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ORIGINAL ARTICLE

Association between telomere length and survival in patients with idiopathic pulmonary fibrosis

JINGHONG DAI,[1,2] HOURONG CAI,[1] HUI LI,[1] YI ZHUANG,[1] HAIYAN MIN,[2] YANTING WEN,[2] JIE YANG,[1] QIAN GAO,[2] YI SHI[3] AND LONG YI[2]

[1]*Department of Respiratory Medicine, Nanjing Drum Tower Hospital Affiliated to Medical School of Nanjing University,* [2]*Jiangsu Key Laboratory of Molecular Medicine, and* [3]*Department of Respiratory and Critical Care Medicine, Jinling Hospital, Medical School of Nanjing University, Nanjing, China*

ABSTRACT

Background and objective: **Short telomere is a crucial risk factor for idiopathic pulmonary fibrosis (IPF). However, little is known about the association between baseline telomere length and survival in IPF. We aimed to determine whether telomere length is associated with survival of IPF.**

Methods: **Leukocyte telomere lengths were measured by quantitative polymerase chain reaction in IPF patients at the time of the initial enrolment assessment in Nanjing Drum Tower Hospital Affiliated to Medical School of Nanjing University. The primary endpoint was the survival of the IPF patients since their initial enrolment assessment.**

Results: **Ninety-four IPF patients were enrolled between 1 January 2012 and 30 June 2014. The mean age-adjusted telomere length of IPF patients (0.85 ± 0.60) was significantly shorter than age-matched controls (1.15 ± 0.60, $P = 0.001$). During the follow-up period, 43 IPF patients died. The mean age-adjusted telomere length of the non-survivors (0.61 ± 0.53) was significantly shorter than that of the survivors (1.03 ± 0.59, $P = 0.005$). The association between telomere length (hazard ratio (HR) 0.470 (95% confidence interval (CI): 0.25–0. 89); $P = 0{\cdot}019$) and survival in patients with IPF was independent of age, sex, forced vital capacity or diffusing capacity of carbon monoxide. After excluding the six patients with telomerase gene mutations, telomere length (HR 0.46 (95% CI: 0.24–0.88); $P = 0{\cdot}018$) remained an independent predictor of survival time in patients with IPF.**

Conclusions: **Short telomere length is independently associated with worse survival in IPF. Future research should focus on the molecular mechanism underlying the shortening of telomere length in IPF.**

Key words: age-adjusted, idiopathic pulmonary fibrosis, survival, telomerase, telomere length.

Correspondence: Long Yi, Jiangsu Key Laboratory of Molecular Medicine, Medical School of Nanjing University, 22 Hankou Road, Nanjing 210093, China. Email: yilong@nju.edu.cn.

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SUMMARY AT A GLANCE

The mean age-adjusted telomere length of IPF patients was significantly shorter than age-matched controls, so was that of non-survivors of IPF patients versus the survivors. Shorter telomere length is independently associated with worse survival in IPF.

Abbreviations: ATS/ERS, American Thoracic Society and the European Respiratory Society; CI, confidence interval; CRP, C-reactive protein; DL_{CO}, diffusing capacity for carbon monoxide; FVC, forced vital capacity; HR, hazard ratio; HRCT, high-resolution computed tomography; IPF, idiopathic pulmonary fibrosis; MMP-7, matrix metalloproteinase 7; PCR, polymerase chain reaction; WBC, white blood counts.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic disease of unknown aetiology with a variable and unpredictable course. Patients may remain stable for several years with a slow and gradual decline in pulmonary function, or experience an accelerated, often fatal course termed acute exacerbations.[1,2] Clinical and physiologic parameters have limited value in predicting development and outcome for the patient.[2] Serum biomarkers including chemokine ligand 18, KL6, surfactant protein A (SFTPA) and SFTPD have been reported to be associated with outcome in IPF.[3,4] Recently, elevated plasma concentrations of matrix metalloproteinase 7 (MMP-7), intercellular adhesion molecule 1 and interleukin 8 have been shown to be associated with poor outcomes in IPF, and when combined with clinical features, to predict mortality in IPF.[5] In addition to the potential for improving prognostic models, identifying genetic and molecular characters which are associated with IPF mortality may provide clues to explore the underlying mechanisms of this fatal disease.

Over the past years, mutations in the telomerase key components in IPF have been related to the

pathogenesis of IPF.[6,7] Telomerase is a remarkable enzyme that adds telomere repeats to the ends of chromosomes. It has two essential components: *TERT*, the telomerase reverse transcriptase, and *TERC*, a specialized RNA that contains a template for telomere repeat addition. *TERT* uses the template within *TERC* to add new (TTAGGG) repeats onto the 3′ end of chromosomes, maintaining the integrity and stability of genome and ensuring the transmission of the total length of the telomere in each cellular division. Telomere shortening occurs with each cell division and therefore reflects the organism ageing at cellular levels.[6,7] Mutations in the essential genes coding for the enzyme telomerase are the most commonly identified genetic risk factors in IPF and these mutations are thought to lead to telomere shortening.[8–10] Some previous studies reported that telomere shortening is a risk factor for the development of IPF.[11]

However, there are few studies which have investigated the effect of telomere length on IPF survival. This current prospective, population-based study aimed to determine whether telomere length, previously reported to be risk factor of IPF, is associated with survival in IPF.

METHODS

Study population

The diagnosis of IPF was made based on criterion for diagnosis in absence of surgical lung biopsy recommended by the American Thoracic Society and the European Respiratory Society (ATS\ERS).[12] Diagnosis criteria comprised: (i) insidious onset of otherwise unexplained dyspnoea on exertion; (ii) bibasilar basal or widespread inspiratory crackles; (iii) high-resolution computed tomography (HRCT) appearances compatible with usual interstitial pneumonia pattern (discussed later); and (iv) patients who had known causes leading to pulmonary fibrosis such as collagen vascular disease and environmental exposure were excluded from our study.

For each patient, medical records were thoroughly reviewed and clinical data (age, sex, smoking history, main symptoms and signs, laboratory data, etc) were extracted. The laboratory data that we recorded were white blood counts (WBC), haemoglobin, erythrocyte sedimentation rate, C-reactive protein (CRP), partial pressure of oxygen and carbon dioxide and lactate dehydrogenase. All patients underwent pulmonary function testing, percent-predicted forced vital capacity (FVC% predicted) and percent-predicted diffusing capacity for carbon monoxide (DL_{CO}% predicted) were recorded. All patients included in this study were prospectively followed up for at least 1 month until death.

As a control group, 85 unrelated age-matched healthy individuals were enrolled at the Center of Physical Examination of Drum Tower Hospital. The individuals in the control group had no current or prior history of lung diseases, as determined by medical questionnaire, physical examination and chest radiograph.

All participants provided informed consent to this study. This study was approved by the Ethical Committee of the Affiliated Drum Tower Hospital of NanJing University Medical School.

Imaging studies

All patients underwent HRCT of the chest when they were admitted to our hospital. Chest HRCT was performed using various computed tomography scanners with the patients in suspended inspiration. Two radiologists, without knowledge of any of the clinical, functional and radiographic findings, independently reviewed the HRCT of all patients. Based on recommendations made by ATS\ERS in 2000,[13] chest HRCT criteria for inclusion in the study comprised: (i) fibrosis predominantly subpleural and basal distribution; (ii) a variable pattern of reticular abnormalities and honeycombing with or without traction bronchiectasis; and (iii) the absence of profuse micronodules, discrete cysts, diffuse air-trapping or other parenchymal abnormalities.

DNA extraction

Venous blood samples were taken at the time of enrolment assessment. Extraction of DNA was performed following standard procedures using Genomic DNA Purification Kit (Life Technology Corporation, Shanghai, China). Sequencing of both *TERT* and *TERC* genes was done as previously described.[14]

Telomere length measurement

The relative repeat copy number of telomere (T) and single copy gene (36B4a) (S) were determined by real-time polymerase chain reaction (PCR) as described previously[15] with power SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) using a Step One Plus TM Real-Time PCR System (Applied Biosystems). The primer sequences were as follows: Telomere—Forward, ACACTAAGGTTTGGGTTTGGG TTTGGGTTTGGGTTAGTGT; Reverse, TGTTAGGTAT CCCTATCCCTATCCCTATCCCTATCCCTAACA; 36B4—Forward, CAGCAAGTGGGAAGGTGTAATCC; Reverse, CCCATTCTATCATCAACGGGTACAA.

Each sample was analysed in triplicate. To perform standard curves, one reference DNA was serially diluted with deionized water by twofold per dilution to create eight concentrations of DNA ranging from 1.875 to 240 ng/μL. The R2 of the standard curves were >98%, and the amplification efficiency of each pair of primers was >98%. The relative telomere length was expressed as the ratio of these two values (T/S ratio), reflecting the average telomere repeat copy number of each DNA sample calculated relative to the reference DNA.

Statistical analysis

Statistical calculations were performed using SPSS version 18.0 (SPSS Inc, Chicago, IL, USA) and adhered to predefined protocols. Relative T/S ratios were logarithm transformed to ensure that the residuals were normally distributed and had constant variance. We

estimated the association between telomere length and age using linear regression in IPF patients and age-matched controls, survivors and non-survivors. We used the estimated regression coefficients to calculate the observed minus expected, or age-adjusted, telomere length for each participant and calculated approximate age-adjusted prediction bands from the linear regression model, as previously described.[16] Continuous variables are presented as means ± standard deviation; difference between survivors and non-survivors was evaluated by unpaired *t*-test. We used Cox proportional hazards regression to test the association between telomere length and survival time in IPF. We stratified patients by quartiles of age-adjusted telomere lengths and plotted the estimated survival functions for each quartile on the basis of the Cox model with values of individual covariates fixed at the sample means. We used a multivariable model, adjusting for age, sex, FVC% predicted and DL_{CO}% predicted in patients including or excluding the patients with telomerase gene mutations. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Patient characteristics

The study included 94 consecutive patients (63 men), with a clinical and HRCT diagnosed IPF, admitted to our hospital from 1 January 2012 to 30 June 2014. The age of patients ranged from 37 to 84 years (median age 66.94 ± 9.96 years). Dyspnoea (77.6%, 73/94) and dry cough (91.4%, 86/94) were the most common symptoms which all the patients had experienced for periods ranging from 30 days to 3 years. Other complaints included expectoration (62.7%, 59/94) and chest pain (21.2%, 20/94). All patients had bibasilar crackles on auscultation and 58.5% (55/94) of patients demonstrated digital clubbing.

The age-matched control (from 45 to 78 years; median age 67.73 ± 10.41 years) consisting of 55 men and 30 women were included in this study.

Telomere length in IPF patients and controls

The mean age-adjusted telomere length of IPF patients (0.85 ± 0.50) was substantially shorter than age-matched controls (1.15 ± 0.60, $P = 0.001$). Telomere length distribution with age is depicted in Figure 1. The decline slope with age in telomere length in IPF patients ($b = -0.016$) was steeper when compared with that of the age-matched controls ($b = -0.005$).

Telomere length in survivors and non-survivors of IPF patients

The median follow-up was 26 months for all IPF patients studied. In total, 43 patients died during the follow-up. The median time to death was 6 months. The clinical characteristics of the survivors and non-survivors were described in Table 1. As shown in Table 1, the non-survivors were older and had

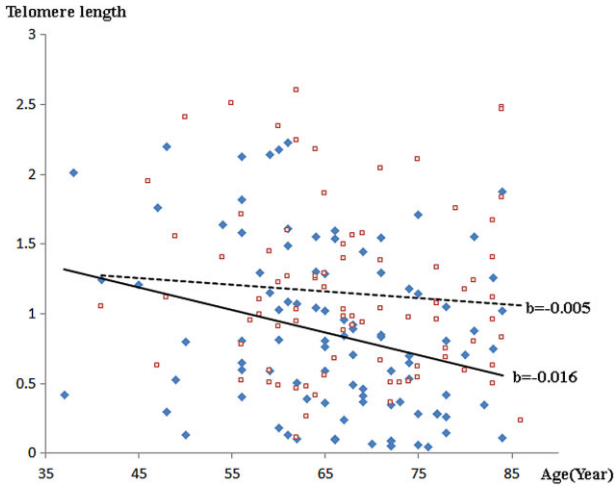


Figure 1 Age-adjusted telomere lengths in patients with idiopathic pulmonary fibrosis (IPF) and controls. The mean age-adjusted telomere length of IPF patients (0.85 ± 0.60) was substantially shorter than the mean age-adjusted telomere length of age-matched controls (1.15 ± 0.60, $P = 0.001$). The decline slope with age in telomere length in IPF patients ($b = -0.016$) was obviously steeper, when compared with that of the age-matched controls ($b = -0.005$). (◆) IPF; (□) control; (—) IPF; (- - -) control.

reduced FVC and DL_{CO} compared with survivors. The differences for CRP and WBC between survivors and non-survivors were significant. Within IPF group, the non-survivors (0.61 ± 0.53) had shorter mean age-adjusted telomere length than survivors (1.03 ± 0.59, $P = 0.005$, Fig. 2). Interestingly, the decline slope of telomere length against age in non-survivors ($b = -0.014$) was also steeper, when compared with survivors ($b = -0.008$).

Association between telomere length and survival

The patients stratified by telomere length quartiles showed a similar stepwise decrease in survival time (Fig. 3). After adjustment for relevant individual covariates (age, sex, FVC% predicted and DL_{CO}% predicted) in Cox proportional hazards model, the telomere length (hazard ratio (HR) 0.47 (95% confidence interval (CI): 0.25–0.89); $P = 0.019$) is independently associated with survival time in patients with IPF. After excluding the six patients with telomerase gene mutations, our data showed that the telomere length (HR 0.46 (95% CI: 0.24–0.88); $P = 0{\cdot}018$) remained as an independent predictor for survival time in patients with IPF (Table 2).

DISCUSSION

Pulmonary fibrosis is the most common manifestation of the telomere-mediated disorders,[8] and previous studies have reported that shortened telomeres, unrelated to germline TERT/TERC mutations, may also be a risk factor for IPF.[11] Current population-

Table 1 Comparison of demographic and clinical variables between the survivors and non-survivors in IPF patients

	IPF patients		
	Survivors Means ± SD (n = 51)	Non-survivors Means ± SD (n = 43)	P value
Sex (Male)	31	32	—
Sex (Female)	20	11	—
Age (years)	62.45 ± 10.97	70.28 ± 8.38	0.000
Telomere length	1.03 ± 0.587	0.61 ± 0.53	0.005
PO2 (mm Hg)	74.30 ± 19.54	70.81 ± 17.92	0.370
PCO_2 (mm Hg)	39.52 ± 6.90	41.99 ± 8.22	0.114
ESR (mm/h)	39.19 ± 30.83	40.05 ± 28.42	0.888
CRP *(mg/L)	18.14 ± 35.86	34.54 ± 45.10	0.050
LDH (u/L)	222.48 ± 61.50	238.14 ± 104.87	0.365
WBC ($\times 10^9/mm^3$)	7.43 ± 3.68	9.08 ± 4.01	0.038
HB (g/L)	125.94 ± 18.45	130.81 ± 18.29	0.200
FVC% predict	66.50 ± 16.66	50.44 ± 20.01	0.000
DL_{CO}% predict	54.14 ± 18.87	40.81 ± 15.20	0.000

CRP, C-reactive protein; DL_{CO}, diffusion capacity for carbon monoxide; ESR, erythrocyte sedimentation rate; FVC, forced vital capacity; HB, haemoglobin; LDH, lactate dehydrogenase; PO_2, partial pressure of oxygen; PCO_2, partial pressure of carbon dioxide; WBC, white blood counts.

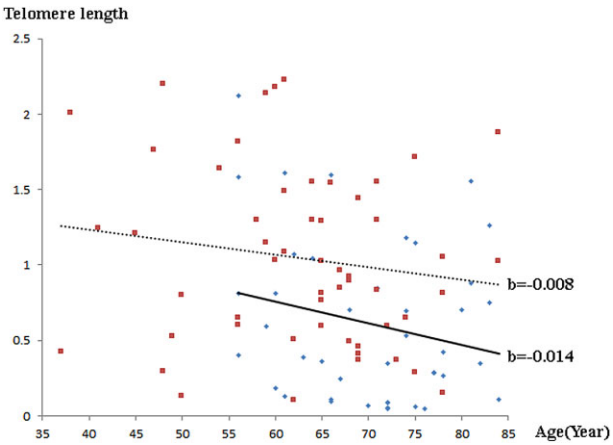
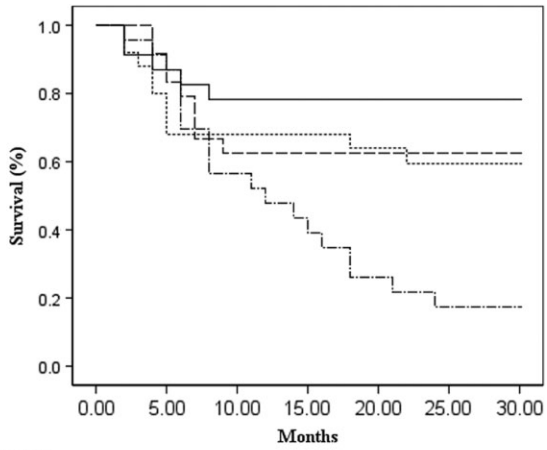


Figure 2 Age-adjusted telomere lengths in survivors and non-survivors of idiopathic pulmonary fibrosis (IPF) patients. Within the IPF group, the non-survivors (0.61 ± 0.53) had shorter mean age-adjusted telomere length than did survivors (1.03 ± 0.59, P = 0.005). The decline slope of telomere length against age in non-survivors (b = –0.014) was also steeper, when compared with survivors (b = –0.008). (◆) Non-survivor; (■) survivor; (—) non-survivor; (- - -) survivor.



Number at risk				
Q1	23	13	6	0
Q2	24	16	15	6
Q3	24	15	15	9
Q4	23	18	18	18

Figure 3 Survival time according to telomere length in patients with idiopathic pulmonary fibrosis (IPF). Survival time was estimated for patients with IPF, stratified by telomere length quartiles. (⌐¬) Q1; (⌐¬) Q2; (⌐¬) Q3; (⌐¬) Q4.

based study demonstrated that (i) the mean age-adjusted telomere length in IPF patients was significantly shorter than age-matched controls. (ii) In IPF group, the mean age-adjusted telomere length of non-survivors was significantly shorter than that of survivors. The decline slope of telomere length against age in IPF patients was steeper, when compared with age-matched controls, so was that of non-survivors of IPF patients versus the survivors, suggesting that the rate of telomere erosion may be accelerated in IPF patients and/or that telomere length may be shorter at a younger age. (iii) After adjustment for age, sex, FVC% predicted and DL_{CO}% predicted, telomere length was identified as an independent predictor for the survival of IPF.

A variety of experimental and genetic studies support the hypothesis that telomere shortening contributes to the pathogenesis of IPF. In a mouse model where telomerase deletion was engineered, it indicated that telomerase itself is not essential.[17,18] No phenotypic defects could be identified when the first

Table 2 Multivariable analysis of survival time in patients with idiopathic pulmonary fibrosis including and excluding the patients with TERT/TERC gene mutations

Cox proportional hazards model	IPF cohort including TERT/TERC gene mutations (n = 94)		IPF cohort excluding TERT/TERC gene mutations (n = 88)	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age	1.03 (0.99–1.06)	0.101	1.04 (0.99–1.07)	0.072
Female sex	0.49 (0.25–0.99)	0.047	0.47 (0.23–0.97)	0.041
FVC% predicted	0.67 (0.55–0.83)	0.000	0.71 (0.58–0.87)	0.002
DL_{CO}% predicted	0.88 (0.70–1.10)	0.272	0.87 (0.69–1.10)	0.250
Telomere length	0.47 (0.25–0.89)	0.019	0.46 (0.24–0.88)	0.018

The reported HRs are for a 1-year difference in age, a 10% difference in FVC and DL_{CO} measurements, and a 1-unit difference in log T/S ratio.

DL_{CO}, diffusion capacity for carbon monoxide; FVC, forced vital capacity; TERC, telomerase RNA; TERT, telomerase reverse transcriptase.

generation mice were null for telomerase. However, after generations breeding successively, mice with short telomeres developed degenerative disease. The phenotypes of telomerase null mice are identical to mice with deleted *TERC* or *TERT* gene, and both *TERC* and *TERT* null mice develop ageing phenotypes in case of telomeres shortening.[17,18] These investigations indicate that telomere length owing to decline in the activity of telomerase mediates degenerative disease.[18] Moreover, in fibroblast, direct telomerase inhibition increases lung fibroblast α-SMA expression *in vitro*, suggesting the potential role of telomerase in the transition of fibroblast to myofibroblast which is believed to be critical for the pathology of IPF.[19] In our study, after adjustment for age, telomere length in IPF patients was significantly shorter than in age-matched controls. In IPF patients, non-survivors had significantly shorter telomere length than survivors. The association between telomere length and survival in IPF was independent of age, sex, FVC and DL_{CO}, which was consistent with previous reports.[11,16] Our study suggested that telomere length may be associated with the pathogenesis and progression of IPF.

The clinical-radiographic-physiologic scoring systems including sex, age, FVC, and DL_{CO} have been reported to be predictive of mortality[2] and decline in FVC over 6- to 12-month period is predictive of survival.[20] In our study, we use the Cox regression model including all relevant variables (sex, age, FVC% predicted and DL_{CO}% predicted) and telomere length was identified as a significant and independent risk factor for survival of IPF. Our results are consistent with a recent study of Stuart *et al.*[16] who reported that the association between telomere length and IPF survival was robust, which was replicated in three independent IPF cohorts with differing baseline demographic, physiological and survival characteristics. Moreover, Stuart *et al.* enrolled not only patients with IPF, but also patients with collagen vascular disease and environmental exposure-associated pulmonary fibrosis, as well as other types of interstitial pneumonia, such as nonspecific interstitial pneumonia and organizing pneumonia. Stuart *et al.* reported that telomere length was not associated with survival in patients with pulmonary fibrosis that are not idiopathic, suggesting that factors other than telomere length might be more relevant to the survival in patients with pulmonary fibrosis that are associated with other diseases.[16]

Over the past decade, mutations in *TERT* and *TERC* were considered to be a risk factor for pulmonary fibrosis considering the inheritance in 8–15% of familial cases[10,21] and 1–3% of sporadic pulmonary fibrosis cases.[22] *In vitro* functional analyses indicated that these mutations led to decreased telomerase activity. Furthermore, many IPF individuals with these mutations had telomere lengths less than 10% predicted value in peripheral blood leukocytes, suggesting that mutations in telomerase exert their effect through telomere shortening.[10] In recent year, Alder *et al.* reported that telomere length is a surrogate for telomerase mutation status in families.[11] We have reported that six sporadic pulmonary fibrosis patients with six novel telomerase gene mutations had shortened telomeres compared with the patients lacking *TERT/TERC* mutation.[14] Considering the possible effect of significant reduction of telomere length in these six IPF patients with telomerase mutations, which may bias the statistical conclusion of the entire IPF group, we excluded the data obtained from these patients and again, we obtained the similar conclusion that telomere length was an independent predictor for the survival of IPF.

The current study has certain limitations. First, in our cohort, patients with IPF were enrolled from a single medical centre of a relatively small size, which in some degree weaken our conclusion. Moreover, the absence of serum or RNA samples restricted our ability to further study the associations between telomere lengths with other biomarkers, such as MMP-7 and interleukin 8.[5]

The clinical course of IPF is variable and no reliable prognostic biomarkers are currently available. Our findings establish a role for telomere shortening in IPF pathogenesis, thus shed light on the understanding of IPF. Future research should focus on the molecular mechanism underlying the shortening of telomere length in IPF and to test the possibility of

blocking the shortening of telomere as a new therapeutic target for this fatal disease.

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