Association of Relative Leucocyte Telomere Length and Gene Single Nucleotide Polymorphisms (*TERT*, *TRF1*, *TNKS2*) in Laryngeal Squamous Cell Carcinoma

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Abstract. *Background/Aim:* The study aimed to evaluate associations of relative leukocyte telomere length (LTL) and polymorphisms of telomere length-associated genes *TERT* (rs2736098), *TERT-CLPTMIL* (rs401681), *TRF1* (rs1545827, rs10107605) and *TNKS2* (rs10509637, rs10509639) in patients with laryngeal squamous cell carcinoma (LSCC). *Materials and Methods:* The study consisted of 300 patients with LSCC and 369 healthy control subjects. Genotyping and relative LTL measuring were carried out using qPCR. *Results:* Relative LTL was statistically significantly shorter in the G3 (tumor differentiation grade) subgroup of patients with LSCC compared to the G1 and G2 subgroups. Significant differences were found in genotype distributions of *TERT* rs401681 and *TNKS2* rs10509639 between the study groups. *TERT* rs401681 C/T and T/T genotypes were associated with approximately 30% decreased odds of LSCC development. *Conclusion:* LTL was shorter in the G3 subgroup compared to the G2 and G1 subgroups of LSCC patients. *TERT* rs401681 and its C/T and T/T genotypes were associated with decreased odds of overall LSCC development.

Laryngeal squamous cell carcinoma (LSCC) represents one of the most common upper respiratory tract tumors and ranks 20th among all oncological diseases in Europe (1). According to published data, LSCC accounts for 2.4% of all

newly diagnosed malignancies, of which more than 95% are diagnosed with squamous cell carcinoma (2). The incidence rate of LSCC has decreased almost twofold from 1995 to 2015 (from 4.6/100,000 inhabitants to 2.4/100,000 inhabitants, respectively), but the mortality rate due to this pathology has not changed during the same period (3). The etiology and pathogenesis of LSCC are complex and still poorly recognized. LSCC represents a heterogeneous disease whose pathogenesis is a multistep and multifactorial process that involves both environmental and genetic factors. Therefore, a better understanding of the LSCC pathogenesis requires a comprehensive research of biological and genetic markers of this disease.

In recent years, attention has been increasingly focused on investigations of telomeres and telomere length regulating genes, due to their importance in aging and development of chronic diseases and malignant tumors (4-7). The name telomere itself originates from the Greek word “telos”, which means “end”, and “meros”, which means “part” (8). In eukaryotic cells, telomeres are located at the ends of chromosomes in association with a protein complex (9). Telomeres are specialized DNA structures composed of several thousand DNA repeats of TTAGGG nucleotides (10). These DNA structures are essential for chromosome integrity and genomic stability through prohibiting nucleolytic degradation, chromosomal end-to-end fusion and irregular recombination (9, 11). Telomere dysfunction takes an important part in the carcinogenesis process. The shortening of telomeres by initiating apoptosis of senescent cells (telomere protection) is thought to be a mechanism of cancer suppression and constitutes a barrier to uncontrolled proliferation of cells (12). Loss of telomere protection can lead to telomere crisis, which can lead to genomic instability and induce carcinogenesis (12). Several studies have shown an association between changes in peripheral venous blood leucocyte telomere length (LTL) and cancer development (13). Raquel *et al.* have found the association between short

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telomeres and head and neck cancer development (14). However, the literature data on this matter are rather controversial. Particularly, there is a lack of data on the role of telomere length in LSCC development.

Several factors are known to determine the function of telomeres, which may also influence carcinogenesis. The telomerase complex and the shelterin complex, as well as tankyrase, are important for telomere function. Extensive studies of telomerase activity revealed that it could be a predictor of cancer risk (15). Telomerase complex counteracts telomere shortening and plays an important role in cells becoming immortal. These structures consist of both a catalytic protein subunit [telomerase reverse transcriptase (*TERT*)] and a human telomerase RNA component that acts as a template for inserting a sequence of the TTAGGG repeats at the 3' end of telomeric DNA (16). Single-nucleotide polymorphisms (SNP) of *TERT* and *TERT-CLPTMIL* demonstrated the relationship with cancer risk, including lung, urinary bladder, prostate, cervix and hepatocellular malignancies (17-21). However, only one paper revealing the association between polymorphisms of the *TERT-CLPTMIL* and pharynx-larynx cancer has been published so far (22).

Telomere binding complexes, such as shelterin, are known to protect telomeres from DNA repair mechanisms, as well as to regulate telomerase activity (23, 24). Six protein subunits (*TRF1*, *TRF2*, *RAP1*, *TIN2*, *TPP1*, and *POT1*) that form the shelterin complex are supposed to be involved in this activity (24). *TRF1* in co-action with both Tankyrase 1 (*TNKS1/ARTD5/PARP5a*) and Tankyrase 2 (*TNKS2/ARTD6/PARP5b*) controls telomere length negatively (telomere shortening is a result of TRF1 overexpression, while telomere lengthening is due to dominant-negative TRF1) (25, 26). Only a few studies have investigated associations between SNPs of *TRF1* and *TNKS2* and different carcinomas (27, 28). Wang *et al.* have found that *TNKS2* rs1340420 SNP was associated with lower non-small cell lung cancer risk, whereas *TNKS2* rs1770474 SNP was associated with higher squamous-cell carcinoma risk, suggesting that these two SNPs may be useful as predictors of risk of developing these malignancies (28). Varadi *et al.* have investigated the relationship between polymorphisms in telomere-associated genes (including *TRF1* and *TNKS2*) and breast cancer. Based on this study, authors concluded that none of the SNPs represents a valuable prognostic marker for breast cancer (27). However, a definitive conclusion on this matter is yet to be reached. To the best of our knowledge, there is no data in the literature regarding the associations between the aforementioned SNPs, LTL and LSCC.

The aim of the present study was to evaluate associations of relative LTL and SNPs of telomere length-associated genes *TERT* (rs2736098), *TERT-CLPTMIL* (rs401681), *TRF1* (rs1545827, rs10107605) and *TNKS2* (rs10509637, rs10509639) in patients with LSCC.

Table I. Demographic characteristics of study subjects.

Characteristic	Group		p-Value
	LSCC n=300	Control group n=369	
Male, n (%)	298 (96.3)	357 (96.7)	0.770*
Female n (%)	11 (3.7)	12 (3.3)	
Age years; mean (SD)	62.8 (8.8)	62.5 (14.3)	0.746**
Stage, n (%)		-	-
I	90 (30.8)		
II	66 (22.6)		
III	53 (18.2)		
IV	83 (28.4)		
T, n (%)		-	-
1	92 (31.5)		
2	66 (22.6)		
3	57 (19.5)		
4	77 (26.4)		
N, n (%)		-	-
0	234 (80.1)		
1	16 (5.5)		
2	42 (14.4)		
M, n (%)		-	-
0	289 (99)		
1	3 (1)		
G, n (%)		-	-
1	72 (25)		
2	192 (65.8)		
3	27 (9.7)		

*Pearson Chi-Square, **Student's *t*-test. SD: Standard deviation; T: tumor size; M: metastasis; N: metastasis to neck lymph nodes; G: tumor differentiation grade.

Materials and Methods

Ethics statement. All study procedures were carried out according to the Declaration of Helsinki, and the study protocol was approved by the Kaunas Regional Ethics Committee for Biomedical Research (authorization number: BE-2-37). Objectives and methods of the study were explained to all study subjects prior to the examination. Written informed consent was obtained from all subjects. The study was conducted in the Department of Otorhinolaryngology, Lithuanian University of Health Sciences (LUHS), Kaunas, Lithuania.

Study protocol/design. This case-control study consisted of 300 first time histologically verified LSCC patients [stage I to IV as defined by the American Joint Committee on Cancer (29)] and 369 healthy subjects as a reference (control) group. The control group consisted of healthy volunteers who agreed to take part in this study. Pregnant or breastfeeding women, patients younger than 18 years, patients with systemic comorbidities [e.g., diabetes mellitus, malignant tumors (except LSCC in the case group)] were excluded from the study. Peripheral venous blood samples were collected from all case group patients before treatment at the Department of Otorhinolaryngology, LUHS. Blood samples of the control group subjects were collected in the Outpatient Clinic of LUHS. The

Figure 1. Relative leukocyte telomere length in the LSCC patient and control groups. *Mann-Whitney U-test.

Figure 2. Relative leukocyte telomere length in LSCC patients by tumor differentiation grade (G). *Mann-Whitney U-test.

control group was formed adjusting to the demographic characteristics of the LSCC patient group (Table I).

DNA extraction and genotyping. DNA was extracted from venous peripheral blood samples using the DNA salting-out method. The genotyping of all six SNPs was performed using TaqMan® Genotyping assays (Applied Biosystems Foster City, CA, USA): C_26414916_20 (rs2736098), C_1150767_20 (rs401681), C_1869846_10 (rs1545827), C_1869856_10 (rs10107605), C_30418896_20 (rs10509637), C_29498647_20 (rs10509639) according to manufacturer's instructions using the real-time polymerase chain reaction (PCR) method.

Relative leukocyte telomere length measurement. Relative LTL for study subjects was determined using modified quantitative real-time PCR (qPCR) protocol (30). The relative LTL for each sample was determined using two separate qPCR runs for each sample, the first to determine the cycle threshold (Ct) value for telomere amplification, and the second one to determine the Ct value for control gene amplification. A standard curve was generated in each run, consisting of a 6-point serial dilution of the reference DNA pool. The Ct data generated in both runs were used to calculate relative LTL values for each sample according to the formula: $T/S = 2^{-\Delta\Delta C_t}$ (31).

Statistical analysis. Statistical analysis was performed using the SPSS/W 20.0 software (Statistical Package for the Social Sciences for Windows, Inc., Chicago, IL, USA). The data of relative LTL are presented as median with interquartile range (IQR) and mean rank. The data of age are presented as mean with standard deviation (SD). Normality of the data distribution was checked using the Shapiro-Wilk test. The Mann-Whitney test was used for comparison of relative LTL between two groups. Student's *t*-test was performed to compare the average age between the study groups. Frequencies of genotypes and alleles, gender, age, distribution of LSCC stage, tumor size, neck lymph nodes, metastasis and tumor differentiation grade are reported using

absolute numbers with percentages. Hardy-Weinberg equilibrium analysis was performed to compare the observed and expected frequencies of *TERT* (rs2736098, rs401681), *TRF1* (rs1545827, rs10107605) and *TNKS2* (rs10509637, rs10509639). The distributions of the genotypes and alleles of *TERT* (rs2736098, rs401681), *TRF1* (rs1545827, rs10107605), *TNKS2* (rs10509637, rs10509639) as well as gender distribution and long and short telomeres in the LSCC and control groups were compared using the χ^2 test. Binomial logistic regression analysis was performed to estimate the impact of genotypes on LSCC development. Odds ratios and 95% confidence intervals are presented. The selection of the best genetic model was based on the Akaike Information Criterion (AIC); therefore, the best genetic models were those with the lowest AIC values. Differences were considered statistically significant when $p < 0.05$.

Results

The total study group consisted of 300 patients with LSCC: 289 (96.3%) males and 11 (3.7%) females with a median age of 62.8 (SD=8.8) years and of 369 healthy subjects: 357 (96.7%) males and 12 (3.3%) females with a mean age of 62.5 (SD=14.3) years. The LSCC patient and control groups were adjusted by age and gender. Demographic characteristics of the total study group are presented in Table I.

Relative LTL was analyzed in 155 healthy subjects: 143 (92.3%) males and 12 (7.7%) females, and in 114 patients with LSCC: 110 (96.5%) males and 4 (3.5%) females. No significant differences in relative LTL between the LSCC and control groups were revealed [relative LTL, median (IQR)=0.5794 (0.421) vs. 0.6306 (0.453), $p=0.162$] (Figure 1).

Comparative analysis of relative LTL in patient subgroups according to the LSCC differentiation grade (G) detected

Table II. The relative leukocyte telomere length distribution as short and long telomeres in the patients with LSCC and control group subjects.

Characteristic	LSCC group N=114	Control group N=155	p-Value
Short telomeres	50 (43.9)	77 (49.7)	0.345*
Long telomeres	64 (56.1)	78 (50.3)	

*Pearson Chi-Square.

Table III. Analysis of Hardy–Weinberg equilibrium in the control group.

SNP	Allele frequencies		Genotype distribution	p-Value
<i>TERT</i> rs2736098	0.79 C	0.21 T	20/117/232	0.305
<i>TERT</i> rs401681	0.55 C	0.45 T	63/204/102	0.023
<i>TRF1</i> rs1545827	0.6 C	0.4 T	64/169/136	0.359
<i>TRF1</i> rs10107605	0.91 A	0.09 C	4/61/304	0.634
<i>TNKS2</i> rs10509637	0.84 A	0.16 G	8/102/259	0.578
<i>TNKS2</i> rs10509639	0.87 A	0.13 G	22/52/295	<0.001

Bold value indicates statistical significance.

statistically significantly shorter relative LTL in patients of LSCC G3 subgroup compared to G1 [relative LTL, median (IQR)=0.4610 (0.4331) vs. 0.6508 (0.668), $p=0.033$] and G2 subgroups [relative LTL, median (IQR)=0.4610 (0.4331) vs. 0.6408 (0.6967), respectively, $p=0.023$] (Figure 2).

The relative LTL was divided into two groups – short and long telomeres, based on the median telomere length of control group subjects. The relative LTL lower than the median telomere length of the control group was considered to represent “short telomeres” and the higher “long telomeres”. There were no statistically significant differences in distribution of short and long telomeres between the LSCC and control groups (43.9% and 56.1% vs. 49.7% and 50.3%, $p=0.345$) (Table II).

Distributions of the analyzed *TERT* rs2736098, *TERT-CLPTM1* rs401681, *TRF1* rs1545827, rs10107605 and *TNKS2* rs10509637 matched the Hardy-Weinberg equilibrium (HWE) ($p>0.001$). Despite the *TNKS2* rs10509639 not matching HWE, we did not exclude this polymorphism from further statistical analysis because such distribution of genotypes and alleles is possible due to the relatively small sample size (Table III).

Distributions of frequencies of the following genotypes and alleles were analyzed in the control and LSCC groups: *TERT* rs2736098, *TERT-CLPTM1* rs401681, *TRF1* rs1545827, rs10107605, and *TNKS2* rs10509637, rs10509639. Significant differences were found in the genotype distributions of *TERT* rs401681 and *TNKS2* rs10509639 between the study groups ($p=0.033$ and $p<0.001$, respectively) (Table IV).

Table IV. Frequencies of genotypes and alleles of *TERT* rs2736098, rs401681, *TRF1* rs1545827, rs10107605, *TNKS2* rs10509637 and rs10509639 in the control and patients with LSCC groups.

Polymorphism	Control group n/%	LSCC n/%	p-Value
<i>TERT</i> rs273609			
C/C	232 (62.9)	171 (57.0)	0.304
C/T	117 (31.7)	110 (36.7)	
T/T	20 (5.4)	19 (6.3)	
Total	369 (100)	300 (100)	
Allele			
C	581 (78.7)	452 (75.3)	0.141
T	157 (21.3)	148 (24.7)	
<i>TERT</i> rs401681			
C/C	102 (27.6)	108 (36.0)	0.033
C/T	204 (55.3)	137 (45.7)	
T/T	63 (17.1)	55 (18.3)	
Total	369 (100)	300 (100)	
Allele			
C	408 (55.3)	353 (58.8)	0.872
T	330 (44.7)	247 (41.2)	
<i>TRF1</i> rs1545827			
C/C	136 (36.9)	97 (32.3)	0.243
C/T	169 (45.8)	157 (52.3)	
T/T	64 (17.3)	46 (15.3)	
Total	369 (100)	300 (100)	
Allele			
C	441 (59.8)	351 (58.5)	0.642
T	297 (40.2)	249 (41.5)	
<i>TRF1</i> rs10107605			
A/A	304 (82.4)	241 (80.3)	0.613
A/C	61 (16.5)	57 (19.0)	
C/C	4 (1.1)	2 (0.7)	
Total	369 (100)	300 (100)	
Allele			
A	669 (90.7)	539 (89.8)	0.616
C	69 (9.3)	61 (10.2)	
<i>TNKS2</i> rs10509637			
A/A	259 (70.2)	196 (65.3)	0.405
A/G	102 (27.6)	96 (32.0)	
G/G	8 (2.2)	8 (2.7)	
Total	369 (100)	300 (100)	
Allele			
A	620 (84)	488 (81.3)	0.197
G	118 (16)	112 (18.7)	
<i>TNKS2</i> rs10509639			
A/A	295 (79.9)	242 (80.7)	<0.001
A/G	52 (14.1)	58 (19.3)	
G/G	22 (6.0)	0 (0.0)	
Total	369 (100)	300 (100)	
Allele			
A	642 (87)	542 (90)	0.057
G	96 (13)	58 (10)	

Bold values indicate statistical significance.

To evaluate the impact of *TERT* rs2736098, rs401681, *TRF1* rs1545827, rs10107605 and *TNKS2* rs10509637 and rs10509639 on the LSCC development, a binomial logistic regression was applied. Our results revealed that the *TERT*

Table V. Binomial logistic regression analysis of *TERT* rs2736098, rs401681, *TRF1* rs1545827, rs10107605, *TNKS2* rs10509637 and rs10509639 in the control and patients with LSCC groups.

Model	Genotype/allele	OR (95% CI)	p-Value	AIC
<i>TERT</i> rs2736098				
Codominant	C/T vs. C/C	1.276 (0.960-1.769)	0.144	921.921
	T/T vs. C/C	1.289 (0.667-2.489)	0.450	
Dominant	C/T+T/T vs. C/C	1.278 (0.936-1.744)	0.123	919.921
Recessive	T/T vs. C/C+T/T	1.180 (0.618-2.254)	0.616	922.051
Overdominant	C/T vs. C/C+T/T	1.247 (0.904-1.719)	0.178	920.489
Additive	T	1.204 (0.936-1.549)	0.149	920.217
<i>TERT</i> rs401681				
Codominant	C/T vs. C/C	0.634 (0.448-0.897)	0.010	917.466
	T/T vs. C/C	0.825 (0.525-1.295)	0.402	
Dominant	C/T+T/T vs. C/C	0.679 (0.489-0.943)	0.021	916.949
Recessive	T/T vs. C/C+T/T	1.090 (0.732-1.625)	0.671	922.121
Overdominant	C/T vs. C/C+T/T	0.680 (0.501-0.923)	0.013	16.1689
Additive	T	0.860 (0.6880-1.074)	0.184	920.530
<i>TRF1</i> rs1545827				
Codominant	C/T vs. C/C	1.303 (0.928-1.828)	0.127	921.472
	T/T vs. C/C	1.008 (0.636-1.596)	0.974	
Dominant	C/T+T/T vs. C/C	1.222 (0.886-1.684)	0.222	920.806
Recessive	T/T vs. C/C+T/T	0.863 (0.571-1.305)	0.486	921.813
Overdominant	C/T vs. C/C+T/T	1.299 (0.957-1.763)	0.093	919.473
Additive	T	1.054 (0.846-1.313)	0.641	922.084
<i>TRF1</i> rs10107605				
Codominant	A/C vs. A/A	1.791 (0.791-1.756)	0.419	923.317
	C/C vs. A/A	0.631 (0.115-3.472)	0.596	
Dominant	A/C+C/C vs. A/A	1.145 (0.775-1.693)	0.497	921.842
Recessive	C/C vs. A/A+A/C	0.612 (0.111-3.367)	0.573	921.969
Overdominant	A/C vs. A/A+A/C	1.184 (0.795-1.764)	0.405	921.610
Additive	C	1.098 (0.763-1.1579)	0.615	922.049
<i>TNKS2</i> rs10509637				
Codominant	A/G vs. A/A	1.244 (0.890-1.738)	0.202	922.498
	G/G vs. A/A	1.321 (0.487-3.583)	0.584	
Dominant	A/G+G/G vs. A/A	1.249 (0.902-1.731)	0.181	920.512
Recessive	G/G vs. A/A+A/G	1.236 (0.458-3.334)	0.675	920.126
Overdominant	A/G vs. A/A+A/G	1.232 (0.883-1.719)	0.220	920.797
Additive	G	1.216 (0.909-1.626)	0.188	920.571
<i>TNKS2</i> rs10509639				
Codominant	A/G vs. A/A	1.360 (0.901-2.051)	0.143	895.366
	G/G vs. A/A	0.000 (0.000-0.000)	0.998	
Dominant	A/G+G/G vs. A/A	0.955 (0.651-1.402)	0.816	922.247
Recessive	G/G vs. A/A+A/G	0.000 (0.000-0.000)	0.998	897.515
Overdominant	A/G vs. A/A+A/G	1.461 (0.970-2.202)	0.070	921.010
Additive	G	0.754 (0.549-1.037)	0.083	921.215

OR: Odds ratio; CI: confidence interval; p-Value: significance level (alpha=0.05); AIC: Akaike information criterion.

Table VI. Frequencies of genotypes and alleles of *TERT* rs2736098, *rs401681*, *TRF1* rs1545827, *rs10107605*, *TNKS2* rs10509637, *rs10509639* in the short and long telomeres subgroups.

Polymorphisms	Short telomeres n/%	Long telomeres n/%	<i>p</i> -Value
<i>TERT</i> rs273609			
C/C	82 (64.6)	81 (57.4)	0.416
C/T	38 (29.9)	53 (37.6)	
T/T	7 (5.5)	7 (5.0)	
Total	127 (100)	141 (100)	
Allele			
C	202 (79.5)	215 (76.2)	0.361
T	52 (20.5)	67 (23.8)	
<i>TERT</i> rs401681			
C/C	42 (33.1)	36 (25.5)	0.325
C/T	63 (49.6)	82 (58.2)	
T/T	22 (17.3)	23 (16.3)	
Total	127 (100)	141 (100)	
Allele			
C	147 (57.9)	154 (54.6)	0.447
T	107 (42.1)	128 (45.4)	
<i>TRF1</i> rs1545827			
C/C	45 (35.4)	60 (42.6)	0.313
C/T	64 (50.4)	58 (41.1)	
T/T	18 (14.2)	23 (16.3)	
Total	127 (100)	141 (100)	
Allele			
C	154 (60.6)	178 (63.1)	0.553
T	100 (39.4)	104 (36.9)	
<i>TRF1</i> rs10107605			
A/A	107 (84.3)	116 (82.3)	0.483
A/C	19 (15.0)	25 (17.7)	
C/C	1 (0.8)	0 (0.0)	
Total	127 (100)	141 (100)	
Allele			
A	233 (91.7)	257 (91.1)	0.805
C	21(8.3)	25 (8.9)	
<i>TNKS2</i> rs10509637			
A/A	81 (63.8)	102 (72.3)	0.182
A/G	39 (30.7)	36 (25.5)	
G/G	7 (5.5)	3 (2.1)	
Total	127 (100)	141 (100)	
Allele			
A	201 (79.1)	240 (85.1)	0.071
G	53 (20.9)	42 (14.9)	
<i>TNKS2</i> rs10509639			
A/A	110 (86.6)	123 (87.2)	0.986
A/G	14 (1)	15 (10.6)	
G/G	3 (2.4)	3 (2.1)	
Total	127 (100)	64 (100)	
Allele			
A	234 (92.1)	261 (92.5)	0.853
G	20 (7.8)	21 (7.5)	

rs401681 C/T genotype was associated with approximately 30% decreased odds of LSCC development in the codominant (OR=0.634; 95% CI=0.448-0.897; $p=0.010$), and overdominant (OR=0.680; 95% CI=0.501-0.923; $p=0.013$)

models; C/T+T/T genotypes were also associated with approximately 30% decreased odds of LSCC development in the dominant model (OR=0.679; 95% CI=0.489-0.943; $p=0.021$) model (Table V).

Analysis of frequency distribution of genotypes and alleles of *TERT* rs2736098, rs401681, *TRF1* rs1545827, rs10107605 and *TNKS2* rs10509637, rs10509639 in the short and long telomeres subgroups revealed no statistically significant differences (Table VI).

Discussion

Results of several studies have shown that telomere aberrations, mainly consisting of shortening, are consistently found in head and neck squamous cell carcinoma (HNSCC) precursors and in the mucosa surrounding pre-neoplastic areas, both in invasive carcinomas and in peripheral venous blood, supporting the hypothesis that genomic instability driven by telomere dysfunction is an early event in the HNSCC oncogenic process (32-36).

Data in literature regarding the associations of relative LTL and development of HNSCC are rather scarce and controversial. Several studies have observed that patients with short telomeres have a higher risk of developing HNSCC (34-37). Namely, short relative LTL was associated with an increased risk of developing oral premalignant lesions and oral squamous cell carcinoma, suggesting that patients with premalignant lesions and short relative LTL face an increased risk to develop this type of malignant tumor (38).

Only a few reports have measured relative LTL in blood cells collected from HNSCC patients. Liu *et al.* have not found a significant association between patients' relative LTL in peripheral blood cells and the risk of developing HNSCC in one large case-control study (39). On the other hand, other studies have revealed shortened telomeres in peripheral blood leukocytes obtained from patients with HNSCC compared to healthy controls. As found by Alves-Paiva *et al.* in a Brazilian cohort of HNSCC patients, the median relative LTL was lower in HNSCC patients compared to age-matched healthy individuals, and this was associated with increased risk of HNSCC (14). Similarly, Barczak *et al.* have reported the presence of shorter telomeres even in early stage HNSCC tumors (40). Xun Zhu *et al.* performed a meta-analysis in 2016, to assess the total cancer risk or cancer-specific risk associated with telomere length. A total of 23,379 cancer cases and 68,792 controls from 51 independent publications were included in this study. They did not find an association between telomere length and overall cancer risk but demonstrated a significant relationship with short telomeres and increased risk of HNSCC (41). The discrepancy between literature data may be explained by significant diversity of malignances united under the umbrella of the HNSCC term,

as this includes malignant tumors of different localizations (oral, pharyngeal, laryngeal regions) as well as of different biological and clinical behavior. All these different tumors have different etiology and pooling all cancer types together may mask the significant associations of relative LTL with individual cancer types (38). In the present study, a pure cohort of LSCC patients was investigated. Results revealed no statistically significant differences in relative LTL between the LSCC patients and control groups. Presumably, these findings reflect the biological and clinical peculiarities of the LSCC tumor.

No data on the relation between relative LTL and tumor differentiation grade in LSCC have been published so far. In the present study, shorter relative LTL in the LSCC G3 subgroup against the G2 and G1 subgroups were detected for the first time. It is generally accepted that G3 tumor differentiation grade is usually associated with the worst prognosis (early metastasis and worst overall survival) when compared to G1 and G2 (42). On the other hand, there is an evidence from various studies that shortened telomeres are related to increased mortality rate in general (43). Therefore, data of the present study on telomere shortening in the subgroup of G3 LSCC patients are in concordance with this concept.

Telomere length regulating factors such as the telomerase complex, the shelterin complex and tankyrase as well as coding genes' polymorphisms are considered important factors in cancerogenesis (15). In the present study, frequencies of genotypes and alleles of *TERT* rs2736098, rs401681, *TRF1* rs1545827, rs10107605, *TNKS2* rs10509637 and rs10509639 were analyzed in the LSCC patients and control groups. Significant differences were found in genotype distributions of *TERT* rs401681 and *TNKS2* rs10509639 between the study groups ($p=0.033$ and $p<0.001$, respectively). Moreover, after performing binomial logistic regression, we found that the *TERT* rs401681 C/T and C/T+T/T genotypes were associated with approximately 30% decreased odds of LSCC development.

According to the literature, SNPs of *TERT* and *TERT* *CLPTMIL* at the 5p15.33 locus have a significant relationship with cancer risk (17-21). A recent study of the Southeast Iranian population has found a protective effect of an allele of *TERT* rs2736098 against breast cancer (44). Yu *et al.* have reported results similar to our study. They performed a case-control study including 490 cases (histologically diagnosed primary squamous cell carcinomas of the pharynx-larynx) and 590 controls. Study results revealed that the CT and CT+CC genotype models of rs401681 were related to a reduced risk of pharynx-larynx cancer (22). The earlier study by Liu *et al.* has found that rs401681 CT+TT genotypes lowered the risk of oropharyngeal cancer for smokers and alcohol users (45). Previous studies have investigated the interactions of other SNPs of the *TERT* gene with head and neck cancer, but no associations have been found (39, 46).

Qu *et al.* have compared the SNPs of *TERT* C228T and C250T between a laryngeal cancer and control group and haven't found any difference; however *TERT* C250T mutation indicated the worst overall survival rate (46). Liu *et al.* have investigated a large group of 888 subjects diagnosed with HNSCC but have not found any associations between SNPs of *TERT* (rs2735940; rs2736098; rs2736109; rs2853669; rs2853677 and rs2853690) and development of this tumor (39). However, results of the present study demonstrated that *TERT* rs401681 CT and C/C+ T/T genotypes were associated with approximately 30% decreased odds of LSCC development.

The strength of the present study consists of a relatively large and homogenous group of pure LSCC patients and age- and gender-matched controls. This peculiarity allowed us to perform a precise analysis of associations between relative LTL and LSCC development, with the latter being a specific tumor in one anatomical region. As generally accepted from clinical practice, LSCC (particularly, the glottis cancer) features a relatively low aggressiveness, i.e. low metastatic and spreading rate compared to other malignancies of head and neck region (47, 48). Therefore, the absence of differences in relative LTL between the low aggressive tumor patient and age-matched control groups is comprehensible. Moreover, an increased aggressiveness of LSCC, as in the G3 LSCC patient subgroup, reflected significantly decreased relative LTL compared to the G1 and G2 subgroups. Of note is the fact that results of the present study revealed that gene polymorphism *TERT* rs401681 and its C/T and C/T+T/T genotypes are associated with approximately 30% reduced odds of LSCC developing. To the best of our knowledge, this is the first report that associates the relative LTL and SNPs of telomere length-associated genes in a pure and homogenous LSCC patient cohort. However, limitations of the present study should be also considered as environmental factors, smoking and alcohol consumption were not analyzed. However, this is foreseen as a task for future research.

Conclusion

Results of the present study revealed no statistically significant differences in relative LTL between the LSCC patient and control groups. However, the relative LTL was shorter in the G3 subgroup compared to the G2 and G1 subgroups of LSCC patients showing their possible role in tumor development. Also, we found that the genetic marker *TERT* rs401681 and its C/T and T/T genotypes are associated with decreased odds of overall LSCC development.

Conflicts of Interest

The Authors declare no potential conflicts of interest associated with this study.

Authors' Contributions

R.L., A.V., G.G. and V.U. designed research; V.L. and V.U. collected patients' samples; P.V., G.G. and A.V. performed research; R.L., A.V., G.G. and P.V. analyzed data; and P.V., R.L., V.L. and V.U. wrote the paper.

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