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Original

The role of TERT-CLPTM1L SNPs, hTERT expression and telomere length in the pathogenesis of oral squamous cell carcinoma

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Abstract: The aim of this study was to assess TERT-CLPTM1L single-nucleotide polymorphisms (SNPs) (rs402710 C/T in the CLPTM1L gene; rs2736100 A/C and rs2736098 G/A in the TERT gene) as risk factors for development of oral squamous cell carcinoma (OSCC), and to investigate the relationship between the analyzed polymorphisms, relative telomere length (RTL), telomerase expression and clinicopathologic characteristics of OSCC in a Serbian population. Paraffin-embedded tumor samples and buccal swabs from cancer-free controls were genotyped using PCR-RFLP, while tumor RTL values and telomerase expression were estimated by real-time PCR and immunohistochemistry, respectively. CLPTM1L rs402710 and TERT rs2736100 polymorphisms were associated with a significantly increased risk of OSCC, and TERT rs2736098 with a significantly decreased risk. No significant association was found between TERT-CLPTM1L polymorphisms, tumor RTL values, telomerase expression, and clinicopathologic features, although a trend towards longer telomeres was evident in telomerase-positive samples and less advanced tumors. Kaplan-Meier survival analysis showed that patients with longer telomeres in their tumors had significantly better overall survival than patients with shorter telomeres. Our research seems to provide strong evidence for an association between CLPTM1L rs402710C/T and TERT rs2736100A/C SNPs and the risk of OSSC, and suggests that higher tumor RTL values and positive hTERT expression may be applicable as early prognostic markers.
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Keywords: OSCC; TERT-CLPTM1L; telomere length; telomerase.

Introduction

Oral squamous cell carcinoma (OSCC) accounts for 90% of all malignancies in the oral cavity (1) and is the eighth most common cancer in Serbia (2). In spite of research and therapeutic advances, OSCC mortality rates remain unchanged (3) and the incidence is increasing, especially in younger persons (4). Although lifestyle choices, such as use of alcohol and tobacco, are important risk factors, oral carcinogenesis is a multi-step process involving distinct genetic changes (5).

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Recent studies have shown that due to their roles in cell replication, telomerase activation and regulation of telomere length also have a central role in carcinogenesis (6). Telomeres are protective caps consisting of DNA tandem repeats (TTAGGG) located at the ends of eukaryotic chromosomes that progressively shorten with each cell division. When a threshold length is reached, cells either undergo replicative senescence or achieve the ability to proliferate indefinitely by activating the enzyme telomerase, as one of two known telomere maintenance mechanisms (7).

Telomerase is a ribonucleoprotein complex consisting of a catalytic protein component (hTERT) and a RNA component (TERC), that compensate for telomere attrition by adding repeats onto chromosome ends (8). Its activity is normally not detectable in most adult tissues, apart from stem cells of proliferative tissues. However, 80-90% of human malignancies show telomerase expression, so its detection could be a useful diagnostic tool for different types of cancer (6).

The TERT gene, located at the 5p15.33 locus, encodes the protein subunit of telomerase and is essential for maintenance of telomere length. The 5p15.33 locus also contains cleft lip and palate transmembrane 1-like (CLPTM1L) gene, whose product is involved in the process of apoptosis (9).

Cancer Genome-wide Association Studies (GWAS) have shown that single-nucleotide polymorphisms (SNPs) at the TERT-CLPTM1L locus, including TERT rs2736100 (A>C), TERT rs2736098 (G>A) and CLPTM1L rs402710 (C>T), are associated with the risk of multiple cancers (10).

The aim of our present study was to assess the potential role of specific TERT-CLPTM1L polymorphisms as risk factors for OSCC development, and to investigate the relationship between these polymorphisms, relative telomere length (RTL), telomerase expression and clinicopathologic characteristics of OSCCs in a Serbian population.

Materials and Methods

Study population

The study was performed according to the ethical principles governing medical research and human subjects as laid down in the Helsinki Declaration (2002 version, www.wma.net/e/policy/b3.htm), and with the approval of the Ethics Committee of the School of Dental Medicine, University of Belgrade, Serbia (No 36/7). All participants were informed of the procedures and furnished written informed consent.

The study population used for polymorphism genotyping included 90 patients with histologically confirmed OSCC, treated at the Clinic for Maxillofacial Surgery, School of Dental Medicine, University of Belgrade, and 100 cancer-free controls recruited at the School of Dental Medicine, University of Belgrade. All study participants were from the same ethnic background. Information on the clinicopathologic characteristics of the tumors (TNM status, stage, histological grade) was obtained from the medical records. As no reliable information on tobacco and alcohol consumption was available, such data were not included in the study. No information about cancer recurrence was obtained. Probably because of the low quality of some of the tumor DNA samples, only 60 OSCCs were genotyped for CLPTM1L. The age distributions of the OSCC patients (61.4 ± 11.1) and the controls (63.1 ± 8.4) were similar. However, the case group included a higher number of men than the control group (79% and 63%, respectively).

Relative telomere length (RTL) measurement was performed in 60 cases, and immunohistochemical staining for TERT was possible in only 34 cases of OSCC, due to the limited amount of paraffin-embedded tumor tissues available.

DNA extraction

DNA in the OSCC group was extracted from formalin-fixed, paraffin-embedded (FFPE) tissues, and in the control group from buccal swabs. All extractions were performed using a KAPA Express Extract Kit (Kapa Biosystems, Inc., Wilmington, MA, USA), as recommended by the manufacturer. The concentration of DNA was measured spectrophotometrically.

Genotyping of polymorphisms

All three SNPs (rs402710C/T in the CLPTM1L gene; rs2736100A/C and rs2736098 G/A in the TERT gene) were genotyped using the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) method, as described previously (11,12). Primers and product lengths are given in Table 1.

After digestion by Pvu II (Thermo Fisher Scientific Inc., Waltham, MA, USA), the CLPTM1L rs402710 product with the C allele remained as one fragment of 473 bp, and the T allele product was divided into fragments of 263 and 210 bp. TERT rs2736100 was digested with restriction enzyme SfcI (BfmI) (Thermo Fisher Scientific Inc.). The A allele produced a single 152-bp fragment and the C allele produced two fragments of 104 and 48 bp. TERT rs2736098 was digested with Bsp120I (Thermo Fisher Scientific Inc.). Two amplification products (289 bp and 90 bp) were generated from the G

Table 1 Primers and product lengths of the PCR assays for detection of polymorphisms in TERT-CLPTM1L loci

Polymorphisms	Primers (5'-3')	Product lengths (bp)
CLPTM1L/rs402710 (C>T)	F:ACATTTGCTTTCAGTGGCTCA	473
	R:CCGTTGGCTTGGTTAGGTT	
TERT/rs2736100 (A>C)	F:CCCCACAAGCTAAGCATTAT	152
	R:GAAGAACCACGCAAAGGAC	
TERT/rs2736098 (G>A)	F:GCCAGACCCGCCGAAGAAG	379
	R:GCGCGTGGTTCCCAAGCAG	

allele, whereas the G>A polymorphism generated only one 379-bp fragment.

All digestion products were loaded on 8% polyacrylamide gel, stained with ethidium bromide, and observed on an ultraviolet fluorescence imaging system.

Relative telomere length (RTL) measurement

The relative telomere length (RTL) of 60 cases of OSCC was assessed by measuring the relative telomere-to-single copy gene (T/S) ratios by qPCR using a previously described method with minor modifications (13). The qPCR was divided into two reactions: telomere PCRs and β-globin (HBG) PCRs. The primer sequences for telomere and β-globin were:
5'CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT3'(Tel1),
5'GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT3'(Tel2),
5'TCTGACACAACTGTGTTCACTAGC3'(HBG1),
5'CACCAACTTCATCCACGTTCACC3'(HBG 2).

Real-time PCR was performed on a Line-Gene K Fluorescence Quantitative PCR Detection System (Hangzhou Bioer Technology Co., Ltd., Shanghai, China). For two separate qPCR runs, the 25-μL volume of reaction mixture consisted of 12.5 μL Maxima (SYBRGreen/ROX, Master Mix 2X, Thermo Fisher Scientific Inc.), the Tel 1 and Tel 2 primers at final concentrations of 100 nM and 900 nM, respectively, or the HBG1 and HBG2 primers at final concentrations of 300 nM and 700 nM, respectively; 10 ng of template DNA was added in each reaction. For each PCR run, serially diluted reference tumor DNA (293T) was included within a range of 2.5-40 ng/μL for generating a standard curve and monitoring the efficiency of the PCR. The temperature profile for the telomere qPCR included 95°C for 5 min, followed by 30 cycles at 95°C for 15 s and 56°C for 1 min. For β-globin (HBG) the cycling profile was 95°C for 5 min, followed by 35 cycles at 95°C for 15 s and 54°C for 1 min. All samples for both of the analyzed genes were run in two replicates, and each sample was analyzed twice. The standard curve slope for both qPCR reactions ranged from −3.2 to −3.7, and the linear correlation coefficient (R^2) for each standard curve was ≥0.98. The mean Ct values for both genes were used to calculate the T/S ratio (telomere repeat copy number/single copy gene number). The relative T/S ratio was determined using the formula $T / S = 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{telomere} - Ct_{\beta\text{-}globin})$ of the sample − $(Ct_{telomere} - Ct_{\beta\text{-}globin})$ of the reference sample (10-ng standard curve point) (13). Using this formula, each sample's RTL was calculated.

Immunohistochemistry

Immunohistochemical analysis of hTERT expression was performed on 34 cases of OSCC. Sections (5 μm thick) from the formalin-fixed, paraffin-embedded tissue samples were deparaffinized and treated with 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. For heat-induced antigen retrieval, tissue sections were immersed in 0.01 mol/L citrate buffer (pH = 6.0) and heated in a microwave oven for 20 min at 620 W. After cooling off for 30 min at room temperature, blocking solution (DAKO, Glostrup, Denmark) was utilized to block any non-specific staining. Thereafter, the sections were incubated overnight at 4°C with a mouse monoclonal primary antibody against elomerase reverse transcriptase (clone 2C4, dilution 1:150; Thermo Fisher Scientific Inc.). As a secondary antibody, peroxidase-conjugated rabbit anti-mouse IgG (dilution 1:500; Thermo Fisher Scientific Inc.) was used. After applying the streptavidin-biotin technique using a DAKO LSAB+ kit (DAKO), the sections were stained with diaminobenzidine (DAB) solution as a chromogen and counterstained with Mayer's hematoxylin. Tissue obtained from tonsils during tonsillectomy was used as a positive control, whereas incubation with the pure antibody diluent (without the primary antibody) served as a negative control.

Expression of hTERT was determined in the nucleus and cytoplasm. All immunostained sections were independently evaluated by two of the authors. Cell counting

Table 2 Genotype frequencies of the TERT and CLPTM1L polymorphisms among OSCC cases and control subjects and their association with the risk of OSCC

Genotypes	Cases *n* (%)	Controls *n* (%)	OR (95% CI)	*P*#
CLPTM1L-rs402710 (C>T)	*n* = 60	*n* = 100		
CC	16 (32.4)	44 (44)	1.00	
CT	24 (38.2)	46 (46)	1.44 (0.67-3.05)	0.45
TT	20 (29.4)	10 (10)	5.50 (2.13-14.23)	<0.001*
CT+TT	44 (67.6)	56 (56)	2.16 (0.86-3.13)	<0.01*
T allele frequency	0.53	0.33	2.29 (1.29-4.06)	<0.01*
TERT-rs2736100 (A>C)	*n* = 90	*n* = 100		
AA	13 (14.4)	30 (30)	1.00	
AC	46 (51.2)	47 (47)	2.26 (1.05-4.86)	<0.05*
CC	31 (34.4)	23 (23)	3.11 (1.36-7.24)	<0.01*
AC+CC	77 (85.6)	70 (70)	2.54 (1.23-5.25)	<0.05*
C allele frequency	0.60	0.47	1.69 (0.96-2.96)	0.089
TERT-rs2736098 (G>A)	*n* = 90	*n* = 100		
GG	38 (42.2)	15 (15)	1.00	
GA	45 (50)	73 (73)	0.24 (0.12-0.49)	<0.001*
AA	7 (7.8)	12 (12)	0.23 (0.08-0.69)	<0.05*
GA+AA	52 (57.8)	85 (85)	0.24 (0.12-0.48)	<0.001*
A allele frequency	0.33	0.48	0.53 (0.30-0.94)	<0.05*

for immunohistochemistry was performed in 10 areas on each slide at magnification of ×40. For nuclear cell counting, the labeling index was applied (14). In all cases, 1,000 nuclei were counted and the percentage of positive nuclei was assessed. For cytoplasmic staining, the number of positive cells was determined using a semi-quantitative method: negative staining (−), fewer than 5% of cells positive; focal expression (+), positive staining of 5-35% of the cells; moderate expression (++), 35-65% of cells positive; diffuse expression (+++), more than 65% of cells positive. Scoring for nuclear staining was: negative staining (−), fewer than 15% of cells positive; positive staining (+), 15% or more of the cells positive. In accordance with previous studies, the cut-off value for telomerase expression was established within the range 5-65%. The cut-off value used in our study was based on both previously published data and hTERT expression in our samples. For statistical analyses, cases with positive cytoplasmic and nuclear staining were combined together into one group and compared with cases showing no hTERT expression.

Statistical analyses

All statistical analyses were done using the Statistical Package for Social Science (SPSS version 17.0; SPSS Inc., Chicago, IL, USA). Mean, median, SD and range were used for description of data. Categorical variables were compared using the chi-squared test (χ^2). Non-parametric data were analyzed using the Kruskal-Wallis and Mann-Whitney tests. Univariate and multivariate logistic regression models were used for dichotomous variables, and univariate and multivariate linear regression models for numeric variables. Pairwise linkage disequilibrium (LD), haplotype frequencies and departure from a Hardy-Weinberg equilibrium were determined using the HAPLOVIEW software package, version 4.2 (Broad Institute of MIT and Harvard, Boston, MA, USA) (15). Haplotype blocks were defined using the "solid spine block" option. Differences at $P < 0.05$ were considered to be statistically significant. Survival analysis was performed using Kaplan-Meier curves.

Results

TERT-CLPTM1L polymorphisms

The genotype and allele frequencies of TERT and CLPTM1L polymorphisms and their associations with the risk of OSCC are summarized in Table 2. Logistic regression analyses and Fisher's exact test showed that CLPTM1L rs402710 polymorphism was significantly associated with an increased risk of OSCC (OR = 5.5, $P < 0.001$ for the TT genotype; OR = 2.16, $P < 0.01$ for the CT+TT genotypes combined; OR = 2.29, $P < 0.01$ for the T allele).

Also, analysis of TERT-rs2736100 polymorphism revealed that both the AC and CC genotypes were more prevalent in patients with OSCC (OR = 2.26, $P < 0.05$ for AC genotype; OR = 3.11, $P < 0.01$ for CC genotype) as well as the AC+CC genotypes combined (OR = 2.54, $P < 0.05$). On the other hand, the A allele of TERT rs2736098 polymorphism was associated with a significantly

Table 3 Association between RTL values and clinicopathologic features of OSCC

Variable	Patients	RTL Mean ± SD	RTL median (range)	$P^{\#}$
Gender				
Male	47 (78.3)	4.19 ± 3.4	3.39 (0.82-17.71)	0.262
Female	13 (21.7)	8.50 ± 12.47	3.22 (1.86-47.41)	
Age, years				
<50	7 (11.7)	2.54 ± 1.19	2.11 (1.63-4.76)	0.034*
50-70	38 (63.3)	4.28 ± 3.62	3.31 (0.82-17.71)	
>70	15 (25)	8.48 ± 11.50	5.43 (1.98-47.41)	
Tumor size				
<2 cm	17 (28.3)	4.26 ± 3.26	3.49 (0.82-15.45)	0.632
2-4 cm	26 (43.3)	6.37 ± 9.19	3.45 (1.34-47.41)	
>4 cm	2 (3.3)	4.12 ± 2.02	4.12 (2.69-5.54)	
Infiltrating	15 (5)	4.08 ± 4.02	2.29 (1.08-16.84)	
Histological grade				
G 1	35 (58.3)	5.36 ± 7.87	3.39 (1.53-47.41)	0.861
G 2	23 (38.3)	4.89 ± 4.62	3.22 (0.82-17.71)	
G 3	2 (3.3)	3.68 ± 1.31	3.68 (2.76-4.61)	
Tumor stage				
I	17 (28.3)	4.45 ± 3.32	3.49 (1.34-15.45)	0.166
II	19 (31.7)	7.62 ± 10.52	4.39 (1.63-47.41)	
III	6 (10)	5.71 ± 5.64	3.91 (1.37-16.84)	
IV	18 (30)	2.93 ± 1.65	2.34 (0.82-6.48)	
Metastasis				
Yes	22 (36.7)	3.13 ± 1.62	2.73 (0.82-6.48)	0.067
No	38 (37.3)	6.28 ± 8.04	3.59 (1.34-47.41)	

n = 60 (%)

#Man-Whitney test for Gender, and Kruskal-Wallis test for other variables.

decreased risk of OSCC (OR = 0.24, $P < 0.001$ for the GA genotype; OR = 0.23, $P < 0.05$ for the AA genotype; OR = 0.24, $P < 0.001$ for the GA+AA genotypes; OR = 0.53, $P < 0.05$ for the A allele).

Before analyzing the associations between the TERT-CLPTM1L polymorphisms and OSCC risk, the Hardy-Weinberg equilibrium of the SNPs was tested in both the case and the control groups. The rs2736098 SNP was in Hardy-Weinberg disequilibrium in the control group, so these results should be taken with consideration Neither of the analyzed SNPs was found to be in linkage disequilibrium (LD), and the frequencies of the identified haplotypes did not show any significant difference in distribution between the patients and the controls (data not shown). Also, TERT-CLPTM1L polymorphisms were not significantly associated with clinicopathologic features of OSCC such as tumor size, histological grade, tumor stage and metastasis (data not shown).

Relative telomere length

Statistical analysis demonstrated no differences in RTL values for tumor samples between males and females ($P = 0.262$, Mann-Whitney test), but significantly higher values were found in the oldest age group relative to the youngest ($P = 0.034$, Kruskal-Wallis test; $P = 0.012$, Mann-Whitney test). Although there was no significant difference between tumor RTL and the clinicopathologic features of OSCC, a trend towards longer telomeres was present in earlier-stage tumors, smaller tumors, tumors with a lower histological grade, and tumors without metastasis. Interestingly, with regard to the dynamics of changes in telomere length, an increase in the mean RTL value from stage I to II was followed by a decrease from stage II to IV. The results are summarized in Table 3.

A univariate linear regression model was used to analyze the association of different variables (age, sex, tumor size, histological grade, tumor stage, metastasis, TERT-CLPTM1L genotype, telomerase expression and overall survival) with tumor RTL. After age and the presence of metastases had been defined as independent variables associated with telomere length, they were entered into the multivariate linear regression model and found to be predictors of telomere length in the analyzed tumors (R square = 0.164, $P = 0.021$, 95% CI = 0.069-0.819).

hTERT expression in OSCC

Among 34 OSCC cases that were immunostained for hTERT, 25 (74%) showed either a nuclear (6 out of 25, 24%) or cytoplasmic (19 out of 25, 76%) staining

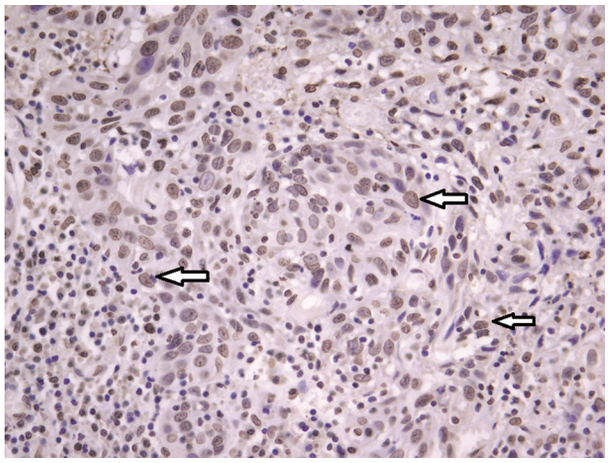

Fig. 1 Positive nuclear staining for hTERT in OSCC. Arrows point to brown-stained nuclei (original magnification ×40).

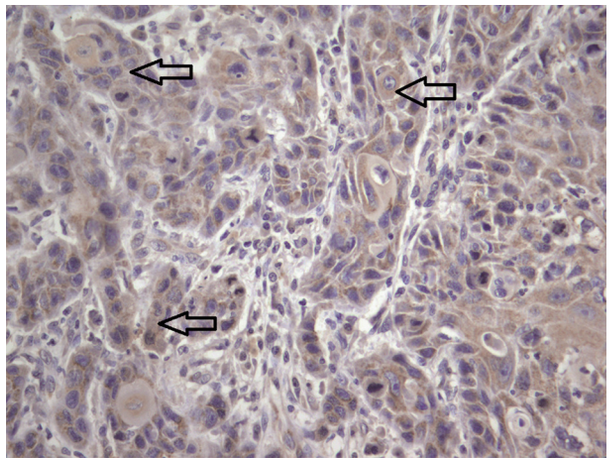

Fig. 2 Diffuse cytoplasmic staining for hTERT in OSCC. Arrows point to brown-stained cytoplasm (original magnification ×40).

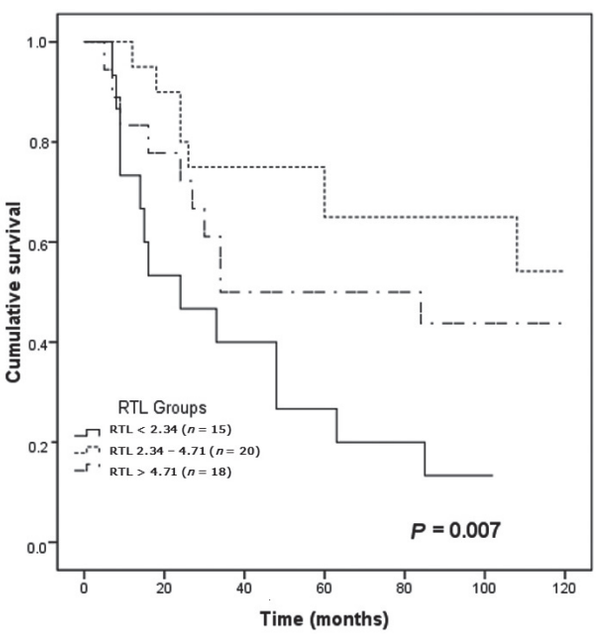

Fig. 3 Survival analysis of patients with different telomere lengths (short <2.34, n = 15; medium 2.34-4.71, n = 20; long >4.71, n = 18) using Kaplan-Meier with log-rank test.

pattern (Figs. 1, 2). The mean RTL value was higher (5.37 ± 4.91) in cases with positive hTERT expression (either cytoplasmic or nuclear) than in cases without any hTERT expression (4.02 ± 2.07), but the difference was not statistically significant (P = 0.73, Mann-Whitney test). Also, there was no significant difference between either nuclear or cytoplasmic hTERT expression and clinicopathologic parameters (data not shown).

The frequency of hTERT-positive cases was higher in the smaller tumor size groups (<2 cm and 2-4 cm) than in advanced lesions (data not shown), perhaps reflecting the increased RTL values we found for smaller oral tumors. The univariate logistic regression model was used to determine predictors of telomerase expression, and among all of the variables mentioned above, only tumor size was found to be a predictor of hTERT expression (P = 0.049, OR = 2.50, 95% CI = 1.00-6.26).

No significant difference was found between hTERT expression, RTL values and the analyzed polymorphisms at TERT-CLPTM1L loci, although trends for higher tumor RTL values and positive hTERT expression were independently demonstrated in less advanced OSCCs.

Survival analysis

Survival analysis was performed using Kaplan-Meier curves and the log-rank test. The duration of survival was measured from the beginning of treatment until the time of death, and data were obtained for 53 cases of OSCC. No significant difference was evident between patients with various genotypes (P = 0.384 for rs402710, P = 0.802 for rs2736100 and P = 0.353 for rs2736098) or hTERT expression (P = 0.408). For Kaplan-Meier analysis of overall survival according to differences in telomere length, patients were divided into tertiles based on their RTL values (short <2.34 (n = 15), medium 2.34-4.71 (n = 20) and long >4.71 (n = 18) telomeres). The analysis revealed that patients with smaller tumor RTL values had significantly shorter overall survival than patients with higher RTL values (Fig. 3). These data appear to provide additional proof of the relationship between shorter telomeres and advanced tumor stage in OSCC.

Discussion

In the present study we investigated the contribution of TERT-CLPTM1L polymorphisms, hTERT expression

and telomere length to the pathogenesis of OSCC.

As noted in the literature, SNPs at the 15p15.33 locus, containing the TERT and CLPTM1L genes, have been associated with the risk of multiple tumors (10), but the results have been somewhat conflicting. One of the most investigated polymorphisms in the gene encoding the key telomerase subunit is TERT rs2736100. Our present study is the first to have investigated TERT rs2736100 and the risk of OSCC, and demonstrated a significantly increased risk in patients with the AC and/or CC genotypes relative to AA carriers. Similarly, numerous studies have demonstrated the same mode of association between rs2736100 and lung cancer (16), gliomas (17), and cancers of the bladder (18), cervix (19), colorectum (20), and testis (21). However, reduced risk of cancer was present in urothelial (22) carcinomas as well as lack of association in breast (23) tumors.

In contrast, the second polymorphism analyzed in our study, TERT rs2736098, was significantly associated with a reduced risk of OSCC. In fact it has been reported that the GA and/or AA genotypes of rs2736098 are associated with a decreased risk of head and neck (9) and breast (24,25) tumors. However, rs2736098 has been mostly associated with an increased risk for a variety of malignancies especially lung cancer (26). On the other hand, studies related to colorectal (27) and esophageal (28) tumors have demonstrated no relationship between TERT-rs2736098 and disease incidence.

To our knowledge, the present study is the first to have provided data regarding CLPTM1L rs402710 and OSCC risk, and to have revealed a significantly higher risk for carriers of the TT and CT+TT genotypes combined. Although similar findings, mostly for lung tumors (29), have been confirmed, some investigations have demonstrated an opposite association (19,30). Taking into account the involvement of CLPTM1L in apoptosis, there is a possibility that, due to exposure to genotoxic stress, genotypes with the T allele might be responsible for a less efficient apoptotic response, consequently increasing the probability of tumor formation.

The partly conflicting results obtained so far could perhaps be explained by factors such as sample size, differences in the types of tumors studied, differences in the ethnicity and genetic background of the study subjects, environmental factors, and involvement of other genetic and epigenetic mechanisms in the process of carcinogenesis. Although a recent study has provided the first evidence of a tumor-related SNP in the TERT-CLPTM1L locus that encodes an alternative splice variant of hTERT yielding an inactive telomerase complex (31), the exact mechanism whereby SNPs influence tumor risk has not yet been completely explained. SNPs are usually chosen not on the basis of biological function, but to provide maximal coverage of other variations in specific regions of the genome. It is therefore unlikely that the causal locus would show the strongest association with certain tumors (32). The previous observations mentioned above might possibly explain the lack of any significant association between the three common SNPs in the TERT-CLPTM1L locus and the clinicopathologic features of OSCCs, telomerase expression or telomere length in our present study. The extent to which expression of the TERT gene can be influenced by a specific polymorphism remains an unresolved issue.

Telomere length in patients with malignant disorders has been extensively studied, not only in tumor cells, but also peripheral blood and normal tissue surrounding the tumor. Most of those studies have shown that telomeres are shorter in tumors than in normal tissues (33-35). However, it can be argued that the genomic stability of a tumor is mostly determined by the length of telomeres in the tumor itself, rather than the tumor: normal tissue telomere length ratio (36). Although our results demonstrated no statistically significant correlation with any clinicopathologic features of OSCCs, longer telomeres in tumors could be considered as an early prognostic marker, since they were present in smaller tumors, tumors with lower stages and histological grades, and tumors without metastases. This finding was supported by the significantly better overall survival of patients with long and medium-length tumor telomeres. Among various clinicopathologic parameters, the relationship of metastasis to telomere length might offer significant evidence of prognostic usefulness, as this was demonstrated in our multivariate linear regression model. These data are in contrast to the general model of the role played by telomeres in tumor development, i.e. telomere shortening as an early event in carcinogenesis (34,37). However, our results are consistent with several previous studies in which longer telomeres have been associated with absence of metastases in OSCC (35), a low grade in intracranial tumors (38), higher overall survival in lung cancer patients (36), and a lower risk of death in patients with prostate tumors (39). Moreover, a lack of correlation between telomere length and clinicopathologic characteristics has also been found in breast (40), colorectal (41), and head and neck (42) tumors.

Telomerase expression, as the main mechanism of telomere maintenance, is upregulated in most malignancies, including OSCC, but the association between clinicopathologic features and telomere length has been less convincing. Although we found no significant associa-

tions among telomerase expression, telomere length and tumor characteristics, our findings revealed a tendency for positive telomerase expression and longer telomeres in less advanced OSCCs, and thus to a certain extent demonstrated the influence of telomerase on telomere maintenance. Moreover, our logistic regression models demonstrated the prognostic significance of tumor size in relation to positive TERT expression. Similarly, Patel et al. found no correlation between telomerase activation and clinicopathologic parameters in head and neck cancer (42), while Pannone et al. reported higher telomerase activity in OSCC than in normal tissue, although this had no clinicopathologic impact (43). The high incidence of hTERT-positive cases in the present study is consistent with other reports (44,45), and the high expression of hTERT in smaller tumors might be indicative of early telomerase activation. Similarly, Chen at al. have concluded that telomerase activation is an early event in OSCC, and have suggested that its degree of expression could be a predictor of cancer progression, recurrence and prognosis (46). Also, Abrahao et al. have reported intense hTERT expression in hyperplastic and dysplastic oral epithelium, and suggested that this could be regarded as additional proof of its involvement in early-stage tumors (44). In line with previous data, several authors have linked the proliferation rate of early-stage cancers to telomerase activation, whereas at later stages, due to attainment of a critical tumor size, down-regulation of hTERT might occur (47,48).

Therefore, the importance of telomere maintenance through telomerase activation during tumor development appears to be supported by our results indicating higher RTL values in TERT-positive cases relative to negative cases. Similarly, a strong association between longer telomeres and higher telomerase activity has been found in hepatocellular carcinoma (49). However, one recent study has noted that the level of telomerase expression was significantly higher in cancers at an advanced stage and those with metastasis, although no such association was demonstrated for telomere length (50). In addition, one comprehensive study of esophageal SCC found no significant difference in telomere length between telomerase-positive and -negative samples (51).

It is clear that since there is not always an obvious correlation between telomere length and telomerase activity in cancer, telomere maintenance has to be also influenced by many additional factors, such as replication rate, regulatory proteins, ALT mechanisms, epigenetics and environmental factors (52). Therefore, when assessing conflicts in the results from different studies, these influences have to be taken into account.

Our research appears to provide strong evidence for the association of CLPTM1L rs402710 (C/T) and TERT rs2736100 (A/C) SNPs with OSSC risk, and suggests that higher RTL values and positive hTERT expression may have potential utility as early prognostic markers.

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Conflict of interest

The authors have no conflicts of interest to declare.

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