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Autoantibodies Targeting Telomere-Associated Proteins in Scleroderma and Idiopathic Pulmonary Fibrosis

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Keywords: telomere, scleroderma, interstitial lung disease, idiopathic pulmonary fibrosis

Abstract

Objectives: Scleroderma is an autoimmune fibrotic disease affecting multiple tissues including the lung. A subset of scleroderma patients with lung disease exhibit short telomeres in circulating lymphocytes, but the mechanisms underlying this observation are unclear.

Methods: Sera from the Johns Hopkins and UCSF Scleroderma Centers were screened for autoantibodies targeting telomerase and the shelterin proteins using immunoprecipitation and ELISA. We determined the relationship between autoantibodies targeting the shelterin protein TERF1 and telomere length in peripheral leukocytes measured by qPCR and flow cytometry and fluorescent in-situ hybridization (Flow-FISH). We also explored clinical associations of these autoantibodies.

Results: In a subset of scleroderma patients we identified autoantibodies targeting telomerase and the shelterin proteins that were rarely present in rheumatoid arthritis, myositis and healthy controls. TERF1 autoantibodies were present in 40/442 (9%) scleroderma patients and were associated with lung disease (OR 2.4, $p=0.04$, Fisher's exact test) and short lymphocyte telomere length. 6/6 (100%) patients with TERF1 autoantibodies in the Hopkins Cohort and 14/18 (78%) patients in the UCSF Cohort had a shorter telomere length in lymphocytes or leukocytes relative to the expected age-adjusted telomere length. TERF1 autoantibodies were present in 11/152 (7%) patients with IPF, a fibrotic lung disease believed to be mediated by telomere dysfunction.

Conclusions: Autoantibodies targeting telomere-associated proteins in a subset of scleroderma patients are associated with short lymphocyte telomere length and lung fibrosis. The specificity of these autoantibodies for scleroderma and IPF suggests that telomere dysfunction may have a distinct role in the pathogenesis of scleroderma and pulmonary fibrosis.

Introduction

Scleroderma is an autoimmune chronic fibrosing disease of unknown etiology that results in vasculopathy and multi-organ fibrosis. The disease is heterogeneous with a wide range of possible clinical manifestations that include skin thickening, interstitial lung disease (ILD), and Raynaud's phenomenon [1]. The majority of scleroderma patients develop ILD [2], which has some clinical similarities with the progressive lung scarring seen in idiopathic pulmonary fibrosis (IPF) [3,4]. Telomere dysregulation has been observed in both scleroderma and IPF [5–7], although it remains unclear if there are common mechanistic pathways underlying telomere dysfunction in these diseases.

Telomeres are repetitive nucleotide sequences that protect the ends of chromosomes from deterioration and fusions with neighboring chromosomes. Telomeres shorten with each cell division, serving as a "molecular clock" for cellular aging [8]. Telomeres are elongated by telomerase containing a telomere-specific reverse transcriptase (hTERT) that adds telomere repeat sequences to the end of telomeres. hTERT is one component of the human telomerase ribonucleoprotein (RNP), which is composed of the telomerase RNA component (hTR), hTERT, and the accessory proteins DKC1, NOP10, NHP2, and GAR1 [9]. Other proteins associate with the telomerase complex and act as regulators of telomerase function, including the six shelterin proteins TERF1, TERF2, POT1, TPP1, TIN2L, and RAP1 [10].

Telomere dysregulation is implicated in lung disease associated with IPF and autoimmune disease including scleroderma [11]. Germline mutations in hTERT or hTR are present in familial clusters of IPF, and patients with such mutations have markedly shortened telomeres [5,12,13]. The literature on telomere dysregulation in scleroderma is conflicting and heterogeneous, in part due to variability in assays used to measure telomere length. Several studies have identified a subgroup of scleroderma patients with markedly short telomeres in lymphocytes [7,14–16] who seem to be at increased risk of ILD [14,17]. The association between germline mutations in telomere-associated genes and IPF, together with the short telomeres observed in some patients with scleroderma-ILD, raises the possibility that the fibrotic lung disease observed in these two patient subgroups might be phenocopies, potentially representing the consequence of inherited and acquired defects in telomere function.

Distinct scleroderma clinical phenotypes have been defined by the presence of specific autoantibodies. These autoantibodies often target intracellular nuclear proteins that maintain chromosome structure and function, including proteins involved in mitosis, DNA replication, and DNA repair [18,19]. Subgrouping scleroderma by autoantibodies has utility in predicting clinical manifestations, and can provide insights into the biological mechanisms underlying this disease [20]. Since telomere lengths are relatively

short in a subset of patients with scleroderma, we hypothesized that this subgroup may be defined by an immune response with autoantibodies targeting the telomerase complex that is associated with a specific clinical phenotype. In this study, we identify autoantibodies targeting multiple telomere-associated proteins in a subset of scleroderma patients and demonstrate an association with shortened peripheral leukocyte telomere length and fibrotic lung disease.

Methods

Patient cohorts

Sera were obtained from scleroderma patients presenting for routine clinical evaluations at the Johns Hopkins (JH) and the University of California, San Francisco (UCSF) Scleroderma Centers. These two independent cohorts have similar databases and collect identical demographic and longitudinal clinical information including pulmonary function test data and organ-specific disease severity assessed by the Medsger Disease Severity Scale (supplemental 1). We also assayed sera from healthy controls and patients with myositis, rheumatoid arthritis (RA), and IPF (supplemental 2). The Institutional Review Board at Hopkins and UCSF approved this study.

Immunoprecipitation Assays for Autoantibody Detection

Cell lysate immunoprecipitation: To determine if patient sera contain autoantibodies targeting hTERT, we developed an immunoprecipitation (IP) assay using a cell lysate overexpressing telomerase. A cell line overexpressing the telomerase RNA component (hTR) and FLAG-tagged human telomerase (hTERT) was generated using a Flp-In T-Rex 293 cell line per the manufacturer's instructions (Thermo Fisher). 50 ug of cell lysate was pre-cleared with protein A beads in NP40 Lysis Buffer (supplemental 3) and immunoprecipitated with 1 ul patient serum. Immunoprecipitates were electrophoresed on SDS-PAGE gels blotted with anti-FLAG antibody (Millipore, Sigma) and visualized using enhanced chemiluminescence (ThermoFisher) in a FluorChem M chemi-luminescence imager (ProteinSimple). The data were quantitated by densitometric scanning of the blots and analyzed using ImageJ [21] . Each sample set was calibrated with the same positive reference IP that was run on each blot. The cut-off for a positive autoantibody was defined as the mean + 4 SD of the healthy controls.

IP using ^{35}S-methionine-labeled proteins: Complementary DNAs for human POT1, TPP1, TIN2L, TERF1, TERF2, RAP1, NHP2 and DKC1 (GenScript) were used to generate ^{35}S-methioinine-labeled proteins by in vitro transcription and translation (IVTT) per the manufacturer's protocol (Promega). The radiolabeled proteins were immunoprecipitated with patient sera in Lysis Buffer, and the products were electrophoresed on SDS-PAGE gels and visualized by fluorography [22].

TERF1 ELISA

The detailed ELISA protocol is in supplemental 3. ELISA plates were coated overnight at 4°C with 200 ng/well of recombinant full-length TERF1 protein (Sino Biological). Patient sera were used at 1:200 dilution and secondary antibodies were horseradish peroxidase-labeled. The color was developed using SureBlue peroxidase reagent (Seracare Life Sciences) and the absorbance was read at 450 nm. The same positive reference serum (with an optical density (OD) in the linear range) was included on every plate as a calibrator. The cutoff for autoantibody positivity was set as the mean plus 4 standard deviations of 50 healthy controls. All positive sera were re-tested by ELISA alongside an uncoated well; ODs of the uncoated wells were subtracted from those obtained with TERF1 coated wells.

Other Autoantibody Assays

The JH scleroderma sera were screened for autoantibodies targeting scleroderma-associated autoantibodies using the line immunoblot platform (EuroImmun: Systemic Sclerosis [Nucleoli] profile). U1RNP autoantibodies were assayed using a commercially available ELISA (Inova Diagnostics, CA). Euroimmun results were considered positive per the manufacturer's guidelines (supplemental 1). Autoantibodies in the UCSF cohort were derived from clinically indicated commercial testing.

Telomere Length Measurements

Two assays were used to measure telomere length: (i) A PCR-based assay measured telomere length in peripheral leukocytes from the UCSF Scleroderma Cohort as previously described [23,24] (supplemental 4); and (ii) Flow-FISH was used to measure telomere length in banked peripheral blood mononuclear cells (PBMC's) prospectively collected from a subset of the JH scleroderma cohort with and without TERF1 autoantibodies. Flow-FISH was done on all samples in batch as previously described [25,26]. Telomere lengths were compared with a validated nomogram of telomere length among healthy controls [26].

Statistics

Fisher's exact test was used to evaluate differences in the frequency of TERF1 autoantibodies between different patient cohorts. The various demographic, clinical, and serologic features of scleroderma, as well as differences in telomere length, were compared between the TERF1 autoantibody-negative and -positive patients using the Wilcoxon rank-sum test or student's t-test for continuous variables and the Fisher's exact test for dichotomous variables. All statistical analyses were 2-sided and were conducted using JMP Version 9 (SAS Institute Inc). $p<0.05$ was considered significant.

Results

Discovery Cohort: Sera from a subset of scleroderma patients IP telomerase and the shelterin proteins

To test whether scleroderma patients have autoantibodies targeting telomerase (hTERT), we screened 200 sera from the JH Scleroderma Cohort for these autoantibodies by IP using lysate made from HEK 293 cells overexpressing FLAG-tagged hTERT and telomerase RNA (hTR) [27]. The IPs were electrophoresed and hTERT was visualized by immunoblotting with anti-FLAG. Of the 200 JH scleroderma sera screened with this assay, 6 (3%) immunoprecipitated hTERT. We did not identify hTERT autoantibodies in 30 healthy control sera (Figures 1A,B).

The same 200 sera from the JH Scleroderma Cohort were screened for autoantibodies targeting the 6 shelterin proteins (POT1, TPP1, TIN2L, TERF1, TERF2, RAP1) by IP using ^{35}S-methionine-labeled protein generated by IVTT as input. 7/200 (4%) scleroderma sera immunoprecipitated either hTERT or one of the shelterin proteins, and many of these patients had multiple telomere-associated autoantibodies (Figure 1C). In contrast, 0/30 healthy controls had a shelterin autoantibody. None of the 7 patients with telomere-associated autoantibodies had autoantibodies targeting DKC1 or NHP2.

Validation Cohort: TERF1 Autoantibodies Detected by ELISA in the JH and UCSF Scleroderma Cohorts

As TERF1 was the most common of the shelterin autoantibodies and overlapped with multiple other telomere-associated autoantibodies, we developed an ELISA to screen for TERF1 autoantibodies. 5/6 patients with TERF1 autoantibodies identified by IVTT IP were positive by ELISA (all except Scl 3, Figure 1). In total the ELISA detected TERF1 autoantibodies in 22/200 (11%) of the JH Cohort. As a validation cohort, we screened by ELISA 242 sera from the UCSF Scleroderma Cohort and identified TERF1 autoantibodies in 18/242 (7%) patients. Table 1 includes demographic and clinical features of both cohorts. We expanded the number of healthy controls screened to 78, and found that the prevalence of TERF1 autoantibodies among scleroderma patients in both cohorts (40/442 [9%]) was significantly higher compared to healthy controls (1/78 [1%]), $p=0.01$).

TERF1 Autoantibodies in Other Rheumatic Diseases

To determine the specificity of TERF1 autoantibodies in scleroderma, we screened 60 RA and 60 myositis sera for TERF1 autoantibodies by ELISA. In each of the RA and myositis cohorts, 1/60 (2%) patients had a positive TERF1 autoantibody, which was similar to healthy controls (Figure 2). TERF1 autoantibodies were significantly more frequent among scleroderma patients

(JH and UCSF combined) compared to RA or myositis (40/442 [9%] vs 1/60 [2%], p=0.05).

TERF1 Autoantibodies and Telomere Length in Leukocytes

We next sought to determine if autoantibodies targeting the telomerase/shelterin complex are associated with abnormalities in telomere length. Telomere length was measured by qPCR in peripheral leukocytes from all UCSF scleroderma patients using the same banked blood draw from which the TERF1 autoantibodies were assayed. In this patient cohort, telomere length was negatively associated with patient age (F ratio = 22.0, $p<0.0001$, $R^2=0.08$) (Figure 3A). Given that telomeres shorten with a constant linear rate in middle-age [28], we calculated each patient's expected telomere length using a linear regression model based on the relationship between age and telomere length among the TERF1 autoantibody-negative patients (expected telomere length (bp)=7028 – 12.62 *[years of age]). The difference between the patient's telomere length and the expected telomere length was then calculated for each patient. Compared to patients without TERF1 autoantibodies, significantly more patients with TERF1 autoantibodies had a shorter telomere length than the expected age-adjusted telomere length (14/18 [78%] vs 96/224 [43%]), p=0.006). Furthermore, the difference between the patient telomere length and the expected age-adjusted telomere length was significantly more negative for patients with TERF1 autoantibodies compared to patients without TERF1 autoantibodies (median -230 [IQR -572 to -18] vs 53 [-272 to 304] bp, p=0.01, Wilcoxon rank-sum) (Figure 3B).

We next sought to confirm the association between TERF1 autoantibodies and short telomeres in the JH scleroderma cohort using the Flow-FISH assay. In addition to producing less variability than qPCR [15], Flow-FISH can also differentiate telomere length in lymphocytes and granulocytes. We identified 6 patients with TERF1 autoantibodies and 10 patients without TERF1 autoantibodies who presented for routine clinical visits and agreed to donate PBMC's. ELISAs performed on serum collected concurrently were used to determine TERF1 autoantibody status. Telomere length was measured on PBMC's using Flow-FISH. The delta TL (telomere length), which is the difference between the patient's telomere length and the median telomere length for a healthy person of the same age, was significantly more negative for the TERF1 autoantibody-positive patients compared to the TERF1 autoantibody-negative patients in lymphocytes (median -1132 [IQR -1552 to -996] vs -254 [-950 to 464] bp, p=0.03, Wilcoxon rank-sum). This difference was not observed in granulocytes (median -706 [IQR -1686 to 22] vs -829 [-1122 to -446] bp, p=0.8, Wilcoxon rank-sum) (Figure 4).

Clinical and Serologic Associations with TERF1 Autoantibodies in Scleroderma

After identifying the existence of TERF1 autoantibodies in scleroderma and demonstrating an association of these autoantibodies with short

telomeres in lymphocytes, we next explored associated clinical and serological features. The JH and UCSF scleroderma cohorts use standardized clinical definitions with harmonization in clinical data acquisition, enabling the evaluation of clinical associations for all 40 TERF1 autoantibody-positive and 402 TERF1 autoantibody-negative patients. Patients with TERF1 autoantibodies were less likely to be white (19/40 [48%] vs 276/399 [69%], p=0.008). The presence of TERF1 autoantibodies was significantly associated with a history of severe lung disease (OR 2.4 [CI 1.2-4.8], p= 0.04) and a lower percent predicted diffusion capacity (DLCO) within one year of serum collection (58.0 vs 67.9, p=0.02, student's t-test). There was also an association with a history of severe muscle disease (OR 3.0 [CI 1.4-6.1], p=0.005) and inflammatory arthritis (OR 2.1 [CI 1.1-4.3], p=0.04). TERF1 autoantibodies were associated with U1RNP autoantibodies in the combined cohorts (OR 4.8 [CI 2.1-10.8], p=0.0006) and Ku autoantibodies in the JH cohort (OR 5.4 [CI 1.4-20.2], p=0.02). Ku autoantibody status was not available for the UCSF cohort.

TERF1 Autoantibodies in Idiopathic Pulmonary Fibrosis

To address whether TERF1 autoantibodies might be present in other syndromes in which telomere dysfunction and lung fibrosis are prominent, we screened 152 patients with IPF and identified TERF1 autoantibodies in 11/152 (7%) patients, compared to only 1/78 (1%) positives among healthy controls (p=0.06) (Figure 6). Further details on the IPF cohort are in supplemental Table 1. The patient in the IPF cohort with the highest TERF1 autoantibody titer had a positive ANA and subsequently developed systemic symptoms of scleroderma approximately 2 years later. It was determined that this patient most likely had scleroderma-ILD rather than IPF, although the TERF1 autoantibody had preceded the other clinical features of scleroderma. The other IPF patients with TERF1 autoantibodies did not have a positive ANA and have not, to our knowledge, developed any features of a systemic autoimmune disease.

Discussion

We describe a subgroup of scleroderma patients with autoantibodies targeting the telomerase and shelterin complex, characterized by short telomeres in lymphocytes and the presence of lung disease. These autoantibodies are also present in a subset of patients with IPF, but are rarely detected in healthy controls, RA, or myositis. While prior studies have demonstrated telomere dysregulation in scleroderma [29], to our knowledge this is the first description of highly specific autoantibodies targeting the telomerase/shelterin complex in a rheumatic disease. The association of these autoantibodies with short telomeres in scleroderma, and the absence of these autoantibodies in other chronic inflammatory diseases such as RA that are also known to have telomere dysfunction [30], suggests that the

mechanism of telomere dysregulation in scleroderma may be distinct from other inflammatory diseases such as RA. The presence of germline mutations in the essential telomerase genes in familial and sporadic IPF supports a causal role for telomere dysfunction in pulmonary fibrosis [5,31]. However, germline mutations account for only a fraction of IPF cases with short telomeres, suggesting there are other mechanisms of telomere dysregulation [32]. Our findings raise the possibility that an immune response directed against telomere-associated proteins may also account for telomere shortening in a distinct subgroup of IPF patients.

Although the presence of autoantibodies targeting the shelterin protein TERF1 were highly enriched among scleroderma patients with short telomeres, the majority of patients with short telomeres did not have these autoantibodies. Therefore there may be multiple distinct mechanisms leading to short telomeres in scleroderma, and the presence of these autoantibodies may be indicative of one such mechanism. We speculate that the subset of patients with TERF1 autoantibodies have abnormal processing and presentation of telomere-associated proteins, leading to an immune response against the multimolecular telomere complex. In support of this hypothesis, patients developed autoantibodies targeting multiple components of the telomerase and shelterin complexes, suggesting loss of tolerance and epitope spreading across multiple related proteins [33].

The association between autoantibodies directed against telomere-associated proteins and short telomeres could also indicate that these autoantibodies exert a directly pathogenic effect on telomeres. We only observed telomere shortening in lymphocytes from scleroderma patients with TERF1 autoantibodies. It is possible that telomeres in granulocytes might be spared from telomere shortening in the setting of a pathogenic autoantibody because of the short life-span of a granulocyte (only a few days), while lymphocytes survive and circulate for weeks to months. Additional research is needed to understand the biology underlying this highly specific association between telomere-associated autoantibodies and shortened telomeres in scleroderma and the significance of these autoantibodies in IPF.

The association between pulmonary fibrosis and TERF1 autoantibodies in scleroderma is consistent with previous studies which have found more severe lung disease among scleroderma patients with short telomeres in lymphocytes, [17,34] and further supports a role for telomere dysfunction in the pathogenesis of scleroderma lung disease. The patient in the IPF cohort with the highest titer TERF1 autoantibody developed systemic symptoms consistent with scleroderma several years after enrollment, suggesting that some patients who meet diagnostic criteria for IPF actually have scleroderma lung disease. TERF1 autoantibodies may serve as an early biomarker to predict subsequent progression to a more definitive diagnosis of scleroderma. This patient was notable for having a high-titer ANA unlike the other IPF patients with TERF1 autoantibodies, suggesting that in most cases

TERF1 autoantibodies may still be an indicator of telomere dysfunction in classic IPF.

TERF1 autoantibodies were associated with anti-Ku and anti-U1RNP, specificities which are predictive of scleroderma overlap syndromes. However, most patients with TERF1 autoantibodies did not have Ku or U1RNP autoantibodies, indicating that TERF1 autoantibodies may provide non-redundant clinical information and could serve as a biomarker for an overlap phenotype as well. The protein Ku is involved in telomere capping [35,36] and it is therefore possible that Ku and TERF1 autoantibodies may both reflect underlying telomere dysfunction.

Strengths of this study are the use of two diverse and well-characterized longitudinal scleroderma cohorts and the use of two different telomere length assays. Limitations of this study are the small sample size for the Flow-FISH assay. We also screened most cohorts only for TERF1 autoantibodies, so it is conceivable that some patients without TERF1 autoantibodies may have other telomere-associated autoantibodies that were not captured.

In summary, we describe a novel subgroup of scleroderma and IPF patients with autoantibodies targeting the telomerase/shelterin complex that in scleroderma is associated with short telomeres in peripheral lymphocytes and the presence of lung disease. Telomere-associated autoantibodies may be pathogenically important in the fibrotic lung diseases with telomere dysfunction.

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	JH Scleroderma Cohort (n=200)	UCSF Scleroderma Cohort (n=242)	P value
Age (years), mean (SD)	57.9 (13.4)	54.6 (13.2)	p=0.009 **
Sex			
Female, n [%]	171 [85%]	207 [86%]	P=1.0
Male, n [%]	29 [15%]	35 [14%]	
Race			
Caucasian, n [%]	153/198 [77%]	142/241 [59%]	P<0.0001 ***
African American, n [%]	36/198 [18%]	23/241 [10%]	
Asian or Indian, n [%]	9/198 [5%]	76/241 [32%]	
Scleroderma Type			
Limited, n [%]	127 [64%]	160 [66%]	P=0.62
Diffuse, n [%]	73 [36%]	82 [34%]	
Disease Duration at time of bleed			
From onset of RP, median (IQR)	12.5 (6.6-21.4)	11.1 (5.4-20.0)	P=0.09
From onset of non-RP symptom, median (IQR)	12.1 (6.1-18.2)	9.4 (4.3-16.3)	P=0.001 **
Autoantibody status			
Centromere, n [%]	62/199 [31%]	60/241 [25%]	P=0.16
U1RNP, n [%]	17/200 [9%]	19/237 [8%]	P=0.86
Scl70, n [%]	44/199 [22%]	64/241 [27%]	P=0.32
RNA polymerase III, n [%]	39/199 [20%]	43/237 [18%]	P=0.81
Ku, n [%]	11/199 [6%]		
Clinical Features			
History of cancer (ever), n [%]	38/200 [19%]	39/242 [16%]	P=0.45
Inflammatory arthritis (ever), n [%]	40/200 [20%]	55/242 [23%]	P=0.56
Digital ulceration or gangrene (ever), n [%]	47/200 [24%]	100/242 [41%]	P<0.0001 ***
Scleroderma renal crisis (ever), n [%]	5/200 [3%]	11/242 [5%]	P=0.31
Myopathy (ever), n [%]	43/200 [22%]	23/242 [10%]	P=0.0005 ***
Max MRSS, mean (SD)	10.9 (10.4)	6.6 (7.2)	P<0.0001 ***
Severe muscle disease (ever), n [%]	51/200 [26%]	18/242 [7%]	P<0.0001 ***
Severe heart disease (ever), n [%]	53/194 [27%]	52/242 [22%]	P=0.18
Severe lung disease (ever), n [%]	59/196 [30%]	92/242 [38%]	P=0.09
Max RVSP (mmHg), mean (SD)	36.2 (11.8)	38.4 (21.3)	P=0.17
Min DLCO % predicted, mean (SD)	64.8 (20.5)	52.3 (21.3)	P<0.0001 ***
Min FVC % predicted, mean (SD)	74.4 (19.2)	70.7 (22.6)	p=0.07

Table 1 Demographics and disease characteristics of patients in the Johns Hopkins (JH) and University of California, San Francisco (UCSF) Scleroderma Cohorts. 200 patients were in the JH cohort and 242 in the UCSF Cohort. Results are depicted as median with interquartile range (IQR) and mean with standard deviation (SD). Ku autoantibody data was not available from the UCSF cohort. MRSS=modified Rodnan Skin Score. Pulmonary function test and echocardiogram data were reported as the maximum (max) right ventricular systolic pressure (RVSP) and the minimum (min) diffusion capacity (DLCO) and forced vital capacity (FVC) recorded in the longitudinal database. Wilcoxon rank-sum test or student's t-test were used for continuous variables and Fisher's exact test for dichotomous variables. * p< 0.05, **p<0.01, ***p<0.001

	TERF1 Ab positive (n= 40)	TERF1 Ab negative (n= 402)	p-value
Age (years), mean (SD)	52.6 (13.7)	56.4 (13.3)	P= 0.10
Sex, female, n [%]	34 [85%]	344 [86%]	P= 1.0
Race, Caucasian, n [%]	19/40 [48%]	276/399 [69%]	P=0.008 **
African American, n [%]	10/40 [25%]	49/399 [12%]	
Asian, n [%]	11/40 [28%]	74/399 [19%]	
Scleroderma Type, limited, n [%]	24 [60%]	263 [65%]	P=0.49
Disease duration			
From onset of RP, median (IQR)	13.9 (7.2-22.5)	11.6 (5.9-20.6)	P=0.08
From onset of non-RP symptom, median (IQR)	12.2 (8.4-17.5)	10.4 (4.5-17.1)	P=0.08
Autoantibody status			
Centromere, n [%]	10/40 [25%]	112/400 [28%]	P=0.85
U1RNP, n [%]	10/40 [25%]	26/397 [7%]	P=0.0006 ***
Scl70, n [%]	11/40 [28%]	97/400 [24%]	P=0.70
RNA polymerase III, n [%]	5/39 [13%]	77/397 [19%]	P=0.39
Ku, n [%]	4/22 [18%]	7/177 [4%]	P=0.02 *
Clinical features (ever, max/min)			
History of cancer (ever), n [%]	7/40 [18%]	70/402 [17%]	P=1.0
Inflammatory arthritis (ever), n [%]	14/40 [35%]	81/402 [20%]	P=0.04 *
Digital ulceration or gangrene (ever), n [%]	12/40 [30%]	140/402 [35%]	p=0.60
Scleroderma renal crisis (ever), n [%]	1/40 [3%]	15/402 [4%]	P=1.0
Myopathy (ever), n [%]	8/40 [20%]	58/402 [14%]	P=0.35
Max MRSS, mean (SD)	8.0 (9.3)	8.6 (9.0)	P=0.44
Severe muscle disease (ever),	13/40 [33%]	56/402 [14%]	P=0.005**

n [%]			
Severe heart disease (ever), n [%]	14/39 [36%]	91/397 [23%]	P=0.08
Severe lung disease (ever), n [%]	20/40 [50%]	131/398 [33%]	P=0.04 *
Max RVSP (mmHg), mean (SD)	39.9 (20.1)	37.3 (17.7)	P=0.46
Min DLCO % predicted, mean (SD)	53.0 (20.6)	58.4 (21.9)	P=0.13
Min FVC % predicted, mean (SD)	66.5 (20.4)	72.9 (21.2)	P=0.07
PFT's within one year of bleed date	TERF1 Ab positive (n=34)	TERF1 Ab negative (n=354)	
DLCO % predicted, mean (SD)	58.0 (22.5)	67.9 (23.4)	P=0.02 *
FVC % predicted, mean (SD)	75.0 (21.3)	80.2 (20.5)	P=0.18

Table 2. Clinical and serologic characteristics among TERF1 autoantibody positive (n=40) and negative (n=402) scleroderma patients from the Johns Hopkins (JH) and University of California, San Francisco (UCSF) Scleroderma Cohorts. Results are depicted as median with interquartile range (IQR) and mean with standard deviation (SD). MRSS=modified Rodnan Skin Score. Pulmonary function test and echocardiogram data were reported as the maximum (max) right ventricular systolic pressure (RVSP) and the minimum (min) diffusion capacity (DLCO) and forced vital capacity (FVC) recorded in the longitudinal database. Wilcoxon rank-sum test or student's t-test were used for continuous variables and Fisher's exact test for dichotomous variables. * $p < 0.05$, ** $p<0.01$, *** $p<0.001$

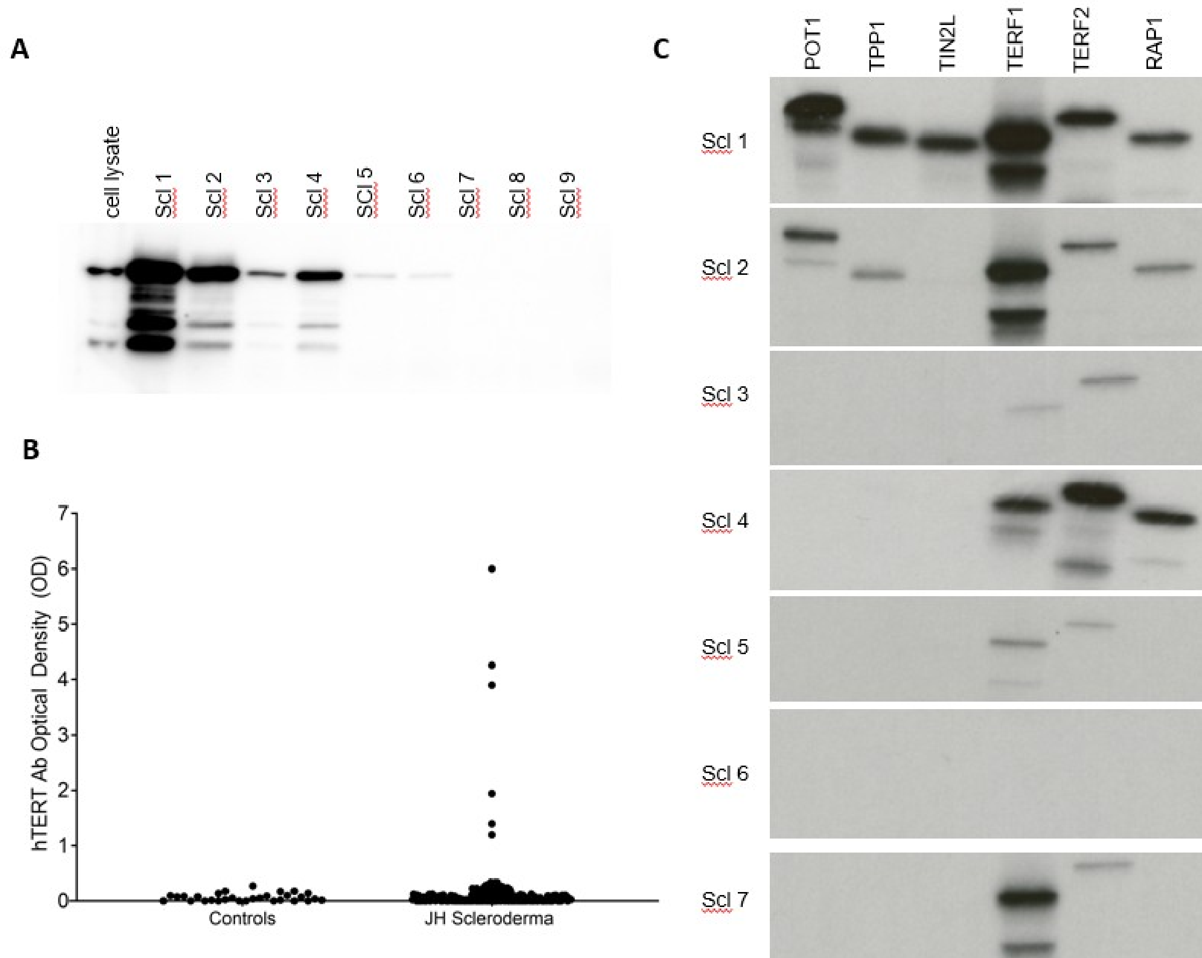


Figure 1. Autoantibodies targeting the telomerase/shelterin complex in scleroderma. A&B: Immunoprecipitations (IPs) were performed with patient sera (JH scleroderma cohort, n=200) using lysate made from HEK293 cells overexpressing hTERT-FLAG as input. IPs were detected by blotting with an anti-FLAG antibody. hTERT autoantibodies were found in 6/200 of the scleroderma patients (Scl 1-6) and 0/30 healthy controls. C: IPs were performed with patient sera using the six ^{35}S-methionine-labeled shelterin proteins generated by in vitro transcription and translation. At least one shelterin autoantibody was detected in 6/200 scleroderma patient sera. In total, 7/200 (4%) scleroderma patients either immunoprecipitated hTERT or had an autoantibody targeting at least one shelterin protein.

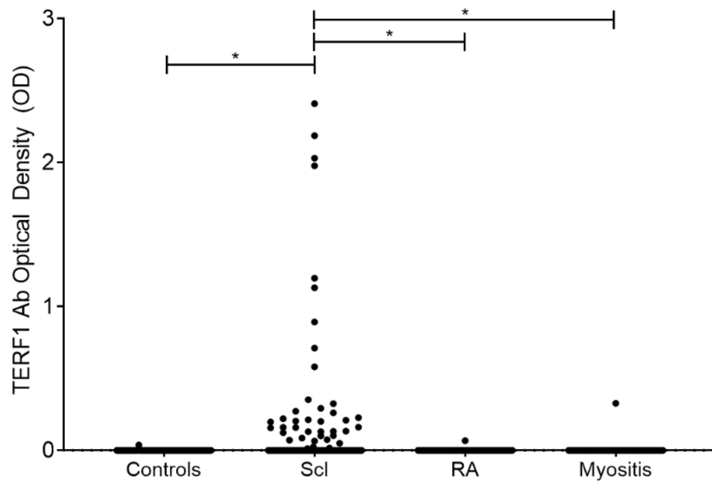
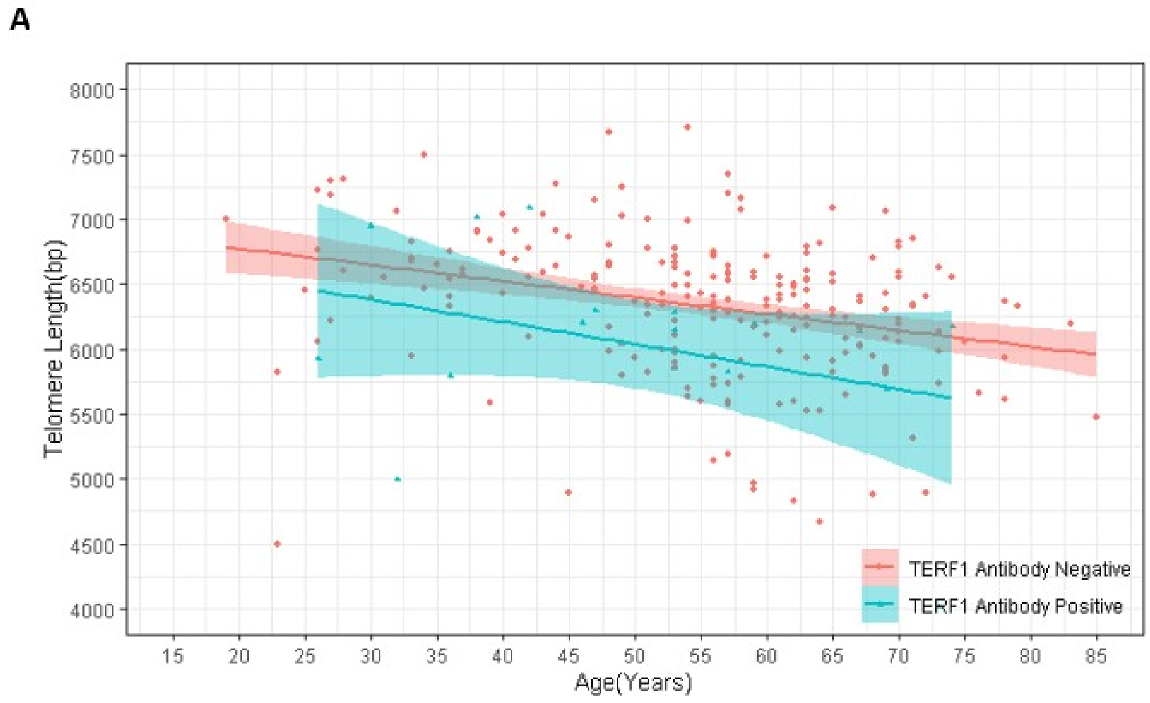


Figure 2. TERF1 autoantibodies detected by ELISA in healthy controls (n=78), the combined JH and UCSF scleroderma cohorts (Scl, n=442), rheumatoid arthritis (n=60), and myositis (n=60). Fisher's exact test was used to compare the frequency of TERF1 autoantibodies between different cohorts. * $p < 0.05$

A



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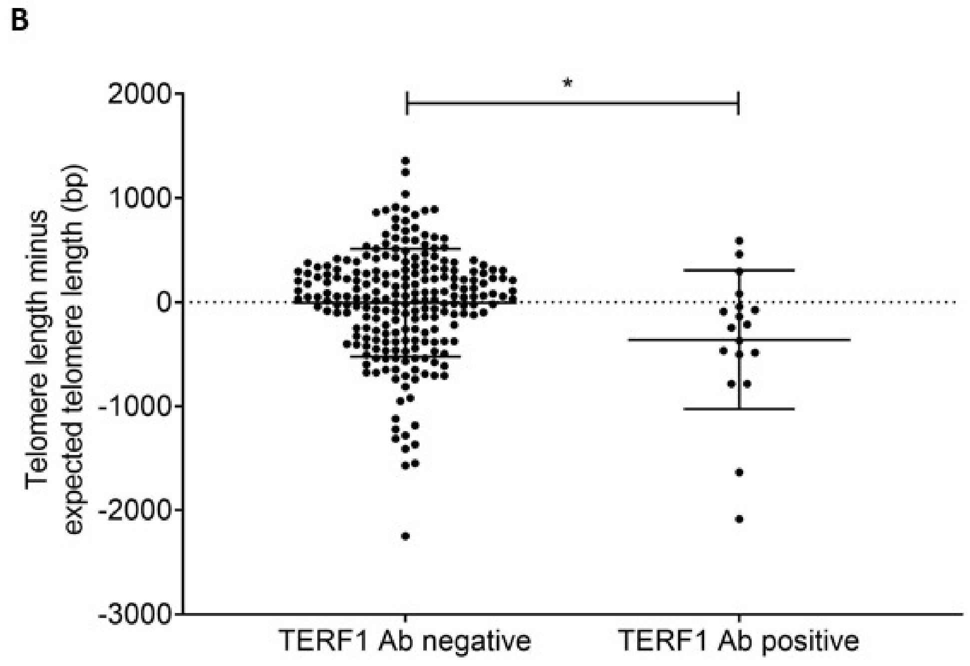

Figure 3. Peripheral blood leukocyte telomere length measured by qPCR in 242 scleroderma patients from the University of California, San Francisco (UCSF) Scleroderma Center. A: Relationship between leukocyte telomere length and age for TERF1 autoantibody positive (n=18) and negative (n=224) patients. B: Patients with TERF1 autoantibodies have a significantly shorter telomere length relative to the expected age-adjusted telomere

length compared to patients without TERF1 autoantibodies. Statistics were performed using Wilcoxon rank-sum test, * p< 0.05.

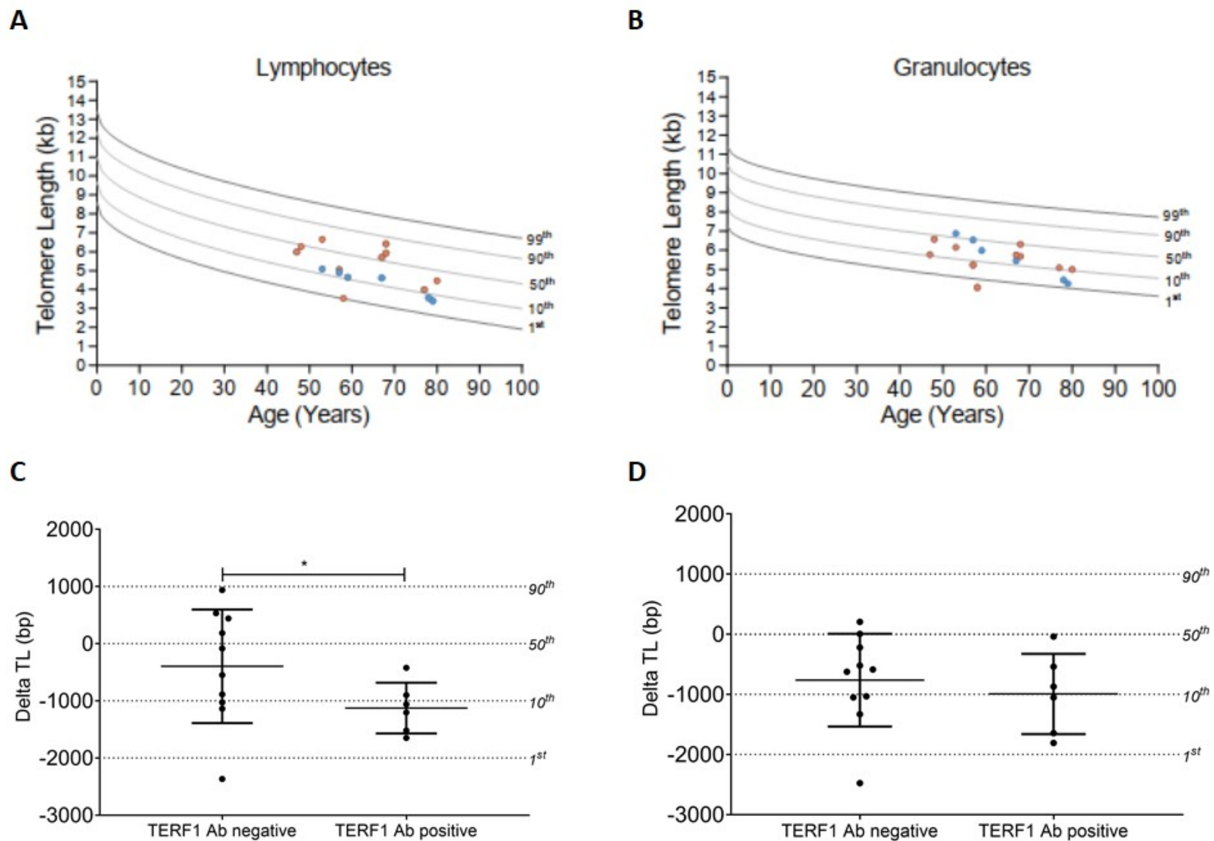

Figure 4. Telomere length measured by Flow-FISH (flow cytometry and fluorescent in-situ hybridization) in lymphocytes and granulocytes of 6 TERF1 autoantibody-positive scleroderma patients and 10 TERF1 autoantibody-negative scleroderma patients. A & B: Nomogram of telomere length relative to age in lymphocytes and granulocytes relative to a healthy control population depicted by percentiles. Patients with TERF1 autoantibodies are depicted in blue, and those without TERF1 autoantibodies are in red. C & D: Patients with TERF1 autoantibodies have shorter telomere lengths in lymphocytes (C) but not granulocytes (D) compared to TERF1 autoantibody-negative patients. Delta TL is the difference between the patient telomere length and the median telomere length of a healthy control population. kb=kilobases, bp=base pairs. Statistics were performed with Wilcoxon rank-sum test, * p < 0.05.

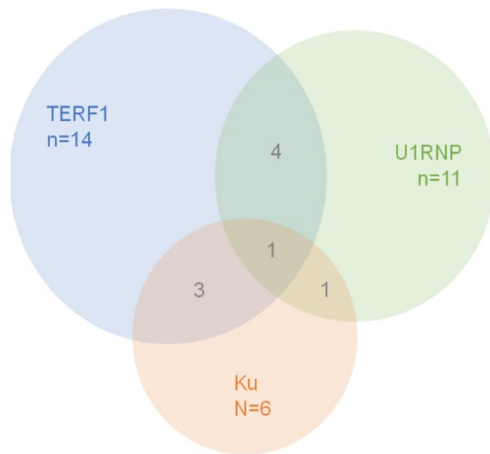

Figure 5. Overlap among TERF1 (n=22), U1RNP (n=17), and Ku (n=11) autoantibodies in the Hopkins (JH) scleroderma cohort. 40/200 (20%) of patients in the JH cohort had at least one of these autoantibodies.

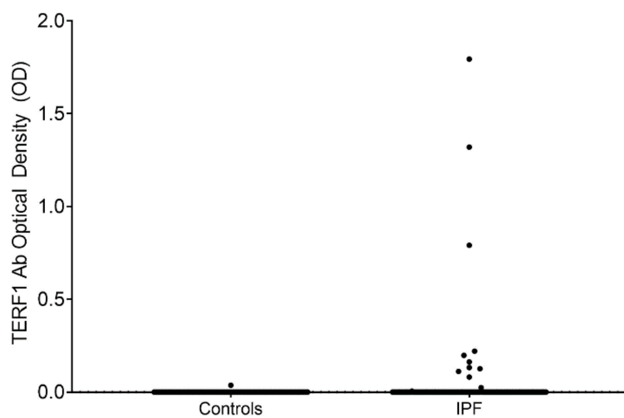

Figure 6. TERF1 autoantibodies detected by ELISA in healthy controls (n=78) and the University of California, San Francisco (UCSF) Idiopathic Pulmonary Fibrosis (IPF) cohort (n=152).

Supplemental Methods:

1. Additional information on the JH and UCSF scleroderma clinical databases: Scleroderma patients were included in this study if they were over 18 years of age and met either the 2013 ACR/EULAR criteria for systemic sclerosis, the 1980 American College of Rheumatology (ACR) criteria, or had at least three of five features of the CREST syndrome (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasias) [37]. Data included in both the JH and UCSF scleroderma databases included date of birth, sex, race, date of onset of first Raynaud's and first non-Raynaud's symptoms, history of cancer, and multiple measures of disease features and severity obtained at the first clinic visit and at 6 month intervals during follow-up visits. Organ-specific disease severity was defined by the Medsger Severity Scale [38]. Skin thickness was quantified using the modified Rodnan skin thickness score (MRSS=[range 0-51]). Longitudinal pulmonary function test (PFT) data standardized by gender and age (diffusion capacity [DLCO], forced vital capacity [FVC]) and the right ventricular systolic pressure (RVSP) measured by echocardiogram were collected. Because clinical features of scleroderma change over time, the most extreme data points recorded (max/min, ever/never) were used to fully capture disease phenotype. PFT data obtained within one year of serum collection was also analyzed.

   Medsger disease severity was defined as follows: Severe cardiac disease was defined by a Medsger score ≥ 1, severe lung disease was defined by a Medsger score ≥ 3, and severe muscle disease was defined by a Medsger score ≥ 1. The presence of myopathy was defined by the presence of muscle weakness (Medsger score ≥ 1 defined by power $\leq 4/5$ in the upper or lower extremities) as well as the presence of at least one of the following: elevation in creatinine phosphokinase (CPK), abnormal electromyography (EMG), muscle edema visualized on magnetic resonance imaging (MRI), or myopathy confirmed on muscle biopsy.

   For the Euroimmune autoantibody profile, indeterminates were considered negative. A positive anti-centromere antibody was defined as reactivity to either centromere A (CENP-A) or centromere B (CENP-B). A positive RNA polymerase III antibody was defined as reactivity to either the RP11 or RP155 subunits.

2. Information on the additional patient cohorts assayed:
   A healthy control serum biobank was collected from donors in the Johns Hopkins rheumatology division. Participants were excluded from the healthy control cohort if they were pregnant, had a history of cancer, autoimmune disease, or chronic infection with hepatitis or HIV. The myositis cohort was randomly selected from all patients in the Johns Hopkins Myositis Center who met Bohan and Peter Criteria for a diagnosis of dermatomyositis or autoimmune necrotizing myopathy [39,40]. Rheumatoid arthritis (RA) sera were obtained from a prospective cohort study of patients seen at the Johns Hopkins Arthritis center [41]. The IPF cohort was obtained from the University of California, San Francisco.

3. Additional details for immunoprecipitation assays:
   NP40 Lysis buffer used: 1% NP40, 20 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA supplemented with protease inhibitor cocktail

   Additional details for ELISA:
   96-well ELISA plates were coated overnight at 4°C with 200 ng/well of recombinant full-length TERF1 protein (Sino Biological). All washes were performed with PBS/0.05% Tween 20 (PBST) and 5% milk was used for blocking for 1 hour at room temperature. Patient sera were used at 1:200 dilution (1.5 hours, room temperature). Secondary antibodies were horseradish peroxidase-labeled (1 hour, room temperature). The color was developed using SureBlue peroxidase reagent (Seracare Life Sciences) and the absorbance was read at 450 nm.

4. PCR-based assay to measure telomere length: Peripheral blood genomic DNA was extracted using Centra Puregene cell kit (Qiagen, Valencia, CA, USA). DNA quality was assessed by agarose gel electrophoresis and degraded DNA was discarded. Peripheral blood leukocyte telomere length was measured in triplicate using quantitative uniplex qPCR relative to the housekeeping gene acidic ribosomal phosphoprotein 36B4.

	TERF1 Ab positive (n=11)	TERF1 Ab negative (n=141)	p-value
Age (years), mean (SD)	71.8 (5.6)	70.9 (8.1)	P= 0.65
Sex, female, n [%]	1 [9%]	27 [19%]	P= 0.69
Race, Caucasian, n [%]	9 [82%]	124 [88%]	P=0.63
Disease duration (years) since blood draw, median (IQR)	4.6 (2.2-7.3)	3.4 (1.4-5.3)	P=0.20
Deceased	8 [73%]	101 [72%]	P=1.0
History of smoking	7 [63%]	103 [73%]	P=0.50
Presence of usual interstitial pneumonia (UIP)	10 [91%]	119 [86%]	P=1.0
PFT's within one year of bleed date			
DLCO % predicted, mean (SD)	53.5 (9.4)	48.1 (17.2)	p=0.11
FVC % predicted, mean (SD)	67.6 (13.9)	72.0 (17.5)	P=0.37
FEV1 % predicted, mean (SD)	76.4 (15.1)	81.3 (19.7)	P=0.35

Supplemental Table 1. Clinical features of patients with idiopathic pulmonary fibrosis (IPF) with and without TERF1 autoantibodies (n=152). Pulmonary function test (PFT) data are presented as the diffusion capacity (DLCO), forced vital capacity (FVC), and forced expiratory volume (FEV1)