DOI 10.4149/BLL_2024_35

Bratisl Med J 2024; 125 (4):
233–238

CLINICAL STUDY

Telomerase activity and *hTERT* gene expression in patients with acute coronary syndrome and stable coronary artery disease

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ABSTRACT
BACKGROUND: In this study, we aimed to examine the telomerase activity and *hTERT* gene expression in patients with acute coronary syndrome (ACS) and those with stable coronary artery disease (SCAD) and compare the results to controls. Additionally, we compared overall mortality rates relative to the telomerase activity.
METHODS: A total of 211 patients (78 ACS and 71 SCAD patients) were included in the study. The telomerase concentration was measured by ELISA and used to determine telomerase activity. The *hTERT* gene expression was determined by real-time PCR.
RESULTS: The serum telomerase enzyme concentration was lower in ACS (36.61 ± 1.54) and SCAD (36.79 ± 1.57) when compared to the control group (37.03 ± 2.25). However, this difference did not reach statistical significance (p = 0.890). The *hTERT* gene expression acting in telomerase enzyme synthesis was 2.7-fold lower in ACS group (p = 0.070) and 2.2-fold lower in the SCAD group (p = 0.101) compared to the control group. Patients were followed for a median of 32 months (minimum: 0.1, maximum: 46.8). The serum telomerase concentrations in patients who died and those survived in the SCAD group (35.98 ± 2.02 vs 36.86 ± 1.52 ng/ml, respectively; p = 0.529) were similar to those in the ACS group (36.39 ± 1.08 vs 36.63 ± 1.60 ng/ml, respectively p = 0.993).
CONCLUSIONS: In the current study, telomerase activity or *hTERT* expression was similar in patients with ACS, SCAD, and controls. Moreover, telomerase activity was not associated with all- cause mortality during the 32-month follow-up *(Tab. 3, Fig. 1, Ref. 29)*. Text in PDF *www.elis.sk*
KEY WORDS: acute coronary syndrome, coronary artery disease, *hTERT* gene expression, stable coronary artery, telomerase activity.

Introduction

Telomeres are tandem repeats of specific DNA sequences located at the ends of chromosomes, while maintaining genomic stability and integrity. Telomeres gradually shorten with each cell division, and therefore, the telomere length is considered as a marker of aging. Apart from aging, the impact of environmental and lifestyle stress factors, including inflammation and oxidative stress accelerate the rate of telomere shortening and lead to cellular senescence (1).

Peripheral leukocyte DNA has been most frequently used in clinical studies to assess the telomere length (TL) (2), while short telomeres have been associated with a variety of diseases related to aging and inflammation, including cardiovascular diseases (CVD). Common conventional risk factors such as smoking, obesity, hypertension and diabetes mellitus (DM) have been coupled with shorter telomeres (3–6). However, the relationship between CVD and short telomeres may not be necessarily due merely to these risk factors. In a meta-analysis with 43,725 participants and 8,400 patients with cardiovascular disease, for the shortest leukocyte telomere length (LTL), the relative risk for coronary heart disease was 1.54 (1.3–1.83) compared to highest third LTL, independently of conventional risk factors (7).

To counteract the telomere shortening, several distinct strategies have evolved in eukaryotic cells. The human telomerase consists of the telomerase RNA component (TERC) and telomerase reverse transcriptase (hTERT), the catalytic component, and balances terminal DNA losses by lengthening the ends of telomeric DNA through addition of tandemly repeated telomeric sequences (1). Nevertheless,

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Acknowledgements: This project was sponsored by Scientific Council of Pamukkale University, Turkey (Grant number 2016TIPF019).

recent studies have also indicated a telomere-independent role for telomerase in cardiovascular disease. Although early studies suggested that *hTERT* expression undergoes silencing in most organ systems, it was later shown that *hTERT* is stringently regulated and induced in response to certain environmental signals (9, 10). Telomerase reactivation can occur in immune cells, which may play critical roles during inflammatory responses (11). Similarly, in the atherosclerotic diseases, telomerase activity can also be upregulated (12, 13). In the Coronary Artery Risk Development in Young Adults (CARDIA) study, higher leukocyte telomerase activity was associated with higher prevalence of calcified atherosclerotic plaque, with a stronger association in persons with short telomeres (14).

There is a paucity of data on telomerase in patients with acute or stable coronary artery diseases. Accordingly, in this study, we aimed to examine the telomerase concentration as a measure of telomerase activity (TA) and hTERT gene expression in patients with acute coronary syndrome (ACS) and those with stable coronary artery disease (SCAD) in comparison to control subjects. We also investigated the all-cause mortality in these patients at a median of 32-month follow-up.

Materials and methods

A total of 211 patients who were aged between 55 and 75 years and underwent coronary angiography were included in the study. Patients were excluded if they met any of the following criteria: medical history of chronic inflammatory disorders, high white cell count and serum c-reactive protein (CRP) level, carcinoma, life expectancy less than six months as a result of a non-cardiac disease, history of coronary artery disease, cardiomyopathy (ischemic, dilated, hypertrophic), congenital heart disease,pregnancy or lactation period, liver disease, chronic kidney failure (glomerular filtration rate < 30 ml / min, serum creatinine > 1.5), serious heart valve disease, active myocarditis or pericarditis, atrial fibrillation, active infection, major metabolic or endocrine diseases and inability to sign the consent form.

The stable coronary artery disease group consisted of 71 consecutive patients who had at least one epicardial coronary arterial stenosis of 50% or greater on angiography. The patients in this group underwent angiography for typical stable angina or evidence of ischemia on exercise test or myocardial perfusion scan. In total, 78 ACS patients with a coronary stenosis of 50% or more were included in the ACS group, while 62 subjects with angiographically normal coronary arteries or with lesions of less than 20% were included in the control group.

Clinical evaluation, serial electrocardiogram (ECG) and cardiac enzyme follow-up were used in the assignment to ACS subgroup to assess ST-segment elevation myocardial infarction (STEMI), non-ST-segment elevation myocardial infarction (NSTEMI) and unstable angina pectoris (UAP). The telomerase concentration was used to determine the telomerase activity. Peripheral blood samples were drawn for routine laboratory tests as well as for determining the *hTERT* gene expression and telomerase concentration before the coronary angiography. Twelve-lead ECG and transthoracic echocardiography were also performed before the coronary angiography. Routine laboratory parameters were recorded from the hospital information system. Informed consent form was obtained from all patients and our study was approved by the Ethics Committee of Pamukkale University Medical Faculty Clinical Research.

RNA isolation, cDNA synthesis and Real-Time PCR assay

Total RNA isolation from blood samples were performed by using Trizol Reagent according to the manufacturer's instructions. Centrifugation with 2,500 rpm for 10 minutes was applied three times with erythrocyte lysis tampon (Red blood cell (RBC); (89.9 g NH_4Cl; 10 g $KHCO_3$, 2 ml 0.5 M EDTA) in 1 ml ddH_2O for leucocyte isolation from blood samples. Total RNA concentration and quality were measured by using NanoDrop spectrophotometer (Thermo Fisher Scientific). The cDNA (complementary DNA) synthesis was performed by Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to manufacturer's protocol. The mRNA expression change of the *hTERT* gene in the control and study groups were analyzed by real-time PCR assay (StepOnePlus system of real-time PCR). Conditions of the RT-PCR were applied as 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, 60°C for one minute. *Beta actin* was used as a housekeeping gene for normalization of PCR data. The *hTERT* and *beta actin* mRNA primer sequences are given in Supplement 1.

Human TERT (telomerase reverse transcriptase) ELISA kit

Human telomerase reverse transcriptase kit was applied to serum samples taken from patient and control groups, while the analyses and determination of concentration in the samples were performed by the sandwich-based ELISA method. Telomerase protein levels were quantified using the Human TERT (Telomerase Reverse Transcriptase ELISA Kit; Fine Test, China). ELISA studies were performed according to the manufacturer's instructions. The absorbances of serum samples in the 96-well plate were measured at 450 nm using a microplate reader (Heales MB-530). The concentrations of telomerase in each well were calculated using the equation of the kit standards. All detected results of telomerase levels were evaluated in both, the control and study groups.

Statistical analysis

The RT-PCR analyses of the findings were performed by evaluating ΔΔCT method and quantitated with a computer program. The comparison of the groups has been performed with the "volcano plot" analysis from "RT^2-Profiles™PCR Array Data Analysis", which is assessed statistically using the "student's t-test". To obtain 90% power with an α error of 0.05, at least 40 subjects needed to be included for a two-tailed comparison. The selected effect size for telomerase concentration was 0.75 ng/ml. All statistical analyses were performed using SPSS 25.0 software. Kolmogorov-Smirnov and Shapiro-Wilk tests were used to determine normal distribution. Continuous variables were defined by the mean ± standard deviation while categorical variables were defined by number and percentage. For independent groups comparisons, we used independent-sample t-test and one-way analysis of variance (post hoc: Tukey test) when parametric test assump-

tions were provided; Mann-Whitney U-test and Kruskal-Wallis analysis of variance (post hoc: Mann-Whitney U test with Bonferroni correction) were used when parametric test assumptions were not provided. The correlation between continuous variables were analyzed by Spearman or Pearson correlation analysis and the differences between categorical variables were examined by chi-square analysis. Kaplan-Meier curves were used for survival probabilities and log-rank test was used for determining the differences between groups. P< 0.05 were evaluated statistically significant.

Results

The baseline characteristics of the patients are shown in Table 1. Mean age was 63.4 ± 6.1 in the ACS group, 63.4 ± 6.4 in the SCAD group, and 61.7 ± 5.6 in the control group (p = 0.200). Seventy-three percent (57) of the ACS patients and 76.0% (54) of the SCAD patients were male (p = 0.677). Forty percent (25) of the patients in the control group were male (p = 0.001).

Serum telomerase enzyme concentration was lower in ACS (36.61 ± 1.54) and SCAD (36.79 ± 1.57) when compared to the control group (37.03 ± 2.25). However, this difference did not reach statistical significance (p = 0.890). The *hTERT* gene expression acting in the telomerase enzyme synthesis was 2.7-fold lower in the ACS group (p = 0.070) and 2.2-fold lower in the SCAD group (p = 0.101) compared to the control group (Tab. 2). When coronary artery patients, i.e., ACS and SCAD groups combined (n = 149), were analyzed, the serum telomerase enzyme concentration was 36.69 ± 1.55 (ng/ml) in the CAD group and 37.03 ± 2.25 (ng/ml)

Table 1. Baseline characteristics of the study subjects.

	ACS (1) (n=78)	SCAD (2) (n=71)	CONTROL (3) (n=62)	p	p (1–2)	p (1–3)	p (2–3)
Clinical and demographic characteristics							
Age (years)	63.4±6.1	63.4± 6.4	61.7± 5.6	0.200			
Male (%)	57 (73.0)	54 (76.0)	25 (40.0)	0.0001*	0.677	0.0001*	0.0001*
Diabetes mellitus (%)	28 (35.9)	28 (39.4)	18 (29.0)	0.447			
Hypertension (%)	25 (32.1)	37 (52.1)	30 (48,4)	0.032*	0.013*	0.049*	0.668
Hyperlipidemia (%)	4 (5.1)	2 (3.1)	0 (0.0)	0.094			
Cigarette smoking (%)	29 (37.2)	19 (26.8)	7 (11.3)	0.002*	0.174	0.0004*	0.025*
Body mass index (kg/m^2)	28.1±4.2	27.0±4.2	28.5±4.0	0.128			
Medication treatment (%)							
Beta-blockers	7 (9.0)	26 (36.6)	17 (27.4)	0.0001*	0.0001*	0.004*	0.258
Calcium blockers	5 (6.4)	10 (14.1)	10 (16.1)	0.162			
ACE inhibitors	8 (10.3)	11 (15.5)	7 (11.3)	0.598			
A2RB	10 (12.8)	12 (16.9)	11 (17.7)	0.683			
Statin	3 (3.8)	5 (7.0)	5 (8.1)	0.528			
Blood results							
Hemoglobin (g/dl)	13.5±1.7	13.5±1.9	13.5±1.3	0.850			
WBC (K/uL)	11.6±4.2	8.2±2,4	7.2±2.0	0.0001*	0.0001*	0.0001*	0.110
Serum creatinine (mg/dl)	0.82±0.21	0.82±0.21	0.72±0.16	0.003*	1.000	0.010*	0.007*
hsCRP (mg/dl)	1.76±3.58	1.43±3.47	0.81±1.59	0.237			
Total cholesterol (mg/dl)	187.5±40.7	188.8±35.2	207.3±40.2	0.007*	0.980	0.010*	0.022*
Triglycerides (mg/dl)	154.7±99.2	159.4±72.4	159.4±80.1	0.371			
HDL cholesterol (mg/dl)	43.0±13.3	42.0±9.6	49.6±15.4	0.006*	1.000	0.016*	0.014*
LDL cholesterol (mg/dl)	114.7±35.4	114.1±27.9	126.2±34.4	0.071			
Echocardiographic findings							
LVEF (%)	48.7±9.4	57.3±6.8	59.2±3.3	0.0001*	0.0001*	0.0001*	0.668
LA (mm)	36.5±7.3	37.1±4.3	36.6±5.7	0.399			
sPAB (mmHg)	10.4±15.5	10.0±13.9	13.3±15.7	0.438			

*ACS – acute coronary syndrome, SCAD – stable coronary artery disease, ACE – angiotensin-converting enzyme, ARB – angiotensin 2 receptor blocker, WBC – white blood cell, hsCRP – high- sensitive c-reactive protein, HDL – high-density lipoprotein cholesterol, LDL – low-density lipoprotein cholesterol, LVEF – left ventricular ejection fraction, LA – Left Atrium, sPAP – systolic pulmonary arterial pressure

Tab. 2. Telomerase enzyme concentration and *hTERT* gene expression results.

	ACS (n=78)	SCAD (n=71)	CONTROL (n=62)	p
Telomerase enzyme concentration (ng / ml)	36.61±1.54	36.79±1.57	37.03±2.25	0.890
Fold-regulation of *hTERT* gene compared to the controls	–2.7064	–	–	0.070
Fold-regulation of *hTERT* gene compared to the controls	–	–2.2896	–	0.101

*ACS – acute coronary syndrome, SCAD – stable coronary artery disease

Tab. 3. Telomerase enzyme concentration.

		Concentration (ng/ml)	p
Gender	Female (n=75)	36.75±1.8	
	Male (n=136)	36.83±1.81	0.669
Diabetes Mellitus	(+) (n=74)	36.97±1.94	
	(–) (n=137)	36.71±1.73	0.711
Smoking	(+) (n=55)	36.73±1.98	
	(–) (n=156)	36.82±1.75	0.412
Age (years)	56–65 years (n=138)	36.8±1.77	
	66–75 years (n=73)	36.79±1.87	0.968

in the control group (p = 0.974). The *hTERT* gene expression was 1.5-fold lower in the CAD group than in the control group. In addition, serum telomerase enzyme concentrations in NSTEMI patients (n = 35) were similar to those of STEMI patients (n = 43) (36.51 ± 1.41 ng / ml vs 36.72 ± 1.71 ng / ml, respectively, p = 0.823). No significant correlation was found in telomerase enzyme concentration measurements according to risk factors; gender, DM, smoking and age groups (Table 3). In addition, no significant correlation was found between telomerase enzyme concentration and CRP, body mass index (BMI), and low-density lipoprotein cholesterol (LDL) in ACS, SCAD and control groups.

Patients were followed for a median of 32 months (minimum: 0.1, maximum: 46.8). During the follow-up, 11 patients (14.1%) died in the ACS group, whereas 6 patients died in the stable CAD group (p = 0.275). No patients died in the control group. In the stable coronary group, serum telomerase concentration was numerically lower in patients who died during the follow-up than in patients who survived. However, the difference was statistically insignificant (35.98 ± 2.02 vs 36.86 ± 1.52 ng/ml; p = 0.529). Likewise, in ACS patients, the serum telomerase concentrations in patients who died were similar to those in the patients who survived (36.39 ± 1.08 vs 36.63 ± 1.60 ng/ml, respectively, p = 0.993). The association between telomerase activity and overall mortality rate was assessed by comparing the groups in tertiles. Neither in ACS group nor in SCAD group, there were differences in all-cause mortality between the highest, middle, or lowest telomerase concentration groups (p = 0.998 in ACS group and p = 0.775 in SCAD group). Similar results were observed when the groups were combined as CAD group (n = 149, p = 0.921 (Fig. 1).

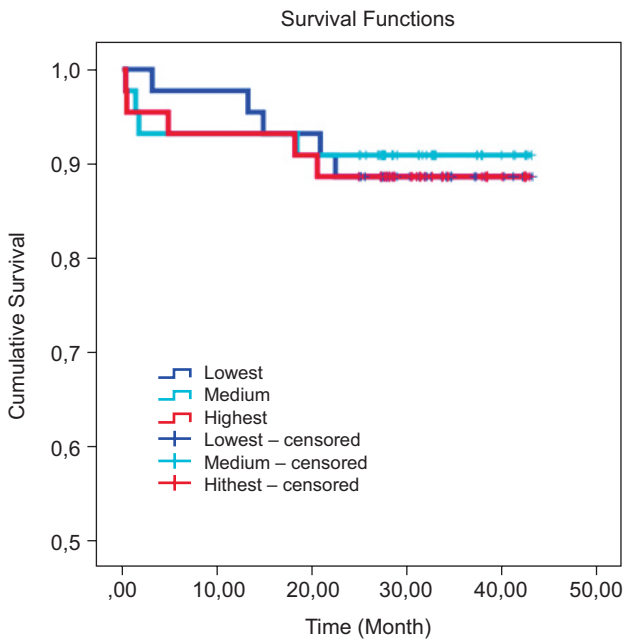

Figure 1. Kaplan–Meier survival curves according to tertiles.

Discussion

In this study, we compared telomerase activity and expression among patients with ACS, SCAD, and controls. Our findings showed that both telomerase activity and expression are comparable between the groups. Moreover, TA was not associated with all-cause mortality during the follow-up.

Telomeres are the nucleoprotein complexes capping and protecting the chromosomes. The telomere length (TL) has generally been considered as a marker for biological aging, and consequently for age-related diseases, including CVD. Lifestyle and environmental factors can also affect TL, and in fact, traditional cardiovascular factors have been associated with shorter telomeres (3–6). Moreover, numerous epidemiological studies have investigated the relation between TL and CVD. In a meta-analysis, authors found a relative risk for CAD of 1.54 (95.0% CI 1.30–1.83) when comparing the shortest and highest LTL tertiles (7). However, despite the significant independent association of shorter LTL with CVD in the studies, the effect size was usually very limited (2). Moreover, large-scale studies such as Asklepios and PESA showed that LTL and short-telomere load were not significant independent determinants of subclinical atherosclerosis (15, 16). On the other hand, individuals with shorter LTL at the time of recruitment had a significantly higher risk of developing subsequent coronary heart disease in the prospective West of Scotland Primary Prevention Study (WOSCOPS) (17).

Counteracting the telomere shortening process, the telomerase enzyme lengthens telomeres by repeatedly adding tandemly repeated telomeric DNA sequences using an RNA template present within the enzyme itself. Moreover, telomerase possesses telomere-independent roles in cardiovascular diseases as shown in recent studies, which includes its roles in the regulation of arterial tone, mitochondrial function, and production of reactive oxygen species (18–20). However, despite the extensive research on TL and CAD, there is a paucity of data on the relationship between telomerase activity and CAD. In a study of 62 heathy women, low telomerase activity in peripheral blood mononucleocytes (PBMCs) was associated with the six major CVD risk factors, namely with smoking, poor lipid profile, high systolic blood pressure, high fasting glucose, and greater abdominal adiposity, which raised the question whether the telomerase activity might be a more direct and potentially earlier predictor of disease processes than LTL (21, 22). In the CARDIA study, higher leukocyte telomerase activity was associated with higher prevalence of calcified atherosclerotic plaque, with a stronger association in persons with short telomeres (14).

Telomere length and TA might also be predictive for mortality. Epel et al examined associations between LTL and changes in LTL with 12-year overall and cardiovascular mortality rates in a subsample of 236 randomly selected Caucasian participants from the MacArthur Health Aging Study. Participants were aged between 70 and 79 years. Investigators found that there were no associations of LTL or % LTL with overall 12-year mortality. However, for women, short baseline LTL was related to higher mortality from CVD, while LT shortening (but not baseline LTL) was related to higher cardiovascular mortality for men (23). Reduced LTL was associated with all-cause mortality in patients with stable CAD in another study (24). Perez- Rivera et al analyzed the prognostic value of LTL in men admitted for ACS and they found a statistically significantly worse prognosis in patients with short telomeres in men aged 50 to 75 years, but not in men over 75 years (25). In a recent study, investigators examined the association of TL and telomerase activity in PBMC with adverse clinical outcomes in older patients (mean age: 81.0) with NSTE ACS underwent an invasive treatment strategy. Neither TL nor TA were found to be associated with adverse outcomes in older patients with non- ST-elevation ACS (26). In our study, we also did not find an association between telomerase activity and all-cause mortality in either of our patient groups (ACS and SCAD patients). However, it is important to note that the current study is not powered for mortality.

Telomerase expression or activity has been shown in human atherosclerotic plaques. Gizard et al showed it in contrast to normal coronary arteries, where the expression was negligible, TERT was significantly expressed in atherosclerotic lesions in coronary artery segments harvested from hearts during autopsy. Moreover, TERT expressions were more pronounced in the macrophage-rich shoulder region of advanced atherosclerotic lesions (27). Liu et al studied coronary arterial segments obtained from heart transplant recipients and found that 70.0% of atherosclerotic coronary arteries exhibited positive TA, and the reactivation incidence reached a 4-fold higher value compared to controls (13). Gupta et al showed TA in 8 of 23 consecutive atherectomy samples and suggested that telomerase may play a role in sustained proliferation of cells causing restenosis (12).

In the previous studies, TL has been generally measured in peripheral blood leukocytes, mostly because it is readily available and there are studies indicating a consistent synchrony between peripheral blood leukocyte and somatic cell TL (1, 2, 28). However, the role of inflammatory cells in atherosclerosis is beyond a mere marker. Telomerase activation has been described during adaptive immune responses, enhances the immune function and may have important implications for the development of atherosclerosis (27). Therefore, leukocyte TA have been investigated as a predictor of atherosclerosis. In fact, as mentioned above, the CARDIA study showed an association between higher leukocyte TA and higher prevalence of calcified atherosclerotic plaque, which usually indicates a stable disease**.** However, in the present study, we failed to show significant differences in TA between patients with normal coronary arteries and those with ACS or stable CAD. In a smaller study, the leukocyte hTERT mRNA level was not different between younger and middle-aged men, but it was significantly lower among older men and even lower among older men with CAD. On the other hand, Narducci et al (29) studied telomerase activity in peripheral blood polymorphonuclear neutrophils (PMN) and PMN isolated directly from coronary atherosclerotic plaques present in the angioplasty balloon washing medium utilized in percutaneous coronary intervention. They found high telomerase activity in PMN from coronary plaque of patients with unstable angina, particularly after a few hours of the last anginal episode, but not from patients with stable CAD or PMN from peripheral blood. Authors suggested that the survival of local activated PMN could be prolonged by telomerase reactivation confined to coronary plaque PMN. These results may partially account for the comparable TA between the groups in the current study, however, they do not provide an explanation for the inconsistency with the CARDIA study.

The main strengths of our study stem from the evaluation of our patients with invasive coronary angiography and inclusion of both the patients with acute coronary syndrome and those with stable CAD. There are also some limitations to the current study. Firstly, despite matching the patients according to age, consecutive recruitment resulted in differences between the groups. These included gender, smoking status and hypertension. Secondly, the inclusion of TL measurement would have provided additional insights to the study, however, in the present study we aimed to evaluate the TA and hTERT expression, independently from TL.

Conclusion

There is a paucity of data on telomerase in patients with acute coronary syndrome and stable coronary artery disease. In the current study, telomerase activity or expression was similar in patients with ACS, SCAD, and controls. Moreover, telomerase activity was not associated with all-cause mortality during the follow-up.

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Received October 7, 2023.
Accepted November 2, 2023.