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hTERT C250T promoter mutation and telomere length as a molecular markers of cancer progression in patients with head and neck cancer

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Abstract. Squamous cell carcinoma of the head and neck (HNSCC) is the sixth leading cause of cancer worldwide, representing over half a million incidents every year. Cancer cells, including HNSCC, are characterized by increased telomerase activity. This enzymatic complex is active in ~90% of all cancer types and is responsible for the lengthening of telomeres. Highly recurrent point mutations in the human telomerase reverse transcriptase (hTERT) promoter have recently been reported in a number of human neoplasms. The aim of the present study was to analyze the prevalence of the hTERT promoter C250T mutation and telomere length in the blood leukocytes of 61 patients with HNSCC and 49 healthy individuals. Quantitative polymerase chain reaction identified the hTERT promoter mutation in 36% of patients with HNSCC. To the best of our knowledge this is first report indicating the presence of shorter telomeres in early stage tumors. In addition, the results suggest that the C250T hTERT promoter mutation and telomere length assessment may serve as important molecular markers of HNSCC progression.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, representing over half a million incidents every year (1,2). Epidemiological studies have revealed an association between exposure to carcinogens (such as tobacco and alcohol), infection by oncogenic human papillomavirus types 16 and 18 (HPV16 and HPV18, respectively) and the increased risk of HNSCC development (3). Currently, the treatment of HNSCC consists of surgery followed by postoperative chemo- and/or radiotherapy (4,5). However, the 5-year mortality rate of patients with HNSCC has not improved, despite the advanced treatment methods (6).

Cancer cells, including HNSCC, are characterized by increased telomerase activity. This enzymatic complex is active in ~80 to 90% of all cancer types and is responsible for the lengthening of telomeres (7). Telomeres, as highly specialized nucleoproteins located at the end of chromosomes, provide genomic stability, integrity and immortalization of cells. The human telomeric sequence is composed of hexamer repeats (5'-TTAGGG-3') at the 3' end strand. Human telomeres have ~500 to 2,000 copies of hexamer repetitions, giving rise to 3,000 to 12,000 base pairs (8-11).

Telomerase activity is controlled by a number of mechanisms, including alternative splicing, posttranslational modifications and activating or inhibiting factors. Thus, the transcriptional regulation of the human telomerase reverse transcriptase (hTERT) gene, followed by the regulation of telomerase activity, is crucial (12). Highly recurrent point mutations in the hTERT promoter have been reported in a number of human malignancies such as melanoma and glioma. This mutation is located at -146 bp (C250T) from the ATG start site of the hTERT gene (13). This mutation generates *de novo* E-Twenty six (ETS)/ternary complex factors

transcription-binding sites. This process results in increased hTERT expression and it has been proposed as a novel mechanism of activating telomerase in malignant cells (13,14). A previous study investigating telomerase and telomere length, indicated that their regulation may be controlled by factors released during carcinogenesis such as hormones and cytokines, including adiponectin and interleukin (IL)-6 (15). These changes, if they are present in the human organism even in the local area (cancer initiation at a single cell level), may be reflected by alterations in the whole organism including leukocytes (16).

Considering the results of our previous studies (16), it was hypothesized that short telomere length and a hTERT mutation in leukocytes may be evaluated as markers of prognosis and tumors presenting at a very early stage of carcinogenesis. Thus, telomere length measurements in leukocytes may be an effective, non-invasive method for the predictive assessment of carcinogenesis. Therefore, the present study focused on the potential application of telomere length and hTERT mutation analysis as prognostic parameters of cancer progression in HNSCC.

Materials and methods

Patients. The study group consisted of 46 patients (31 males and 15 females; age 21-88 years, median=67, mean=63) who were histologically diagnosed with HNSCC at various stages, and different anatomical sites based on World Health Organization criteria (Table I). All patients were recruited between April 2015 and June 2016 from the Department of Head and Neck Surgery at the Poznan University of Medical Sciences and The Greater Poland Cancer Centre in Poznan, Poland. The control group comprised of 49 healthy blood donors from Poznan Regional Blood Center. The patients were Caucasians and the majority were from the same region of Poland (Greater Poland). The study protocol was approved by the Ethics Committee of the Poznan University of Medical Sciences (Decision no. 446/15) and written informed consent was provided by the participating individuals.

Table I. Characteristics of the present study's cohort.

Characteristic	Total number (n)
Healthy individuals	49
TERT promoter status [n(%)]	
T/T	13 (26.5)
C/T	29 (59.2)
C/C	7 (14.3)
Cancer patients	61
Age (years)	
Median	63
Mean	62
Range	37 to 88
Sex [n(%)]	
Male	49 (80.3)
Female	12 (19.7)
Tumor stage (TNM classification) [n(%)]	
T1	11 (18.0)
T2	22 (36.1)
T3	17 (27.9)
T4	11 (18.0)
Anatomical site (n)	
Mouth (including lip)	25
Voice box (larynx)	25
Nose and sinuses	5
Throat (pharynx)	6
TERT promoter status [n(%)]	
T/T	23 (37.7)
C/T	16 (26.2)[a]
C/C	22 (36.1)[b]
Total study cohort (n)	110

TERT, telomerase reverse transcriptase; C/C, homozygous for wild-type C allele; C/T, heterozygous C/T allele; T/T, homozygous for mutant T allele; [a]P<0.0001 vs. healthy group; [b]P<0.0001 vs. healthy group.

Exclusion criteria. Following the study protocol, patients with local recurrences, second primary tumors and were HPV positive were excluded from experimental groups. Patients with a previous history of chemo- or radiotherapy were also excluded.

DNA isolation. DNA was extracted from 300 μl peripheral blood mononuclear cells (PBMCs) using a Wizard Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA) according to manufacturer's protocol. DNA was then stored at -20˚C until analysis.

Quantitative polymerase chain reaction (qPCR)

Assessment of relative telomere length. Telomere length was assessed using 2 pairs of primers, one telomere-specific and one a single copy gene-specific (albumin) (Table II), as previously described (16-18). Briefly, specific primers with an efficiency close to 100% (98±2%), were used in a SYBR Green-based assay with FastStart Essential SYBR Green I Master (Roche Applied Science, Penzberg, Germany). Initial denaturation and polymerase activation (hot start) was performed at 95˚C for 10 min. The signal was detected during 45 cycles of 95˚C for 10 sec, 61˚C for 10 sec and 72˚C for 10 sec. Melting analysis (65 to 95˚C; 0.2˚C resolution) was performed at the end of the reaction to verify specificity of the product. Telomere length was assessed using a qPCR system (LC 96; Roche Diagnostics, Basel, Switzerland), and calculated using the $2^{-\Delta\Delta Cq}$ method (19).

High resolution melting (HRM) mutation analysis. To identify the C250T hTERT promoter mutation, HRM analysis using specific primers was performed (sequences listed in Table II). The reaction was optimized to 10 ng genomic DNA, 0.8 μM of each primer, 2.5 mM magnesium chloride and 5 μl of LightCycler 480 High Resolution Melting Master Mix (2X; Roche Applied Science, Penzberg, Germany), in a total volume of 10 μl. The initial denaturation and polymerase activation (hot start) was performed at 95˚C for 10 min. The signal was detected during another 45 cycles of 95˚C for

Table II. Primer sequences for quantitative polymerase chain reaction.

Primer name	Sequence (5'-3')	Amplicon length (base pairs)
ALB_F	TTTGCAGATGTCAGTGAAAGAGA	69
ALB_R	TGGGGAGGCTATAGAAAATAAGG	
Telo_F	ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT	91
Telo_R	GTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA	
TERT_HRM_F	CTGCCCCTTCACCTTCCAG	159
TERT_HRM_R	AGCGCTGCCTGAAACTCG	

ALB, albumin; Telo, telomere; TERT, telomerase reverse transcriptase; HRM, high resolution melting; F, forward; R, reverse.

10 sec, 57˚C for 15 sec and 72˚C for 10 sec. HRM analysis (65 to 95˚C; 0.07˚C resolution) at the end of the reaction was performed to identify different variants of the hTERT mutation. The HRM analysis was performed using a qPCR system (LC 96; Roche Diagnostics, Basel Switzerland).

Sequencing. A total of 10% of samples were verified by sequencing using the BigDye v3.1 kit according to the manufacturer's instructions (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and separation by ethanol extraction using the ABI Prism 3130XL (Applied Biosystems; Thermo Fisher Scientific, Inc.) in a 36 cm capillary and a POP7 polymer.

Statistical analysis. Statistical analysis was performed using Student's t-test, two-way analysis of variance, χ^2 and Fisher's exact tests, which were calculated using GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA). Data is presented as the mean ± standard error of the mean. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Telomere length assessment. The average relative telomere length (AU) in the cancer and control groups (14.06±2.109 and 19.06±1.801, respectively) was evaluated, and no significant difference was observed (P=0.787; Fig. 1A). Telomere length in leukocytes in relation to tumor invasion according to the TNM Classification of Malignant Tumors (TNM) was also determined. Telomeres in leukocytes from individuals with T2 HNSCC cancer were significantly shorter when compared with those of healthy individuals (6.329±1.864 and 19.06±1.801, respectively; P=0.0001; Fig. 1B). There was also a significant difference in telomere length between T2 and T3 patients (6.329±1.864 and 16.94±3.301, respectively; P=0.0063; Fig. 1B), and T2 and T4 patients (6.329±1.864 and 26.3±7.615, respectively; P=0.0028; Fig. 1B). In addition, there was trend towards shortened telomeres in the first two tumor invasion stages (P=0.0003; Fig. 1B).

The analysis of telomere lengths in different anatomical sites of patients with HNSCC demonstrated that there was a significant difference between healthy individuals and patients affected by mouth cancer (19.06±1.801 and 10.76±2.365, respectively; P=0.0083; Fig. 1C) and pharyngeal cancer (19.06±1.801 and 3.985±1.03, respectively; P=0.0063; Fig. 1C).

TERT promoter C250T mutation assessment-TNM classification. The hTERT promoter mutation was identified in 36% of the patients with HNSCC, while 27% of healthy individuals also carried the mutation (Table I and Fig. 2A). However, the heterozygous variant was observed in 26% of the HNSCC group and 59% of the control group (P<0.0001; Table I and Fig. 2A). Notably, when compared with the control group (14%), 38% of the cancer types had the C wild-type allele (P≤0.0001; Table I and Fig. 2A).

The analysis of the C250T mutations indicated that there is a significant association between the frequency of the homozygous mutation and the grade of the tumor (T1=27%; T2=36%; T3=35%; T4=46%; P≤0.0001; Fig. 2B).

An opposite trend was identified in the case of the wild-type allele as there was a decreasing frequency of the wild type C allele with increasing tumor advancement (P≤0.0001), and a significantly higher frequency of the wild type allele in T1=55%, T2=36%, T3=35% and T4=27% when compared with the control group (14%; P≤0.0001; Fig. 2B).

TERT promoter C250T mutation assessment-anatomical sites. The C250T mutation was identified in 40% of patients with mouth cancer (P=0.001; Fig. 2C) and in 20% of patients with nose and sinuses cancer (P=0.0018; Fig. 2C), compared with 27% of healthy individuals. In addition, a nonsignificant trend for increased incidence of the C250T mutation was observed in pharyngeal cancer (50%) and laryngeal cancer (32%) compared with healthy individuals (Fig. 2C).

Differences in C allele status were also revealed. In the control group, 14% of samples contained the wild type homozygous allele (Fig. 2C); by comparison, 44% of mouth cancer (P=0.001), 80% of nose and sinuses cancer (P=0.0018), 33% of pharyngeal cancer and 24% of laryngeal cancer were homozygous for the wild type allele (Fig. 2C).

Comparisons between C250T variant and telomere length revealed no significant differences between patients with HNSCC and the healthy control group (Fig. 3A). However, it was revealed that telomeres were significantly shorter in patients with hTERT-wild and heterozygous mouth cancer than in the control group (P=0.0401 and P=0.0252, respectively; Fig. 3B). Significantly shorter telomeres were also

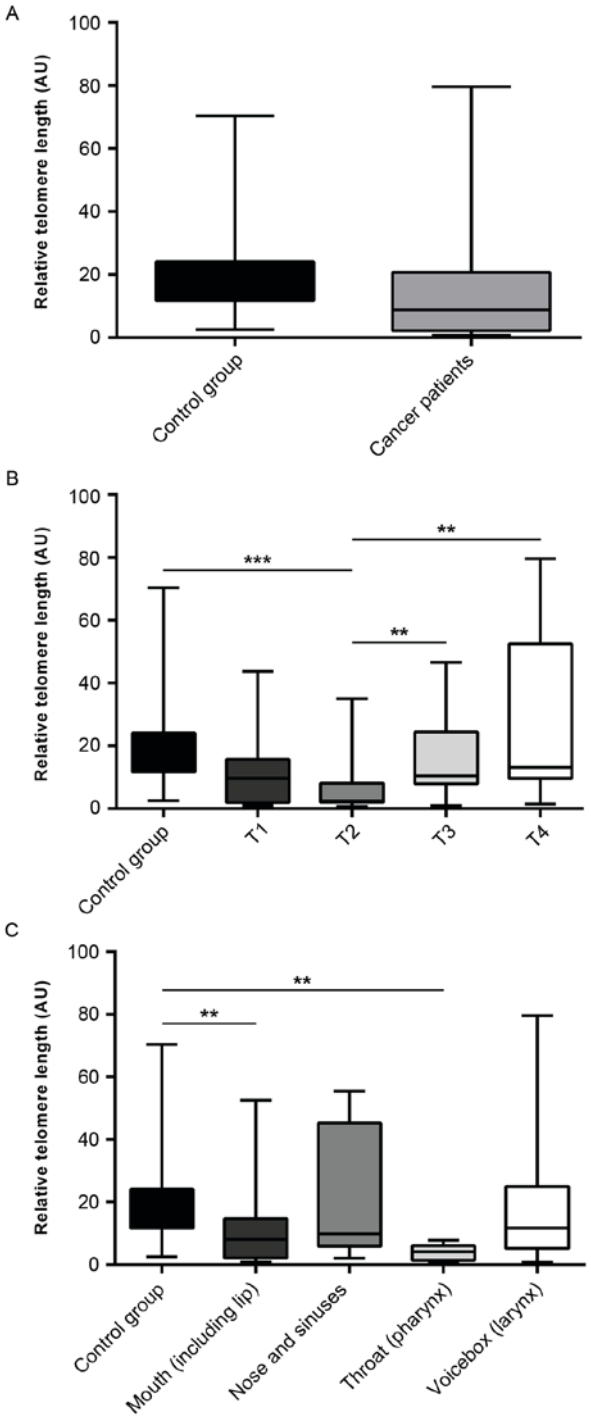

Figure 1. Quantitative analysis of relative telomere length (AU) in peripheral blood leucocytes. (A) Relative telomere length in the study (patients with HNSCC) and control (healthy individuals) groups. (B) Comparisons of relative telomere length in peripheral blood leucocytes in relation to TNM classification. (C) Comparisons of relative telomere length in peripheral blood leucocytes in relation to the anatomical sites of HNSCCs. Data is presented as the mean ± standard error of the mean. **P<0.01 and ***P<0.001, with comparisons indicated by lines. HNSCC, head and neck squamous cell carcinoma; TNM, TNM Classification of Malignant Tumors.

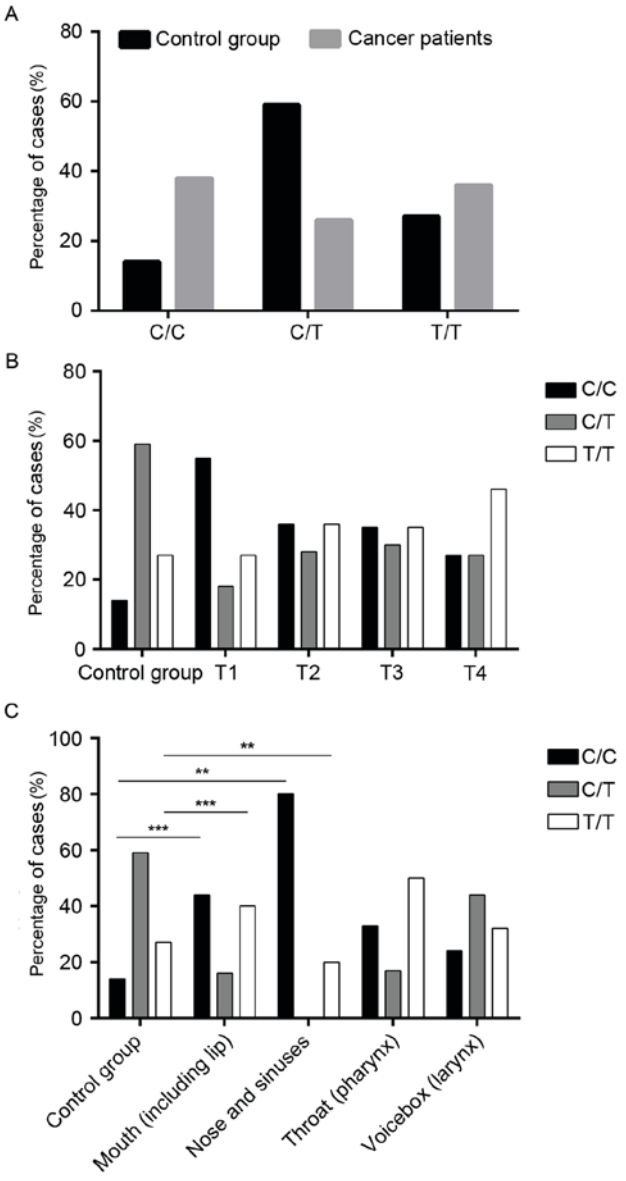

Figure 2. Frequency of C250T TERT promoter mutation in patients with HNSCC. (A) Frequency of TERT promoter mutations in the study (patients with HNSCC) and control (healthy individuals) groups. Statistical analysis of results count by χ^2 test. P≤0.0001; χ^2=25,17; df=2. (B) Comparisons of TERT promoter mutation frequencies in peripheral blood leucocytes in relation to TNM classification. Statistical analysis of results count by χ^2 test P≤0.0001; χ^2=63,87; df=8. (C) Comparisons of TERT promoter mutation frequencies in peripheral blood leucocytes in relation to the anatomical sites of HNSCCs. **P<0.01 and ***P<0.001, with comparisons indicated by lines. TERT, telomerase reverse transcriptase; HNSCC, head and neck squamous cell carcinoma; C/C, homozygous for wild-type C allele; C/T, heterozygous C/T allele; T/T, homozygous for mutant T allele; TNM, TNM Classification of Malignant Tumors.

observed in hTERT-wild (P=0.026) pharyngeal cancer. The results exhibited a trend suggesting that hTERT-mutant laryngeal tumors have longer telomeres than the control group, however the differences were not significant (28.861±8.198 and 17.849±3.115, respectively; P=0.195; Fig 3B).

Discussion

In the present study, the frequency of the C250T hTERT promoter mutation in patients with HNSCC, identification of the mutation associated with telomere length and the association between cancer advancement and telomere length and mutation status was determined.

There have been a number of studies that have correlated telomere length in leukocytes and cancer risk, however only

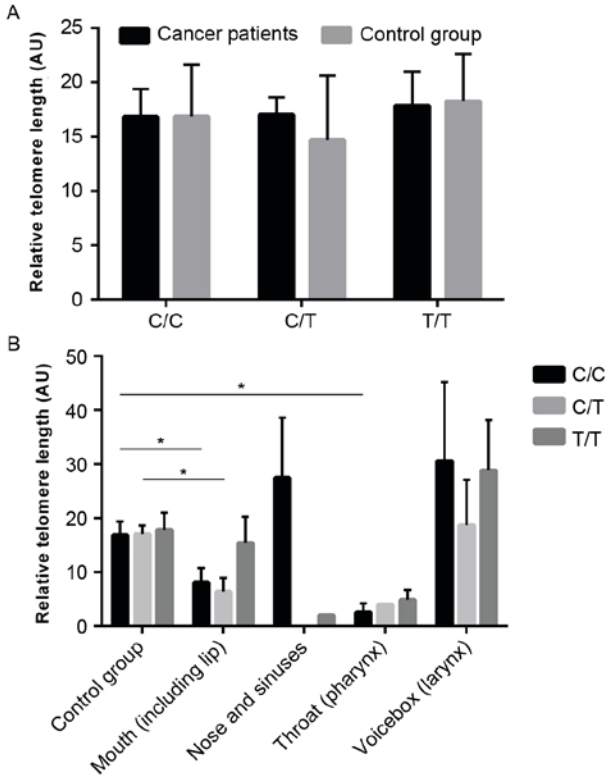

Figure 3. Assessment of relative telomere length (AU) in peripheral blood leucocytes relative to TERT promoter mutation (C250T) status. (A) Relative telomere length in the study (patients with HNSCC) and control (healthy individuals) groups. (B) Comparisons of relative telomere length in peripheral leucocytes in relation to the anatomical sites of HNSCCs. Data is presented as the mean ± standard error of the mean. *P<0.05, with comparisons indicated by lines. TERT, telomerase reverse transcriptase; HNSCC, head and neck squamous cell carcinoma; C/C, homozygous for wild-type C allele; C/T, heterozygous C/T allele; T/T, homozygous for mutant T allele.

a few focused on head and neck cancer. Zhang *et al* (20) demonstrated that short telomere length in PBMCs is strongly associated with a moderately increased risk of oropharyngeal squamous cell carcinoma however, not with increased risk of oral cavity cancer. Associations between telomere length and a higher risk of HPV16-positive oropharyngeal carcinoma and tumor HPV16 status were also revealed (20). Bau *et al* (21) observed that short leukocyte telomere length was associated with an increased risk of developing oral premalignant lesions and precursors of oral squamous cell carcinoma. However, the opposite was revealed by Liu *et al* (22) who reported that relative telomere length may not be important in HNSCC carcinogenesis. A correlation between mouth cancer and pharyngeal cancer, and significantly shorter telomeres when compared to the control group was identified in the present study. Similar results were obtained by Bau *et al* (21) and Zhang *et al* (20). These studies demonstrated that short telomeres were a consistent feature of oral and pharyngeal cancer. Notably, the present study demonstrated that leukocytes from individuals with early stage T2 HNSCC tumors are characterized by shorter telomeres than leukocytes from healthy donors. The expected increase in the length of telomeres was observed in leukocytes from T3 and T4 patients when compared with T2 individuals. There is evidence that suggests that the advancement of the tumors may correlate with long telomeres and poor prognosis (23). Increased telomere length indicates that telomere maintenance may also be important for the progression of HNSCC.

The presence of the C250T hTERT promoter mutation results in increased hTERT expression, for which a novel mechanism of activating telomerase in malignant cells has been proposed (24). Only a few studies have described the correlation between hTERT mutation status and the risk of HNSCC. In the present study, hTERT promoter mutations were identified in 36% of patients with HNSCC. Similar results have been obtained by Vinothkumar *et al* (25). The correlation between two hot spot mutations and the patient's clinicopathological phenotype was analyzed by the authors and a relatively high frequency of hTERT hot spot mutations in oral squamous cell carcinoma was identified. Qu *et al* (26) observed hTERT promoter mutations in a large group of laryngeal cancers. A strong association between these mutations and poor survival rates in patients with laryngeal cancer was demonstrated as an independently variable with respect to tumor localization or tumor invasion. An increased frequency of hTERT mutations in laryngeal and pharyngeal cancers was also established in the present study. A significantly higher frequency of the hTERT mutation was revealed in patients with mouth cancer. Notably, the opposite result was observed in nose and sinuses cancer. In addition, a significantly higher frequency of the hTERT mutation was observed in more advance tumors with increasing tendency. The results highlight the potential influence of the hTERT promoter mutation in the progression of HNSCC. Notably, trends towards the decreasing frequency of wild allele with increasing tumor advancement, and a significantly higher frequency of C allele in T1, T2 and T3 tumors when compared to the control group were also described.

By contrast with the present study, the majority of previous studies in the head and neck area were focused on thyroid cancer. Vingare *et al* (24) demonstrated that the hTERT promoter mutations are relatively frequent in specific types of human cancers including thyroid cancer. Liu *et al* (27) also revealed that hTERT promoter mutations are common, particularly in follicular thyroid cancer and BRAF mutation-positive papillary thyroid cancer, and are associated with aggressive clinicopathological phenotypes. In addition, the mutation in the hTERT promoter was associated with decreased survival in patients with papillary thyroid carcinoma (28), and the hTERT promoter mutation is particularly prevalent in aggressive thyroid cancers (29). Notably, the present study revealed a trend with an increased frequency of the wild allele in mouth, nose and sinuses, pharyngeal and laryngeal cancers when compared to healthy individuals.

In addition, the results revealed significantly shorter telomeres in hTERT-wild and heterozygous mouth cancer, and in hTERT-wild pharyngeal cancer, when compared with the control group. To the best of our knowledge this association has not previously been identified in HNSCC. These results indicate that the hTERT promoter mutation may increase or decrease telomere length depending on the tumor localization and advancement of cancer.

In conclusion, the results demonstrated that the C250T hTERT promoter mutation may represent a common event during carcinogenesis in patients with HNSCC, which together with telomere length assessment may be one of the molecular markers of HNSCC progression. The present study

also revealed an association between short telomeres and the presence of the C250T mutation, which depends on the anatomical site of cancer and tumor invasion status. However, long or short telomeres in the PBMCs of patients with HNSCC do not necessarily indicate the presence or absence of the hTERT mutation, therefore the two parameters should be considered to characterize patient status. The present study has highlighted that there should be a greater emphasis on using the length of telomeres in PBMCs as biomarkers of HNSCC. Future studies should be performed with long-term follow ups and larger study groups to determine the clinical importance of C250T hTERT promoter mutation status in the absence and/or presence of short telomeres.

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