Early View

Original article

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Telomere shortening and DNA damage in culprit cells of different types of progressive fibrosing interstitial lung disease

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Take home message: In patients with IPF telomere shortening and accumulation of DNA damage is primarily affecting AT2 cells, while in fHP the particularly high telomere-independent DNA damage signals in club cells, underscores it’s bronchiolocentric pathogenesis.
Abstract

Pulmonary fibrosis is strongly associated with telomere shortening and increased DNA damage. Key cells in the pathogenesis involve alveolar type 2 (AT2) cells, club cells and myofibroblasts, however to which extend these cells are affected by telomere shortening and DNA damage is yet unknown. We sought to determine the degree of, and correlation between telomere shortening and DNA damage in different cell types involved in the pathogenesis of progressive fibrosing interstitial lung disease. Telomere length and DNA damage were quantified, using combined fluorescence in situ hybridization and immunofluorescence staining techniques, in AT2 cells, club cells and myofibroblasts of controls and patients with pulmonary fibrosis and a telomerase reverse transcriptase mutation (TERT-PF), idiopathic pulmonary fibrosis (IPF) and fibrotic hypersensitivity pneumonitis (fHP). In IPF and TERT-PF lungs, AT2 cells contained shorter telomeres and expressed higher DNA damage signals than club cells and myofibroblasts. In fHP lungs, club cells contained highly elevated levels of DNA damage, while telomeres were not evidently short. In vitro, we found significantly shorter telomeres and higher DNA damage levels only in AT2 surrogate cell lines treated with telomerase inhibitor BIBR1532. Our study demonstrated that in IPF and TERT-PF lungs, telomere shortening and accumulation of DNA damage is primarily affecting AT2 cells, further supporting the importance of AT2 cells in these diseases, while in fHP the particularly high telomere-independent DNA damage signals in club cells, underscores it’s bronchiolocentric pathogenesis. These findings suggest that cell type-specific telomere shortening and DNA damage may aid to discriminate between different drivers of fibrogenesis.
Introduction

Progressive fibrosing interstitial lung disease (ILD) is a group of devastating disorders characterized by scarring of the epithelium and reduced survival (1, 2). Although the pathogenesis is incompletely understood, evidence is growing that processes associated with accelerated aging, such as telomere shortening and genetic instability, play a causative role in the destruction of the lung epithelium and subsequent fibrosis (3, 4).

Telomeres are DNA-protein complexes at the end of chromosomes which act as a buffer in cell-cycle dependent DNA shortening, thereby protecting the genetic information of the genome (5-7). Shortening of telomeres is associated with several forms of progressive fibrosing ILD, such as idiopathic pulmonary fibrosis (IPF) and fibrotic hypersensitivity pneumonitis (fHP) (8-11). In a subset of these patients, disease is caused by genetic mutations in telomere-related genes such as telomerase reverse transcriptase (TERT), the catalytic subunit of telomerase involved in telomere elongation and maintenance (12-16). Furthermore, we have previously shown that telomere length in lung tissue of sporadic IPF patients is significantly reduced and associated with poorer survival (8, 17).

Critical telomere shortening is recognized as DNA damage similar to a double-strand DNA break. This results in the phosphorylation of H2A histone family member X (γH2AX) initiating the DNA damage response. In healthy circumstances, double-strand DNA breaks take approximately 72 hours to resolve (18). However, if not fixed properly in time, the DNA damage response becomes persistent and eventually leads to cellular senescence (19-21), a process associated with fibrogenesis in IPF lungs and fibrotic mouse models (22, 23).

Previous studies using mice models with a telomere-repeat factor-1 (Trf1) knockout in alveolar or bronchiolar epithelial cells demonstrated telomere shortening and increased DNA damage foci in both cell types (24, 25). Similar results were found in TRF2-inactivated human cell lines showing an elevated amount of damage foci at uncapped telomeres (26). Furthermore, we previously found a significant inverse correlation between γH2AX signals
and telomere length in alveolar epithelial cells of a patient with pulmonary fibrosis harbouring a PARN mutation (27). In contrast, other studies reported that, even though DNA damage signals were increased, no such correlation between average whole lung telomere length and DNA damage signals was found in IPF lungs (28). However, extensive cell-type specific measurements are missing in pulmonary fibrosis.

Several cell types have been associated with the pathogenesis of tissue remodelling underlying pulmonary fibrosis. Alveolar type 2 (AT2) cells, progenitor cells responsible for maintenance and renewal of the alveolar compartment, are generally considered to play a fundamental role in the onset of fibrogenesis in the lung (24, 29, 30). Loss of functional AT2 cells result in an impaired renewal capacity of alveolar cells and the production of pro-fibrotic factors, subsequently leading to activation of myofibroblasts and extracellular matrix deposition (31). However, there has been emerging attention for a role of club cells in pulmonary fibrosis. Similar to AT2 cells in alveoli, club cells are progenitor cells responsible for maintenance and renewal of bronchiolar epithelium. In pulmonary fibrosis, club cells drive bronchiolization, a process in which bronchiolar epithelial cells migrate to and repopulate the alveoli (32, 33). A third cell type, i.e. the myofibroblast is the main source of collagen deposition, thereby driving fibrogenesis. Clusters of myofibroblasts forming fibroblast foci are a histological hallmark of IPF (1, 34). We previously found that in IPF lungs, telomeres in AT2 cells were shorter than in other, as yet unclassified cells surrounding these AT2 cells (8). However, telomere length in the two other cell types involved in fibrogenesis has never been studied specifically in IPF or other types of pulmonary fibrosis such as fHP. Moreover, a possible quantitative and cell-type specific relation between telomere shortening and persistent activation of the DNA damage response remains to be elucidated.

To mimic lung-specific telomere shortening in an experimental setup, lung cell lines can be treated with BIBR1532, a telomerase inhibitor which binds noncompetitively to the active site of the telomerase protein (35). Previously, BIBR1532-dependent telomere shortening in a A549 carcinoma cell line, which is derived from the alveolar epithelium and closely resemble
AT2 cells, showed that after 140 population doublings telomeres were shorter in these cells (36). However, to date telomere shortening in surrogate cell lines of club cells and myofibroblasts with BIBR1532 treatment and its correlation with DNA damage was not assessed.

Material and Methods

Patient selection

Lung material of three patient groups with progressive fibrosing ILD were included in the analysis, consisting of 32 patients with IPF, 17 patients with pulmonary fibrosis and a TERT mutation (TERT-PF), and 9 patients with fHP (Table S1). The study was approved by Medical research Ethics Committees United of the St Antonius Hospital (approval number W14.056 and R05-08A) and all patients provided written informed consent.

Cell culture

To investigate the relation between telomere shortening and DNA-damage, non-small-cell lung cancer cell lines A549 (cultured in Dulbecco’s Modified Eagles Medium (DMEM)) and NCI-H460 (cultured in Roswell Park Memorial Institute (RPMI) 1640 medium), bronchial epithelial cell line 16HBE (cultured in Minimum Essential Medium (MEM)) and lung fibroblast cell line MRC5 (cultured in Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12)) were treated with 0, 10 or 25 μM telomerase inhibitor 2-[(E)-3-naphtalen-2-ylbut-2-enoylamino]-benzoic acid (BIBR1532; Selleckchem, Munich, Germany). Additional detail on cell culture experiments is provided in an online data supplement.

Whole biopsy telomere length measurements in lung tissue

Whole biopsy telomere length in DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue was measured by monochrome multiplex quantitative polymerase chain reaction (MMqPCR) as described previously (8, 17, 37). 13 controls, 17 TERT-PF, 32 IPF
and 9 fHP lung biopsy samples were included. Additional detail on telomere length measurements by MMqPCR is provided in an online data supplement.

**Cell type-specific telomere and DNA damage staining**

Subsequent FFPE tissue slides of 4 µm were prepared and stained for telomere length and DNA damage analysis in specifically labelled AT2 cells, club cells and myofibroblasts as described previously (27). To investigate cell type-specific telomere length, we performed a fluorescence *in situ* hybridization (FISH) analysis in a random sub selection of control (n = 8), TERT-PF (n = 6), IPF (n = 10) and fHP (n = 5) lungs. Additional detail on telomere length and γH2AX DNA damage measurements by FISH and immunofluorescence (IF) is provided in an online data supplement.

**Statistical analysis**

Statistical significances were computed using non-parametric tests in GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). Telomere length and DNA damage signal differences were determined by Mann-Whitney tests and combined Kruskall-Wallis and Dunn’s multiple comparisons tests. First, we used the data to compare differences in the signal between the different groups of patients (controls, TERT-PF, IPF and fHP), then we used the data to compare differences between the different types of cells (AT2, Club cells and myofibroblasts). Spearman’s rank coefficient was used to calculate correlations per disease group between telomere length and γH2AX signal in all cell-types together. Next, we investigated if the correlation that was present in TERT-PF lungs would fit observed values in the specific cell-types AT2, Club cells or myofibroblasts in IPF or fHP lungs. The cell-type specific observed telomere length was used in the equation representing the correlation in TERT-PF lungs to calculate the cell-type specific expected DNA damage value. Statistical differences between observed and expected values for each cell-type were computed with the Mann-Whitney tests.

**Results**
No difference in whole biopsy telomere length between IPF and fHP lungs

Analysis of whole biopsy telomere length measured by MMqPCR showed that telomeres in the control group were significantly longer than in the patient groups (p < 0.05). Furthermore, comparison between patient groups showed that telomere length in TERT-PF lungs was significantly shorter than in IPF and fHP groups (p < 0.05). No difference was found between whole biopsy telomere length of IPF and fHP groups (Table 1).

Table 1. Group comparison of telomere length and DNA damage in lung tissue

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>TERT-PF</th>
<th>IPF</th>
<th>fHP</th>
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<tr>
<td><strong>Average TL (T/S ratio)</strong> of whole biopsy by MMqPCR (IQR)</td>
<td>0.932&lt;sup&gt;a&lt;/sup&gt; (0.909-0.947)</td>
<td>0.772&lt;sup&gt;b&lt;/sup&gt; (0.734-0.803)</td>
<td>0.862 (0.810-0.959)</td>
<td>0.849 (0.778-0.884)</td>
</tr>
<tr>
<td><strong>AT2 cell</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γH2AX signal (IQR)</td>
<td>1005 (246-3354)</td>
<td>4253&lt;sup&gt;c&lt;/sup&gt; (3019-6390)</td>
<td>3923 (2005-5364)</td>
<td>3661 (2028-4749)</td>
</tr>
<tr>
<td>FISH TL (IQR)</td>
<td>1764&lt;sup&gt;a&lt;/sup&gt; (999-2484)</td>
<td>464&lt;sup&gt;b&lt;/sup&gt; (226-703)</td>
<td>729&lt;sup&gt;a&lt;/sup&gt; (342-1101)</td>
<td>1049 (679-1605)</td>
</tr>
<tr>
<td><strong>Club cell</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γH2AX signal (IQR)</td>
<td>1303 (248-3782)</td>
<td>1879 (902-4624)</td>
<td>1745 (487-3407)</td>
<td>6769&lt;sup&gt;c&lt;/sup&gt; (4025-8827)</td>
</tr>
<tr>
<td>FISH TL (IQR)</td>
<td>1650&lt;sup&gt;a&lt;/sup&gt; (769-2600)</td>
<td>813&lt;sup&gt;b&lt;/sup&gt; (467-1147)</td>
<td>1405 (821-1949)</td>
<td>1155 (847-1864)</td>
</tr>
<tr>
<td><strong>Myofibroblast</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γH2AX signal (IQR)</td>
<td>NA</td>
<td>752 (0-1637)</td>
<td>388 (9.2-2691)</td>
<td>2080 (681-7381)</td>
</tr>
<tr>
<td>FISH TL (IQR)</td>
<td>NA</td>
<td>924&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1518</td>
<td>1513</td>
</tr>
</tbody>
</table>
TERT-PF = Patients with a TERT mutation and pulmonary fibrosis; IPF = Idiopathic Pulmonary Fibrosis; fHP = fibrotic Hypersensitivity Pneumonitis; AT2 = Alveolar Type 2; TL = Telomere Length; IQR = Inter Quartile Range; NA = Not Applicable. Numbers describe median telomere length or DNA damage signal.

a = Telomeres were significantly longer than in the other study groups (p < 0.05).

b = Telomeres were significantly shorter than in the other study groups (p < 0.05).

c = DNA damage signal was significantly higher than in the other study groups (p < 0.05).

d = Telomeres in AT2 cells of IPF lungs were significantly shorter than in AT2 cells of fHP lungs (p < 0.0001).

**AT2 cells of TERT-PF and IPF lungs have the shortest telomeres**

To determine cell type-specific telomere length, we stained telomeres by FISH together with cell type markers by IF (Fig1). Comparison between cell types showed that in controls there was no difference between telomere length in AT2 cells and club cells (p = 0.961, Fig 2a). In TERT-PF and IPF lungs, telomere length in AT2 cells was significantly shorter than in club cells (p < 0.0001, Fig 2b and c) and in myofibroblasts (p < 0.0001, Fig 2b and c), while in fHP lungs telomere length of AT2 cells was not different from that of club cells (p = 0.168, Fig 2d), and slightly shorter than that of myofibroblasts (p = 0.0002, Fig 2d). In all three patient groups, no difference in telomere length was found between club cells and myofibroblasts. Comparison between groups showed that telomere length in AT2 cells and club cells of control lungs were significantly longer than those of TERT-PF, IPF and fHP lungs (Table 1). In contrast, telomere length in AT2 cells, club cells and myofibroblasts of TERT-PF lungs were significantly shorter than those of control, IPF and fHP lungs (Table 1). Interestingly, telomeres in AT2 cells of IPF lungs were significantly shorter than in AT2 cells of fHP lungs (p < 0.0001), while telomere length in club cell and myofibroblasts did not differ between IPF and fHP (Table 1).

**Increased DNA damage in club cells of fHP lungs**
To determine cell type-specific DNA damage, we used immunofluorescent staining of γH2AX together with cell-type specific markers. Comparison between cell types showed that in TERT-PF and IPF lungs, γH2AX signal in AT2 cells was significantly higher than in club cells (p < 0.0001, Fig 2f and 2g) and in myofibroblasts (p < 0.0001, Fig 2f and 2g). In contrast, in fHP lungs γH2AX signal in club cells was higher than in AT2 cells (p < 0.0001, Fig 2h) and myofibroblasts (p < 0.0001, Fig 2h). Moreover, comparison between study groups showed that club cells in fHP lungs contained a significantly higher γH2AX signal than club cells in control (p < 0.0001), TERT-PF (p < 0.0001) and IPF lungs (p < 0.0001, Table 1). In AT2 cells of TERT-PF lungs, γH2AX signal was higher than in the other study groups (controls: p <0.0001, IPF: p = 0.038 and fHP: p = 0.005, Table 1) while no difference in γH2AX signal of AT2 cells between fHP and IPF lungs was detected (p = 0.248, Table 1).

**DNA damage in club cells and myofibroblasts of fibrotic lungs is higher than expected from telomere shortening alone**

Next, using data from all cell-types, we analysed the association between FISH telomere length and γH2AX signal and found in TERT-PF lungs a moderately strong correlation (r = -0.689, p = 0.003, Fig 3a). The correlation between telomere length and DNA damage in TERT-PF lungs, in which a causative mutation in the TERT gene is underlying telomere shortening and disease, shows that telomere shortening is likely the cause of the observed increase in DNA damage. However, using data from all cell-types, such a correlation was not found in the IPF or fHP groups. Next, we investigated whether a correlation exists for specific cell-types in IPF and fHP, rather than in cell types combined. Therefore, we used the equation representing the correlation between telomere length and DNA damage found in TERT-PF lungs to calculate the expected DNA damage for each cell-type based on the observed telomere length for each cell-type in IPF and fHP. The expected and observed DNA damage signals per cell type are presented in Fig 3b – 3g. In AT2 cells of IPF and fHP no difference between expected and observed DNA damage signals was present (Fig 3b and 3e). However, in club cells and myofibroblasts of IPF and fHP lungs, observed DNA
damage signals were significantly higher than the expected values \((p < 0.05, \text{Fig 3c, 3d, 3f and 3g)}\).

**Induced telomere shortening and increased DNA damage in AT2 surrogate cell lines**

In order to experimentally study if telomere shortening causes an increase in DNA damage, we added BIBR1532, a highly specific telomerase inhibitor to cultures of surrogate lung cell lines A549 and NCI-H460 for AT2 cells, 16HBE for club cells and MRC5 for myofibroblasts. Inhibition of telomerase showed that only in AT2 surrogate cell lines A549 and NCI-H460 telomeres shortened significantly with 25μM BIBR1532 \((p < 0.05, \text{Fig 4a and 4b)}\) and that the level of DNA damage increased significantly compared with no BIBR1532 \((p = 0.0001, \text{Fig 4a, 4b, 4e and 4f)}\). In 16HBE and MRC5 cells treated with 25μM BIBR1532, however, telomeres did not shorten \((\text{Fig 4c, 4d)}\), while DNA damage increased to very high levels when compared with no BIBR1532 \((p = 0.0001, \text{Fig 4g and 4h)}\).

**Discussion**

In this study, telomere length and DNA damage were investigated for the first time in different cell types involved in the pathogenesis of progressive fibrosing ILD. In AT2 cells of patients with IPF and patients with a \textit{TERT} mutation we detected the shortest telomeres and highest DNA damage signals when compared to club cells and myofibroblasts. However, telomere length in AT2 cells of fHP lungs was not evidently short, while very high DNA damage signals were present in club cells. The observed increase of DNA damage in AT2 cells may be caused by telomere shortening. This was experimentally replicated in two AT2 surrogate cell lines, which showed BIBR1532-induced telomere shortening together with increased DNA damage. However, the level of DNA damage in club cells and myofibroblasts of IPF and fHP lungs could not be explained by telomere shortening alone.

Cell-type specific analysis is most important to better understand processes in fibrotic lungs. Our results demonstrate that although no differences in average whole lung biopsy telomere length was present between IPF and fHP, significant differences between cell-types exist. In
both TERT-PF and IPF lungs, telomere length in AT2 cells was most affected, confirming the
important role of AT2 cell telomere shortening in IPF pathogenesis (8, 10, 24, 30). Moreover,
yH2AX signals were significantly elevated in AT2 cells of these groups. However, only in
TERT-PF lungs, in which a causative mutation in the TERT gene is underlying telomere
shortening and disease, a correlation between telomeres and yH2AX-related DNA damage
exists, suggesting that telomere shortening causes DNA damage in these patients. This is
consistent with a previous report showing that in IPF lungs no correlation was found between
average whole lung telomere length and DNA damage signals (28) and supports the notion
that DNA damage in IPF may be caused by other factors than telomere shortening. To
investigate if a decrease in telomere length induces an increase in DNA damage for each
cell-type in IPF and fHP lungs, we tested if observed DNA damage signals deviated from
expected DNA damage signals based on the observed telomere length. In AT2 cells,
observed and expected DNA damage signals were similar, while in club cells and
myofibroblasts the amount of DNA damage was significantly higher than expected. These
data imply that in AT2 cells of IPF and fHP lungs telomere shortening may be the primary
cause of DNA damage, while in club cells and myofibroblasts other processes may be
involved.

Progressive telomere shortening and accumulation of DNA damage are prominent features
of aging and may eventually lead to cellular senescence or apoptosis (19-21). Our data
demonstrates that telomere shortening in TERT-PF lungs and in AT2 cells of IPF and fHP
lungs is associated with elevated DNA damage signals. This suggests that AT2 cells in
fibrotic lungs are prone to become senescent or apoptotic. Senescent and apoptotic AT2
cells have been observed in IPF lung (38-40) but not in fHP. Accumulation of senescent cells
has been associated with progressive pulmonary fibrosis in IPF lungs and fibrotic mouse
models (22, 23, 41, 42) and was postulated to drive pulmonary dysfunction in IPF. Whether
the high level of DNA-damage observed in club cells of fHP lungs associates with
senescence or apoptosis, remains to be investigated. This is of special interest because,
treatment with senolytic dasatinib in combination with quercetin was proven to be effective in eliminating cultured senescent human lung cells (43) and recent clinical trials with these drugs demonstrated physical alleviation in patients with IPF (44).

In fHP, club cells most prominently contained highly elevated DNA damage signals, but showed no excessive telomere shortening. The causal trigger of fHP is a sustained allergic reaction against an extrinsic antigen, but how this allergic reaction leads to telomere-unrelated DNA damage in club cells is unclear. A possible cause may be the inflammation-induced accumulation of reactive oxygen species (ROS), a group of highly reactive, DNA damage-inducing molecules that are also associated with other allergic diseases, such as asthma (45, 46). Because fHP is characterized by inhaled antigens that, due to size, strand in the bronchioles (47), it is possible that the accumulation of DNA damage in club cells of fHP lungs at this location is caused by ROS, and not by telomere shortening. This is in line with a previous study that showed that bronchoalveolar lavage of fHP patients contained significantly higher carbonylated protein levels, a marker of ROS, compared to IPF and controls (48). However, another study showed that in mice with a club cell-specific knock out of telomere repeat-binding factor-1 (Trf1), rapid aging of club cells by telomere dysfunction alone was sufficient to induce DNA damage and subsequent bronchiolocentric fibrosis (25). In addition, it was reported that in 25% of the cases with an initial diagnosis of IPF, bronchiolocentric fibrosis is indicative for a revised diagnosis of fHP (49). The excess DNA damage in fHP club cells might therefore, regardless of the cause, be suggestive of an important role of these cells in disease development and is in congruence with the localization of fibrosis in fHP.

Next, we showed for the first time that experimental inhibition of telomerase resulted in telomere shortening in AT2 cell surrogate cell lines A549 and NCI-H460, suggesting that telomeres in these cells, similar to AT2 cells in diagnostic biopsies of pulmonary fibrosis, are most sensitive to telomerase dysfunction. This is in congruence with previous experiments where telomere shortening in BIBR1532-treated NCI-H460 cells was observed (36).
Moreover, in both *in situ* and *in vitro* experiments a decrease in telomere length in AT2 cells is associated with an increase in DNA damage, underlining the telomere-dependent accumulation of DNA damage in AT2 cells. Furthermore, in surrogate club cell and myofibroblasts cell lines 16HBE and MRC5 treated with BIBR1532 no evident telomere shortening was found, while these cells showed high levels of DNA damage. This corresponds with the finding that club cells and myofibroblasts in IPF and fHP tissue accumulate DNA damage independent of telomere shortening. However, it is unclear why BIBR1532-treated 16HBE and MRC5 cells present with high telomere-independent levels of DNA damage.

Strengths of this study comprise of the detailed assessment of cell type-specific telomere length and DNA damage in a broad spectrum of progressive fibrosing ILD patients, including those harbouring a *TERT* mutation (TERT-PF), IPF and fHP. However, some limitations are worth noting. The data presented here are based on associations; no causative links could be concluded from telomere length and DNA damage analysis in human tissue samples. Also, control tissue was obtained from various sources, such as residual lung resected from tissue next to a tumour. Furthermore, in contrast to the other cell lines, MRC5 cells are mortal foetal cells. Even though the MRC5 cells used in this study were still actively replicating, no definitive conclusions can be drawn on telomere length or DNA damage signals compared to other cell lines. Finally, use of primary cells instead of surrogate cell lines for AT2 and club cells would have been optimal to investigate a relation between telomere shortening and DNA damage.

In conclusion, this is the first study addressing in detail telomere status and DNA damage signals in AT2 cells, club cells and myofibroblasts in different types of progressive fibrosing ILD. In IPF and TERT-PF lungs, telomere shortening and accumulation of DNA damage is primarily affecting AT2 cells, further supporting their central role in fibrogenesis of these groups, while the remarkably high DNA damage in club cells of fHP lungs underscores the more bronchiolocentric fibrogenesis and a prominent role for club cells and DNA damage in
fHP (Fig 5). To further elucidate the link between club cells and the pathogenesis of fHP, future studies should focus on cellular aging due to a sustained allergic reaction and DNA damage in these cells.

References


Figure 1. Telomere and DNA damage in fibrotic lung tissue measured by combined FISH and immunofluorescence staining techniques. (a) Hematoxylin and Eosin (H&E) staining representing a typical IPF lung biopsy. A = Alveolus, B = Bronchiolus and FF = Fibroblast Focus. (b-d) Representative fluorescent stained examples of boxed areas in image a, containing pro-Spc-positive AT2 cells (green), CC10-positive club cells (light blue) and αSMA-positive myofibroblasts (purple), respectively. (e-g) FISH-stained telomere signals (red dots) and (h-j) IF-stained γH2AX signals (yellow dots) in magnified boxed areas of images b-d, respectively. (k-m) Overlay pictures of telomere, γH2AX and DAPI stainings. All fluorescent pictures were captured and Z-stacked using a LSM700 laser scanning confocal microscope. Abbreviations: FISH, fluorescence in situ hybridization; IF, Immunofluorescence; AT2, alveolar type 2 pneumocyte; Spc, surfactant protein C; CC10, club cell protein 10; αSMA, alfa smooth muscle actin; DAPI, 4′,6-diamidino-2-phenylindole; γH2AX, phosphorylated histone protein from the H2A family.
Figure 2. Cell-specific quantification of telomere and DNA damage signals in control and fibrotic lungs. Bar charts of telomere length (measured by FISH) and DNA damage signals (measured by IF) in AT2 cells, club cells and myofibroblasts in lungs of (a, e) 8 controls, (b, f) 6 patients with a TERT mutation, (c, g) 10 IPF and (d, h) 5 fHP patients. Controls contained long telomeres and low DNA damage signals in AT2 cells and club cells. In TERT-PF and IPF lungs, telomere length in AT2 cells was shorter than in club cells and myofibroblasts (p < 0.0001) and DNA damage signals in AT2 cells were significantly higher than in club cells and myofibroblasts (p < 0.0001). In fHP lungs, DNA damage signals in club cells were highly elevated compared to AT2 cells and myofibroblasts (p < 0.0001), while telomere length in club cells was comparable with AT2 cells and myofibroblasts. Boxes represent medians and whiskers extend up to values within the 3rd quartile. Abbreviations: FISH, Fluorescence in situ hybridization; IF, Immunofluorescence; γH2AX, phosphorylated histone protein from the H2A family; Ctrl, Controls; TERT-PF, Patients with a TERT mutation and pulmonary fibrosis; IPF, Idiopathic pulmonary fibrosis; fHP, fibrotic hypersensitivity pneumonitis; NA, Not applicable. Asterisks indicate significant differences calculated by Kruskal-Wallis multiple comparison tests (**** = p < 0.0001).
Figure 3. Observed and expected DNA damage signals in AT2 cells, club cells and myofibroblasts of IPF and fHP lungs. (a) Inverse spearman correlation ($r = -0.689$, $p = 0.003$) of median telomere length and median γH2AX signals in AT2 cells, club cells and myofibroblasts per biopsy specimen in 6 TERT-PF lungs resulting in a linear regression line with equation $y = -4.23x + 5727$. This equation was used to calculate expected DNA damage values from observed telomere length signals in (b) IPF AT2 cells (c) IPF club cells, (d) IPF myofibroblasts, (e) fHP AT2 cells (f) and fHP club cells, (g) fHP myofibroblasts. In IPF and fHP, club cells and myofibroblasts showed lower expected than observed DNA damage signals, while in AT2 cells no significant difference was found between expected and observed DNA damage signals. Statistical differences were computed by Mann-Whitney tests (* = $p < 0.05$, ** = $p < 0.01$). Abbreviations: TERT-PF, Patients with a TERT mutation and pulmonary fibrosis; IPF, Idiopathic pulmonary fibrosis; fHP, Fibrotic hypersensitivity pneumonitis AT2, Alveolar type 2 pneumocytes; Exp, Expected; Obs, Observed.
Figure 4. Experimental induction of telomere shortening and DNA damage signals by inhibition of telomerase by BIBR1532. Bar charts of telomere length (measured by FISH) and DNA damage signals (measured by IF) in cytospins of (a, e) A549, (b, f) H460, (c, g) 16HBE and (d, h) MRC5 cell lines. Induced telomere shortening with 25µM BIBR1532 was only observed in A549 (p = 0.004) and H460 cells (p = 0.013) compared to untreated cells, while in all cell lines increased DNA damage was found. Telomeres in MRC5 cells treated with 25µM BIBR1532 were significantly longer than untreated cells (p = 0.0001). Boxes represent medians and whiskers extend up to values within the 3rd quartile. Abbreviations: FISH, Fluorescence in situ hybridization; IF, Immunofluorescence; γH2AX, phosphorylated histone protein from the H2A family; BIBR1532, 2-[(E)-3-naphtalen-2-yl-but-2-enoylamino]-benzoic acid. Asterisks indicate significant differences calculated by Kruskal-Wallis multiple comparison tests (* = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001).
Figure 5. Graphical abstract summarizing the main message of this study. The extent to which key cells in progressive fibrosing lung disease are affected by telomere shortening and DNA damage is yet unknown. This study revealed that in patients with idiopathic pulmonary fibrosis (IPF) telomere shortening and accumulation of DNA damage is primarily affecting AT2 cells, further supporting the importance of AT2 cells in this disease, while in fibrotic hypersensitivity pneumonitis (fHP) the particularly high telomere-independent DNA damage signals in club cells, underscores it’s bronchiolocentric pathogenesis.
Supplementary material

Telomere shortening and DNA damage in culprit cells of different types of progressive fibrosing ILD

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Supplementary methods

Tissue selection

Diagnostic surgical lung biopsies, taken between 1994 and 2015, were reviewed by an experienced pathologist (MFMvO) in accordance with the ATS/ERS/JRS/ALAT guidelines (1, 2). Furthermore, we cross-referenced data with 13 age matched control lung tissue specimens, which were obtained during post-mortem examination (n = 3), from residual donor lobes (n = 3) and from normal appearing lung resection tissue next to a tumour (n = 7).

Cell culture

All media were supplemented with pen/strep and 10% FCS. Proliferative cultures were incubated at 37°C in a humidified 5% CO2 incubator. Cultures were split twice a week. To subculture the cell monolayers, they were washed with phosphate buffered saline (PBS) without calcium and magnesium before cells were detached from the culture glass using a Trypsin/EDTA solution at 37°C and seeded into fresh flasks. Trypsin was inactivated by the addition of growth medium. All media and supplements have been obtained from Thermofischer (Waltham, Massachusetts, USA). To achieve continuous exposure, the final concentration of 10 or 25 µM BIBR1532 was added after each passage. These concentrations were reported to be below levels of toxicity (3). Cells were cultured with the
telomerase inhibitor BIBR1532 for 22 days before they were spun down on glass slides and stained for telomeres and DNA double strand breaks using FISH and a γH2AX antibody.

**Whole biopsy telomere length measurements by MMqPCR in FFPE tissue**

In short, three sequential sections were cut from tissue samples and used for MMqPCR and FISH. DNA was isolated from the first section using an AllPrep DNA/RNA FFPE Kit (Qiagen, Hilden, Germany) according to manufacturer instructions. Whole biopsy telomere length assessment was performed using a monochrome multiplex qPCR (MMqPCR) on a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) (4-6). The relative telomere length for each sample was calculated from the ratio telomere repeat copy number (T) to a single human β-globin gene copy number (S) (T/S ratio), using standard curves from a serial dilution of a genomic DNA-pool. Samples were analysed in triplicate in at least two runs, of which only coefficients of variation below 10% were included. The overall mean coefficient of variation was 2.5.

**Cell type-specific telomere and DNA damage staining**

For a more detailed description of the methods on cell-specific telomere length and DNA damage measurements using combined FISH and immunofluorescence staining techniques, we refer to our methodological article (7). In short, slides were incubated with a telomere-Cy3 peptide nucleotide acid (PNA) probe (2.70 μg/ml, F1002; Panagene, Daejeon, South Korea). Excess probe was cleared with a PNA wash solution to subsequently stain for γH2AX (1:100, 05-636-I; Merck Millipore, Darmstadt, Germany) and specific cell markers with immunofluorescence antibodies; AT2 cells (rabbit anti-human pro-Spc, 1:100, AB3786; Merck Millipore, Darmstadt, Germany), club cells (rabbit anti-human CC10, 1:100, sc-25554; Santa Cruz Biotechnology, Dallas, TX), Myofibroblasts (mouse anti-human αSMA, 1:50,
A2547; Sigma-Aldrich, Darmstadt, Germany) and corresponding secondary antibodies. Lastly, nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; 25 μg/ml). Pictures were taken with a LSM700 laser scanning confocal microscope (Zeiss, Jena, Germany) and images were analysed using the image analysis Telometer plugin (available at http://demarzolab.pathology.jhmi.edu/telometer/index.html) of ImageJ (http://rsb.info.nih.gov/ij/). To this end the nuclear surface area was manually demarcated for the cell of interest. The telometer plugin provides the intensity of each fluorescent cluster within a demarcated area and the surface of this area. All clusters were manually summed up to calculate the total telomere or γH2AX fluorescence signal within a cell nucleus. To adjust the total signal intensity for the amount of DNA the cell’s total telomere or γH2AX signal was divided by the nuclear surface area.

**Supplementary tables**

**Table S1. Baseline characteristics of study groups**

<table>
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<tr>
<th></th>
<th>Controls</th>
<th>TERT-PF</th>
<th>IPF</th>
<th>fHP</th>
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<tbody>
<tr>
<td>N</td>
<td>13</td>
<td>17</td>
<td>32</td>
<td>9</td>
</tr>
<tr>
<td>Male/Female</td>
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<td>14/3</td>
<td>29/3</td>
<td>6/3</td>
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<tr>
<td>Mean Age at time of biopsy (SD)</td>
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<td>58.7 (9.8)</td>
<td>60.1 (9.5)</td>
<td>60.1 (10.8)</td>
</tr>
<tr>
<td>Mean FVC%predicted (SD)</td>
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<td>70.5 (21.6)</td>
<td>75.3 (27.5)</td>
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<tr>
<td>Mean DLCO%predicted (SD)</td>
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<td>46.5 (16.4)</td>
<td>46.5 (19.3)</td>
</tr>
<tr>
<td>Smoking status (CS:FS:NS:U)</td>
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<td>0:14:3:0</td>
<td>3:20:6:3</td>
<td>0:7:2:0</td>
</tr>
<tr>
<td>Pack years (SD)</td>
<td>NA</td>
<td>18 (15.5)</td>
<td>20.2 (19.4)</td>
<td>8.7 (12.4)</td>
</tr>
</tbody>
</table>

TERT-PF = patients with a TERT mutation and pulmonary fibrosis; IPF = Idiopathic Pulmonary Fibrosis; fHP = fibrotic Hypersensitiviy Pneumonitis; FVC = Forced Vital Capacity; DLCO = Diffusing Capacity of the Lungs for Carbon Monoxide. CS = Current Smoker; FS =
Former Smoker; NS = Never Smoker; U = Unknown; NA = Not Available. No significant differences in baseline characteristics were observed between the study groups (Kruskal-Wallis multiple comparison tests).

**Supplementary references**


