Telomere Length in Cystic Fibrosis Patients - Are Patients with CF Ageing Too Quickly?

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ABSTRACT

Life expectancy for patients living with Cystic Fibrosis (CF) is increasing year on year and there is growing interest in the ageing process in CF. Telomeres are repetitive sequences of DNA that cap the ends of eukaryotic chromosomes and shorten with ongoing cell division, thus providing a marker of replicative history and biological ageing. We aimed to investigate whether telomere length as a function of age differs between patients with CF and healthy individuals and whether telomere length is associated with severity of the patient’s CF condition.

Peripheral blood samples and demographic data were collected from 47 consenting patients (age 1 to 57 years) with CF attending their routine annual review appointment at the All Wales Adult CF Centre and Noah’s Ark Children’s Hospital in Cardiff, UK. Telomere length profiles were assessed from peripheral blood samples, using the high resolution single telomere length analysis technique (STE-LA) and compared to healthy control telomere length data.

Patients with CF had significantly shorter telomere lengths than healthy individuals, when adjusting for age (p<0.001). Telomere length is decreasing 70% more quickly in the CF cohort than healthy controls. Telomere length does not appear to correlate with markers of disease severity.

Telomere lengths are significantly shorter in individuals with CF than in the age-adjusted healthy population. This is suggestive of premature biological ageing of peripheral blood leukocytes in CF patients.

Keywords: Ageing, telomere, inflammation
Introduction

Cystic fibrosis (CF) is the most common fatal inherited condition in Caucasians. With novel therapies and increasing numbers of lung transplantations being performed in recent years, there has been a significant improvement in the life expectancy of individuals with CF. Studies predict that babies born in the 21st century with CF have a median survival at least into their 50s [1, 2]. With this shift to an older population, we are starting to see a rise in age-related diseases, such as cancers, in CF [3]. This has resulted in an increased interest in studying ageing in CF.

Telomeres are repetitive sequences of DNA that cap the ends of eukaryotic chromosomes and telomere length is inextricably linked to ageing and carcinogenesis in humans. Telomeres prevent the natural ends of the chromosomes from being recognised as double-stranded DNA (dsDNA) breaks, preventing the activation of genome damage checkpoints [4]. Due to end-replication losses, telomeres shorten with each cycle of cell division, ultimately reaching a length where they become dysfunctional, triggering either cell cycle arrest, or cell death. Once dysfunctional, the unprotected chromosome ends can be subjected to fusion with other telomeres and non-telomeric dsDNA breaks [5, 6]. The ensuing genome instability can lead to large-scale genomic mutation such as non-reciprocal translocations that can drive early-stage tumour development [7-10]. Telomere length is maintained in the germline by activity of telomerase which synthesises telomere repeats de novo [11] thereby maintaining telomere length between generations. Telomere length is a heritable trait [12] and telomeres are longer in females than in males [13].

In somatic tissues, telomerase activity is limiting and thus with ongoing cell division throughout life, telomeres progressively erode as a function of age [14]. Telomere length has therefore, been widely studied as a marker of biological age and disease [15]. However it is becoming increasingly apparent that telomere dysfunction may underlie age-related tissue deterioration, pathology and longevity [14]. Given the increased longevity of CF patients, together with their concomitant frailties, we sought to investigate telomere length dynamics as a putative biomarker in CF and to evaluate its potential to assess the ageing process in these patients.

Materials and Methods

CF patients

47 patients (34 adults, 13 paediatrics), attending the All Wales Adult CF Centre (AWACFC) and Noah’s Ark Children’s’ Hospital for Wales (NACHfW) for annual review, gave written informed consent for a peripheral blood sample to be collected, in addition to the clinical sample taken as part of their standard care. Ethical approval was given by North of Scotland Research Ethics Committee (15/NS/0047). Normal peripheral blood samples were a composite of locally obtained samples, together with an additional 21 samples obtained from the Caerphilly Prospective Study (CaPS) [16]. Ethical approval for CaPS was provided by the Ethics Committee of the Division of Medicine of the former South Glamorgan Area Health Authority.

Data collection

Data collected from patient records at annual review included: age, sex, Forced Expiratory Volume 1 second percentage predicted (FEV1 %) [in patients over six years of age able to perform reliably], Body Mass Index (BMI), oxygen requirement, number of days of intravenous (IV) antibiotics in previous 12 months, C Reactive protein, pancreatic status, duration of pseudomonas aeruginosa infection and the presence of: CF-related diabetes, liver disease, CF related low bone mineral density pancreatic insufficiency or lung transplantation.

DNA extraction and single telomere length analysis

DNA was extracted from peripheral blood samples using a standard proteinase K/phenol/chloroform protocol. Telomere length analysis was performed at the XpYp telomere using the single telomere length analysis
(STELA) assay, as previously described [17]. DNA concentrations were quantified using Hoechst 33258 fluorometry, performed in duplicate. Samples were diluted to 10 ng/μl with 10 mmol/l Tris-HCl (pH 8.0). DNA was diluted further to 250 pg/μl in a volume of 40 μl, consisting of 1 μl Tel2 and 37 μl Tris-HCl. Six PCR reactions were performed per DNA sample, each run consisting of 10 μl: 1 μl 250 pg DNA, 4.98 μl H2O, 0.12 μl 100 mM NTPs, 1 μl Taq 10x buffer, 0.8 μl 25 mM MgCl2 and 0.1 μl of a 10:1 mixture of Taq and Pwo. Six polymerase chain reactions (PCR) were carried out per sample, using a Tetrad2 thermocycler (BioRad). PCR conditions were: 22 cycles of 94°C for 20 s, 65°C for 30 s and 68 oC for 8 min. DNA fragments were separated using tris-acetate-EDTA (TAE) 0.5% agarose gel electrophoresis, and detected following Southern hybridisation with a random-primed α-33P labelled TTAGGG repeat probe, along with a probe to detect the 1 Kb (Stratagene) and 2.5 Kb (Bio-Rad) marker. Hybridised fragments were detected using a Typhoon phosphorimager (GE Healthcare) and molecular weights were calculated using the Phoretix 1D quantifier (Nonlinear Dynamics, Newcastle-upon-Tyne, UK). Data on the longest, shortest, median and mean telomere length, as well as the standard deviation, were recorded for each sample.

Statistical methods
Statistical analysis was carried out using SPSS (Version 23.0) and R (Version 3.4.1). P <0.05 was considered statistically significant. Graphs were created using R. Telomere lengths were compared with patient demographics and measures of severity of their CF. Patient telomere length profiles were also compared to a group of 61 healthy individuals (controls) of a similar age. Linear regression was used to compare the relationship between age and telomere length between the CF and control groups.

Results
Patient demographics

Samples from 47 (25 male) patients with CF were analysed. Patient ages ranged from 1 to 57 years (median: 27.5 years, mean (SD) 25.7 years (12.6)). The FEV1 % of predicted ranged from 20-105% (median: 61%, mean (SD 60.9 (25.0)). Patient BMI ranged from 15.3-32.2 Kg/m² (median: 21.3, mean 22.2 (3.16)). Of the 47 patients 36 (77%) had pseudomonas lung colonisation with duration ranging from 1.0 to 30.0 years (median 12.0 years). Four patients had pulmonary colonisation with burkholderia cepacia. The number of days of IV antibiotics per annum ranged from 0 to 200 days (median 19.0 days). C Reactive protein level at time of sampling of telomere length ranged from 1 to 130 (median 4.5 (mean 12.5 with SD 25.4).

Telomere length analysis
We determined the telomere length profiles of the CF patient cohort (n=47) and normal controls (n= 61) using STELA at the XpYp telomere. Consistent with previous reports in the normal population patients with CF had significantly shorter telomere lengths than healthy individuals, when adjusting for age (p<0.001). There were gender differences in telomere length in the CF cohort; females had longer mean telomere lengths than males (mean length= 5.8 Kb Vs. 5.1 Kb, respectively (p<0.01). With an estimated mean telomere length at birth of 7.58 kb, and an age-related coefficient of -0.07 in CF patients and -0.04 in healthy individuals (adjusted R-squared = 0.479), a significant (P<0.001) rate of change was shown to occur in the erosion of telomere length (Figure. 1 and Table 1).

Figure 1. A predicted multiple linear regression model plot for the CF Patient group and normal controls. Age (years) and Telomere length (kb) X and Y-axis respectively.
The rate of telomere erosion was faster in the CF patient cohort (74 base pairs/year) compared to normal controls (40 base pairs/year; P<0.001). The rate of decrease in telomere length was 70% greater in patients with CF in comparison to healthy individuals. There was no clear correlation between telomere length and lung function (FEV1 %), BMI, genotype classification, number of IV antibiotics per annum, duration of pseudomonas colonisation or CRP (p>0.05).

Table 1: Illustrates the regression coefficients for Figure 1

| Term                          | Coef  | Se Coef | T      | P        |
|-------------------------------|-------|---------|--------|----------|
| Intercept                     | 7.582540 | 0.205453 | 36.906 | < 2e-16 *** |
| Control: Age                  | -0.043952 | 0.004840 | -9.081 | 7.06e-15 *** |
| Patient: Age                  | -0.074469 | 0.008519 | -8.742 | 4.02e-14 *** |
| Age:Methods == "Patient"TRUE | -0.030517 | 0.006943 | -4.396 | 2.65e-05 *** |
Discussion

Patients with CF displayed significantly shorter mean telomere lengths than the age-adjusted healthy population. We found CF patient telomere lengths tended to be equivalent to those observed in normal individuals at least 20 years older than themselves. This is suggestive of premature ageing in adults with CF, putting them at increased risk of age-related diseases, including cancers. Premature ageing in CF has previously been suggested \[18\]. Patients with CF are known to be at risk of premature vascular ageing \[18,19\], showing early signs of large vessel hardening approximately a decade in advance of what would be expected for chronological age. Furthermore, not only are CF patients at increased risk of malignancies, but they also tend to present at younger ages. For example, Parkins et al. \[18\] found that patients developed gastrointestinal malignancies approximately 26 years younger than in the general Caucasian population.

In the absence of a mechanistic link between the CF mutation and constitutive telomere length, we made the assumption that telomere length at birth was unlikely to be different in CF patients compared the normal population. Our data indicated two scenarios to explain the shorter telomere length profiles observed in CF patients: firstly, we considered that telomere erosion may occur more rapidly early in the life of CF patients, perhaps coincident with the time of colonisation, and that once a shorter telomere length was established, the rates of telomere erosion may be consistent with the normal population; secondly, we considered that telomere erosion was elevated in CF patients throughout their life, with an increasing difference in telomere length as a function of age. Our modelling data are consistent with the latter hypothesis, indicating an elevated rate of telomere erosion of 0.07bp/year CF patients compared to 0.04bp/year in the normal population. The mechanisms underlying the elevated rates of telomere erosion in CF patients are not clear. However we speculate that low-level, chronic systemic inflammation, commonly seen in CF due to bacterial infections such as pseudomonas aeruginosa colonisation, will drive elevated rates of telomere erosion in peripheral blood sample. Telomere length was not associated with level of CRP in this study but we acknowledge that numbers in the study are small and CRP may not reflect historical inflammatory drive but is more reflective of current inflammatory or infective status.

Chronic inflammation is a likely driver of telomere erosion as determined from peripheral blood leukocytes across a broad range of conditions \[20,21\] largely due to the increased rate of cell turnover. However, telomere driven replicative senescence has been characterised as a pro-inflammatory state \[22\] and this is particularly true of senescent CD 8 T-lymphocytes that become resistant to apoptosis and secrete pro-inflammatory cytokines, including TNFα and IL-6 \[20\]. Moreover, senescent lymphocytes are functionally compromised, by their reduced ability to express IFN, the anti-viral cytokine, and the loss of the co-stimulatory molecule CD28 required for the immune synapse. Thus telomere driven replicative senescence in lymphocyte populations, can exacerbate the inflammatory process and hasten immune-senescence. Such immune senescence may itself lead to ineffective and poorly controlled airway inflammation that subsequently fails to abolish or adequately suppress airway pathogens. This could trigger the initiation of a vicious cycle of ineffective, poorly controlled airway inflammation and immune senescence.

During normal ageing, tissue-residing senescent cells tend to accumulate, and might negatively impact their microenvironment by displaying pro-inflammatory characteristics \[22\]. It is conceivable that due to uncontrolled inflammation this ageing process is expedited in patients with CF where senescent cells fail to recruit the immune system to facilitate their own removal and actually become proinflammatory. Such senescent cells are most abundant at sites of age-related
pathologies, including degenerative disorders and malignancies and studies on progeroid mice indicate that selective elimination of such senescent cells can delay age-related deterioration [22]. This would suggest that chronic inflammation induced by senescent cells may drive pathologies such as cancer and vascular ageing which are seen more prematurely in patients with Cystic Fibrosis [18]. This is of great interest as it would suggest that modulating immune response in patients with CF may have significant systemic benefits long term.

**Limitations:**
A key weakness of this hypothesis generating study is the lack of longitudinal data. The rate of decrease in telomere length of the cohort has been derived from individuals’ single telomere reading and on the assumption that patients with CF are born with equivalent telomere lengths to the general population. We acknowledge that the study numbers are small and that the underlying aim of the study was to generate a hypothesis and to provide a basis for a wider study.

**Declarations:**

**Ethics approval and consent to participation:**
Ethical approval was given by North of Scotland Research Ethics Committee (15/NS/0047) and CF patients provided written informed consent.

**Consent for publications:** The manuscript does not contain details, images or videos relating to an individual person requiring consent for publication.

**Availability of data and material:** The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**
All authors: no reported conflicts

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**Authors Contributions**
IA, LT, JF, JAD, AS, DP and JD all contributed to the study design. All authors participated in data analysis/interpretation and critical review. IA, AS, DB and JD participated in data management and statistical analysis.

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**List of abbreviations:**
BMI: Body mass index
CF: Cystic Fibrosis
CRP: C Reactive Protein
dsDNA : Double stranded DNA
FEV1%: Forced Expiratory Volume 1 second percentage predicted
IV: Intravenous
STELA: Single telomere length analysis technique

**References**

[1]. Dodge JA, Lewis PA, Stanton M, Wilsher J. Cystic fibrosis mortality and survival in the UK: 1947-2003. Eur Respir J. 2007;29:522-6.

[2]. Simmonds NJ. Ageing in cystic fibrosis and long-term survival. Paediatr Respir Rev. 2013;14 Suppl 1:6-9.

[3]. Maisonneuve P, Marshall BC, Knapp EA, Lowenfels AB. Cancer risk in cystic fibrosis: a 20-year nationwide study from the United States. J Natl Cancer Inst. 2013;105:122-9.

[4]. de Lange, T. Shelterin: the protein complex that shapes and safeguards human telomeres. Genes Dev 2005 19, 2100-2110

[5]. Liddiard K, Ruis B, Takasugi T, Harvey A, Ashelford KE, Hendrickson EA, et al. Sister chromatid telomere fusions, but not NHEJ-mediated inter-chromosomal telomere fusions, occur independently of DNA ligases 3 and 4. Genome Res. 2016;26:588-600.

[6]. Jones RE, Oh S, Grimstead JW, Zimbrick J, Roger L, Heppel NH, et al. Escape from Telomere-Driven Crisis Is DNA Ligase III Dependent. Cell reports. 2014;8:1063-76.

[7]. Artandi SE, Chang S, Lee SL, Alson S, Gottlieb GJ, Chin L, et al. Telomere dysfunction
promotes non-reciprocal translocations and epithelial cancers in mice. Nature. 2000;406:641-5.

[8]. Lin TT, Norris K, Heppel NH, Pratt G, Allan JM, Allsup DJ, et al. Telomere dysfunction accurately predicts clinical outcome in chronic lymphocytic leukaemia, even in patients with early stage disease. Br J Haematol. 2014;167:214-23.

[9]. Roger L, Jones RE, Heppel NH, Williams GT, Sampson JR, Baird DM. Extensive telomere erosion in the initiation of colorectal adenomas and its association with chromosomal instability. J Natl Cancer Inst. 2013;105:1202-11.

[10]. Jones CH, Pepper C, Baird DM. Telomere dysfunction and its role in haematological cancer. Br J Haematol. 2012;156:573-87.

[11]. Kolquist KA, Ellisen LW, Counter CM, Meyerson M, TanLK, Weinberg RA, Haber DA, Gerald WL. 1998. Expression of TERT in early premalignant lesions and a subset of cells in normal tissues. Nat Genet 19: 182-186

[12]. Andrew T, Aviv A, Falchi M, et al. Mapping genetic loci that determine leukocyte telomere length in a large sample of unselected female sibling pairs. Am J Hum Genet. 2006;78:480-486

[13]. Vasan RS, Demissie S, Kimura M, et al. Association of leukocyte telomere length with circulating biomarkers of the renin-angiotensin-aldosterone system: the Framingham Heart Study. Circulation. 2008;117:1138-1144

[14]. Sabharwal S, Verhulst S, Guirguis G, Kark JD, Labat C, Roche NE, et al. Telomere length dynamics in early life: the blood-and-muscle model. FASEB J. 2018;32:529-34.

[15]. Blasco MA. Telomeres and human disease: ageing, cancer and beyond. Nat Rev Genet. 2005;6:611-22.

[16]. Caerphilly and Speedwell collaborative heart disease studies. The Caerphilly and Speedwell Collaborative Group™. Journal of Epidemiology and Community Health. 38 (3): 259–62. September 1984.

[17]. Capper R, Britt-Compton B, Tankimanova M, Rowson J, Letsolo B, Man S, et al. The nature of telomere fusion and a definition of the critical telomere length in human cells. Genes Dev. 2007;21:2495-508.

[18]. Parkins MD, Parkins VM, Rendall JC, Elborn S. Changing epidemiology and clinical issues arising in an ageing cystic fibrosis population. Ther Adv Respir Dis. 2011;5:105-19.

[19]. Hull JH, Garrod R, Ho TB, Knight RK, Cockcroft JR, Shale DJ, et al. Increased augmentation index in patients with cystic fibrosis. Eur Respir J. 2009;34:1322-8.

[20]. Effros R.B. Telomere/telomerase dynamics within the human immune system: Effect of chronic infection and stress. Exp. Gerontol. 2011;46:135–140

[21]. Kordinas V, Ioannidis A, Chatzipanagiotou S. The Telomere/Telomerase System in Chronic Inflammatory Diseases. Cause or Effect? Genes (Basel). 2016;7.

[22]. Ovadya Y, Krizhanovsky V Senescent cells: SASPected drivers of age-related pathologies Biogerontology. 2014 Dec;15(6):627-42