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Leukocyte telomere length and mitochondrial DNA copy number association with colorectal cancer risk in an aging population

Sofia Malyutina[1]*, Vladimir Maximov[1], Olga Chervova[2], Pavel Orlov[1], Vitaly Voloshin[3], Andrew Ryabikov[1], Mikhail Voevoda[1], and Tatiana Nikitenko[1]

[1]Research Institute of Internal and Preventive Medicine - Branch of Institute of Cytology and Genetics SB RAS, Novosibirsk 630089, Russia

[2]UCL Cancer Institute, University College London, London WC1E6BT, UK

[3]Royal Botanical Gardens Kew, London TW9 3AE, UK

Abstract

In this study, we evaluated the association of blood leukocyte telomere length (LTL) and mitochondrial DNA copy number (mtDNA-CN) with the risk of incident colorectal cancer (CRC). We studied and followed-up a cohort of Russian men and women (aged 45 – 69 years, *n* = 9360, 54% female) from the HAPIEE study for 15 years. Using the nested case-control design, we selected cases with incident CRC among those free from any baseline cancer (*n* = 146) and sex- and age-stratified controls among those free from baseline cancer and cardiovascular disease and alive at the end of the follow-up (*n* = 799). We employed multivariable-adjusted logistic regression to estimate the odds ratios (ORs) of CRC per 1 decile of LTL or mtDNA-CN. We observed an inverse association of LTL and mtDNA-CN baseline values with the 15-year risk of incident CRC. Carriers of shorter telomeres had an increased 15-year risk of incident CRC with adjusted OR 3.2 (95% CI: 2.56 – 3.87, $P < 0.001$) per 1 decile decrease in LTL, independent of baseline age, sex, smoking, body mass index, blood pressure, lipid levels, and education. Similarly, lower mtDNA-CN was associated with the higher risk of incident CRC with adjusted OR 1.7 (95% CI: 1.12 – 1.89, $P < 0.001$) per 1 decile decrease in mtDNA-CN, independent of the aforementioned factors. Using the modified values of LTL and mtDNA-CN adjusted for multiple factors and their interactions with a case–control status, the ORs of CRC were 2.53 and 1.52 per 1 decile decrease in adjusted baseline LTL and mtDNA-CN, respectively. In conclusion, LTL and mtDNA-CN were independent inverse predictors of the 15-year risk of CRC in the Russian cohort. These findings highlight the relevance for subsequent research to exploit the mechanisms through which LTL and mtDNA-CN may reflect human health.

Keywords: Leukocyte telomere length; Mitochondrial DNA copy number; Aging; Cancer; Case–control; Population

***Corresponding author:**
Sofia Malyutina
(smalyutina@hotmail.com)

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1. Background

The world population is expected to reach 8.6 billion people by 2030, with about 1.4 billion being over 60 years old[1]. An increase in life expectancy goes hand-in-hand with the aging of population and the aggregation of age-related diseases.

Cancer primarily represents an age-related group of pathologies and is among the top 20 causes of death[2]. Colorectal cancer (CRC) is the third most common type of cancer in men and the second in women worldwide, with a mortality rate of 11.0 and 7.2/100,000, respectively[3].

Environmental factors contribute significantly to the risk of CRC development; however, there are also non-modifiable risk factors, such as older age, family history of cancer[4], and molecular genetic contributors[5].

Considering the multifaceted process of aging accompanied by health decline, the molecular markers of "biological age" may reflect the signals of the pace of aging. Among the potential markers of biological age, telomere length and mitochondrial DNA copy number (mtDNA-CN) have been extensively studied. Telomeres are nucleoprotein complexes that are located at chromosome ends and support chromosomal stability by protecting against DNA degradation[6]. Shortened telomeres eventually lead to cellular senescence, and telomere length is regarded as a likely biomarker of a history of replication and cumulative oxidative stress[6]. When telomeres become critically short, the life cycle of cells stops[7]. A shorter leukocyte telomere length (LTL) has been found to be associated with age[8,9], male sex[10], age-related risk factors for cardiovascular disease (CVD) and non-communicable disease[11,12], and all-cause mortality[9,13-15]. These associations are independent of chronological age, which points at an extra value of telomere length as a sign of cellular or biological aging.

In multiple cellular processes such as lipid metabolism, apoptosis, and cell differentiation, mitochondria are engaged in energy production (oxidative phosphorylation) and also participate in the generation of reactive oxygen species (ROS), which is the crux of the free-radical theory of aging[16]. According to experimental results, ROS could act as "mediators" of the stress response to the damage induced by aging[17]. MtDNA-CN is a marker of mitochondrial replication and cellular energy reserve[18]. Although mtDNA-CN is an indirect indicator of mtDNA damage, it is associated with mitochondrial enzyme activity and adenosine triphosphate production[19].

The evaluation of the possible association between LTL and the risk of cancer[14,20-23] and that between alternations in mtDNA-CN and cancer[24-27] has shown inconsistent findings.

We aimed to study the association of LTL and mtDNA-CN with the risk of incident CRC during a 15-year follow-up period.

2. Methods

2.1. Study participants

A random population sample of men and women aged 45 – 69 years old was examined at baseline in 2003/05 (n = 9360, mainly Caucasoid) and re-examined in 2006/08 and 2015/18 in Novosibirsk (Russia) in the frame of the HAPIEE study (Health, Alcohol and Psychosocial Factors in Eastern Europe; http://www.ucl.ac.uk/easteurope/hapiee-cohort.htm). The baseline cohort was followed-up for cancer, CVD, and all-cause mortality until December 31, 2019, for an average of 15.6 years (standard deviation, SD 0.70; median 15.6; range 14.5 – 17.0), calculated across those alive until censoring date.

In the present analysis, we focused on the most common cancer: CRC. Data on CRC (ICD-10: C18 – C20) in the cohort were collected, including fatal and non-fatal events. For cancer events ascertainment, we used the Cancer Register of Novosibirsk city. In addition, the following sources were used to collect information on all-cause and cause-specific mortality: The Bureau of Population Registration (ZAGS), the State Statistical Bureau of the Novosibirsk Region, and the data obtained from additional sources at two re-examinations of the cohort (including the address bureau and proxy-information on deceased respondents of the study).

2.2. Ethical approval and consent

The study was conducted in accordance with the guidelines of the Declaration of Helsinki. The ethical approval for the study was from the Ethics Committee of IIPM – Branch of IC&G SB RAS (Institute of Internal and Preventive Medicine – Branch of Federal State Budgeted Research Institution, "Federal Research Center, Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences") (protocol #1, March 14, 2002, and protocol #12, December 8, 2020). All the study participants signed an informed consent.

2.3. Sample selection

Among 9360 people, 160 events of CRC were ascertained during a 15-year follow-up period, including repeated events in several participants. The present study was designed as a nested case-control. We selected incident CRC cases among those free from baseline cancer of any site and with available DNA samples (n = 154) for analysis. The universal control group for the present study was formed based on certain criteria; we excluded those with baseline cancer or CVD, or those who died within the follow-up period. These exclusion criteria were applied to generate universal controls suitable for several outcomes.

We randomly selected controls whose age and sex frequency matched the CRC cases (n = 806). The average follow-up of the studied sample was 15.9 years (SD = 0.74; median = 15.9; range 14.5 – 17.0). After excluding samples with inadequate DNA quality or inappropriate genotyping of LTL and mtDNA-CN, our dataset consisted of 146 CRC cases and 799/785 controls for LTL/mtDNA-CN. The characteristics of the groups are shown in Table 1.

2.4. Data collection

In the frame of the HAPIEE study, baseline data were collected using standardized structured interview, physical examination, and the collection of blood and DNA samples. Trained and certified stuff conducted the interview and assessed the participants' medical history in relation to hypertension, diabetes mellitus, CVD, and other chronic diseases; health characteristics; as well as behavioral and socio-economic factors. Briefly, the physical examination included measurements of blood pressure (BP), anthropometry, physical performance, and other optional ones. The protocol details were previously reported[28].

We classified smoking status into three categories (current smoker, former smoker, and never smoked); education level into four categories (primary or less than primary, vocational, middle, and high); and marital status into two categories (married/cohabiting and single/divorced or widow/widower).

We measured BP in sitting position after a 5-min rest over the right arm three times (Omron M-5, OMRON Healthcare) about 2 min apart. For current analysis, we used the average of three BP readings. Height and weight were measured to the nearest 1 mm and 100 g, respectively; body mass index (BMI) was defined as weight/squared height, kg/m^2.

Venous blood samples were taken from the cubital vein using the Vacuette blood collection system after an overnight fast (minimum 8 h). After centrifugation (3,000 rate/min, 20 min), the blood cells were separated and the aliquots of serum, plasma, and blood cells were stored in a deep freezer (−80°C). The levels of lipids and glucose were measured within a month from the time blood was drawn. Serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and glucose levels were measured on Konelab 300i Autoanalyzer (Thermo Fisher Scientific Inc., USA) with an enzymatic approach using kits from Thermo Fisher Scientific. Friedewald formula was applied to obtain the low-density lipoprotein cholesterol (LDL-C) values.

DNA samples were isolated from blood cells by phenol-chloroform extraction[29] within 1 month from the time

Table 1. Distribution of basic parameters of incident CRC cases and controls (HAPIEE study, Russian cohort, men and women, baseline survey 2003 – 2005, 45 – 69 years old)

Covariates	Cases (incident CRC)	Controls	*P*
	Mean (SD)/n(%)		
Observed, *n*	146	799	
Baseline age, years (mean, SD)	60.8 (6.69)	55.7 (6.51)	<0.001
Women, *n* (%)	70 (47.9)	476 (59.6)	0.009
Systolic blood pressure, mmHg (mean, SD)	141.8 (20.86)	137.6 (22.58)	0.035
Diastolic blood pressure, mmHg (mean, SD)	89.1 (10.94)	88.2 (12.86)	0.422
Heart rate, per min	70.5 (11.27)	70.1 (10.37)	0.680
Body mass index, kg/sqm (mean, SD)	28.7 (5.12)	27.9 (4.84)	0.101
Waist/hip ratio, unit (mean, SD)	0.91 (0.09)	0.88 (0.08)	<0.001
Total cholesterol mmol/L (mean, SD)	6.38 (1.23)	6.31 (1.21)	0.512
LDL cholesterol, mmol/L (mean, SD)	4.13 (1.07)	4.08 (1.08)	0.642
HDL cholesterol, mmol/L (mean, SD)	1.534 (0.40)	1.55 (0.34)	0.565
TG, mmol/L (mean, SD)	1.57 (0.84)	1.47 (0.74)	0.149
Glucose, plasma, mmol/L (mean, SD)	6.03 (1.17)	5.79 (1.07)	0.017
Hypertension (HT), *n* (%)	103 (70.5)	436 (54.6)	<0.001
HT treatment (among HT), *n* (%)	56 (54.4)	206 (47.2)	0.193
Type 2 diabetes mellitus (T2DM), *n* (%)	21 (14.4)	46 (5.8)	<0.001
T2DM treatment (among T2DM), *n* (%)	4 (19.0)	10 (21.7)	0.802
Menopause (women), *n* (%)	65 (92.9)	363 (76.3)	0.005
Smoking, *n* (%)			0.600
Present smoker	30 (20.5)	184 (23.0)	
Former smoker	25 (17.1)	114 (14.3)	
Never smoked	91 (62.3)	501 (62.7)	
Frequency of drinking, *n* (%)			0.664
5+/week	2 (1.4)	16 (2.0)	
1 – 4/week	39 (26.7)	185 (23.2)	
1 – 3/month	32 (21.9)	216 (27.0)	
<1/month	58 (39.7)	310 (38.8)	
Non-drinker	15 (10.3)	72 (9.0)	
Education, *n* (%)			0.006
Primary	11 (7.5)	20 (2.5)	
Vocational	43 (28.8)	192 (24.0)	
Middle	47 (32.2)	297 (37.2)	
High	46 (31.5)	290 (36.3)	

(*Cont'd...*)

Table 1. (*Continued*)

Covariates	Cases (incident CRC)	Controls	*P*
	Mean (SD)/n(%)		
Marital status, *n* (%)			0.605
Single	38 (26.0)	152 (24.0)	
Married	108 (74.0)	607 (76.0)	
LTL, unit	0.61 (0.31)	1.39 (0.39)	<0.001
mtDNA-CN, unit	0.87 (0.29)	1.30 (0.49)	<0.001

P value, Fisher's analysis of variance (ANOVA) or Pearson Chi-square test. CRC: Colorectal cancer, CVD: Cardiovascular disease, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, LTL: Leukocyte telomere length, mtDNA-CN: Mitochondrial DNA copy number, SD: Standard deviation, TG: Triglyceride.

blood was drawn. After isolation from blood cells, genomic DNA samples were stored at −70°C.

2.5. Measurement of biomarkers

The assessment of LTL and mtDNA-CN was performed by batches within 6 months from the time blood was drawn with quantitative real-time polymerase chain reaction (qPCR) using StepOnePlus™ System (Applied Biosystems, Thermo Fisher Scientific Inc., USA). The details of these measurement techniques are reported elsewhere[30,31].

2.5.1. LTL measurement

LTL analysis was conducted with the qPCR-based method[32,33]. We performed the quantitative reactions for telomeres and β hemoglobin gene in separate pairs of 96-well plates. The plates included a series of DNA dilutions (0.5, 1, 2, 5, 10, 20, and 30 ng); based on these serial data, a standard calibration curve and the computation of each sample were carried out. Each reaction was done with 10 ng of DNA. The composition of the reaction mixture for telomere analysis included 270 nM tel1b primer (5'-CGGTTT(GTTTGG)5GTT-3'), 900 nM tel2b primer (5'-GGCTTG(CCTTAC)5CCT- 3'), 0.2X SYBR Green I, 5 mM dithiothreitol (DTT), 1% dimethyl sulfoxide (DMSO), 0.2 mM of each deoxynucleoside triphosphate (dNTP), 1.5 mM magnesium chloride ($MgCl_2$), and 1.25 U DNA polymerase. The reaction mixture for β hemoglobin gene contained 300 nm Hbg1 primer (5'-GCTTCTGACACAACTGTGTTCACTAGC-3'), Hgb2 primer (5'-CACCAACTTCATCCACGTTCACC-3'), 0.2X SYBR Green I, 5 mM DTT, 1% DMSO, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, and 1.25 U DNA polymerase. The standard amplifier software was used for calculations. The ratios (T: S) based on single-copy gene were calculated to determine the relative LTL value. We rejected the sample if the SD in three replications was above 0.5. Each plate contained a sample with short LTL and three control samples. We checked the relative signal rates from the controls to ensure the comparability of the plates.

2.5.2. mtDNA-CN measurement

The analysis of mtDNA-CN value was performed by using the qPCR technique based on the modified method of Ajaz *et al.*[34]. Beta-2-microglobulin (B2M) served as a single-copy reference gene (refDNA). We set quantitative reactions separately for mtDNA-CN and B2M in duplicate 96-well plates at identical positions. The plates included a series of DNA dilutions (1.25, 6.25, 25, and 100 ng); these serial data were used to construct a calibration curve and quantitatively process each sample. In each reaction, 10 ng of DNA was taken. The reaction mixture for mtDNA-CN analysis contained the following reagents: 270 nM hMitoF3 primer (5'-CTAAATAGCCCACACGTTCCC-3'), 900 nM hMitoR3 primer (5'-AGAGCTCCCGTGAGTGGTTA-3'), 0.2 Ч SYBR Green I, 5 mM DTT, 1% DMSO, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, and 1.25 U DNA polymerase in a final volume of 15 µL of PCR buffer. The reaction mixture for B2M analysis contained the following reagents: 300 nM hB2MF1 primer (5'-GCTGGGTAGCTCTAAACAATGTATTCA-3'), hB2MR1 primer (5'-CCATGTACTAACAAATGTCTAAAATGGT-3'), 0.2 Ч SYBR Green I, 5 mM DTT, 1% DMSO, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, and 1.25 U DNA polymerase in a final volume of 15 µL buffer.

We used the standard amplifier software for computation. The quality control and calculation of mtDNA/refDNA ratio were performed. We excluded the sample from further analysis if the amplification curves of a sample in three replicates had SD > 0.5. A universal control DNA sample was used for all plates; for comparability of plates, we tested the relative rates of the signal from a control. mtDNA-CN was estimated by the parameter of the threshold cycle (Ct; which represents the intersection point of the DNA accumulation schedule and the threshold line). This procedure allows the estimation of the initial mtDNA-CN and comparison between samples.

2.6. Statistical analysis

SPSS (v19.0) was used for statistical analysis. The dataset included 146 CRC cases and 799/785 controls (for LTL and mtDNA-CN, respectively).

In the first step, we conducted a descriptive analysis comparing the general characteristics of the studied groups (using ANOVA for continuous variables, and cross-tabulation techniques for categorical variables). Second, logistic regression was used to evaluate the odds

ratios (ORs) of CRC per 1 decile decrease in LTL and mtDNA-CN as continuous variables. Incident CRC case status was the dependent variable. Model 1 was adjusted for baseline age and sex; Model 2 included additional adjustments for smoking, BMI, systolic blood pressure (SBP), and TC; Model 3 was additionally controlled by the level of education.

Third, we conducted several sensitivity analyses. Analysis stratified by sex was repeated based on the same models, and we conducted analyses separately for CRC at different sites (colon and rectal). To eliminate the potential reverse effect of an early cancer stage on the reduction of biomarkers' values, we excluded cancer cases with onset (i) within the first 2 years from the baseline and (ii) within the first 8 years from the baseline (i.e., below the median period value), and repeated two variants of logistic regression analysis using the same covariates and models as above.

The adjusted LTL and mtDNA-CN values were then generated to incorporate the possible non-linear joint influence of the covariates and case–control status. This was done by adding extra interaction terms between case–control and every covariate into the linear regression analyses of LTL or mtDNA-CN (dependent variable); for example, case-control status, baseline age, and the interaction term between case-control and age. This was repeated for every covariate, including baseline age, sex, smoking, BMI, SBP, TC, education, waist-hip ratio (WHR), and glucose level. The adjusted LTL and mtDNA-CN values were generated (*i.e.*, adjusted for baseline age and sex; controlled for age, sex, smoking, BMI, SBP, TC, education; and, additionally adjusted for WHR and glucose level). Subsequently, we applied logistic regression to evaluate the ORs of CRC risk per 1 decile decrease in adjusted LTL and mtDNA-CN as continuous variables.

3. Results

3.1. Basic phenotype characteristics of studied groups

Table 1 presents the basic characteristics of the studied case and control.

Participants with incident CRC were somewhat older; they had higher SBP, serum glucose level, and waist/hip ratio but similar BMI; they were also more likely to have hypertension and diabetes mellitus type 2; in addition, their level of education was lower compared with the control group; and women with CRC were more likely to be in menopause.

In a structure of incident CRC, the proportion of colon cancer was 66%, rectal cancer was 32%, and the combination of both sites was 2%. The mean (SD; median) onset age of cancer as registered was 68.5 (8.01; 69.7); the period between the time blood was drawn and the identification of incident CRC was 7.74 years (4.53; 7.92).

The mean (SD; median) of baseline LTL and mtDNA-CN was 1.27 (0.48; 1.26) and 1.23 (0.49; 1.12), respectively. The baseline LTL and mtDNA-CN values were lower among cases compared to controls: 0.61 (0.31) versus 1.39 (0.39), $P < 0.001$ for LTL; and 0.87 (0.29) versus 1.30 (0.49), $P < 0.001$ for mtDNA-CN (Figure 1). Both biomarkers were correlated well between themselves and negatively correlated with baseline age. The correlation coefficient between LTL and baseline age was −0.211, $P < 0.001$; between mtDNA-CN and age was −0.086,

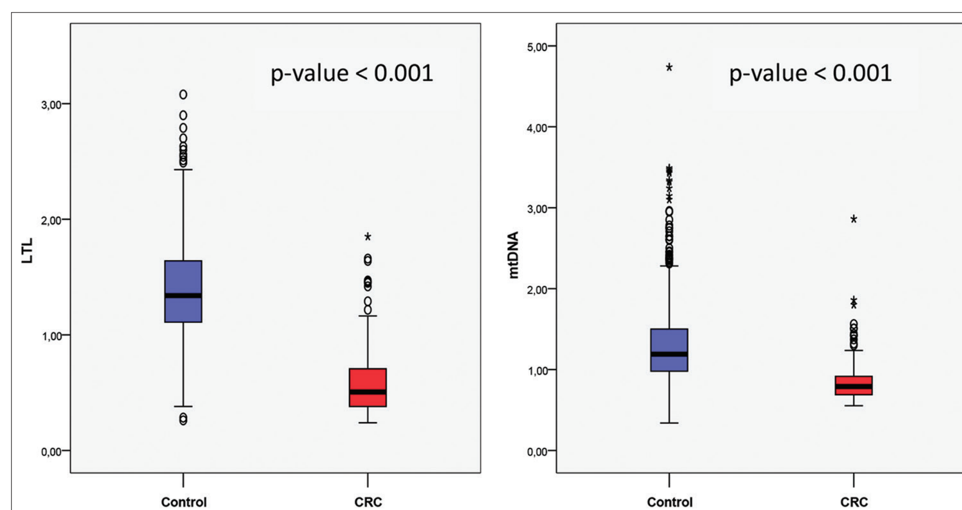

Figure 1. Boxplot of leukocyte telomere length (LTL) and mitochondrial DNA copy number (mtDNA-CN) values in cases of colorectal cancer and control groups (cases/controls: n = 146/799 for LTL and n = 146/785 for mtDNA-CN).

P = 0.009; and between LTL and mtDNA-CN was 0.437, P < 0.001. Scatterplots of LTL and mtDNA-CN versus age are presented in Figure 2.

3.2. Association between LTL and risk of CRC

Table 2 shows the ORs of incident CRC per 1 decile decrease in baseline LTL. In the model adjusted for age and sex, the OR of CRC per 1 decile decrease in LTL was 3.10 (95% CI: 2.54 – 3.79). The association was independent of smoking, biological covariates, and education; it remained significant in multivariable analyses (fully adjusted Model 3), in which the OR was 3.15 (95% CI: 2.56 – 3.87).

Table 2 (bottom) presents the results separated into men and women. The relationship between LTL and CRC had the same directionality compared to the pooled results; however, the risk of CRC in relation to shorter telomeres was slightly higher in women compared to men. In fully adjusted Model 3, the OR was 3.39 (95% CI: 2.51 – 4.58) in female and 2.96 (95% CI: 2.23 – 3.95) in male.

3.3. Association between mtDNA-CN and risk of CRC

Table 3 shows the ORs of incident CRC per 1 decile decrease in baseline mtDNA-CN. In the model adjusted for age and sex, the OR of CRC per 1 decile decrease in mtDNA-CN was 1.68 (95% CI: 1.55 – 1.86). Similarly, in multivariable analyses, the association remained significant, and the OR was 1.71 (95% CI: 1.54 – 1.89) in fully adjusted Model 3 (Table 3).

Table 3 (bottom) presents the results separated into men and women. The relationship between mtDNA-CN and CRC had again the same directionality, with very close ORs in men and women compared to the pooled dataset results.

For sensitivity analyses, we assessed the association of CRC with LTL and mtDNA-CN measures in a cohort excluding early cancer cases that occurred during the first 2 years after baseline examination (Table 4). The results were similar but somewhat weaker, with OR of CRC 3.02 (95% CI: 2.46 – 3.70) per 1 decile decrease in baseline LTL. The relationship between CRC and baseline mtDNA-CN and OR remained practically the same as in the full sample. We also repeated this analysis in a cohort excluding cancer cases that occurred during the first 8 years after baseline examination (below the median of the time between the baseline and onset of CRC). The results were slightly attenuated with OR of CRC 2.90 (95% CI: 2.25 – 3.74) per 1 decile decrease in baseline LTL; for mtDNA-CN, the OR remained close to the results in the full sample (1.74 [95%CI: 1.51 – 2.00]) (Supplementary File, Table S1).

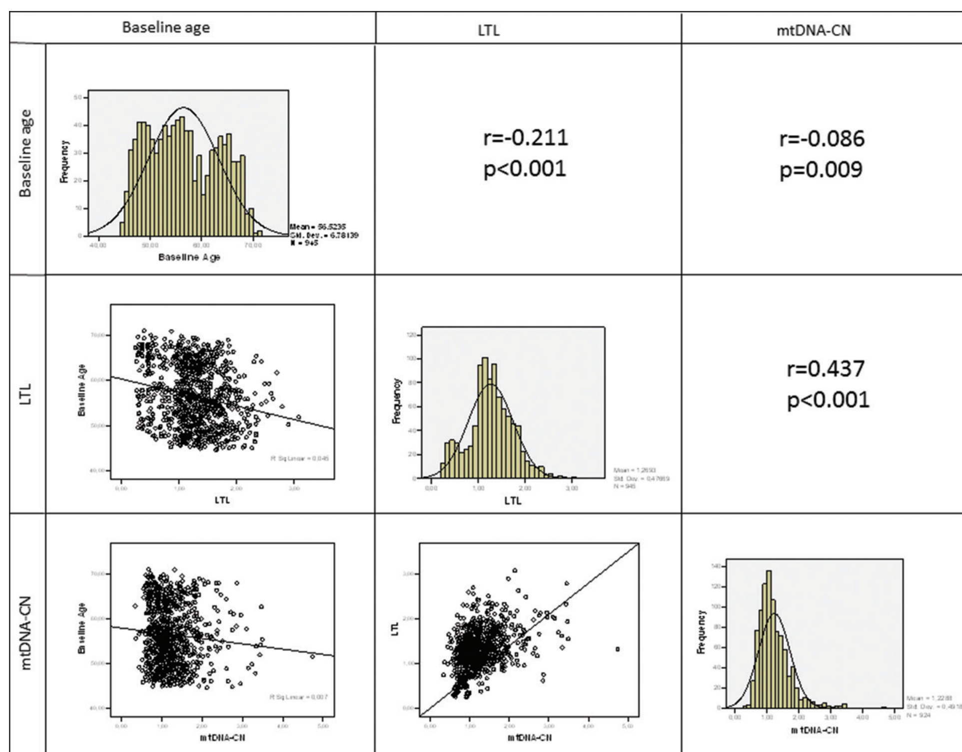

Figure 2. A correlation chart for baseline age, leukocyte telomere length, and mitochondrial DNA copy number values, showing distributions of each variable (on the main diagonal); scatterplots with fitted lines for each pair of the variables (below the diagonal); Pearson correlation coefficients (r) and significance level (P-value) for each pair of the parameters (above the diagonal).

Table 2. Relationship between CRC and LTL, per 1 decile decrease in LTL (cases, *n*=146; controls, *n*=799; men and women, 15-year follow-up)

Biomarker	Cases/control, *n*	Model 1	Model 2	Model 3
		OR (95% CI), *P*	OR (95% CI), *P*	OR (95% CI), *P*
LTL, unit per 1 decile	146/799	3.10 (2.54 – 3.79)	3.17 (2.58 – 3.89)	3.15 (2.56 – 3.87)
P value for trends		<0.001	<0.001	<0.001
Men*				
LTL, unit per 1 decile	76/323	2.90 (2.20 – 3.82)	2.99 (2.25 – 4.00)	2.96 (2.23 – 3.95)
P value for trends		<0.001	<0.001	<0.001
Women*				
LTL, unit per 1 decile	70/476	3.34 (2.49 – 4.48)	3.40 (2.51 – 4.59)	3.39 (2.51 – 4.58)
P value for trends		<0.001	<0.001	<0.001

Model 1: Adjusted for age and sex; Model 2: Adjusted for age, sex, smoking, body mass index (BMI), systolic blood pressure (SBP), and total cholesterol (TC); Model 3: Adjusted for age, sex, smoking, BMI, SBP, TC, and education; *Models 1, 2, and 3 stratified by sex (sex excluded from covariates). LTL: Leukocyte telomere length, OR: Odds ratio, CRC: Colorectal cancer

Table 3. Relationship between CRC and mtDNA-CN, per 1 decile decrease in mtDNA-CN (cases, *n*=146; controls, *n*=785; men and women, 15-year follow-up)

Biomarker	Cases/controls, n	Model 1	Model 2	Model 3
		OR (95% CI), *P*	OR (95% CI), *P*	OR (95% CI), *P*
mtDNA-CN, unit per 1 decile	146/785	1.68 (1.55 – 1.86)	1.70 (1.53 – 1.88)	1.71 (1.54 – 1.89)
P value for trends		<0.001	<0.001	<0.001
Men*				
mtDNA-CN, unit per 1 decile	76/311	1.72 (1.48 – 2.00)	1.72 (1.48 – 2.01)	1.76 (1.50 – 2.06)
P value for trends		<0.001	<0.001	<0.001
Women*				
mtDNA-CN, unit per 1 decile	70/474	1.67 (1.46 – 1.91)	1.70 (1.48 – 1.95)	1.71 (1.49 – 1.97)
P value for trends		<0.001	<0.001	<0.001

Model 1: adjusted for age and sex; Model 2: adjusted for age, sex, smoking, body mass index (BMI), systolic blood pressure (SBP), and total cholesterol (TC); Model 3: adjusted for age, sex, smoking, BMI, SBP, TC, and education; *Models 1, 2, and 3 stratified by sex (sex excluded from covariates). Abbreviations: mtDNA-CN, mitochondrial DNA copy number, OR: Odds ratio

Table 4. Relationship between CRC and LTL and mtDNA-CN, per 1 decile decrease in each biomarker in a cohort, excluding early cancer cases within 2 years from baseline (cases, *n*=131; controls, *n*=799 for LTL/785 for mtDNA-CN; men and women, 15-year follow-up)

Biomarker	Cases/controls, n	Model 1	Model 2	Model 3
		OR (95% CI), *P*	OR (95% CI), *P*	OR (95% CI), *P*
LTL, unit per 1 decile	131/799	3.00 (2.45 – 3.66)	3.04 (2.48 – 3.73)	3.02 (2.46 – 3.70)
P value for trends		<0.001	<0.001	<0.001
mtDNA-CN, unit per 1 decile	131/785	1. 71 (1.54 – 1.89)	1.71 (1.54 – 1.91)	1.72 (1.54 – 1.92)
P value for trends		<0.001	<0.001	<0.001

Model 1: Adjusted for age and sex; Model 2: Adjusted for age, sex, smoking, body mass index (BMI), systolic blood pressure (SBP), and total cholesterol (TC); Model 3: Adjusted for age, sex, smoking, BMI, SBP, TC, and education. LTL: Leukocyte telomere length, mtDNA-CN: Mitochondrial DNA copy number, OR: Odds ratio

In addition, we estimated the association of LTL and mtDNA-CN with cancer of the colon and rectal sites separately. The associations were very close to the pooled dataset results with somewhat higher ORs for the risk of rectal cancer compared to colon cancer, both for LTL and mtDNA-CN (Supplementary File, Table S2).

Finally, we assessed the association of CRC with generated LTL and mtDNA-CN values, preliminary adjusted for multiple covariates, and their interaction with case–control status. The results were weaker with OR of CRC 2.53 (95% CI: 2.16 – 2.97) per 1 decile decrease in fully adjusted baseline LTL (Supplementary File, Table S3) and with OR of CRC 1.52 (1.39 – 1.65) per 1 decile decrease in fully adjusted baseline mtDNA-CN (Supplementary File, Table S4).

4. Discussion

The present nested case-control study included individuals with incident CRC (cases) and age and sex frequency-matched controls from a Novosibirsk population cohort (Russia). The carriers of shorter telomeres had an increased 15-year risk of incident CRC with adjusted OR 3.2 per 1 decile decrease in LTL. Lower mtDNA-CN was associated with an increased risk of incident CRC with adjusted OR 1.7 per 1 decile decrease in mtDNA-CN. The risk coefficients of CRC were attenuated to OR 2.53 per 1 decile decrease in adjusted baseline LTL and OR 1.52 per 1 decile decrease in adjusted baseline mtDNA-CN.

A number of epidemiological studies, with varying samples and designs, have explored the associations between LTL and the risk of cancer, but the results are inconsistent[14,20-23]. Specifically for CRC incidence and mortality, both negative and positive relationships, U-shaped, and absence of association with LTL have been reported.

Our findings of an inverse relationship between baseline LTL and CRC incidence are consistent with the prospective Bruneck study[35], a case–control study in China[36], and are in line with the CRC mortality findings in two meta-analyses[37,38]. In the Bruneck study with a 10-year follow-up, the adjusted hazard ratio was 1.56 (95% CI: 1.32 – 1.85) for CRC incidence and 1.88 (95% CI: 1.48 – 2.40) for CRC mortality per 1 SD shorter baseline LTL[35]. In a case-control study of a Chinese population, shorter LTL was modestly associated with higher risk of CRC (OR per LTL tertile 1.13; 95% CI: 1.00 – 1.28)[36]. In a study conducted by Kroupa *et al.*, shorter telomeres were found in CRC tumor tissue than in adjacent mucosa[39]. This finding is supported by evidence from recent studies[40]. Zhang *et al.*, in a meta-analysis based on 45 prospective studies of incident cancer (2 for CRC), have shown that short telomeres are related to increased CRC mortality, with an RR of 2.54 (95% CI: 1.73 – 3.72) for short telomere length compared to long telomere length as dichotomized variable[37]. In another meta-analysis of prospective and retrospective studies, short LTL was associated with poorer survival for CRC (HR = 2.01; 95% CI: 1.46 – 2.77)[38]. Wentzensen *et al.*, in their meta-analysis, have indicated an inverse association between LTL and CRC risk in a retrospective case–control study[41].

On the other hand, our findings are opposite to the data obtained from a prospective study in Singapore (n = 26.000, 12-year follow-up)[42], which has reported a positive association between LTL and CRC risk, with a HR of 1.32 (95% CI: 1.08 – 1.62) for the top quartile of telomere length versus bottom quartile. In a meta-analysis of 28 prospective studies (2 on CRC), no associations were reported between LTL and CRC risk[21]. Similarly, the pooled results of eight prospective studies have revealed that LTL is associated with neither a better or poorer prognosis of CRC patients[43]. Interestingly, in a Mendelian randomization study (MRS) of the UK Biobank data (7.5-year follow-up, n = 261,837), genetically determined longer telomeres were found to be associated with a modestly elevated risk of pooled cancer. However, the risk of CRC was found to be below 1 among those with longer telomeres, while remaining statistically insignificant[22]. A recent MRS using genetic risk score for LTL has found that short LTL score is related to a reduced risk of nine types of cancer (not including CRC) in the UK Biobank dataset, but there has been an observation that is suggestive of an association between short LTL score and a high death hazard of rectum adenocarcinoma in The Cancer Genome Atlas (TCGA) dataset[23], of which the latter is in line with our findings.

Concerning the alternations of mtDNA in relation to cancer, different results have been reported[24]. For a number of cancer types, an inverse, multidirectional, or nonlinear relationship with mtDNA has been reported (colorectal, breast, kidney, and lung)[25,27]; the absence of distinction between tumor tissue and adjacent unaffected tissue has also been reported (colorectal, kidneys, pancreas and thyroid glands, prostate, stomach, and uterus)[26].

Our results of an inverse association between baseline mtDNA-CN and CRC incidence are in line with the Shanghai Women's Health Study[44] and, partly, with a recent prospective Swedish study[45]. In a case–control design for CRC established on a population sample (n= 444/1,423 women, >9 years follow-up) under the Shanghai Women's Health Study, the researchers found an inverse association between baseline mtDNA-CN and incident CRC with adjusted OR = 1.26 (95% CI: 0.93 – 1.70) and 1.44 (95% CI: 1.06 – 1.94) for the middle and bottom tertiles of mtDNA-CN values, respectively[44]. In a prospective Swedish study (women, 15.2 years follow-up), baseline LTL and mtDNA-CN were not associated with the prevalence and incidence of a digestive cancer as a cumulative category (including CRC). However, mtDNA-CN (but not

LTL) was found to be inversely associated with mortality from digestive cancer with OR = 1.53 (95% CI: 1.02 – 2.28) per 1 SD decrease in mtDNA-CN[45]. Van Osch *et al.*, in combined CRC cases from hospital series and Netherlands Cohort study, discovered lower mtDNA-CN in primary CRC tissue compared to a resected one as well as an inverse U-shaped relationship between CRC survival and mtDNA-CN[46].

On the other hand, several studies have shown an association between higher mtDNA-CN and risk of incident CRC[47] or CRC progression[48]. In a meta-analysis, Mi *et al.* failed to find any association between mtDNA-CN and several digestive system cancers (including CRC)[49].

The mechanisms of the relationship between LTL and mtDNA-CN and CRC have not been fully understood. A growing body of evidence has suggested the dual role of telomeres (or telomere paradox) in carcinogenesis[39,50]. Tumor cells with increased proliferation undergo faster attrition of telomeres than non-affected cells. Telomere shortening works as a tumor-suppressing mechanism. On the other hand, for cell clones that escape the crisis, critically short telomeres contribute to the mechanisms related to telomere maintenance, including the reactivation of telomerase, which stabilizes short telomeres[50], or rarer alternative telomere lengthening[45,51], thus preventing cell death and promoting tumor invasion. Critically short telomeres also affect genomic aberrations and chromosome instability, which, along with microsatellite instability[40], represent important pathways in the genesis of CRC[39]. The recently discovered telomere-driven chromothripsis (complex chromosomal rearrangement) is a widespread mutational phenomenon found in diverse tumor types[51].

MtDNA-CN, as a proxy for mitochondria function, differs between cancer tissue and non-affected tissue for a number of cancer types[45]. The impact of mitochondria metabolism on tumor onset and progression is heterogeneous by cancer type, and it has been shown to be related to CRC initiation[52,53]. Existing data have suggested that mtDNA-CN changes depend on nuclear or mtDNA mutations and serve as an adaptive response toward these mutations for certain cancer types[52,54]. A recent data have supported the role of mtDNA mutations in metabolic alterations (oxidative phosphorylation defects), facilitating colon tumor[55], and suggested that somatic mutations in mtDNA control region may be shaped by tumor-specific selective pressure and involved in tumorigenesis[56]. Telomere attrition is involved in the regulation of mitochondrial genesis and function in the aging process, leading to their dysfunction and ROS formation; in particular, the role of telomere-p53-mitochondrial axis in cancer has been demonstrated[45].

The heterogeneity of the results for studied biomarkers might be due to the variability across cancer sites, study designs (retrospective or prospective studies with early follow-up may reflect reverse causation), age and other covariates impact, accuracy of LTL or mtDNA-CN measurement, and risk assessment for incident events or cancer progression and mortality[21].

5. Limitations and strengths of the study

Our findings should be interpreted within the context of their potential limitations. First, we have to mention the modest sample size. Although our nested case-control analysis involved the complete set of incident CRC cases that developed in a large-scale cohort (9,360) within a long-term follow-up period of 15 years, we ruled out only a few CRC cases (because of the absence of DNA material or insufficient quality of LTL and mtDNA-CN genotyping). The controls satisfied strict exclusion criteria and were frequency-matched to cases by age and sex. We believe that this design likely covers a representative sample of incident CRC in this population.

In addition, to ensure the completeness of registration, we checked multiple sources of information for case ascertainment (including the Cancer Register, Mortality Register, and repeated examinations of the cohort).

Another potential limitation might be related to the effect of cancer per se or cancer treatment on studied biomarkers[57]; thus, retrospective design or early follow-up may reflect reverse causation. To avoid this shortage, we used a prospective design and included only incident cancer cases. Besides that, we fulfilled two sensitivity analyses with the exclusion of CRC cases that occurred within the first 2 years or within the first 8 years after the baseline blood was drawn, which did not substantially alter the results.

Another concern is the sex-dependent variance in LTL and mtDNA-CN (due to the known higher values of both biomarkers in women compared to men). To overcome this potential limitation, we kept a similar sex distribution among cases and controls (nearly 50%–60%), and the estimates were adjusted for sex. In addition, we obtained ORs that were comparable to the combined dataset results after splitting the analysis by sex.

Taking into account the reported difference in the relationship between LTL and subtypes of CRC[42,58], we analyzed the risk of colon cancer and rectal cancer separately. This additional stratification did not materially affect the results.

Furthermore, our results are based on the investigation of a Caucasoid population in Novosibirsk (West Siberia),

and thus have limited generalizability and may not be applicable to another populations. The geographical and ethnic variations as well as the difference in CRC rate, related risk factors, and lifestyle profile between populations might contribute to the population-specific predictive role of studied molecular markers for CRC risk.

Aside from limitations, this study has its strengths. For the first time, this prospective case–control study investigated the links between LTL and mtDNA-CN as well as the risk of incident CRC in the Russian population and altogether the Eastern European population.

It is noteworthy that we used two molecular markers with established association with mortality and several age-related diseases[9,14,15,18,52]. We showed a close correlation between these markers themselves and with age.

Finally, our findings from a Russian cohort offer the first evidence of the value of the association of LTL and mtDNA-CN with CRC in the studied population. Our findings may be useful for further integration with data from different populations for large-sample integrative analysis of the association of LTL and mtDNA-CN with CRC.

6. Conclusion

In our nested case–control study, LTL and mtDNA-CN were independent inverse predictors of the 15-year risk of CRC in the middle-aged and elderly (Caucasoid) population cohort in Novosibirsk, West Siberia. These molecular markers may become useful predictors for CRC risk and may have potential implications in cancer prevention. At the same time, our findings highlight the relevance for further research to uncover the mechanisms behind the association of LTL and mtDNA-CN with human health.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Conceptualization: Sofia Malyutina, Vladimir Maximov, Olga Chervova

Formal analysis: Sofia Malyutina, Olga Chervova, Vitaly Voloshin

Investigation: Vladimir Maximov, Pavel Orlov, Andrew Ryabikov, Tatiana Nikitenko

Methodology: Sofia Malyutina, Vladimir Maximov, Olga Chervova, Andrew Ryabikov, Mikhail Voevoda, Tatiana Nikitenko

Validation: Sofia Malyutina, Vladimir Maximov, Olga Chervova, Andrew Ryabikov, Mikhail Voevoda, Tatiana Nikitenko

Writing – original draft: Sofia Malyutina, Olga Chervova

Writing – review & editing: Sofia Malyutina, Vladimir Maximov, Olga Chervova, Pavel Orlov, Vitaly Voloshin, Andrew Ryabikov, Mikhail Voevoda, Tatiana Nikitenko

All authors have contributed substantially to this work and consented to submit the paper to *Global Translational Medicine*, and to the best of the authors' knowledge, the entire research and paper writing process were carried out in adherence to the highest academic conduct standards and publishing ethics code.

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of IIPM – Branch of IC&G SB RAS (Institute of Internal and Preventive Medicine – Branch of Federal State Budgeted Research Institution, "Federal Research Center, Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences"), Protocol #1 from March 14, 2002 and Protocol #12 from December 8, 2020. The study was observational and did not involve experiments on humans, animals, or cell lines. Informed consent was obtained from all subjects involved in the study.

Consent for publication

Informed consent was obtained from all subjects (for study participation, anonymized data analysis and publication).

Availability of data

The data presented in this study are available in tabulated form on request. The data are not publicly available due to ethical restrictions and project regulations.

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