variant Server Database (http://evs.gs.washington.edu/EVS). The a priori designed filtering strategy is summarized in Supplemental Figure 1. Candidate variants that fulfilled our filtering criteria from the exome data were confirmed by Sanger sequencing as previously described (22). We also manually sequenced *TR* in COPDGene subjects by PCR because of low coverage (ref. 22 and Supplemental Figure 4).

Targeted sequencing

To screen for telomerase mutations in Lung Health Study subjects, we designed and validated a TruSeq Custom Amplicon probe set (Illumina) that included the coding and flanking sequences of *TERT*, *TR*, as well as exon 6 of *SERPINA1* containing the PI*Z allele, that accounts for more than 90% of alpha-1 antitrypsin deficiency cases (3). Libraries were generated from 250 ng DNA and analyzed on a MiSeq sequencer (Illumina). Of 83 samples sequenced, 7 samples (8%) had suboptimal coverage (less than 50% at 8× depth); the coverage for the 76 samples that passed quality control is summarized in Supplemental Figure 3. Supplemental Table 3 lists common *TERT* variants found in the COPDGene and Lung Health Study subjects along with their respective dbSNP identifiers and accession numbers.

Telomere length measurement

Telomere length was measured on peripheral blood lymphocytes by flow cytometry and FISH as previously outlined (22). For deceased subjects, telomere length was measured using archived DNA by quantitative PCR (37). For these studies, control and COPDGene genomic DNA was extracted from whole blood using the Gentra Puregene method (QIAGEN). Each run included three replicates, and the mean from three independent runs was calculated.

Telomerase activity assay

The functional consequences of all the rare variants from the COPDGene and Lung Health Studies were examined using the direct telomerase activity assay. Wild-type and variant telomerases were reconstituted in vivo in 293FT cells (Invitrogen) by transient transfection with pcDNA-3xFLAG-hTERT and pBS-U1-hTR (38). The reconstituted telomerase was then immunopurified from cell lysates and analyzed by the direct primer-extension assay at previously validated physiologic nucleotide concentrations (29, 38). The 10 μl direct primer-extension reaction contained 5 μM dTTP, 5 μM dATP, 5 μM dGTP, 0.165 μM α-^{32}P-dGTP (3,000 Ci/mmol, 10 mCi/ml, Perkin-Elmer), and 1 μM $(TTAGGG)_3$ DNA primer in 1× telomerase reaction buffer (50 mM Tris-HCl pH 8.3, 2 mM DTT, 0.5 mM $MgCl_2$, and 1 mM spermidine). Comparable wild-type and mutant telomerase expression in transfected cells was confirmed by Western blotting for the FLAG-tagged TERT protein (anti-FLAG, clone M2, Sigma-Aldrich) and GAPDH (clone 6C5, Ambion) as an internal control (39). Comparable immunopurification efficiency was also confirmed by Northern blotting of TR extracted from immunopurified telomerase (40). Telomerase activity was determined by measuring the total intensity of telomerase-generated products on the gel and normalizing against the internal loading control (^{32}P end-labeled 18-mer oligonucleotide) and the TR level measured by Northern blotting from immunopurified telomerase (40). Quantification was based on 4 activity assays using cell lysates from 2 independent transfections.

Statistics

Means were compared using Student's *t* test. The Fisher's exact test was utilized to test the significance of categorical data. A 2-sided *P* value of less than 0.05 was considered statistically significant, and all *P* values shown are 2-sided.

Study approval

The study was reviewed and approved by the Johns Hopkins Medicine Institutional Review Board.

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