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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 the essential telomerase genes TERT and TR cause familial pulmonary fibrosis; however, in telomerase-null mice, short telomeres predispose to emphysema after chronic cigarette smoke exposure. Here, we tested whether telomerase mutations are a risk factor for human emphysema by examining their frequency in smokers with chronic obstructive pulmonary disease (COPD). Across two independent cohorts, we found 3 of 292 severe COPD cases carried deleterious mutations in TERT (1%). This prevalence is comparable to the frequency of alpha-1 antitrypsin deficiency documented in this population. The TERT mutations compromised telomerase catalytic activity, and mutation carriers had short telomeres. Telomerase mutation carriers with emphysema were predominantly female and had an increased incidence of pneumothorax. In families, emphysema showed an autosomal dominant inheritance pattern, along with pulmonary fibrosis and other telomere syndrome features, but manifested only in smokers. Our findings identify germline mutations in telomerase as a Mendelian risk factor for COPD susceptibility that clusters in autosomal dominant families with telomere-mediated disease including pulmonary fibrosis.

Conflict of interest: None of the authors have a direct conflict of interest that is relevant to this study. Outside the content of the study, Robert A. Wise has received consulting fees from GlaxoSmithKline, Boehringer Ingelheim, Pfizer, Merck, Genentech, MedImmune, Mylan, Janssen, Novartis, Forest Laboratories, Suneovian, Imedex, and Pulmonx. He has received grant support from GlaxoSmithKline, Boehringer Ingelheim, Forest Laboratories, and Pearl Therapeutics. In the past three years, Edwin K. Silverman has received honoraria and consulting fees from Merck and grant support and consulting fees from GlaxoSmithKline.

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

<table>
<thead>
<tr>
<th>Subjects</th>
<th>COPDGene</th>
<th>Lung Health Study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls (n = 206)</td>
<td>Cases (n = 209)</td>
</tr>
<tr>
<td>Age at FEV1, assessment, mean yr (range)</td>
<td>70.4 (65–81)</td>
<td>57.4 (46–63)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>92</td>
<td>109</td>
</tr>
<tr>
<td>Female</td>
<td>114</td>
<td>100</td>
</tr>
<tr>
<td>Ethnicity (self-reported)</td>
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<td>European American</td>
</tr>
<tr>
<td>Smoking history, mean pack-years (range)</td>
<td>50.0 (25–120)</td>
<td>50.1 (10–117)</td>
</tr>
<tr>
<td>Smoking status&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>41</td>
<td>50</td>
</tr>
<tr>
<td>Former</td>
<td>165</td>
<td>159</td>
</tr>
<tr>
<td>FEV1 baseline, l/s (range)</td>
<td>2.60 (1.41–4.27)</td>
<td>0.93 (0.30–1.98)</td>
</tr>
<tr>
<td>FEV1 5-year visit, l/s (range)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>GOLD stage&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>206</td>
<td>0</td>
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<td>3</td>
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<td>4</td>
<td>0</td>
<td>105</td>
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</table>

<sup>a</sup>Smoking status and GOLD stage are from year 5 data for the Lung Health Study subjects. FEV<sub>1</sub>, 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, Arg-653Cys, 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<sub>1</sub>) 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 fibrosis. We determined 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).
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 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.

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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 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

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**Table 2. Characteristics of consecutive telomere syndrome cases with parenchymal lung disease (n = 50)\(^a\)**

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Age at diagnosis, mean yr (range)</td>
<td>53.1 (32–77)</td>
<td>54.2 (34–68)</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never-smokers</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>Smokers</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Lung disease type to smoking history</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smokers with emphysema</td>
<td>1</td>
<td>6(^a)</td>
</tr>
<tr>
<td>Smokers with fibrosis</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Never-smokers with emphysema</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Never-smokers with pulmonary fibrosis</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>Smoking history, mean pack-years (range)</td>
<td>24 (10–41)</td>
<td>30 (25–37)</td>
</tr>
</tbody>
</table>

\(^a\)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). \(^P = 0.015\) for proportion of female smokers with emphysematous changes relative to males (Fisher’s exact test, 2-sided).
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 telomerases 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 telomerases 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 telomerases 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...
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 phs002273.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-

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**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.
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 TrueSeq Custom Amplicon probe set (Illumina) that included the coding and flanking sequences of TERT, TR, as well as exon 6 of SERPINA1 containing the PI5’ 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 DNA was extracted from whole blood using the Gentra Puregene quantitative PCR (37). For these studies, control and COPDGene genomic subjects, telomere length was measured using archived DNA by quantitative PCR because of low coverage (ref. 22 and Supplemental Figure 4).

Telomerase activity assay
The functional consequences of all the rare variants from the COPD Gene 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 μM direct primer-extension reaction contained 5 μM dTTP, 5 μM dATP, 5 μM dGTP, 0.165 μM α-32P-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 MgCl2, 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 (32P 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 were 2-sided.

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

Acknowledgments
We are grateful to all the study participants and to the investigators who contributed to these projects: COPDGene, Lung Health Study, and the NHLBI Lung GO Exome Sequencing Project. We are grateful to Carol Greider for critical comments on the manuscript. We acknowledge David Mohr and the Johns Hopkins Genetics Core Facility for assistance with the sequencing. We thank Joseph Potee for technical assistance. This work was supported by NIH grants R01 GM094450 (to J.J. Chen), K99 HL113105 (to J.K. Alder), R01 HL089856 (to E.K. Silverman), U01 HG004738 and UC2 HL102923 (to K.C. Barnes), and R01 CA160433 and R01 HL119476 (to M. Armanios); and grants from the Mary Beryl Patch Turnbull Scholar Program (to K.C. Barnes), the Flight Attendants Medical Research Institute (to M. Armanios), and the Commonwealth Foundation (to M. Armanios). S.E. Stanley received support from NIH grant T32GM007309.

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6. Armanios M. Telomeres and age-related disease: how telomere biology informs clinical para...
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