Pack Years and Lower Lung Function is Associated With Ultra-short Telomeres in Copd: Evidence From Lung Tissue

Huseyin Cagsin
Near East University
Nedime Serakinci (nedime.serakinci@neu.edu.tr)
Near East University https://orcid.org/0000-0002-1884-0839
Ali Uzan
Near East University
Finn Rasmussen
Near East University

Research

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Abstract

Background

Chronic obstructive pulmonary disease (COPD) is driven by a complicated mix of factors such as lifestyle and environmental exposures. Tobacco smoking is the main risk factor for forming chronic inflammation in COPD. Association between cigarette smoking and the role of telomere shortening in COPD has been studied mainly based on the assessment of mean telomere length on leukocytes instead of lung tissue where the primary damage occurs. Here we investigate this association in bronchoalveolar samples by using a new assay that specifically evaluates critically short telomeres, namely, ultra-short telomeres that have sizes less than 1.5kb.

Methods

The study was carried out on materials from the patients eligible for bronchoscopy as well as mild to severe persistent airway obstruction, defined as a post-bronchodilator ratio of less than 70%. Bronchial washing (BW) and leukocyte samples were collected from 32 patients diagnosed with COPD. Telomere length evaluation was done with isolated DNA using Universal STELA to specifically identify the presence of ultra-short telomeres in samples. A t-Student, ANOVA, Chi^2, and Paired Sample *T*-test were used to test differences in means and proportions in statistical analysis. Two-tailed *p*-values ≤ 0.05 were considered significant

Results

The location of BW did not show a significant difference when compared in terms of the presence of ultra-short telomeres ($p>0.05$). Higher total pack-years was found amongst patients with ultra-short telomeres (32 packyears versus 16 packyears; $p=0.045$), lower lung function (FEV1%) (51% versus 82%; $p<0.001$) when compared with subjects with telomere length more than 1.5kbs in BW. An increasing number of total pack-years, older age and lower FEV1% was observed through the groups comprising subjects with ultra-short telomeres in both BW and leukocytes, subjects with ultra-short telomeres only in BW and subjects with telomeres longer than 1.5kbs(all $p<0.01$)

Conclusions

Our results emphasize the role of ultra-short telomeres in COPD, in vivo, especially when the lung tissue instead of leukocytes is investigated. Additionally, our results demonstrated a dose-response association between pack-years of smoking, low lung function, and ultra-short telomere length in COPD.

Background

Cigarette smoking has long been recognized as the leading cause of the development of chronic obstructive pulmonary disease (COPD). Destruction of the lung tissue (alveoli septae) with the development of emphysema is one of the cornerstones of COPD disease. The most widely recognized

explanation for this phenomenon is that smoking activates inflammatory cells which with their proteolytic enzymes destroy alveoli septae [1]. This assumption is supported by the fact that individuals with α1-antitrypsin deficiency have an increased risk of developing emphysema at earlier ages [2], as these individuals have a lower level of enzymes that protect against degradation. Since the risk of developing COPD is also closely related to age, some pathogenesis of COPD could be a smoking-induced accelerated "aging" of the lung epithelial cells of particularly sensitive individuals with increased cell death, apoptosis [3]. Lung tissue fails to reconstruct itself after a gradually increased number of senescent cells. This may also be contributed by the short telomeres which promote cellular senescence [4]. Both apoptosis and senescence are triggered as a DNA damage response through the p53 pathway if the damage is beyond repair [5]. These DNA damage results from genotoxic effects from the environment. In lung epithelium, several genotoxic components are found in cigarette smoke, and in connection with studies of the etiology of lung cancer, the accumulation of DNA damage in lung cells in tobacco smokers has been demonstrated [6].

Tobacco smoking is the main risk factor for forming chronic inflammation in COPD. This inflammation gradually results in structural changes in the small airway, and thereby persistent airway obstruction through accelerated aging in lung tissue [7]. Aging, even in non-smoking subjects, is related to a decreasing forced expiratory volume in 1 second (FEV1)/ forced vital capacity (FVC) (FEV1/FVC) ratio which is a sign of increased airway limitation [8]. It is thought that chronic inflammation by other environmental exposures or cellular aging itself might be a reason for the airway limitation. The associations between telomeres and inflammatory diseases like COPD and aging-related diseases are often studied on leukocytes [9]. This might be one of the reasons that telomere length associations with COPD remain unclear.

COPD is mainly a disease in the lung. COPD is a progressive disease that seems to affect the body with many co-morbidities such as heart and musculoskeletal problems. The development of COPD affects humans very differently therefore individual's regenerative ability of lung tissue is important. a decrease in repair and regenerative ability likely leads to loss of lung cells and persistent airway obstruction [10]. A decrease in regenerative ability likely to be explained by telomere dysfunction which also takes place in normal aging and smoke-induced inflammations. There is also partly non-telomere-associated DNA damage that takes place [11-12].

COPD is driven by a complicated mix of factors such as lifestyle and environmental exposures that contribute to telomere shortening. Telomere length has been highlighted as a biomarker of aging and aging-related diseases. As aging takes place, telomeres gradually shorten because of cell division [13]. Furthermore, shorter telomeres have been identified as potential markers for several health problems in a wide range of diseases such as cardiovascular disease [14], neurodegenerative disorders [15], and cancers [16]. Often, telomere shortening is the biological effect of exogenous and hazardous exposures to chemicals like asbestos, smoking, and air pollution. Such exposures can cause DNA damage on the whole genome including telomeres which induces double-strand breaks in DNA thus increasing potential shortening of telomeres [17-18].

To date, there is no direct evidence of whether telomere length is a causal factor in mechanisms of how smoking affects lung function, particularly in patients diagnosed with COPD. In this study, we investigated the telomere length in lung epithelial cells that is isolated from bronchial washings (BW) that is related to smoking-induced COPD. In this connection, we group the study subject's samples according to clinical markers for disease stage (severity), tobacco use, and age.

Methods

Collection of biological samples

The hypothesis is tested on materials from the patients eligible for bronchoscopy as well as mild to severe persistent airway obstruction, defined as a post-bronchodilator ratio of less than 70%. Lung function was expressed as a post-bronchodilator predicted value of forced expiratory volume in second (FEV1%) according to the European Respiratory Society (ERS) [19]. This served also as a COPD grading 1 to 4 according to the Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD) [20]. One packyear was defined as the average use of 20 cigarettes/day for one year. Patients were recruited from the pulmonary medicine Department at Erciyes University Hospital Bronchoscopy Unit. 32 subjects were included. 17 patients (53%) were diagnosed with lung cancer, 1 patient had lung metastasis (3%) and 14 patients (44%) no abnormalities were found in the bronchoscopy. The recruitment of cells for telomere measurement was done at the end of the bronchoscopy. BW was done in the opposite bronchial tree than the routine samples in which sampled with a pre-wash and then applying 20ml isotonic NaCl. Figure 1 shows where the sampling was done. A peripheral blood sample for leukocyte telomere length was also taken from the subjects after the sampling with the bronchoscopy.

Telomere length evaluation

DNA from both BW and leukocytes were extracted by commercial kits (RTA technologies). Universal-STELA was carried out as described previously [20] with minor alterations. To summarize, 1 µg DNA was digested in a 50 µl reaction containing 1 µl MseI, 0.5 µl NdeI and 5 µl cutsmart buffer in 1 hour at 37^{o}C. MseI and NdeI were subsequently inactivated at 65^{o}C in 20 minutes. 0.05 µg of digested DNA is added to 3 µl of 12 mer and 42 mer panhandles in 7 µl of volume. The reaction temperature is decreased from 65^{o}C to 16^{o}C in 49 minutes. 20 units of T4 DNA ligase with 1.5 µl of T4 DNA ligase buffer were quickly added to the reaction and volume was increased to 15 µl with 6 µl dH2O at 16^{o}C. Reactions were incubated overnight. After incubation 20 units of T4 DNA ligase and 2.5 µl telorette working solution was added with 1 µl of T4 DNA ligase buffer and volume was increased to 25 µl with 6 µl dH2O. Reactions were incubated at 35^{o}C overnight and were inactivated by 20 minutes incubation at 65^{o}C. This was then followed by a PCR reaction which was exact to the previously described [20]. Universal STELA amplicons were separated by gel electrophoresis on a 0.8% agarose gel at 70 V for 3 hours. The separated DNA was transferred to a positively charged nylon membrane (Amersham). DNA fragments in the blot were hybridized to the DIG-labeled telomeric probe overnight at room temperature and incubated with a DIG-specific antibody with AP fragments. Chemiluminescence was detected with CDP-Star (Roche). All

experiments were triplicated. Ultra-short telomeres were defined as telomeres shorter than 1.5 kb as established previously. Telomeres that are not shorter than 1.5kb were defined as not critically short telomeres.

Statistics

A t-Student, ANOVA, Chi^2, and Paired Sample *T*-test were used to test differences in means and proportions. Normality was tested by visual inspection of curves and by homogeneity testing. Two-tailed *p*-values ≤ 0.05 were considered significant. Statistical analysis was performed with the SPSS, IBM Co software ver. 23 (IBM Inc., New York, USA).

Results

The section of the lung in BW does not influence telomere length measurement

To investigate whether the BW localization is significant in telomere length measurement, the different locations in the lung were compared with respect to the presence of ultra-short telomeres. The location of BW did not show a significant difference when compared in terms of the presence of ultra-short telomeres ($p>0.05$) (Figure 1). The median age of the 32 subjects was 63 years, range [31 to 73 years]. The median total pack-years 25 (20cig/day/year) range [0-106] and the median FEV1% was 47%, range [19-86%]. 24 (75%) of 32 subjects were males.

The presence of ultra-short telomeres was found in 26 out of 32 (81%) in BW samples whilst ultra-short telomeres were found only 3 out of 32 (9%) of leukocytes samples of same subjects. There was no case with ultra-short telomeres that were observed only in leukocytes. 23 out of 32 (72%) subjects showed presents of ultra-short telomeres only in the bronchial washing and 6 out of 32 (19%) had telomeres longer than 1.5kbs.

Higher pack-years and lower lung function is associated with the presence of ultra-short telomeres in lung tissue

COPD grade 1 was seen in 8 of the subjects (25%), grade 2 in 7 (22%), grade 3 was 14 (44%) and grade 4 was 3 (9%) of the 32 subjects, respectively. Older age was significantly associated with COPD severity ($p=0.005$), increasing total pack-years was not significantly associated with COPD grade ($p=0.88$).

Higher total pack-years was found amongst patients with ultra-short telomeres (32 packyears versus 16 packyears; $p=0.045$), lower lung function (FEV1%) (51% versus 82%; $p<0.001$) when compared with subjects with telomere length more than 1.5kbs in BW (figure 2a).

Patients with ultra-short telomeres were older when compared to patients with telomere length above 1.5kbs (63 years versus 49 years) in BW but this association did not reach formal statistical significance ($p=0.06$).

Samples from leukocytes, only age (72 years versus 59 years; p<0.001) were significantly associated with ultra-short telomeres when compared to telomeres longer than 1.5kbs. A similar result was found for total pack-years (77 years versus 24 years; p=0.058), and FEV1% (37% versus 58%; p=0.12) between ultra-short telomeres in leukocytes and not critically short telomeres (figure 2b).

Incidence of total pack-years, age, and lung function

The incidence of total pack-years, age, and lung function (FEV1%) was compared among three distinct groups; patients with ultra-short telomeres in both BW and leukocytes, patients with ultra-short telomeres only in BW and subjects with no critically short telomeres. An increasing number of total pack-years, older age and lower FEV1% was observed through the groups comprising subjects with ultra-short telomeres in both BW and leukocytes, subjects with ultra-short telomeres only in BW and subjects with telomeres longer than 1.5kbs(all p<0.01) (figure 3).

Discussion

To our knowledge, this is the first study reporting the association between ultra-short telomeres and smoking in lung tissue in patients diagnosed with COPD concerning total pack-years. The results of this study are not influenced by the location of BW. The results suggest a dose-response relationship between pack-years of smoking, low lung function in COPD, and ultra-short telomere length. This implies a link between ultra-short telomere length, total tobacco smoke exposure, presence of persistent airway limitation as well as aging-related disease.

Previous reports on associations between telomere length with smoking were with leukocytes [21], lung tissue from mice [22], and from people who were not diagnosed with COPD [23]. Here in this study, there were no associations between telomere length and total pack-years and lung function parameters in leukocytes in our study group. In addition to this, there were not any subjects who had ultra-short telomeres in leukocytes and lack in lung tissue, whereas 72% of the COPD subjects only had ultra- short telomere in the lung tissue. Our results indicate that measuring ultra-short telomeres in lung tissue may detect smoking-related lung damage earlier than in leukocytes. Our finding suggests that abrupt telomere shortening is an important DNA damage indicator of the effects of cigarette smoke on lung tissue from patients diagnosed with COPD. Thus, the evaluation of telomere length in patients diagnosed with COPD is strongly suggested to be carried out in the lung tissue.

We confirmed previous findings that telomere length was shorter with older age in the leukocytes of the patients diagnosed with COPD [22,24-25]. As natural or accelerated aging occurs in the lungs, telomeres are expected to be shorter [22]. COPD risk factors contribute to cellular aging and result in reduced telomere length. The wide range of age in patients involved in the current study increases the representativeness of the results.

In a previous study, a sample of more than 45000 individuals, telomere length was moderately associated with FEV1 in leukocytes [26]. This is also the case when other pulmonary diseases are included [27]

although telomere length and FEV1 association is stronger in COPD. Albeit a significantly smaller sample size, here we report a significant association between ultra-short telomeres and FEV1 in the lung tissue. This may be attributed to the lung being the site of injury in COPD may increase the likelihood of having ultra-short telomeres.

The main limitation of this study was the small sample size. Thus, sex-specific effects were not possible to be evaluated. Due to our cross-sectional approach, longitudinal effects between telomere length, total pack-years, and lung function cannot be studied.

Conclusions

We conclude that ultra-short telomeres are associated with increased pack-years, age, and decreased lung function in patients diagnosed with COPD irrespective of BW location. Pack-years is an important variable in telomere shortening and thus lower lung function in COPD. The effects of smoking can be determined at an earlier stage if the telomere evaluation is carried out in the lung tissue.

Abbreviations

1. Chronic obstructive pulmonary disease- COPD
2. Bronchial washing- BW
3. Forced expiratory volume in 1 second- FEV1
4. Forced vital capacity - FVC
5. predicted value of forced expiratory volume in second- FEV1%
6. European Respiratory Society- ERS
7. Global Initiative for Chronic Obstructive Pulmonary Disease - GOLD

Declarations

Ethics approval and consent to participate

Near East University granted Ethical approval to carry out the study within its facilities (Ethical Application Ref: YDU-2015/30-202) with informed consent from participants.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interest

The authors declare that they have no competing interest

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Region Syddanmarks Forskningspulje, “Telomerforkortning og DNA-skader i lungevæv ved KOL”

Region Syddanmarks Forskningspulje, “Telomere shortening in lung epithelial cells in patients with chronic obstructive pulmonary disease (COPD)”Authors’ contributions

Authors’ contributions

HC performed the laboratory work for telomere length evaluation, contribute to generation of raw data, interpretation of data and writing of manuscript. NS supervised the study, contribute to study design, telomere length evaluation, interpretation of data and writing and reviewing of this manuscript. AU collected patient samples and clinical information of patients. FR is a contribute to study design, evaluate the patient’s clinical data, carried out the statistical analysis and contribute to interpretation of result and writing and reviewing the manuscript.

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Not Applicable

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Figures

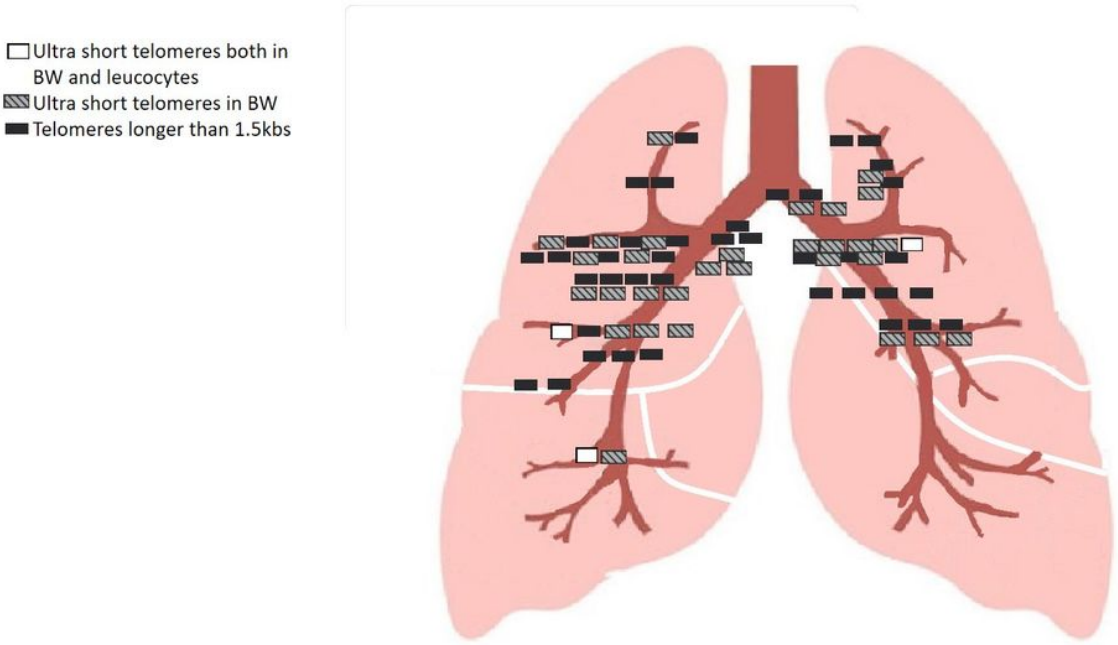

Figure 1

Bronchial tree showing the different locations of the bronchial washing sample (BW) location Presence of ultra-short telomeres in both BW and leukocytes (white bars), ultra-short telomeres only in BW (shade bars), and only telomeres longer than 1.5kbs (black bars).

Figure 2

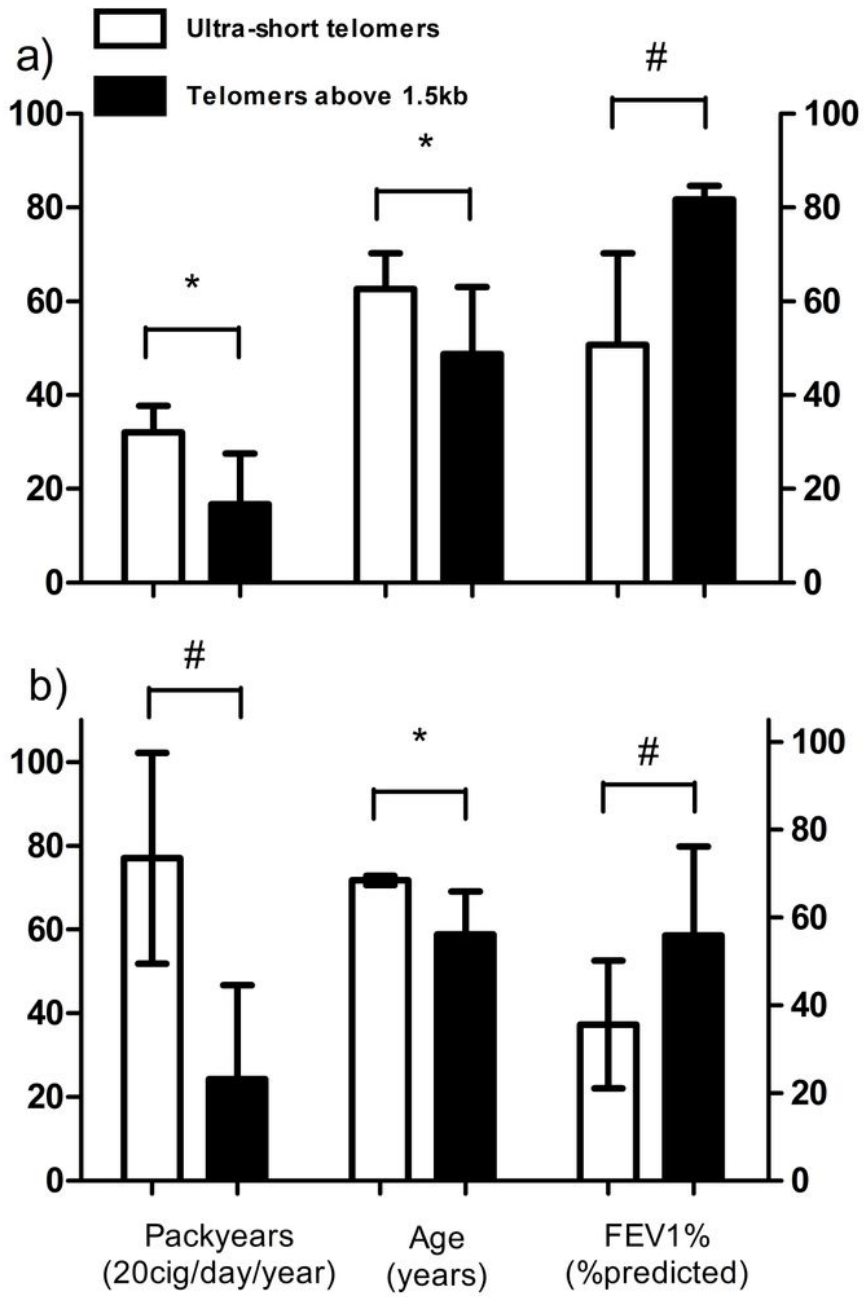

Figure 2

Distribution of total pack-years, age and lung function with respect to ultra-short telomeres Ultra-short telomeres (white bars) and telomeres longer than 1.5kbs (black bars) in bronchial washing samples (a) and leukocyte samples (b). *=p<0.05, #=p>0.05, FEV1%= predicted value of Forced Expiratory Volume in 1 second.

Figure 3

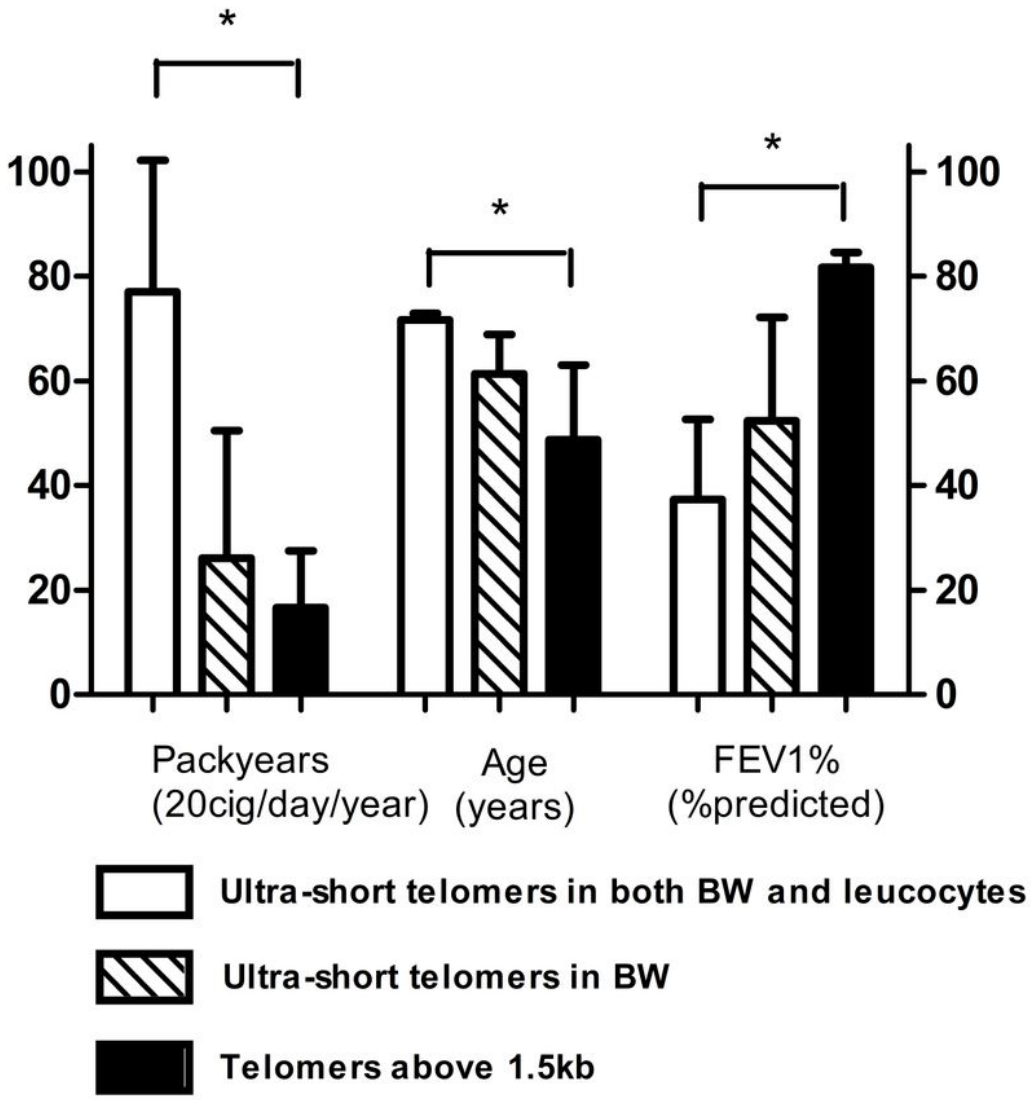

Figure 3

Incidence comparison of total pack-years, age, and lung function Subjects with ultra-short telomeres compared to subjects with telomeres longer than1.5kbs in both bronchial washing samples and leukocyte samples(white bars); subjects with ultra-short telomeres only in bronchial washing samples (shaded bars) and subjects with telomeres longer than1.5kbs (black bars). *=$p<0.05$, FEV1%= predicted value of Forced Expiratory Volume in 1 second.