## Original Article
# Human telomerase reverse transcriptase protein expression is associated with survival in patients with oral squamous cell carcinoma

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**Abstract:** Despite improvements in diagnosis and treatment, the 5-year survival of oral squamous cell carcinoma (OSCC), no matter the location, remains low, averaging 50%. Telomerase is expressed in 85% of malignancies and may play an important role in human carcinogenesis. Its catalytic component is human telomerase reverse transcriptase (hTERT), which has been thought, but not proven, to be involved in survival with OSCC. We investigated whether hTERT protein was a prognostic factor in OSCC by evaluating its association with clinicopathologic findings and OSCC survival. We found that in comparison to patients with high hTERT expression, patients with low hTERT expression survived significantly longer, including a longer 5-year overall survival. In addition, overall survival was significantly correlated to hTERT expression and the histologic grade and N status of the tumor. Disease-free survival was significantly related to hTERT expression, the histologic grade and N status of the tumor, and mode of invasion. These results suggest that hTERT protein is involved in cervical lymph node metastasis, that its levels may be increased during carcinogenesis, and that it may influence tumor invasion. We believe that this study is the first to demonstrate that OSCC with high hTERT expression carries a worse prognosis than cases with low hTERT expression.

**Keywords:** Human telomerase reverse transcriptase, oral squamous cell carcinoma, 5-year survival, 5-year disease-free survival

## Introduction

Oral squamous cell carcinoma (OSCC) is the eighth most prevalent malignancy worldwide and the most common malignant tumor of the oral cavity. Despite improvements in diagnosis and treatment, the 5-year survival rate remains low, averaging 50% for all OSCC locations [1]. The common sites of OSCC are the tongue, lower lip, buccal mucosa, gingiva and floor of mouth [2]; and the most common location, with the worst prognosis, seems to be the tongue, although not all studies have confirmed this [3]. The poor outcomes are explained by the tumor's propensity for local invasion and by high rates of cervical lymph node metastasis. Because of these factors, and the lack of reliable prognostic markers, decisions about treatment are made with difficulty. This situation emphasizes the need to identify biologic correlates of tumor behavior to improve outcome prediction and therapeutic decision making in affected patients.

One of the hallmarks of malignant tumors is the potential for chaotic growth based on telomere maintenance [4]. Telomerase, a ribonucleoprotein complex enzyme, caps the ends of linear chromosomes. It consists of two main components: the catalytic component, human telomerase reverse transcriptase (hTERT), and human telomerase RNA molecule (hTR), in addition to telomerase associated proteins, human telomerase associated protein 1 (hTEP1), Hsp90, p23, and dyskerin [5]. Telomerase adds telomere (TTAGGG) repeats to the 3' ends of

chromosomes and is expressed in 85% of malignancies, but it is absent or almost undetectable in most normal human somatic cells. However, germ cells, stem cells, and cardiovascular cells express significant detectable levels of telomerase [6]. Consequently, telomerase might serve as an important biomarker for human malignancy and play an important role in human carcinogenesis. Although hTR and hTEP1 are expressed ubiquitously, hTERT is strictly regulated at the transcriptional and post-translational levels, and telomerase activity is believed to be regulated at the level of hTERT [7]. hTERT overexpression correlates with clinical aggressiveness of patients with OSCC and plays an important role in the oral carcinogenic process [8]. Therefore, the expression of hTERT is thought to play an important role in the survival of patients with OSCC; however, to date, there is a lack of research on the pattern of hTERT expression and its association with clinicopathologic values and survival in patients with OSCC. To examine whether hTERT level could be a prognostic factor in OSCC, we evaluated the relationship of expression of hTERT with clinicopathologic findings and OSCC survival rate.

## Materials and methods

*Patients' characteristics and tumor samples*

The study protocol was approved by the Ethics Committee of Kyushu Dental University, Kitakyushu, Japan (approval No. 20-52). Patients with a biopsy-based diagnosis of invasive OSCC and who subsequently underwent resection surgery were eligible to participate. Exclusion criteria were the presence of carcinoma in situ by biopsy, patients who received preoperative radiation or chemoradiation therapy of the head and neck, and patients who were treated with chemoradiotherapy. A total of 53 paraffin-embedded tissues from clinical OSCC tumor samples and 12 samples of normal oral mucosa (NOM) were included in this study. All patients with OSCC who visited the Oral and Maxillofacial Surgery Department at Kyushu Dental University Hospital between February 2013 and January 2015 were evaluated retrospectively. Of the 53 samples of OSCC, 36 were from the tongue, 13 from the gingiva, and 4 from the buccal mucosa. NOM samples were obtained from noninflamed buc-

cal mucosa of patients who had impacted lower third molars without any oral mucosal diseases. Clinical records and slides stained with hematoxylin and eosin were reviewed in relation to clinicopathologic values, such as age, sex, tumor location, histological grade, T status, N status, tumor stage, and mode of invasion. Overall survival (OS) was defined as being alive for a certain period (the follow-up period in this study) after diagnosis of or beginning of treatment for OSCC. Disease-free survival (DFS) was defined as the time from tumor resection to the date of death or recurrence. The tumor stage was defined according to the 8th edition of the American Joint Commission on Cancer staging manual [9].

*Immunohistochemistry for hTERT expression*

To detect hTERT, the streptavidin-biotin method was carried out with routinely processed formalin-fixed, paraffin-embedded 4-μm-thick sections, as previously described [10], and mouse anti-hTERT monoclonal antibody (clone 2C4; GeneTex, Inc., Irvine, CA, USA) was used at a dilution of 1:40.

Cells immunohistologically positive for hTERT were those that showed brown staining within the nucleus. Among at least 1000 cells in each section, the number of immunostaining-positive cells were counted under a light microscope, and the nuclear positivity index was defined as the ratio of immunostaining-positive cells to the total number of cells. The percentages of immunostaining-positive cells of hTERT were grouped as follows: Sections with more than 50% positive cells were considered to have high hTERT expression, and those with fewer than 50% positive cells were considered to have low hTERT expression.

*Statistical analysis*

We used Student's *t*-test to analyze differences between the two independent groups, and we used analysis of variance to perform multiple comparisons between groups. We estimated OS and DFS by using the Kaplan-Meier method, and we used the log-rank test to compare the estimated curves between groups. The Cox proportional hazards regression model was applied to determine which variables affected OS and DFS. A *P*-value of less than 0.05 was deemed significant.

**Table 1.** Association between clinicopathologic characteristics and hTERT expression in OSCC

| | n | Mean nuclear labeling indices of hTERT: LI ± SD (%) | p-value |
|---|---|---|---|
| Age | | | |
| < 60 years | 18 | 58.79 ± 15.67 | 0.006 |
| ≥ 60 years | 35 | 46.82 ± 13.70 | |
| Sex | | | |
| Male | 28 | 48.94 ± 17.27 | 0.333 |
| Female | 25 | 53.07 ± 12.87 | |
| Location | | | |
| Tongue | 36 | 50.67 ± 16.67 | > 0.05 |
| Gingiva | 13 | 54.57 ± 12.34 | |
| Buccal mucosa | 4 | 40.88 ± 6.41 | |
| Histological grade | | | |
| Well | 35 | 46.39 ± 15.36 | 0.002 |
| Moderate/Poor | 18 | 59.62 ± 11.26 | |
| T status | | | |
| T1 + T2 | 43 | 47.16 ± 14.48 | < 0.001 |
| T3 + T4 | 10 | 66.92 ± 5.64 | |
| N status | | | |
| N0 | 37 | 44.67 ± 12.87 | < 0.001 |
| N1 + N2 + N3 | 16 | 66.51 ± 6.59 | |
| Clinical staging | | | |
| early stage (stage I + II) | 36 | 43.27 ± 12.11 | < 0.001 |
| advanced stage (stage III + IV) | 17 | 67.02 ± 6.16 | |
| Mode of invasion | | | |
| INF a + b | 41 | 47.47 ± 15.58 | 0.015 |
| INF c | 15 | 57.53 ± 11.85 | |

OSCC: oral squamous cell carcinoma; hTERT: human telomerase reverse transcriptase; INF: infiltrative growth factor.

## Results

### Patients characteristics

The patients' characteristics are shown in **Table 1**. Of the 53 patients with OSCC included in this study, 28 were men and 25 were women. The average age was 64.5 years (median: 64.0 years, range: 33.0-93.0 years); 35 patients were 60 years of age or older, and 18 were younger than 60 years. In this study, 36 tumors were located on the tongue, 13 in the gingiva, and 4 in the buccal mucosa; 35 (66.0%) were well differentiated, and 18 (34.0%) were moderately or poorly differentiated. In T status, 43 OSCCs were T1 and T2 tumors, and 10 were T3 and T4 tumors. Cervical lymph node metastasis was present in 16 patients. In tumor stage, 36 had early-stage (stage I or II) disease, and 17 had advanced-stage (stage III or IV) disease. Regarding the mode of invasion, 41 had infiltrative growth factor (INF) a and b and 15 had INF c. We also studied 12 NOM samples (7 from men and 5 from women). The average age of healthy volunteers was 57.9 years (median: 63.5 years; range: 33.0-72.0 years).
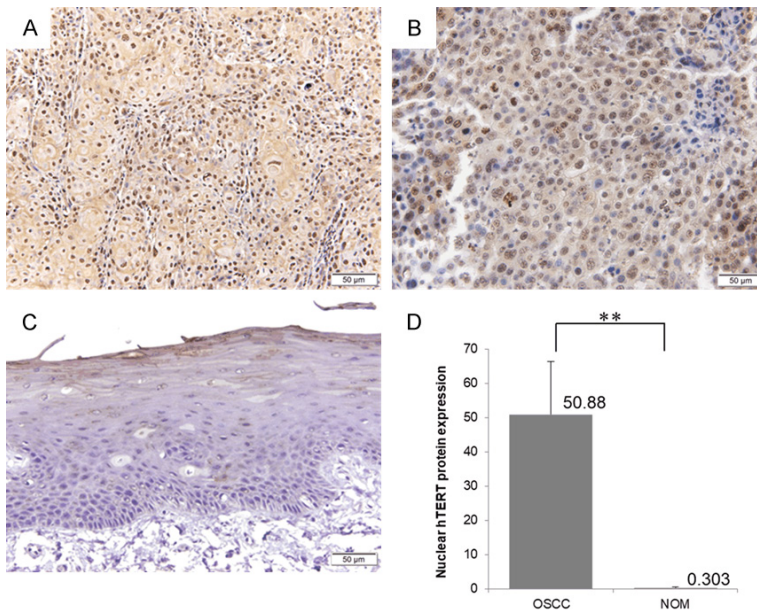
### Immunohistochemical staining characteristics of hTERT

Representative microphotographs of OSCC and NOM samples immunostained for hTERT are shown in **Figure 1A-C**. hTERT protein immunoreactivity was positive to varying degrees in epithelial cells in almost all the OSCC samples (**Figure 1A**, **1B**). However, in the NOM samples, hTERT immunoreactivity was usually completely absent or only slightly present within the nuclei of the basal layer and parabasal layer but not in the spinous layer (**Figure 1C**). The mean positive nuclear index of hTERT was 50.89 ± 15.35% (range: 18.9-79.5%) in OSCC samples and 0.30 ± 0.25% (range: 0.00-0.89%) in the NOM samples (**Figure 1D**). The comparison of nuclear hTERT staining between the OSCC and NOM samples revealed significant differences (*P* < 0.001).

The relationship between hTERT expression and histopathologic findings in patients with OSCC is illustrated in **Table 1**. The levels of hTERT expression in OSCC differed significantly with age (< 60 years: 58.79 ± 15.67%; ≥ 60 years: 46.82 ± 13.70%; *P* = 0.006), with histological differentiation (well differentiated: 46.39 ± 15.36%; moderately and poorly differentiated: 59.62 ± 11.26%; *P* = 0.002), with T status (T1 and T2: 47.16 ± 14.48%; T3 and T4:

**Figure 1.** Immunohistochemical staining for hTERT. A: Well differentiated OSCC exhibiting moderate nuclear hTERT staining in approximately 45% epithelial cells (IHC, ×40). B: Approximately 60% cells in moderately differentiated OSCC exhibited nuclear hTERT staining in epithelial cells (IHC, ×40). C: NOM showing weak or negative nuclear hTERT staining in basal layer and parabasal layer (IHC, ×40). D: Mean positive nuclear index of hTERT was 50.89% in OSCC samples and 0.30% in NOM samples (**$P < 0.01$). OSCC: oral squamous cell carcinoma; hTERT: human telomerase reverse transcriptase.
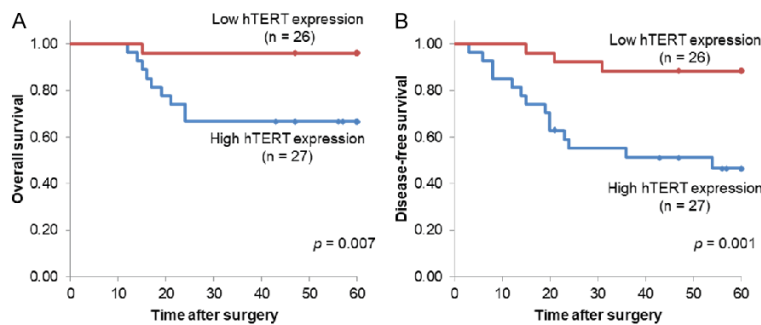


**Figure 2.** Kaplan-Meier analysis for (A) overall survival (OS) and (B) disease-free survival (DFS) in patients with OSCC. High hTERT expression was associated with shorter OS ($P = 0.007$) and DFS ($P = 0.001$). OSCC: oral squamous cell carcinoma.

va: 54.57 ± 12.34%; buccal mucosa: 40.88 ± 6.41%; $P > 0.05$).

*Value of hTERT expression in predicting the clinical outcome in patients with OSCC*

Of the 53 patients included in this study, 36 (67.9%) survived without any cancer-related death, cervical lymph node metastasis, or tumor recurrence. Among the other 17 (32.1%), subsequent metastasis occurred in 4 patients (7.5% of the total), tumors recurred in 2 (3.8%), and death, including cancer-related death, was recorded in 9 (17.0%) during the follow-up period.

We conducted Kaplan-Meier survival analysis to examine rates of 5-year OS and 5-year DFS. According to Kaplan-Meier curves for OS based on hTERT expression level in OSCC (**Figure 2A**), rates of 5-year survival were significantly lower among patients with high hTERT expression (66.7%) than among patients with low hTERT expression (96.2%; $P = 0.007$). Multivariate analysis (Cox regression) revealed that OS was related to hTERT expression ($P = 0.034$, hazard ratio [HR] = 10.075, and 95% confidence interval [CI] = 1.195-84.968 for hTERT levels of ≥ 50%, whereby levels of < 50% served as the reference), histologic grade ($P = 0.024$, HR = 1.346, and 95% CI = 1.039-1.744 for moderately and poorly differentiated tumors, whereby well differentiated tumors served as the reference), and N status ($P = 0.010$, HR = 7.435, and 95% CI = 1.613-34.281 for N positive tumors, whereby N0 tumors served as the reference), as documented in **Table 2**. According to Kaplan-Meier curves for DFS that were based on the hTERT expression level of OSCC (**Figure 2B**), rates of

66.92 ± 5.64%; $P < 0.001$), with N status (N0: 44.67 ± 12.87%; N positive: 66.51 ± 6.59%; $P < 0.001$), with tumor stage (early-stage: 43.27 ± 12.11%; advanced-stage: 67.02 ± 6.16%; $P < 0.001$), and with mode of invasion (INF a and b: 47.47 ± 15.58%; INF c: 57.53 ± 11.85%; $P = 0.015$). Expressions of hTERT did not differ significantly by sex (male: 48.94 ± 17.27%; female: 53.07 ± 12.87%; $P = 0.333$) or by tumor location (tongue: 50.67 ± 16.67%; gingi-

**Table 2.** Multivariate Cox regression analysis for the values associated with OS

| Factor | Hazard ratio | 95% CI | p-value |
|---|---|---|---|
| Nuclear labeling index of hTERT protein (hTERT positive [≥ 50%] vs. negative [< 50%]) | 10.075 | 1.195-84.968 | 0.034 |
| Age (≥ 60 years vs. < 60 years) | 1.045 | 0.994-1.084 | 0.088 |
| Histologic grade (Well vs. Moderate + Poor) | 1.346 | 1.039-1.744 | 0.024 |
| N status (N0 vs. N1 + N2 + N3) | 7.435 | 1.613-34.281 | 0.010 |

hTERT: human telomerase reverse transcriptase; OS: overall survival.

**Table 3.** Multivariate Cox regression analysis for the values associated with DFS

| Factor | Hazard ratio | 95% CI | p-value |
|---|---|---|---|
| Nuclear labeling index of hTERT protein (hTERT positive [≥ 50%] vs. negative [< 50%]) | 5.763 | 1.579-21.027 | 0.008 |
| Location (Gingiva + Buccal mucosa vs. Tongue) | 4.906 | 1.368-17.602 | 0.015 |
| Histologic grade (Well vs. Moderate + Poor) | 1.445 | 1.119-1.867 | 0.005 |
| N status (N0 vs. N1 + N2 + N3) | 3.020 | 1.046-8.719 | 0.041 |
| Mode of invasion (INF a + b vs. INF c) | 5.153 | 1.721-15.423 | 0.003 |

hTERT: human telomerase reverse transcriptase; INF: infiltrative growth factor; DFS: disease-free survival.

5-year DFS were significantly lower among patients with high hTERT expression (54.2%) than among patients with low hTERT expression (88.5%; P = 0.001). Multivariate analysis (Cox regression) revealed that DFS was significantly related to hTERT expression (P = 0.008, HR = 5.763, and 95% CI = 1.579-21.027 for hTERT levels of ≥ 50%, whereby levels of < 50% - served as the reference), location (P = 0.015, HR = 4.906, and 95% CI = 1.368-17.602 for tongue, whereby gingiva and buccal mucosa served as the reference), histologic grade (P = 0.005, HR = 1.445, and 95% CI = 1.119-1.867 for moderately and poorly differentiated tumors, whereby well differentiated tumors served as the reference), N status (P = 0.041, HR = 3.020, and 95% CI = 1.046-8.719 for N positive tumors, whereby N0 tumors served as the reference), and mode of invasion (P = 0.003, HR = 5.153, and 95% CI = 1.721-15.423 for INF c, whereby INF a and b served as the references), as documented in **Table 3**.

## Discussion

OSCC is caused by a multistep carcinogenesis process that involves gene mutation and chromosomal instability [11]. The transition from normal oral mucosa (NOM) to oral epithelial dysplasia and OSCC results from accumulated genetic and epigenetic alterations [12]. In addition, OSCC features cellular and subcellular alterations, which are considered to lead to dedifferentiation and growth. These changes,

in turn, are associated with genetic changes that affect cell cycle, apoptosis, angiogenesis, and telomere length. Telomeres, the protective regions found at the ends of linear chromosomes, are essential for chromosomal stability and cell survival. With each cell division, telomeres are shortened because in the DNA polymerase protein complex, the sequences at the ends of the chromosomes cannot be replicated completely. This process is counteracted by telomerase, which maintains telomere length by adding the hexametric TTAGGG repeats to the 3' ends of chromosomes [13]. The expression of telomerase in various cancer cells contributes to the growth and progression of cancer by compensating for the telomeres lost when the cancer cells actively grow.

In most cells and tissues, hTERT is responsible for controlling the activity of telomerase complex, and the significant increase in hTERT expression during tumorigenesis is correlated with increased proliferative potential of cancer cells [14]. Previous studies have shown that hTERT expression increases gradually during malignant transformation from NOM to oral epithelial dysplasia and to OSCC [15]. Similar findings have been reported for laryngeal cancer [16], colorectal cancer [17], hepatocellular carcinoma [18], and vulvar cancer [19]. These findings suggest that hTERT expression plays an important role in the human carcinogenic process. The mechanism of the transcriptional regulation of hTERT and its role in carcinogen-

esis has been reported in several studies, but these aspects have not been previously fully clarified.

hTERT expression is controlled by the chromatin environment and epigenetic regulation during cell development and differentiation. In addition, hTERT transcription is regulated by the binding of transcription factors to the hTERT promoter. The hTERT promoter contains abundant sites of potential transcription factor binding, and these sites may play important roles in hTERT transcription in response to changes in physiologic conditions and during tumorigenesis. Researchers have identified several sequence-specific transcriptional factors that bind directly to the hTERT promoter, such as c-myc, specificity protein 1 (SP1), upstream stimulatory factors (USFs), E2F1, activator protein, Ets, and estrogen receptor [13, 20]. Most of the transcription factor-binding motifs are conserved between hTERT core promoters. In this study, too, the level of hTERT expression was significantly higher in the OSCC samples than in the NOM samples. Therefore, in OSCC, hTERT expression may be increased by the process of carcinogenesis, and cells are immortalized by the increase in telomerase activity; however, further studies are needed to clarify the details.

Cervical lymph node metastasis is the most reliable prognostic factor in OSCC. In this study, the expression level of hTERT protein in patients with cervical lymph node metastasis was significantly higher than in those without cervical lymph node metastasis. This result suggests that hTERT may be involved in cervical lymph node metastasis. To date, some studies have demonstrated the role of hTERT in invasion and metastasis in several tumor types, such as melanoma [21], gastric cancer [22], and urological tumors [23], but very few have focused on the mechanism underlying this observed pattern. Some research has revealed that hTERT plays a role in tumor invasion and metastasis by promoting epithelial mesenchymal transition (EMT). In addition, hTERT may contribute to the maintenance of cancer stem cells by activating the WNT/β-catenin signaling pathway [24]. Similarly, Liu et al. stated that hTERT induces EMT through transforming growth factor (TGF) β1, resulting in the increased invasive and metastatic potential of cancers [22]. Furthermore, a correlation of hTERT with integrin β1 expression was associated with invasion, migration, angiogenesis, and proliferation--all of which are essential for metastasis formation [22].

Regarding the mode of invasion, patients with INF c are generally reported to have a poorer prognosis compared to patients with INF a and b [25]. In this study, hTERT protein expression in INF c was significantly higher than that in INF a and b, and patients with INF c had a poorer prognosis than patients with INF a and b. In addition, the findings suggest that hTERT may influence tumor invasion. The invasive potential of cells depends on their ability to degrade the extracellular matrix, which is mainly achieved by different extracellular proteases, such as those of the members of the matrix metalloprotease (MMP) family. In other studies, hTERT has been associated with expression levels of several MMPs in many types of tumors. For example, Park et al. reported that in OSCCs, a decrease in hTERT levels leads to reduction in MMP2 and MMP9 expression levels, thereby inhibiting invasiveness [26].

Our study showed that the level of hTERT expression in moderately and poorly differentiated OSCCs was significantly higher than that in well differentiated OSCCs. According to previous reports, the degree of differentiation was significantly correlated with the occurrence of cervical lymph node metastasis. Suresh et al. revealed that majority (78.8%) of patients with well differentiated OSCCs showed no lymph node metastasis, whereas in patients with moderately and poorly differentiated OSCCs, the rate of metastasis was high (55%) [27]. The results of their univariate analysis indicated that histologic grade was associated with prognosis, but multivariate analysis demonstrated that it was not an independent poor prognostic factor. This may be explained by the correlation between histologic grade and hTERT expression, whereby higher-grade tumors are more likely to be hTERT-positive. Moreover, Raghunandan et al. reported that hTERT expression gradually increased as the degree of differentiation of OSCCs decreased [28].

This study proved that hTERT expression was significantly higher according to tumor size or invasion depth. In multivariate analysis, Saeednejad et al. found that tumor size was an independent prognostic indicator [29]. Sharma

et al. found a positive correlation between tumor size and cervical lymph node metastasis, reporting that the larger the tumor, the higher the rate of cervical lymph node metastasis [30]. Eckert et al. also reported that tumor size and cervical lymph node metastasis affected patient survival [31]. These results suggest that tumor size affects the rates of cervical lymph node metastasis and survival, which indicates the involvement of hTERT expression and telomerase activity.

In this study, the OS and DFS of patients with high hTERT expression were statistically different from that of patients with low hTERT expression. We believe that our study is the first to show that OSCCs with higher hTERT expression tend to carry a worse prognosis than do those with low hTERT expression. In addition, patients in whom OSCC expressed high hTERT levels had lower rates of 5-year OS and 5-year DFS than did patients in whom OSCCs had low hTERT expression. This research suggests that hTERT overexpression activates EMT and WNT/β-catenin pathway by affecting MMP family and TGF β1. As a result, it was considered that hTERT overexpression was involved in tumor infiltration, metastasis, differentiation, and growth, causing a poor prognosis in OSCC patients. However, further research is needed to support these results.

In particular, the present study evinced the following findings.

1. Telomerase is involved in cell immortalization, and hTERT overexpression maintains telomere length and causes cell immortalization.

2. A small amount of hTERT protein expression was observed in basal and parabasal cells in the NOM, suggesting that hTERT may also be involved in self-renewal.

3. hTERT overexpression was correlated with tumor size, suggesting that hTERT is involved in cell proliferation.

4. Because hTERT protein expression is associated with cervical lymph node metastasis, it may also be associated with tumor cell infiltration and metastasis.

5. The high hTERT expression group had significantly lower 5-year OS and 5-year DFS compared to the low expression group, suggesting

that hTERT expression is also involved in the prognosis of OSCC patients.

To the best of our knowledge, this is the first study to show that hTERT protein expression is a prognostic marker in OSCC. Although further studies are needed, the prognosis of patients with OSCC might possibly be estimated by measuring the level of hTERT expression. If so, hTERT levels may be useful for determining the method of treatment for OSCC, such as postoperative chemoradiotherapy.

### Conclusions

The level of hTERT expression in OSCC was significantly higher than that in normal oral mucosa (NOM). Moreover, both OS and DFS were significantly poorer in patients with high hTERT expression than in those with low hTERT expression. Increased hTERT expression may indicate more aggressive tumor behavior and more advanced disease in cases of OSCC. Thus, evaluation of hTERT expression can be useful for predicting tumor invasiveness in patients with OSCC. In this study, high hTERT expression was considered a poor prognostic factor and a useful biomarker in OSCC.

### Disclosure of conflict of interest

None.

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