Telomere shortening in mucosa surrounding the tumor: Biosensor of field cancerization and prognostic marker of mucosal failure in head and neck squamous cell carcinoma

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Objectives: The aim of the present study was to investigate the pattern of telomere length and telomerase expression in cancer tissues and the surrounding mucosa (SM), as markers of field cancerization and clinical outcome in patients successfully treated for head and neck squamous cell carcinoma (HNSCC).

Materials and methods: This investigation was a prospective cohort study. Telomere length and levels of telomerase reverse transcriptase (TERT) transcripts were quantified by real-time PCR in cancer tissues and SM from 139 and 90 patients with HNSCC, respectively.

Results: No correlation was found between age and telomere length in SM. Patients with short telomeres in SM had a higher risk of mucosal failure (adjusted HR = 4.29). Patients with high TERT levels in cancer tissues had a higher risk of regional failure (HR = 2.88), distant failure (HR = 7.27), worse disease-specific survival (HR for related death = 2.62) but not mucosal failure. High-risk patients having both short telomeres in SM and high levels of TERT in cancer showed a significantly lower overall survival (HR = 2.46).

Conclusions: Overall these findings suggest that telomere shortening in SM is a marker of field cancerization and may precede reactivation of TERT. Short telomeres in SM are strongly prognostic of mucosal failure, whereas TERT levels in cancer tissues increase with the aggressiveness of the disease and are prognostic of tumor spread.

Introduction

Head and neck cancer was estimated to be the sixth most common malignancy in Europe in 2012, affecting approximately 140,000 new patients and resulting in nearly 65,000 deaths [1]. About 90% of head and neck cancers are squamous cell carcinomas (HNSCC), which develop in the epithelial lining of the upper aero-digestive tract (UADT) after exposure to tobacco, alcohol, or persistent oral infection by human papillomavirus (HPV) [2].

Despite improvements in functional outcomes due to developments in the field of radiotherapy, chemotherapy, surgical and imaging techniques, with the exception of the oral cavity and tonsillar cancers, survival has only marginally improved over the past two decades [3].

Patients with HNSCC who received successful curative treatment are in fact reported to have high propensity for mucosal failure [2,4]. Intensive investigations have demonstrated that mucosal failure may result from either outgrowth of histopathologically undetectable residual tumor cells or development of a second field tumor (SFT) following crucial genetic hits occurring in a pre-neoplastic field which may persist after treatment of both surgical and non-surgical treatment of the primary tumor [5–9]. For accurate risk assessment, disease monitoring and early
diagnosis, it is of particular interest to identify molecular markers of pre-neoplastic fields in tumors surrounding epithelium.

Telomeres are highly repetitive G-rich DNA sequences located at the end of chromosomes maintained by telomerase, a ribonucleoprotein complex containing an internal RNA component [telomerase RNA (TR)] and a catalytic protein with telomerase-specific reverse transcriptase activity [telomerase reverse transcriptase (TERT)]. TERT, which synthesizes de novo telomere sequences by using TR as a template, is the rate-limiting component of the telomerase complex, and its expression is correlated with telomerase activity [10]. The telomere/telomerase complex is a key element in determining the replicative potential of cells. On one hand, maintenance of telomere length by telomerase is critical for preserving the replicative potential of cancer cells; on the other, telomere erosion may impair their function in protecting chromosome ends, resulting in genetic instability, a crucial step in the initiation of carcinogenesis [11]. Many studies, including ours on colorectal carcinomas [12,13], have demonstrated that neoplastic cells generally have shorter telomeres than their adjacent non-tumoral counterparts, suggesting that telomere attrition precedes the reactivation of telomerase. As telomere attrition is considered to be an early event in human carcinogenesis, and as telomerase activation is crucial for tumor formation [13–17], studying them as potential markers of field cancerization and disease outcome appears to be important.

Previous studies on HNSCC have shown that telomere attrition [18–25] and telomerase activation [18,20,22,26–35] are frequent events in this malignancy. However, several studies have analyzed only small numbers of patients, few studies have evaluated both telomere length and telomerase activation in the same samples [18–22,34], and only two research groups have examined matched tumoral and adjacent tissues [22,34]. Few studies have evaluated the relationship between telomere length/telomerase activation and clinical outcomes [20,31–33], and no studies have analyzed the significance of telomere and telomerase as markers of field cancerization in HNSCC.

The aim of the present study was to investigate the pattern of telomere length and telomerase expression in cancer tissues and the surrounding mucosa (SM) as markers of field cancerization and clinical outcome in patients successfully treated for HNSCC.

Materials and methods

Patients

In total, 139 untreated consecutive patients with histologically confirmed SCC of the UADT were included in this prospective cohort study (Supplementary Tables S1 and S2). Patients underwent treatment from 2009 to 2012. Patients with nasopharyngeal carcinoma were excluded from this study.

A multidisciplinary team decided on treatment planning, mainly according to TNM staging, irrespective of HPV status. Most T1 and T2 SCC were treated with functional-preserving surgery or definitive radiotherapy, while most patients with T3 or T4 SCC underwent radical surgery often followed by post-operative (chemo)-radiotherapy or concurrent chemoradiation. The local institutional review board approved the study protocol, and all patients gave their informed consent. Two samples were collected from each patient at the time of surgical resection or biopsy, one from a non-necrotic area of the carcinoma, and the other from SM.

Only patients who underwent complete surgical resection with clear margins (distance > 5 mm between carcinoma and margins) and achieved complete clinical and radiological response to (chemo)-radiotherapy were included in the study. The routine follow-up program consisted of locoregional examination at 2-month intervals during the first year, 3-month intervals in the second year, 4-month intervals between the third and fifth years, and every 6 months thereafter.

The median age of patients was 66 (range 27–95 years); 103 were men (74.1%) and 36 women (25.9%). The site of origin of the carcinoma was the larynx in 46 cases (33.1%), the oral cavity in 45 (32.4%), the oropharynx in 30 (21.6%) and the hypopharynx in 18 (12.9%). One hundred and three patients (74.1%) were ever smokers. According to the American Joint Committee on Cancer TNM 2002 [36], 91 patients (65.5%) had advanced disease (stage III or IV). All tumors were tested for HPV DNA sequences by PCR assays, as previously described [37], and nine (6.5%) were high-risk HPV-positive (HPV-16 in eight cases and HPV-58 in one). In all patients with HPV-positive tumors, the SM was HPV-negative.

Tissue samples

Surgeons were requested to biopsy the surrounding epithelium in the uninvolved mucosa at 4–8 cm from the tumor margins depending on anatomical condition. Precautions were taken not to contaminate the SM with tumor samples by changing surgical blades each time before cutting tissue. Both samples were snap-frozen in liquid nitrogen and stored at −80 °C until analysis. Cryostat sections, 6 μm thick, from each tissue sample were prepared using a 1720 Digital cryostat (Leitz, Germany). One section of each sample was stained with haematoxylin-eosin for histopathology. Surrounding mucosa was histologically assessed and in all cases no histopathological alterations were found. DNA was extracted by the standard phenol/chloroform method and RNA was extracted with Trizol reagent (Invitrogen).

Telomere length analysis

Telomere length was determined by real-time polymerase chain reaction (PCR), exactly as previously described [38,39]. Briefly, two PCRs were performed for each sample, one to determine the cycle threshold (Ct) value for telomere (T) amplification with the primer pair TEL1B and TEL2B [40], and the other to determine the Ct value for the amplification of a single-copy (S) control gene (acidic ribosomal protein P0, RPLP0) with the primer pair RPLP01 and RPLP02 [41]. Each sample was run in triplicate and each PCR reaction was performed using 10 μl of DNA sample (1 ng of DNA per μl) in 50 μl final reaction volume. A reference curve, consisting of fivefold serial dilutions of the reference DNA from the RAJI cell line, was generated at each PCR run, as previously described [38]. All PCR reactions were carried out in 96-well plates using the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). Intra- and inter-assay reproducibility of both telomere and RPLPO PCR results have been evaluated using dilutions of the reference curve [38]. Mean Ct values were used to calculate the relative telomere length using the telomere/single copy gene ratio (T/S) according to the formula: ΔCt sample = Ct telomere − Ct control, ΔCt = ΔCt sample − ΔCt reference curve (where ΔCt reference curve = Ct telomere − Ct control) and then T/S = 2−ΔΔCt [38,40].

Quantification of TERT transcripts

Sample sizes from both cancers and SM were sufficient to allow RNA extraction in 90 cases (Supplementary Tables S1 and S2). RNA samples were reverse transcribed on cDNA using the SuperScript TM III RNase reverse transcriptase assay (Invitrogen) [42]. The expression of TERT transcripts was quantified by real-time PCR using an ABI prism 7900 HT Sequence Detection System (Applied Biosystems). Absolute quantification was carried out with fivefold dilutions of TERT ampiclon as a reference curve for TERT copies, and fivefold dilutions of housekeeping hypoxanthine-guanine
phosphoribosyl transferase 1 (HPRT1) amplexon for HPRT1 copies \[42,43\]. Each sample was run in triplicate and cDNA derived from telomerase-positive (BL41) and telomerase-negative (U2OS) cell lines was included in each plate. TERT values were then normalized for 10 × 5 copies of HPRT1.

Quantification of telomerase activity by real-time polymerase chain reaction

Quantification of telomerase activity by real-time polymerase chain reaction (PCR) method was performed as previously described \[39\]. Briefly, frozen tissue sections were lysed in 50 μL of CHAPS buffer (0.5% CHAPS, 10 mM Tris HCl, pH 7.5, 1 mM MgCl2, 1 mM EGTA, 0.1 mM phenylmethyl-sulfonyl fluoride, 5 mM β-mercaptoethanol, and 10% glycerol) at 4 °C for 30 min. The protein concentration was measured by NanoDrop spectrophotometry (ND-1000; Cabbage) and telomerase activity was evaluated by a real-time PCR method, with 250 ng of cellular protein extract for each sample. Threshold cycle values (Ct) of the samples were plotted against a standard curve generated from serial fivefold dilutions starting from 1250 ng protein extract from telomerase-positive BL41 cells. Each sample was analyzed in triplicate and lysate derived from telomerase-negative U2OS cells was included in each plate. Values were then expressed as relative units.

Statistical analysis

The relationship between telomere length in cancer tissues and SM (data available for all patients), TERT levels in cancer tissues and SM (data available for 90 patients) and age was explored with Spearman’s rank correlation coefficient. The Wilcoxon signed rank test was used to analyze differences in telomere length and TERT levels in paired SM and tumor tissues. Comparisons of telomere length and levels of TERT according to categorical variables (gender, smoking status, alcohol consumption status, HPV-status, tumor site, T-status, N-status, overall stage, and grade of differentiation) were performed using the Kruskal–Wallis test. Computation of 95% Confidence Intervals (CI) for median values was based on distribution-free method. In order to determine the specific effect of each variable, data were dichotomized by the median value. In time-to-event analysis, unadjusted and adjusted hazard ratios (HR) were estimated by Cox’s proportional hazards regression according to the forward stepwise method. Survival curves were generated with the Kaplan–Meier method. Mucosal failure was defined as reappearance of SCC at the UADT site after curative treatment. Death from any cause was considered as a recurrence outside the UADT and regional lymph nodes. For overall survival (OS) analysis, death from any cause was considered as an event; for disease-specific survival (DSS), events considered were death following mucosal or regional failure and distant progression. All tests were two-tailed and the levels of statistical significance were calculated at the 5% level of probability (P < 0.05). Statistical analyses were conducted using SAS statistical software release 9.3 (SAS Institute, Cary, NC).

Results

Telomere length in cancer tissues and SM

The median level of telomere length in cancer tissues (median T/S values 1.05, 95% CI 1.01–1.12) did not differ significantly from that of SM (median T/S values 1.09, 95% CI 0.98–1.17) (P = 0.535, Wilcoxon signed rank test). However, the relative difference between telomere length in cancer tissues and in SM showed great variability (median –0.03, 95% CI –0.11 to 0.09), with 74 cases (53.2%) having longer telomeres in SM and 65 (46.8%) in cancer tissues. No correlation was found between age and telomere length in either cancer tissues (P = 0.932) or SM (P = 0.356).

Telomere length and tumor characteristics

Data on telomere length and tumor characteristics are described in Table 1. Telomere length in tumor tissues did not significantly differ between early and late stage (P = 0.692). Similarly, telomere length in tumor tissue did not significantly differ with tumor grading (P = 0.511). Moreover, median telomere length did not significantly differ according to tumor site (P = 0.437) and HPV-status (P = 0.572).

TERT expression and tumor characteristics

Quantification of TERT transcripts in both cancer tissues and SM was possible for 90 patients (Table 1; Fig. 1A–D). TERT levels in cancer tissues (median 1288 copies, 95% CI 1037–1660) were significantly higher than those in SM (median 393 copies, 95% CI 335–465) (P < 0.001, Wilcoxon signed rank test). Levels of TERT mRNA significantly correlated with telomerase activity (Supplementary Fig. S1; P < 0.001). TERT levels in tumor tissues significantly differed according to tumor stage (Fig. 1B). In particular, high TERT levels were associated with regional lymph node metastases (Table 1; Fig. 1A; P < 0.001), TERT levels differed according to tumor grading (Table 1; Fig. 1C). A significant association was found between TERT levels and HPV-status (Table 1; Fig. 1D). Notably, of the 6 HPV-positive cases in which TERT levels were assessed, 4 fell in the highest quartile with the HPV-58 positive cancer showing the highest level of TERT (11694 copies).

Telomere length and time-to-event analysis

Median follow-up time in survivors was 24 months (interquartile range, 19–30 months). Two patients were lost to follow-up. After complete clinical and radiological response to treatment, 35 patients had mucosal failure, 28 regional failures and 9 distant metastases. Fifteen patients developed second primary tumor outside the UADT. In all, 46 of the 139 patients died; of these, 35 died of disease and 11 of other unrelated causes. Table 2 lists the univariate HRs for the various endpoints. We explored the impact on mucosal failure of dichotomization according to the median value of telomere length in SM (Fig. 2A). Remarkably, in both univariate and multivariate analyses (Table 3), patients with telomere length in SM below the median value had significantly worse local control than those with long telomeres (HR = 4.29, 95% CI, 1.07–4.48; P = 0.049). TERT levels in SM showed a trend of association with time-to-event endpoint (Table 2).
level in cancer tissues (1288 copies). Due to the relatively limited sample size and the lower number of events, multivariate analysis was not attempted. Patients with high TERT levels in cancer tissues (above the median value) showed significantly worse regional control (regional failure HR = 2.88, 95% CI, 1.02–8.08; \( P = 0.045 \)) (Table 2; Fig. 2B). Patients with high TERT levels also exhibited a significantly worse DSS (HR = 2.62, 95% CI, 1.06–6.47; \( P = 0.037 \)), a trend toward a higher distant failure (HR = 7.27, 95% CI, 0.87–60.71; \( P = 0.067 \); Fig. 2C) and worse OS (HR = 1.98, 95% CI, 0.95–4.10; \( P = 0.067 \)). When univariate analysis was restricted to patients with HPV-negative HNSCC, the result was significantly worse OS in patients with high TERT levels (HR = 2.14, 95% CI, 1.02–4.48; \( P = 0.044 \)). Instead, TERT levels in SM were not associated with time-to-events (Table 2).

**Risk factor stratification**

Telomere length and TERT level data were combined for risk stratification. The high-risk group with short telomeres in SM and high TERT levels in cancer tissue showed significantly poorer OS (HR 2.46; 95% CI, 1.18–5.10; \( P = 0.016 \)) compared with other groups (Fig. 2D).

**Discussion**

In patients with HNSCC, substantial concern exists regarding the importance of identifying molecular signatures indicating increased risk of mucosal failure and metastatic spread of tumor cells [7,44,45]. The present study was attempted to investigate the role of telomere erosion and TERT expression as molecular markers of field cancerization and disease severity. No correlation between age and telomere length in SM was found. This was unexpected as telomere length in non-cancerous cells, including those from cancer-adjacent normal tissues, is inversely correlated with age [12,46,47]. In addition, unlike results found in other studies, including those on head and neck cancers reporting that telomeres are shorter in tumors than in their normal counterparts [18,20], in this study median telomere level in cancer tissues did not differ significantly from that of SM, as about half of the patients had shorter telomeres in cancer tissues and the others had shorter telomeres in SM. Interestingly, the patients with short telomeres in SM had a highly significant increased risk of mucosal failure. All the above observations indicate that SM may not be a biologically normal tissue. Previous studies have provided evidence that primary HNSCC and subsequent relapses are frequently the result of independent consecutive critical genetic alterations within the same field of clinically and histologically normal mucosa altered by exposure to environmental carcinogens [6,7]. These fields of apparently normal UADT epithelium may spread over several centimeters [6]. Genetic and cytogenetic studies, as well as more recent proteomic analysis, have demonstrated dramatic tumor-related aberrations, transcriptional activity, loss of heterozygosity, chromosomal aneuploidy, and abnormal proteomic profiling in tumor–adjacent and tumor–distant UADT mucosa without histological aberrations [7,44,45,48]. Nevertheless, the most extensively studied molecular marker, the TP53 gene, has provided conflicting results [45,48]. The distance from the tumor where the SM was biopsied may, in part, account for the discrepancy of results among different studies. In this work, we sampled SM over an area which has been demonstrated to be the approximate extent of genetic-altered fields [6]. Data from our analysis support the hypothesis that in some cases SM constitutes a telomere-shortened epithelial field prone to genetic instability. Therefore, telomere attrition in SM may represent a molecular marker of genetically altered fields and thus useful in predicting mucosal failure.

In agreement with other studies, including those on head and neck cancer [31,32,34,49], we found that TERT levels in cancer tissues increase with the aggressiveness of the disease. Conversely, telomere length in cancer tissues does not increase with stage. These data support the concept that the rising levels of TERT observed in advanced stage carcinomas balance the enhanced replication activity, resulting in maintenance rather than elongation of telomere length during cancer progression [50]. In time-to-event analysis, patients with high TERT levels showed significant worse regional control and DSS with a trend toward higher distant failure. Other studies have reported that telomerase activity is an independent prognostic factor for OS in patients with...
**Fig. 1.** TERT levels and tumor characteristics. (A) TERT levels in tumor according to N status. (B) TERT levels in tumor according to stage. (C) TERT levels in tumor according to grade of tumor differentiation (G1, well-differentiated carcinoma; G2, moderately differentiated carcinoma; G3, poorly differentiated carcinoma). (D) TERT levels in tumor according to HPV status. Boxes and whiskers: 25th–75th and 10th–90th percentiles, respectively; median is the central line in each box.

**Table 2**
Telomere length/TERT levels and outcome: univariate analysis.

<table>
<thead>
<tr>
<th>TL in SM &gt; 1.09</th>
<th>TL in SM ≤ 1.09</th>
<th>TL in tumor &gt; 1.05</th>
<th>TL in tumor ≤ 1.05</th>
<th>TERT levels in tumor &lt; 1288</th>
<th>TERT levels in tumor &gt; 1288</th>
<th>TERT levels in SM ≤ 393</th>
<th>TERT levels in SM &gt; 393</th>
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<td>n</td>
<td>Mucosal failure</td>
<td>Regional failure</td>
<td>Distant failure</td>
<td>DSS</td>
<td>OS</td>
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<tr>
<td>68</td>
<td>1</td>
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<tr>
<td>71</td>
<td>4.25 (1.86 – 9.74)</td>
<td>1.84 (0.85 – 3.99)</td>
<td>1.78 (0.42 – 7.45)</td>
<td>2.19 (1.07 – 4.48)</td>
<td>1.63 (0.89 – 2.95)</td>
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<td>0.491</td>
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<td>0.110</td>
<td>0.942</td>
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<tr>
<td>69</td>
<td>0.02 (0.52–1.99)</td>
<td>1.30 (0.61–2.75)</td>
<td>1.03 (0.26–4.14)</td>
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<td>0.673</td>
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<td>70</td>
<td>1.02 (1.86–9.74)</td>
<td>2.88 (1.02–8.08)</td>
<td>7.27 (0.87–60.71)</td>
<td>2.62 (1.06–6.47)</td>
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<tr>
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<tr>
<td>P-value</td>
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<td>1.25 (0.54–2.91)</td>
<td>2.88 (1.02–8.08)</td>
<td>7.27 (0.87–60.71)</td>
<td>2.62 (1.06–6.47)</td>
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<td>45</td>
<td>1.83 (0.77–4.35)</td>
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<td>0.72 (0.16–3.21)</td>
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TERT: telomerase reverse transcriptase; HR: hazard ratio; CI: confidence interval; DSS: disease specific survival; OS: overall survival; TL: telomere length; SM: surrounding mucosa.
HNSCC [31,32]. In the present study, high TERT levels strongly correlated with worse OS in HPV-negative tumors. It has been demonstrated that the E6 oncoprotein of high-risk HPV types increases cellular telomerase activity by activating transcription of the TERT gene and by post-transcriptional mechanisms [51]. Interestingly, although the quantification of TERT transcripts was demonstrated that the E6 oncoprotein of high-risk HPV types increases cellular telomerase activity by activating transcription of the TERT gene and by post-transcriptional mechanisms [51]. Interestingly, although the quantification of TERT transcripts was determined in a small number of HPV-positive SCC, median TERT levels in HPV-positive tumors were significantly higher than in HPV-negative cases, most probably as a consequence of the E6-mediated transcription of the TERT gene. Patients with HPV-induced HNSCC have significantly better OS than patients with HPV-unrelated tumors [2]. Therefore, high levels of TERT expression could have different prognostic significance in HPV-positive and HPV-negative SCC. As HPV-negative cancers still remain the majority of HNSCC, quantification of TERT levels may be useful to stratify prognosis in this larger subset of patients.

Unlike telomere shortening in SM, which was related to local mucosal failure, high levels of TERT expression appear to reveal the metastatic propensity of tumor cells. The greatest difference in OS were observed when patients were stratified according to both telomere length in SM and TERT levels in cancer tissues; patients with short telomeres in SM and high TERT levels in cancer tissue had a significantly higher risk of death.

Although at very significant lower levels, TERT expression was also detected in SM in both this and other studies [27,28,30,34]. This was not unexpected, as telomerase expression is compatible with the nature of the UADT epithelium, which contains a normal proliferative progenitor compartment with stem cells and undergoes renewal cycles of proliferation and differentiation [52]. Telomerase-positive lymphocytes are also normal constituents of UADT and HNSCC, which frequently develop within a context of chronic inflammatory cell infiltrate [30,53]. However, TERT levels in SM were not predictive of mucosal failure; this observation may support the concept that erosion of telomeres precedes telomerase activation in the oncogenic process.

In conclusion, quantitative analyses of telomere and telomerase in matched tumors and SM allowed us, for the first time, to identify short telomeres in tumor SM as highly significant prognostic

**Table 3**

Mucosal failure: univariate and multivariate analysis.

<table>
<thead>
<tr>
<th>Univariate</th>
<th>HR (95% CI)</th>
<th>P-value</th>
<th>Multivariate</th>
<th>HR (95% CI)</th>
<th>P-value</th>
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<tbody>
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<td>Age (continuous variable)</td>
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<td>T3</td>
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<tr>
<td>T4</td>
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<td>T category</td>
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<td>Treatment</td>
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<tr>
<td>Surgery</td>
<td>1.11 (0.55–2.23)</td>
<td>0.772</td>
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<td>TL in SM</td>
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<tr>
<td>&gt;1.09</td>
<td>4.25 (1.86–9.74)</td>
<td>&lt;0.001</td>
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<td>≤1.09</td>
<td>1</td>
<td></td>
<td></td>
<td>4.29 (1.87–9.87)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

HR: hazard ratio; CI: confidence interval; NS: not significant; NT: not tested; TL: telomere length; SM: surrounding mucosa.
marker of mucosal failure. High levels of TERT transcripts in cancer tissues are predictive of regional and distant spread of the disease. While telomere shortening in SM should be considered as a biosensor of peritumoral field cancerization, increasing TERT level in tumoral tissue is a biomarker of cancer progression (Fig. 3). If these data are validated in larger series, they may lead to new perspectives in post-therapeutic surveillance strategies, moving toward more personalized follow-up. Patients identified as at risk of tumor field cancerization, increasing TERT level in cancer tissues are predictive of regional and distant spread of the disease.

**Conflict of interest statement**

None declared.

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**Appendix A. Supplementary material**

Supplementary data associated with this article can be found, in the online version.

**References**


Liu Y, Dong XL, Tian C, Liu HG. Human telomerase RNA component (hTRC) gene amplification detected by FISH in precancerous lesions and carcinoma of the larynx. Diag Pathol 2012;7:34.
