Original Article

Correlation of Short Leukocyte Telomeres and Oxidative Stress with the Presence and Severity of Lung Cancer Explored by Principal Component Analysis

(lung cancer / peripheral blood telomere length / oxidative stress)

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Abstract. Lung cancer (LC) is the second most common malignancy and leading cause of cancer death. The potential "culprit" for local and systemic telomere shortening in LC patients is oxidative stress. We investigated the correlation between the peripheral blood leukocyte (PBL) telomere length (TL) and the presence/severity of LC and oxidative stress, and its usefulness as LC diagnostic marker. PBL TL was measured in 89 LC patients and 83 healthy subjects using the modified Cawthon RTq-PCR method. The relative PBL TL, found to be a potential diagnostic marker for LC with very good accuracy (P < 0.001), was significantly shorter in patients compared to the control group (CG) (P < 0.001). Significantly shorter telomeres were found in patients with LC TNM stage IV than in patients with stages I-III (P = 0.014), in patients without therapy compared to those on therapy (P = 0.008), and in patients with partial response and stable/progressive disease compared to those with complete response (P = 0.039). The total oxidant status (TOS), advanced oxidation protein products (AOPP), prooxidant-antioxidant balance (PAB) and C-reactive protein (CRP) were significantly higher in patients compared to CG (P < 0.001) and correlated negatively with TL in both patients and CG (P < 0.001). PCA showed a relation between PAB and TL, and between the EGFR status and TL. Oxidative stress and PBL telomere shortening are probably associated with LC development and progression.

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Abbreviations: AOPP – advanced oxidation protein products, CRP – C-reactive protein, EGFR – epidermal growth factor receptor, FEV1 – forced expiratory volume in one second, FVC – forced vital capacity, IMA – ischaemia-modified albumin, LC – lung cancer, LTL – leukocyte telomere length, NSCLC - non-small cell lung cancer, $O_2^{\cdot-}$ – superoxide anion radical, PAB – prooxidant-antioxidant balance, PBL(s) – peripheral blood leukocyte(s), PCA – principal component analysis, qPCR – quantitative polymerase chain reaction, TL – telomere length, TOS – total oxidant status.

Introduction

According to World Health Organization (WHO), lung cancer is the second most common malignancy worldwide, with 2.21 million new cases in 2020, out of which 1.80 million resulted in death, making it the leading cause of cancer death (Ferlay et al., 2021). In the same year, in Serbia, lung cancer had both highest incidence, with 8,048 new cases (16.4 % of all new cases of

cancer), and highest mortality, with 7,084 deaths (25.2 % of all cancer deaths) (Ferlay et al., 2021). Multiple risk factors, either individually or jointly, contribute to lung cancer development in general, including demographic/genetic, behavioural (both active and passive smoking, including cannabis and electronic cigarettes) and environmental (radon, asbestos, air pollutants) exposure, as well as persistent infections (with, for example, oncogenic viruses, such as human immunodeficiency virus (HIV)) and chronic inflammation (Dela Cruz et al., 2011; Effros, 2011; de Groot et al., 2018). Tuberculosis and acute respiratory Chlamydia pneumonia infection-caused inflammation are suspected to be able to contribute to LC pathogenesis, the latter hypothesized to trigger reactive oxygen species (ROS) production and cause DNA damage and genomic instability (Dela Cruz et al., 2011; de Groot et al., 2018). Information gained in the period 1990–2017 showed that men were more prone to developing lung cancer than women (Fitzmaurice et al., 2019). However, during that time and later, the rates of male lung cancer started decreasing, while the rates of female lung cancer started increasing, which is mostly attributed to changes in smoking habits and which led to a later peak in lung cancer incidence in women (de Groot et al., 2018). Other factors seem to have a greater impact on female lung cancer incidence, such as indoor air pollution, occupational exposures, genetic mutations, higher family risk of lung cancer, and possibly even the hormonal influence (de Groot et al., 2018). Currently valid histological classification of lung cancer was given by WHO in 2021 (Nicholson et al., 2022).

Emerging evidence indicates that cancer has multiple molecular pathways in common with the natural process of ageing (Bernardes de Jesus and Blasco, 2013), both resulting in a decline of immune system functions. The competence of the immune system is known to be dependent on the cellular survival, renewal, and clonal expansion of immune cells (Qian et al., 2016). Senescence of the immune cells alters and impairs their functions, which can cause genomic instability and potentially lead to cancer development (Fali et al., 2019; Kachuri et al., 2019; Lim et al., 2020). Immunodeficient individuals have been shown to be at increased risk of developing malignancies, including lung cancer (Vesely et al., 2011; Gonzalez at al., 2018). Conversely, chronic antigenic stimulation, such as the one occurring in cancers, accelerates immune cells' differentiation into effector/memory cells, their turnover, replication and, consequently, progressive immunosenescence and exhaustion (Vesely et al., 2011; Fali et al., 2019). The latter, along with the other mechanisms of tumour-mediated immunosuppression, facilitates tumour progression (Burkholder et al., 2014; Qian et al., 2016; Gonzalez at al., 2018; Lim et al., 2020; Hiam-Galvez et al., 2021). So far, a correlation was found between multiple immune system parameters, such as the presence and number of tumour-infiltrating lymphocytes (TILs), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-regulated immune response genes and programmed death-ligand 1 (PD-L1) expression, and non-small cell lung cancer (NSCLC) outcome (Mu et al., 2011; Hopewell et al. 2013). Additionally, immune changes were also found in pulmonary premalignancy and early-stage lung cancer (Lim et al., 2020). In accordance with the fact that cancer is a systemic disease (Burkholder et al., 2014; Hiam-Galvez et al., 2021), changes were reported in both peripheral and tumour leukocyte counts in NSCLC patients (Brahmer, 2013; Hiam-Galvez et al., 2021).

Telomeres are nucleoprotein complexes stabilizing the coding sequences at the ends of somatic chromosomes. Telomeres are normally progressively lost with each cell division as a part of the cell ageing process (Qian et al., 2016). Multiple factors can make telomeres dysfunctional, leading to genomic instability and, subsequently, cancer development (Heaphy and Meeker, 2011). Chronic antigenic stimulation occurring in cancer accelerates immune cells' proliferation upon which their telomeres shorten (Burkholder et al., 2014). To preserve the immune function, lymphocytes tend to upregulate telomerase, the enzyme which prolongs and thus protects telomeres (Qian et al., 2016). So far, several studies have shown positive association of longer white blood cell telomere length with the lung cancer risk (Seow et al. 2014; De-Torres et al., 2017), in some of which only association with adenocarcinoma histological subtype was confirmed (Yuan et al., 2018; Kachuri et al., 2019). However, meta-analysis of nine studies showed positive association between short leukocyte telomere lengths and the risk of lung cancer (Karimi et al., 2017). Also, shorter peripheral blood T-lymphocyte telomere length was found in patients who already developed lung cancer in comparison to healthy controls. Based on the results from the same study, shorter telomere length was supposed to be related with the increased stage of lung cancer (Qian et al., 2016).

The balance between prooxidants and antioxidants in each cell is vital for its normal functioning, due to the prooxidants' roles in signal transduction, gene expression, etc. (Valavanidis et al., 2013; Gómes et al., 2016; Morry et al., 2017). Dysregulation of this balance in favour of prooxidants (which can be triggered by multiple endogenous and exogenous factors, including, but not limited to, ageing and inflammation) leads to oxidative stress, which has a high potential of having deleterious effects on essential cellular macromolecules – proteins, lipids and nucleic acids (Valavanidis et al., 2013; Gómes et al., 2016; Morry et al., 2017). DNA damage is especially important, as oxidative stress implication in modulation of multiple genes' expression and signal transduction pathways, as well as in the genetic damage and replication blockage, can lead to cellular degeneration, carcinogenesis and ageing (Valavanidis et al., 2013; Morry et al., 2017). Telomeres seem to be particularly sensitive to oxidative DNA damage, which can accelerate their shortening and thus contribute to cellular senescence (Effros, 2011; Valavanidis et al., 2013).

In this study, we sought to investigate whether shorter telomere length (TL) in peripheral blood leukocytes (PBLs) is associated with the presence and severity of lung cancer and the overall response to therapy. PBL TL was also assessed as a diagnostic marker for LC. In addition, the relationship between PBL TL and the presence and epigenetic effects of oxidative stress was explored.

Material and Methods

This study involved 89 lung cancer outpatients paired by gender with a control group of 83 healthy subjects. Lung cancer patients were recruited in the period between March 16, 2017, and July 17, 2017, at the Pulmonology Clinic, Clinical Center of Serbia, with the approvals of the Ethical Committee of the Faculty of Medicine, University of Belgrade (No. 29/V-15) and Collegium of Pulmonology Clinic, Clinical Center of Serbia (No. 478/2). Approval for use of the specimens from the control group subjects was obtained from the Ethical Committee of the General Hospital "Medigroup", Belgrade, Serbia (No. 1050/1/15).

Among 89 lung cancer patients, 76 had NSLSC (40 adenocarcinoma, 31 squamous-cell carcinoma, 5 large-cell carcinoma), while 13 had small-cell lung carcinoma. Patients were classified according to the 8th edition of tumour, nodes, metastases (TNM) Classification of Malignant Tumours (Planchard et al., 2018). Out of 89 LC patients, 46 (52 %) had distal metastases and 78 (88 %) LC patients received therapy (77 patients received chemo/targeted therapy and one patient received radiotherapy of endocranium). Therapy included paclitaxel/platinum, etoposide/platinum (EP), EP in adjuvant regimen, vinorelbine/platinum (VP), gemcitabine/platinum (GP), pemetrexed/platinum, Giotrif (afatinib), Iressa (gefitinib), paclitaxel/carboplatin, cisplatin/doxorubicin/cyclophosphamide (CAP), or GP with EP in adjuvant regimen. According to WHO categorization of therapy responses, the complete response is defined as disappearance of all target lesions; progressive disease as ≥ 25 % increase in the size of measurable lesions, appearance of new lesions, or unequivocal progression of non-target lesions; partial response as ≥ 50 % decrease in target lesions, without a 25 % increase in any target lesion or new lesions, and stable disease as neither partial response nor progressive disease ("no change" per the WHO criteria) (Nishino et al., 2014).

Peripheral blood was collected in 10 ml ethylenediaminetetraacetic acid (EDTA) vacutainers after 8-hour fasting. The samples were kept and transported at +4 °C within a 60-minute period. All the samples were then centrifuged for 10 minutes at 1,500 *g* and the buffy coat was collected and stored at –80 °C until DNA isolation. After quick thawing of the buffy coat in a 37 °C water bath (stored on ice afterwards), genomic DNA for telomere length measurement was isolated using a FlexiGene DNA kit (Qiagen, Düsseldorf, Germany*)*. Absorbance was measured at the wavelengths of 230, 240, 260, 280 and 300 nm in a Shimatzu UV spectrophotometer UV-1800 and the concentration of the isolated DNA was calculated. Acceptable purity ratio A260/A280 (indicating the presence of protein and/or phenol) was 1.7 to 2.0 and A260/230 (indicating the contamination with organic compounds) 2.0 to 2.2. The remaining isolated DNA samples were then stored at –80 °C until further analysis.

The telomere length in peripheral blood leukocytes was measured using the modified Cawthon real-time quantitative polymerase chain reaction (RTq-PCR) amplification method (Cawthon, 2002), quantifying fluorescent signal proportional to the mean telomere length (TL) in the sample, compared to the "single nuclear gene copy number" (S). In this study, albumin was used as a standard (reference) single-copy gene (SCG) for normalization. qPCR was performed using the Applied Biosystems 7500 Real Time PCR System (Thermo Fisher Scientific, Waltham, MA). The samples were pooled and used for the calibration curve. Four 4-fold standard dilutions (50, 12.5, 3.125 and 0.78125 ng/µl) were used for construction of the standard curve. Each sample was run in triplicate. qPCR mixes for both telomere and albumin were prepared from 4 µl of PCR clean water, 3 µl of 5 × HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia), 2 µl of sample (DNA concentration 5 ng/µl) and 3 µl of 400 nM of each primer ('ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3' partially complementary sequence was used as a forward primer and 5'-TGTTAGGTATCCCTATCCCTATCCCTAT CCCTATCCCTAACA-3' as a reverse primer for telomere and 5'-CGGCGGCGGGCGGCGCGGGCTGGGCGGAAATGCTGCACAGSSTCCTTG-3' as a forward primer and 5'-GCCCGGCCCGCCGCGCCCGTCCCGCCGGAAAAGCATGGTCGCCTGTT-3' as a reverse primer for albumin). For telomeres, the protocol in the real-time system was set to one cycle of initial activation at 95 ℃ for 12 minutes, four cycles of 15 seconds at 95 ℃ and 20 seconds at 49 ℃, and 40 cycles of denaturation for 15 seconds at 95 ℃, annealing for 10 seconds at 60 ℃ and elongation for 35 seconds at 72 ℃. For the standard gene, it was set to one cycle of initial activation at 95 ℃ for 12 minutes and 40 cycles of denaturation for 15 seconds at 95 ℃, annealing for 10 seconds at 60 ℃ and elongation for 35 seconds at 87 ℃. The threshold cycle (Ct) value was read at the third point of the elongation cycle. DNA concentration was read from the Ct vs log (quantity of specimen DNA) standard curve graph. The relative, mean telomere length was calculated as the ratio between the telomere : albumin gene concentration (T/S) of the specimen and T/S of the reference DNA pool used on the same plate as a calibrator.

Parameters of the oxidative stress status, including prooxidant-antioxidant balance (PAB), total oxidant status (TOS), advanced oxidation protein products (AOPP), ischaemia-modified albumin (IMA) and superoxide anion radical (O_2^-), and inflammation (C-reactive protein (CRP)) were also assessed using plasma, which was separated and stored at –80 °C after centrifugation of the

samples, as a biologic material. PAB, TOS, AOPP, IMA, $O_2^{\cdot-}$ and CRP were assessed by the methods optimized in the laboratory of the Department of Medical Biochemistry, Faculty of Pharmacy, University of Belgrade, Serbia and published by Kotur-Stevuljevic et al. (2015). IMA was assessed using the spectrophotometric method with cobalt chloride and dithiothreitol, originally established by Bar-Or et al. (2000), in an ELISA reader (Pharmacia LKB, Wien, Austria).

Statistical analysis

Normality of data distribution was tested with the Shapiro-Wilk's test. Normally distributed data were presented as mean and standard deviation. Data that did not follow normal distribution were presented as median with 25^{th}–75^{th} percentiles. Comparisons between the tested groups were done by Student's *t*-test and Mann-Whitney test, respectively. Categorical data were given as absolute frequencies and compared by χ^2 test for contingency tables. Correlations between clinical data were tested by Spearman's bivariate correlation analysis. The discriminative ability of TL was assessed by receiver operating characteristic (ROC) curves as global measures of diagnostic accuracy. According to established criteria, the area under the curve (AUC) was used for evaluation of diagnostic accuracy. Principal component analysis (PCA) was performed using the varimax-normalized rotation method. This was done to streamline a substantial number of estimated variables into a chosen set of factors, each characterized by similar variability in parameters. Factors extracted by PCA were determined according to preselected eigenvalues > 1. Variables with factor loadings larger than 0.5 were used in subsequent analysis and factor interpretation. Statistical analyses were done by using the IBM®SPSS®model 22.0 (IBM Corporation, Armonk, NY). Differences at $P < 0.05$ were considered as significant.

Results

Characteristics of patients with LC and healthy controls are presented in Table 1. LC patients were significantly older ($P < 0.001$) and had a higher percentage of smokers ($P < 0.001$). There was no significant difference in gender distribution between the two groups ($P = 0.081$). Relative TL in peripheral blood leukocytes was significantly shorter in patients with LC compared to CG ($P < 0.001$, Fig. 1). This difference remained significant after adjustment for age using the Quade's test ($P < 0.001$). In CG, females had significantly longer

Table 1. Characteristics of lung cancer patients and controls

	Control group N = 83	**Lung cancer patients N = 89**	**P**
Age[a]	50 ± 10	65 ± 7	< 0.001
Gender (m/f)[b]	47/36	62/27	0.081
Smoking status (yes/no)[b]	16/67	83/6	< 0.001
Oxidative stress markers			
TOS (μmol/l)[c]	10.3 (6.0–19.9)	32.74 (19.7–51.7)	< 0.001
AOPP (μmol/l)[c]	34.0 (29.7–38.5)	64.1 (51.1–79.2)	< 0.001
PAB (IU)[c]	67.7 (54.7–92.3)	175.3 (159.2–196.3)	< 0.001
$O^{2\cdot-}$ μmol/l NBT/min/l[c]	2.5 (2.0–13.5)	493.0 (387.0–592.0)	< 0.001
IMA[c]	0.088 (0.0760–0.1193)	0.522 (0.321–0.752)	< 0.001
CRP (mg/l)[c]	0.50 (0.20–1.40)	15.70 (6.75–21.08)	< 0.001
Type of lung cancer:			
Small-cell lung carcinoma	N/A[d]	13	N/A
Large-cell carcinoma	N/A	5	N/A
Adenocarcinoma	N/A	40	N/A
Squamous-cell carcinoma	N/A	31	N/A
Stage of cancer[e]:			
Stage I	N/A	9	N/A
Stage II	N/A	5	N/A
Stage III	N/A	29	N/A
Stage IV	N/A	46	N/A
Metastasis (yes/no)	N/A	43/46	N/A
Chemotherapy (yes/no)	N/A	78/11	N/A

a – variables were tested with Student's *t*-test; b – variables were tested with χ^2 test; c – variables were tested with Mann-Whitney test; d – N/A – not applicable to the control group of healthy subjects; e – TNM staging of cancer

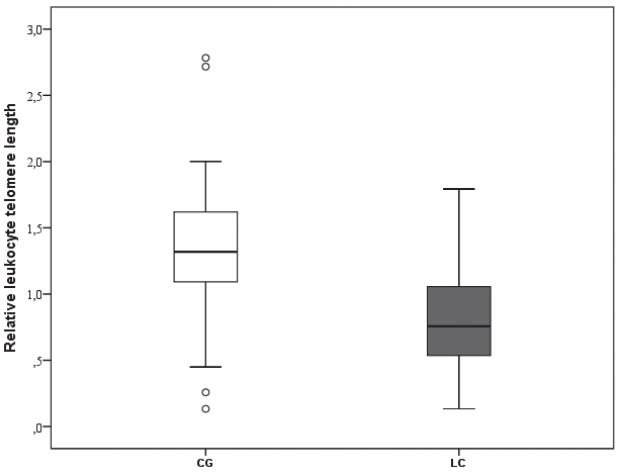

Fig. 1. Relative TL in peripheral blood leukocytes of healthy subjects (CG) and LC patients

telomeres compared to males (1.463 (1.256–1.737) vs 1.164 (0.988–1.485), P = 0.001), while there was no significant difference in TL between males and females with LC (0.536 (0.339–0.773) vs 0.529 (0.437–0.740), P = 0.443). To explore whether telomere shortening is related to the severity of LC, patients were classified according to TNM. TL was not associated with the tumour size and the degree of spread to regional lymph nodes. On the other hand, patients with the presence of distant metastases had significantly lower relative TL compared to patients without distant metastases (P = 0.014). Furthermore, when patients were categorized according to TNM staging of cancer, patients with stage IV had significantly lower TL compared to patients in other groups (I, II, or III) (P = 0.014, Fig. 2A), while there was no difference according to the groups' age, gender distribution or smoking status.

The TL ability to discriminate between healthy subjects and LC patients was also analysed (Fig. 3). According to the established criteria, AUC between 0.7 and 0.8 defines good diagnostic accuracy; AUC between 0.8 and 0.9 designates very good diagnostic accuracy, while AUC between 0.9 and 1.0 is a marker of excellent diagnostic accuracy (Hosmer and Lemeshow, 1989). Our results demonstrated that TL has very good accuracy as a potential diagnostic marker for LC (AUC = 0.835 (0.772–0.898), P < 0.001). In addition, the ability of TL to discriminate between patients with different stages of cancer was estimated. Patients with stage IV had significantly lower TL compared to patients in other groups (I, II, or III) and TL was shown to have satisfactory discriminatory potential (AUC = 0.652 (0.538–0.765), P = 0.014). In the case of patients with complete response to therapy compared to patients either in the progressive disease, partial response, or stable disease group, we found that TL had good discriminatory potential (AUC = 0.705 (0.503–0.907), P = 0.039).

LC patients on therapy had significantly longer telomeres compared to those without therapy (P = 0.008). In

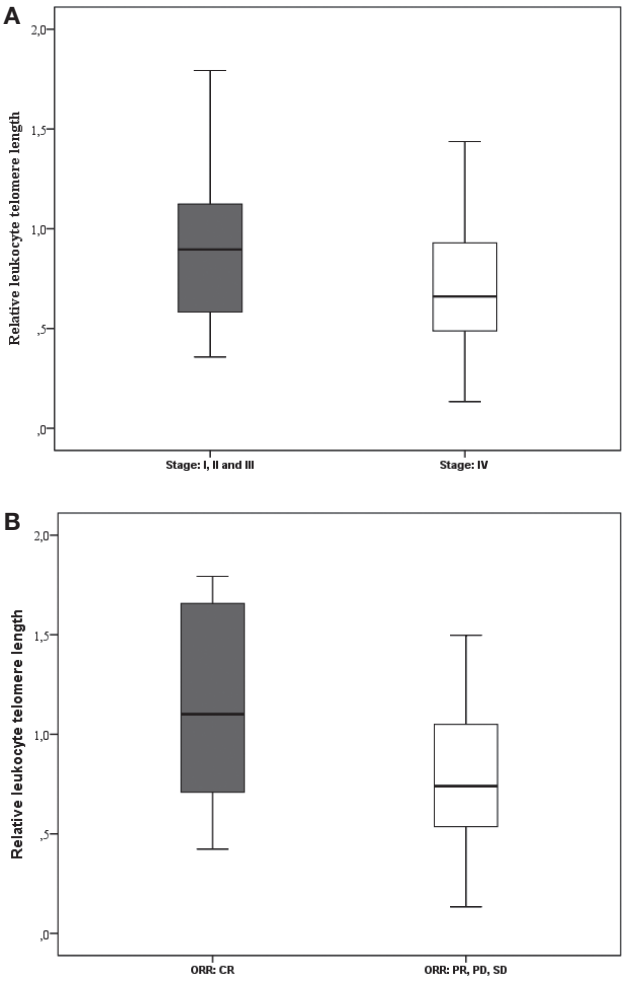

Fig. 2. Relative TL in peripheral blood leukocytes of LC patients according to: (**A**) TNM staging of cancer and (**B**) the overall response rate (complete response, progressive disease, partial response, and stable disease).

addition, TL was significantly longer in patients with complete response compared to patients with progressive disease, partial response, or stable disease (P = 0.039, Fig. 2B).

The measured prooxidant parameters (TOS, PAB), product of prooxidants' activity (AOPP) and CRP values were all significantly higher in LC patients (32.74 (19.70–51.75), 175.33 (159.20–196.26), 64.1 (51.1–79.2), and 15.70 (6.75–21.08), respectively) compared to the control group (10.30 (6.00–19.90), 34.0 (29.7–38.5), 67.26 (54.69–92.33) and 0.50 (0.20–1.40), respectively) (P < 0.001). Additionally, a significant negative correlation was found between the relative, mean telomere length and TOS (P = –0.374, P < 0.001), AOPP (P = –0.398, P < 0.001), PAB (P = –0.510, P < 0.001) and CRP (P = –0.438, P < 0.001). It is important to outline that such results were obtained when the correlation was assessed in both healthy CG subjects and LC patients together, while no significant correlation was found when assessed in each group of subjects individually, indicating natural interdependence between TL and each of the

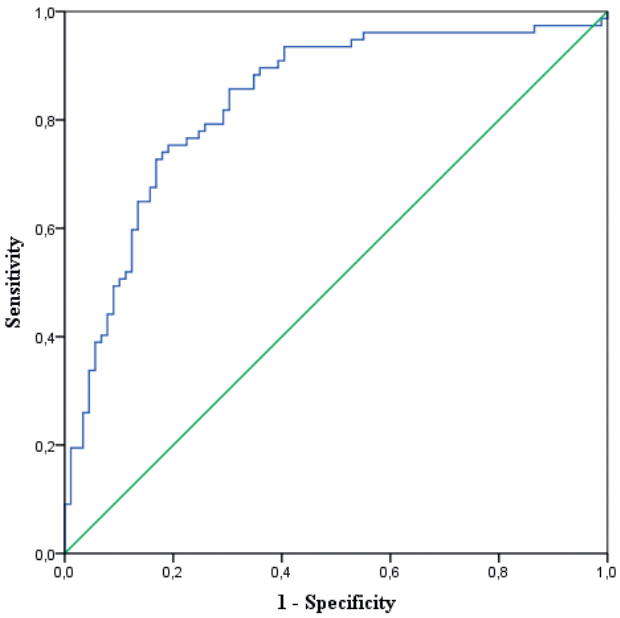

Fig. 3. Diagnostic accuracy for the TL as a marker of LC (AUC = 0.835 (0.772–0.898), P < 0.001).

above-mentioned parameters (TOS, AOPP, PAB and CRP), and thus requiring assessment of both low and high values for a correlation to be observed.

PCA was applied to leukocyte telomere length (LTL) together with two groups of parameters, first the redox status group of parameters and second the clinical and lung cancer disease-related group of parameters. The first group of parameters plus LTL explained about 69 % (Kaiser-Meyer-Olkin (KMO)) measure of sampling adequacy = 0.602 and Bartlett's test of sphericity P < 0.001), while the second group of parameters explained 77 % of the variance (KMO = 0.585, Bartlett's test of sphericity P < 0.001) in all examined parameters. The detailed percentage of variance by distinct factors is presented in Table 2. Redox status parameters are grouped in three factors which we have named as follows: Oxidatively modified lipids and proteins-related factor (AOPP and IMA), Prooxidant factor (TOS and $O_2^{\cdot-}$) and Prooxidant balance – telomere-related factor (PAB and LTL). Clinical-lung cancer disease parameters consisted of four factors: Clinical – patient-related data (FVC, FEV1 and gender), Patients' survival – metabolic-related factor (alive/deceased status, survival after diagnosis and CRP concentrations), Disease characteristics-related factor (initial metastases and disease staging) and DNA-related factor (epidermal growth factor receptor (EGFR) polymorphism and LTL). It should be stressed that regarding the similar variability grouping, among the redox marker group, LTL was related to PAB, and among the clinical parameter group, LTL was found related with EGFR polymorphism presence, both parameters being of DNA origin.

Discussion

In our study, peripheral blood leukocyte TL was found to be shorter in LC patients compared to healthy controls. The same results were obtained in the study in which the same biological material was used as in ours (Xue et al., 2020), as well as in a study in which only peripheral blood T lymphocytes were used (Qian et al., 2016). Telomeres are expected to be shorter in the cells exposed to antigenic stimulation, as occurring in cancer if, as suggested, they shorten due to proliferative stress (Yang et al., 2013). In addition, our results demonstrated that TL is a very accurate potential diagnostic marker for LC. No other study, according to our knowledge, has so far assessed its diagnostic potential in lung cancer. However, a study conducted in renal cell carcinoma (RCC) patients did suggest potential involvement of short peripheral blood leukocyte TL in the cancer development and also indicated it as a potentially useful marker (when combined with other markers) for early RCC detection (Park et al., 2019). It would be important to find out whether telomere shortening starts some time before lung cancer diagnosis, but this would be a subject of another, prospective study.

Table 2. Principal component analysis-extracted factors in LC patients connected with telomeres

Factors (redox status parameters + LTL)	Included variables with loadings	Factor variability	Factors (clinical-lung cancer disease parameters + LTL)	Included variables with loadings	Factor variability
1. Oxidatively modified lipids and protein-related factor	AOPP (0.689) IMA (0.520)	36 %	**1. Clinical – patient-related data**	FVC (L) (0.939) FEV1 (L) (0.901) Gender (m/f) (–0.794)	32 %
2. Prooxidant factor	TOS (0.889) $O_2^{\cdot-}$ (0.699)	17 %	**2. Patients' survival – inflammation-related factor**	Alive/deceased (0/1) (–0.901) Survival (months) (0.886) CRP (mg/l) (–0.536)	21 %
3. Prooxidant balance – telomere-related factor	PAB (0.815) LTL (–0.576)	16 %	**3. Disease characteristics-related factor**	Initial metastases (0/1) (0.904) Disease stage (I–IV) (0.900)	14 %
			4. DNA-related factor	EGFR status (0/1) (0.848) LTL (0.716)	10 %

Shorter telomeres found in cancer patients' peripheral blood leukocytes might be explained by their enhanced immunosuppressive status. Changes in both immunosenescence-specific immune cells and cytokines were found in cancer patients with shorter peripheral blood leukocyte telomere length (Chen et al., 2014; Qu et al., 2015), suggesting possible contribution of short telomere length to the decline in immune cells' function (Chen et al., 2014; Qu et al., 2015; Qian et al., 2016). The immune system's competence is dependent on clonal expansion and cell renewal of T and B cells, making them highly sensitive to telomere shortening (Qian et al., 2016). Evidence exists that immunocompromised individuals (such as transplant recipients using immunosuppressants and acquired immune deficiency syndrome (AIDS) patients) are more prone to cancer development (Vesely et al., 2011). Immunocompromisation and cancers, both associated with human ageing, are shown to be associated with short peripheral blood cell TL (Blackburn et al., 2015). Short telomeres, whose length represents the status of cell proliferation, growth, senescence, and apoptosis, cause quantitative and qualitative defects in haematopoietic stem cells, leading to their exhaustion and decrease in mature blood cell number (Qu et al., 2015). This defective lymphopoiesis further reflects in the decrease in protective immune functions, one of the major factors contributing to changes in age-associated incidence and severity of cancer. Vaccination aimed at prevention of infections, which can, in combination with other factors, potentially lead to cancer development, was shown to be significantly less effective in the elderly. Once the cancer develops, constant exposure to tumour-specific antigen triggers massive cell division and clonal expansion of the immune cells with adequate receptors, generated during transition of haematopoietic stem cells into mature lymphocytes. This long-term antigen-driven extensive proliferation ultimately leads to the end-stage replicative senescence, reflecting in the accumulation of senescent lymphocytes in certain cancers. Oxidative stress, shown to accelerate telomere shortening in cell culture, also contributes to T-lymphocyte replicative senescence and stimulates production of the NF-κB ligand (Effros, 2011).

In our study, we found that not only three of the oxidative stress markers measured (TOS, AOPP and PAB) but also the inflammatory parameter, CRP, were significantly higher in LC patients compared to the control group, but each of them also correlated negatively with the relative, mean telomere length when assessed in both LC patients and CG together. Such findings could be explained by a known interplay between inflammation, oxidative stress and DNA damage (Gómes et al., 2016). Inflammation is both a risk factor for cancer development and a consequence of cancer cell oncogenic changes, in the sense that DNA damaged by ROS and reactive nitrogen species (RNS) produced by inflammatory cells (Gómes et al., 2016; Morry et al., 2017) can induce further inflammatory cells' ROS/RNS production and chronic inflammatory conditions (Gómes et al., 2016). Specifically, telomeres were shown to be sensitive to shortening caused by increased oxidative stress and its epigenetic effects (Valavanidis et al., 2013). When parameters were grouped into factors by PCA selection based on their similar variability, PAB was found to correlate with LTL. Additionally, the EGFR polymorphism presence was found to be related with LTL. A study conducted by Yuan et al. (2019) has previously indicated the existence of an interaction between telomerase reverse transcriptase (TERT) and EGFR in telomere biology of NSCLC patients. A Weng et al.'s (2018) review article also outlined ROS and EGFR separate contributions to tumour progression, dysregulation of the latter being an especially important mechanism of NSCLC progression, as well as the existence of a correlation between these two parameters. Oxidation of both EGFR and its downstream pathways was found to promote tumour progression by enhancing EGFR-mediated signalling.

When we categorized patients according to TNM, TL was not associated with the tumour size and the degree of spread to regional lymph nodes; however, significantly lower relative TL was found in patients with distant metastases (stage IV) compared to the patients without metastases (groups I, II and III), regardless of their age, gender distribution or smoking status. Other studies, conducted in lung (Qian et al., 2016) and breast (Barczak et al., 2016) cancer patients, also showed shorter peripheral blood TL to be associated with a more severe TNM stage. In gastric and colorectal cancer patients, shorter leukocyte relative TL seemed to be an even more efficient indicator of worse prognosis when combined with advanced TNM stage (Chen et al., 2014; Qu et al., 2015). Most studies have so far indicated peripheral blood leukocyte relative telomere length as a useful predictor of clinical outcome (Qu et al., 2015), with short peripheral blood leukocyte relative telomere length found to be associated with poor cancer survival in patients with gastric, colorectal, renal, and breast carcinoma and glioma (Chen et al., 2014; Chen et al., 2015; Qu et al., 2015; Zhang et al., 2015; Ennour-Idrissi et al., 2017; Chen et al., 2020). Meta-analysis from 2015 which included studies with telomere length measured in both blood and tumour tissue showed no association between telomere length and lung cancer prognosis (Zhang et al., 2015). However, overall, it has indicated the telomere length as a better prognostic factor when measured in blood lymphocytes than in tumour tissue cells, which could possibly be explained by blood cells' TL having a stronger relationship with age than TL in other tissues (Zhang et al., 2015). Although leukocyte TL is widely used for estimating the human biological age, it is still questionable whether and to what extent it is affected by a specific disease in addition to the ageing process itself (Vaiserman and Krasnienkov, 2021). This is additionally complicated by the ability of various stress factors to trigger a redistribution of leukocyte subtypes, affecting the composition of blood leukocyte cell type populations, which is highly heterogeneous and

variable even among healthy individuals (Dhabhar et al., 2012; Semeraro et al., 2020). Furthermore, the telomere length is heritable to a significant extent (Samavat et al., 2021; Vaiserman and Krasnienkov, 2021). Contrary to the above-mentioned studies in which TL was measured in peripheral blood, a more recent study in which TL was measured in cancer cells and cancer-associated fibroblasts (CAFs) found longer telomeres to be associated with worse prognosis in adenocarcinoma and squamous cell carcinoma and acknowledged it as a novel biomarker for the diagnosis of aggressive cancers with poor prognoses (Matsuda et al., 2023). In studies exploring the role of cancer cell telomere length in cancer risk, both shorter and longer telomere lengths have been found to be associated with increased cancer risk (Chen et al., 2014), the possible explanation of the latter suggested to be lying in the germline mutations of the telomere maintenance genes causing telomerase up-regulation (Chen et al., 2014). Supporting this, circulating TERT mRNA (telomerase catalytic subunit) was shown to be an independent prognostic marker for lung cancer (Miura et al., 2006). The dual role of telomeres in carcinogenesis, reflected in both shortening and elongation of telomeres being able to increase cancer risk, can be explained by longer telomeres' higher proliferative potential making them more prone to mutations and the short telomeres' increased genomic instability favouring carcinogenesis (Nelson and Codd, 2020). A meta-analysis conducted by Zhu et al. (2016) showed significant association of short telomeres with the risk of only certain types of cancer, but non-significant association of short telomeres and the overall cancer risk, indicating diverse telomere roles among different cancers.

Our study showed that LC patients in therapy had significantly longer telomeres compared to those without therapy. Additionally, TL was found to be significantly longer in patients with complete response compared to patients with partial response, stable and progressive disease. This might be attributed to the immune cells' capability to adequately respond to therapy aimed to regulate their function (Zhang et al., 2020). Systematic review of the literature by Gallicchio et al. (2018) gave no definitive conclusions regarding the effects of cancer treatments on telomere length and found these effects to be dependent on both cancer and treatment type, as well as other factors. A study in which lymphopoenia, primarily reflected in a decline in the T lymphocyte number and exhaustion, was found in COVID-19 patients indicated that certain TL is required for the lymphocyte replicative response and immune system recovery, partially attributing this lymphopoenia to a short LTL (Aviv, 2020). However, the mean lymphocyte percentage (33.43 %) among LC patients from this study was within the reference range (18–54 %) (Valiathan et al., 2014; Aviv, 2020).

Limitations

The main conclusions of this study are limited by a relatively small number of patients and a small number of patients in certain subgroups. There was also a difference between LC patients and CG in demographic characteristics (age and smoking status). However, the differences in relative TL remained significant after adjustment for age using the Quade's test. In addition, when we compared TL between LC patients and CG subjects in men and women separately, the same results were obtained, suggesting that the gender distribution within the LC patients group and CG did not affect our results.

Conclusion

The study revealed a connection between short telomeres in peripheral blood leukocytes and the existence and severity of lung cancer. Additionally, there was an association with the use of chemotherapy, targeted therapy, or radiation, along with the level of treatment response. This implies a potential impact of PBL TL on the development and progression of lung cancer. Furthermore, PBL TL demonstrated high accuracy as a potential diagnostic marker for lung cancer. Future studies with more subjects in different subcategories could enable drawing more definitive conclusions. We have also found both main inflammatory and certain oxidative stress markers to be increased in LC patients compared to CG and a negative correlation of each of these parameters with relative, mean LTL, when observed in both LC patients and CG together. In addition, PCA also showed a correlation of prooxidant-antioxidant balance, as well as the presence of EGFR polymorphism with LTL, indicating not only a significant role of oxidative stress in the overall DNA damage, but also a possible interplay between telomeres and EGFR and/or a common mechanism of oxidative stress influence on both parameters.

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