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Lymphocyte telomere length predicts clinical outcomes of HPV-positive oropharyngeal cancer patients after definitive radiotherapy

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Abstract

Because lymphocyte telomere length (LTL) plays critical roles in the maintenance of genomic stability and integrity, LTL thus may influence the etiology and prognosis of squamous cell carcinoma of the oropharynx (SCCOP). However, given the association between LTL and risk of human papillomavirus (HPV)-associated SCCOP and between LTL and tumor HPV status of SCCOP, we hypothesized that LTL is associated with SCCOP prognosis, particularly in HPV-positive patients after definitive radiotherapy. LTL and tumor HPV type 16 (HPV16) status were determined in 564 incident SCCOP patients before radiotherapy or chemoradiation. Both univariate and multivariable Cox regression analyses were performed to estimate the association between LTL and prognosis. Eighty-five percent patients had HPV16-positive tumors. Patients with shorter telomeres had significantly better overall, disease-specific and disease-free survival than did those with longer telomeres (log-rank $P < 0.001$). Moreover, patients with shorter telomeres had significantly lower risk of death overall [hazard ratio (HR) = 0.2; 95% confidence interval (CI) = 0.1–0.4], death due to SCCOP (HR = 0.2; 95% CI = 0.1–0.4) and SCCOP recurrence (HR = 0.3; 95% CI = 0.2–0.5) after adjusting for other important prognostic confounders. Finally, we found more pronounced effects of LTL on survival in HPV16-positive SCCOP patients after stratified analysis according to tumor HPV status. These findings indicate that LTL plays a significant role in the survival of patients with SCCOP, especially HPV16-positive patients who undergo definitive radiotherapy. Therefore, pretreatment LTL may be an independent prognostic biomarker for HPV16-positive SCCOP. Prospective studies with larger sample sizes are needed to confirm these findings.

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Abbreviations

CI	confidence interval
Ct	threshold cycle
DFS	disease-free survival
DSS	disease-specific survival
HBG	human β2-globulin
HPV	human papillomavirus
HPV16	human papillomavirus type 16
HR	hazard ratio
LTL	lymphocyte telomere length
OS	overall survival
PCR	polymerase chain reaction
SCCOP	squamous cell carcinoma of the oropharynx
TL	telomere length

Introduction

In addition to the traditional risk factors of alcohol and tobacco consumption, human papillomavirus (HPV) infection, particularly HPV type 16 (HPV16) infection, is now recognized as an etiological factor driving carcinogenesis in the majority of squamous cell carcinoma of the oropharynx (SCCOP) cases in North America and Europe (1). A meta-analysis of population studies from North America and Europe indicated that the overall prevalence of HPV infection in SCCOP patients increased considerably over time (before 2000 = 40.5%; 2000–2004 = 64.3%; 2005–2009 = 72.2%) (2), which was consistent with the epidemiological trend of increasing incidence of SCCOP from the 1980s to the present (1,3,4). Reports of multiple trials published over the past decade present strong evidence that HPV infection is an independent marker of favorable prognosis in SCCOP and is associated with improved response to treatment and survival (5–10).

Because patients with HPV-associated SCCOP are anticipated to live longer, they are at greater risk of experiencing the long-term toxic effects of radiotherapy (11). Therefore, developing novel biomarkers for HPV-associated SCCOP is important to identify patients who would benefit from less-intensive treatment and to define a subset of patients with poor prognoses who should be treated more aggressively (12).

Telomeres protect the ends of chromosomes and contain tandem repeats of the TTAGGG sequence, a single-stranded 3′G-rich overhang, and several generic DNA-binding proteins, tankyrases and telomere-binding proteins (13) Human telomeres are ~9–15 kb long and are found in somatic cells. The telomeres shorten with each cell division, resulting in a decrease in telomere length (TL) with increasing age (14,15). Many different endogenous and environmental factors, such as aging, oxidative stress, an unhealthy lifestyle and genotoxic stress (16), can modify TL, influencing cell fate and leading to disease occurrence. Researchers have detected significant associations between TL and cancer incidence (17–21). Moreover, meta-analyses and a large prospective study have confirmed the association between TL and cancer survival, suggesting that TL is a predictive marker of cancer prognosis (22,23).

The HPV16 E6 and E7 proteins in patients with high-risk HPV infection can affect telomerase reverse transcriptase (TERT) expression, telomerase activity and TL (24–27), suggesting an association between TL and HPV-associated SCCOP. In our previous study of 188 patients with SCCOP, short lymphocyte TL (LTL) proved to be significantly associated with increased risk of SCCOP as well as with the SCCOP HPV status (28). However, the association between LTL and prognosis for SCCOP has yet to be reported. Given the association between LTL and risk of HPV-associated SCCOP and between LTL and tumor HPV status of SCCOP, we hypothesized that LTL is associated with prognosis for SCCOP, particularly in HPV-positive patients after definitive radiotherapy. Therefore, in this study, we evaluated the association between LTL and prognosis in SCCOP, further stratifying our analysis according to tumor HPV status.

Materials and methods

Study subjects

Patients with SCCOP were recruited consecutively from January 2000 to May 2013 as part of an ongoing molecular epidemiological study of squamous cell carcinoma of the head and neck at The University of Texas MD Anderson Cancer Center. All patients had newly diagnosed, histopathologically confirmed, untreated SCCOP and were recruited without any restrictions on age, sex, ethnicity, cancer stage or histology. As shown in Figure 1, all SCCOP patients in this study had SCCOP. These eight cases presented the imaging with different staining for the patients with SCCOP. Furthermore, all patients provided informed consent before enrollment. This study was approved by the MD Anderson Cancer Center Institutional Review Board. During the study recruitment, all patients completed a questionnaire to provide information on epidemiological,

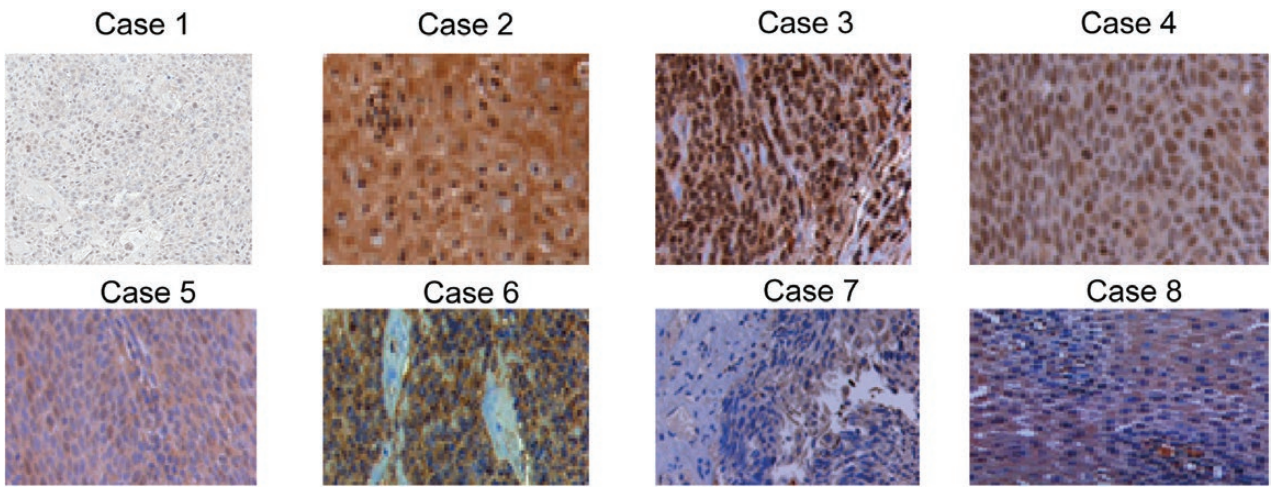

Figure 1. Imaging illustration of squamous cell carcinoma of the oropharynx of study patients.

demographic and risk factors, including smoking and alcohol status. Before treatment, each patient also had blood drawn (30 ml) for LTL determination. More details about this information are described in our previous report (29).

Clinical data were obtained *via* review of the patients' medical records. The sixth edition of the *American Joint Committee on Cancer* tumor-node-metastasis (TNM) staging system was used for determining SCCOP stage at the time of presentation. Medical comorbidities were classified according to a modification of the Kaplan–Feinstein comorbidity index (Adult Comorbidity Evaluation 27) (30), which scores comorbidities as none, mild, moderate or severe on the basis of the severity of individual organ decompensation and prognosis. The overall comorbidity score was assigned on the basis of the highest ranked single ailment. Patients were classified as ever-smokers if they had smoked ≥100 cigarettes in their lifetimes and as never-smokers if they had smoked <100 cigarettes in their lifetimes. Patients who had drunk at least one alcoholic beverage per week for ≥1 year were defined as ever-drinkers, whereas the rest were defined as never-drinkers.

Patients were monitored throughout their treatment and posttreatment courses using regularly scheduled clinical and radiographic examinations. Because universal standards for imaging were lacking, patients typically underwent routine serial imaging, and follow-up imaging was performed only when indicated by symptoms or findings of physical examinations. All of the patients received SCCOP treatment with curative intent at MD Anderson.

Tumor HPV16 status determination

For most of the SCCOP patients, DNA was extracted from paraffin-embedded tumor samples, and the presence of HPV16 in tumors was determined using PCR and *in situ* hybridization methods as described for our previous studies (31). For a small portion of the patients, tumor HPV16 status was determined on the basis of *in situ* hybridization and p16 immunohistochemical data from their clinical records in the pathology laboratory at MD Anderson. Personnel in this department routinely classify the HPV status of all SCCOP specimens as a standard clinical practice.

Measurement of pretreatment LTL

The relative mean LTL in each patient was determined using SYBR Green quantitative real-time PCR-based measurement of the ratio of telomere repeat units to a single-copy gene, as described previously (32,33). In brief, for each blood sample, genomic DNA was amplified for telomeric DNA and for human β2-globulin (HBG), a single-copy control gene that was used as an internal control to normalize the initial amount of DNA, using an Applied Biosystems 7900HT thermocycler (Foster City, CA) in a 384-well format. The telomere reaction mixture contained 5 ng of genomic DNA, 2× SYBR Green Master Mix, 200 nM Tel-1 primer (GGTTTTTGAGGGTGAG GGTGAGGGTGAGGGTGAGGGT) and 200 nM Tel-2 primer (TCCCGACTATC CCTATCCCTTCCCTATCCCTATCCCTA). The PCR analysis ran for one cycle at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and then 56°C for 1 min. The HBG reaction mixture consisted of 2× SYBR Green Master Mix, 200 nM HBG-1 primer (GCTTCTGACACAACTGTGTTCACTAGC) and 200 nM HBG-2 primer (CACCAACTTCATCCACGTTCACC). The HBG PCR analysis ran for one cycle at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and then 58°C for 1 min. Both telomeric DNA and HBG reactions were performed in duplicate.

For each assay, the fractional PCR cycle at which each reaction crossed a predefined fluorescence threshold was determined [the threshold cycle (Ct) value]. The initial template amount of genomic DNA and HBG was expected to be proportional to 2-Ct value. To correct for variations in genomic DNA concentration, the single-copy gene Ct value was subtracted from the telomere repeat unit Ct value (ΔCt). The relative telomere copy number per genome for each sample was then proportional to 2-ΔCt value. Each plate contained randomly selected samples to ensure equal representation of samples. The laboratory personnel were blinded to study patients' vital status. In each PCR run, negative (water) and positive controls, a calibrator DNA and a standard curve were included. The positive controls contained two telomeres of 1.2 kb and 3.9 kb, respectively, from a commercial TL assay kit (Roche Applied Science, Penzberg, Germany). For the standard curve, one reference DNA sample (the same DNA sample from a healthy control for all runs) was diluted using a 2-fold serial dilution to produce a 6-point standard curve between 20 ng and 0.625 ng of DNA in each reaction. The R^2 correlation for each standard curve was > 0.99, with an acceptable standard deviation of 0.25 for the Ct values. If the result was outside the acceptable range, the sample was repeated. To examine the interassay variation, two samples with relatively long and short TLs were tested using three different runs with an interassay variation < 3.6%. The $2^{\Delta Ct}$ method was used to calculate relative TL values in peripheral blood lymphocytes, and a standard curve was created in each PCR run to monitor PCR efficiency (34,35). We used the mean of TL among all patients who were alive as the cutoff point for the TL [(long (LTL ≥ 0.99) versus short telomeres (LTL < 0.99)]. Among the patients who died, the mean ± SD was: 0.97 ± 0.04, whereas for patients who were alive after final follow-up, the mean ± SD was: 0.99 ± 0.05.

Statistical analysis

Recurrent SCCOP was defined as the appearance of a new lesion of the same histology as the initial primary tumor (verified using incisional, excisional or needle biopsy analysis). Time to recurrence was the time from the end of treatment to the last follow-up examination or clinically detectable local, regional or distant recurrence. Patients who did not have recurrences or were lost to follow-up were censored. The overall survival (OS) duration was defined as the time from initial diagnosis to death of any cause or last follow-up examination. Patients who were alive at the end of the study or lost to follow-up also were censored. The disease-specific survival (DSS) duration was defined as the time from initial diagnosis to death due to SCCOP or last follow-up examination.

In a univariate analysis, epidemiological variables of the study patients assessed at the time of diagnosis, including age, ethnicity, sex, smoking and alcohol status, HPV status, and clinical characteristics such as index tumor stage, comorbidities, and treatment, were evaluated. Although the results of the univariate prognostic analysis were not statistically significant for some variables, these variables were retained in the main effects and final multivariable models owing to epidemiological and clinical considerations in building the model. Also, the Kaplan–Meier method was used to compare survival in patients with different LTLs, and the log-rank statistic was used to test the hypothesis that survival differed between the LTL groups. Next, how LTL affected survival and whether it was statistically associated with survival in SCCOP patients were investigated by fitting a Cox proportional hazards model that included age, sex, ethnicity, smoking history, alcohol consumption, disease stage, comorbidities and treatment as covariates. A similar multivariable analysis stratified according to tumor HPV status was performed. The SAS software program (version 9.4; SAS Institute, Cary, NC) was used for all statistical analyses. In all analyses, statistical significance was set at $P < 0.05$, and all tests were two-sided.

Results

Patient characteristics

Table 1 shows the demographic, epidemiological and clinical characteristics; outcomes and tumor HPV status for the 564 study patients. The median age at diagnosis was 55 years (range = 28–82 years). The patients were predominantly male (87%) and non-Hispanic white (92%). Of them, 50% and 67% were ever-smokers and ever-drinkers, respectively, and 94% had late-stage disease. Eighty-five percent of the patients had HPV16-positive SCCOP. All of the patients received radiotherapy, with 133 (24%) undergoing radiotherapy alone and 431 (76%) undergoing chemoradiation. The patients' median follow-up time after treatment was 34.8 months, with 64 patients having SCCOP recurrences and 78 patients dying of any cause (44 died of SCCOP).

Association between LTL and survival

Kaplan–Meier analysis demonstrated that patients with shorter LTLs had significantly better OS, DSS and disease-free survival

Table 1. Characteristics of the study patients with SCCOP (*N* = 564)

Characteristic	No. of patients (%)
Age	
≤57 years	343 (61)
>57 years	221 (39)
Sex	
Male	491 (87)
Female	73 (13)
Ethnicity	
Non-Hispanic white	518 (92)
Other	46 (8)
Smoking	
Never	280 (50)
Ever	284 (50)
Alcohol use	
Never	187 (33)
Ever	377 (67)
Index cancer stage	
I or II	36 (6)
III or IV	528 (94)
Comorbidities	
None or mild	511 (91)
Moderate to severe	53 (9)
Treatment	
X	133 (24)
X+C	431 (76)
Death, any cause	
Yes	78 (14)
No	485 (86)
Death due to SCCOP	
Yes	44 (8)
No	519 (92)
SCCOP recurrence	
Yes	64 (11)
No	500 (89)
Tumor HPV status	
Positive	482 (85)
Negative	82 (15)

X, radiotherapy; and C, chemotherapy.

(DFS) than did those with longer LTLs (log-rank $P < 0.001$; Figure 2). The distribution of the patients according to LTL and the associations between LTL and survival in the patients are shown in Table 2. After adjustment for other important prognostic confounders, including age, sex, ethnicity, smoking status, alcohol status, disease stage, comorbidities, HPV status and treatment, multivariable Cox regression analysis demonstrated that patients with short LTLs had a significantly lower risk of death overall [hazard ratio (HR) = 0.2; 95% confidence interval (CI) = 0.1–0.4], death due to SCCOP (HR = 0.2; 95% CI = 0.1–0.4) and SCCOP recurrence (HR = 0.3; 95% CI = 0.2–0.5) than did those with long LTLs after treatment.

Association between LTL and survival stratified according to tumor HPV16 status

Because we previously reported that LTL was significantly associated with tumor HPV status, with shorter LTLs being more likely than longer ones in HPV16-positive SCCOP patients (28), and because the majority of patients in this study had HPV16-positive SCCOP, we analyzed the association between LTL and survival separately in HPV16-positive and HPV16-negative cases. As shown in Figure 3, univariate survival analysis demonstrated that HPV19-positive SCCOP patients with shorter LTLs had significantly better OS, DSS and DFS than did those with longer LTLs (log-rank $P < 0.001$). However, we did not observe any significant survival differences in HPV16-negative SCCOP patients because of the small sample size or small number of outcome events in these patients. The associations between LTL and OS, DSS and DFS in the 482 HPV16-positive SCCOP patients and 82 HPV16-negative patients in our multivariable analysis are shown in Table 3. These association estimates were adjusted for potential confounders, including age, sex, ethnicity, smoking and alcohol status, disease stage, comorbidities and treatment. Compared with patients with long LTLs, those with short ones had a 5- to 10-times lower risk of death overall (HR = 0.1; 95% CI = 0.0–0.3), death from SCCOP (HR = 0.1; 95% CI = 0.0–0.3) and recurrence of SCCOP (HR = 0.2; 95% CI = 0.1–0.4; Table 3). We observed no such significant associations in the HPV16-negative SCCOP patients.

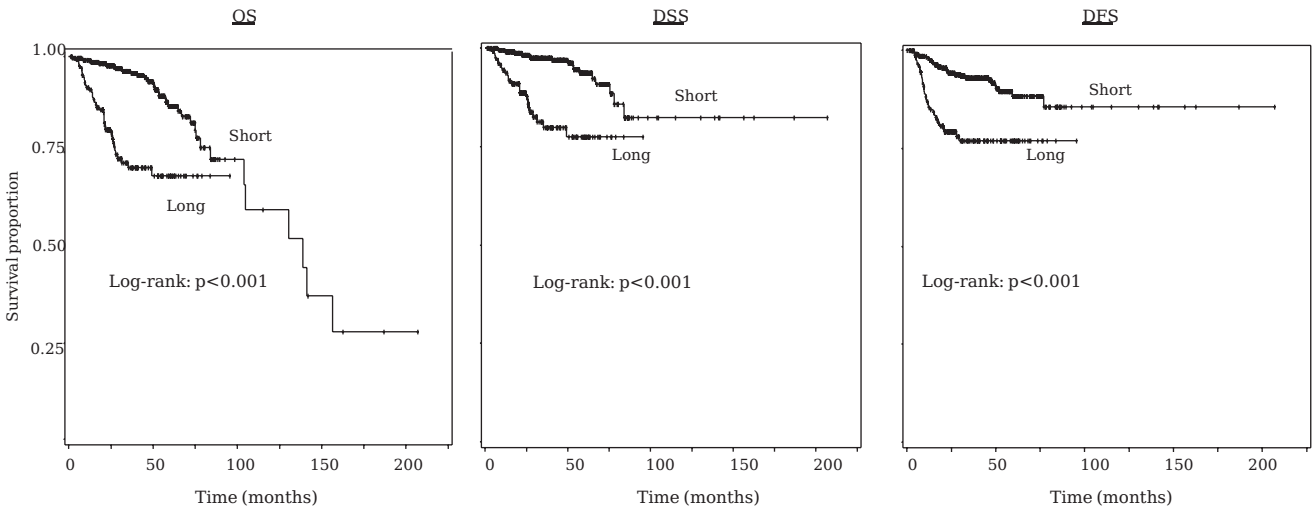

Figure 2. Survival according to LTL in all SCCOP patients (*N* = 564).

Table 2. Association of LTL with OS, DSS, and DFS in SCCOP patients (*N* = 564)

	OS			DSS			DFS		
LTL	Overall deaths/total (*N*)	Log-rank *P*	HR[a] (95% CI)	Deaths due to SCCOP/total (*N*)	Log-rank *P*	HR[a] (95% CI)	SCCOP recurrences/ total (*N*)	Log-rank *P*	HR[a] (95% CI)
		<0.001			<0.001			<0.001	
Long LTL[b]	40/171		1.0	26/171		1.0	34/171		1.0
Short LTL	38/393		0.2 (0.1–0.4)	18/393		0.2 (0.1–0.4)	30/393		0.3 (0.2–0.5)

[a]Adjusted for age, sex, ethnicity, smoking status, alcohol status, stage, comorbidities, HPV status and treatment.
[b]Reference group.

Discussion

In this study, we found that LTL was significantly associated with survival in SCCOP patients, particularly those with HPV-positive tumors. This suggests that LTL is a prognostic biomarker for HPV16-positive SCCOP.

TL causes chromosomal instability through the breakage-fusion-bridge cycle, which in turn leads to carcinogenesis in affected cells (36,37). Previous studies demonstrated that HPV16 E6/E7 increased telomerase activity in tumor cells by inducing telomerase hTERT expression (24,25,38,39). Also, we previously observed that LTL was significantly associated with risk of HPV-associated SCCOP but not risk of non-HPV-associated head and neck cancers and that SCCOP patients with shorter LTLs were more likely than those with longer LTLs to have HPV16-positive tumors (28). These results support the idea that the interaction between LTL and HPV affects SCCOP risk and prognosis. In this study, we found that patients with shorter LTLs had better outcomes than did those with longer LTLs, which is consistent with our previous report that SCCOP patients with shorter LTLs were more likely than those with longer LTLs to have HPV16-positive tumors and that patients with HPV16-positive SCCOP had better prognoses than did patients with HPV16-negative SCCOP.

The incidence of HPV-associated SCCOP, which is biologically and clinically distinct from tobacco- and alcohol-related SCCOP, is increasing. Whereas individuals with HPV-positive SCCOP generally have significantly better prognoses than do their HPV-negative counterparts, HPV-associated SCCOP is heterogeneous with regard to its biological and clinical behavior. Thus, patients with HPV-positive SCCOP may have different responses to chemoradiation, leading to different prognoses (1,12,40–43). Historically, TNM stage and smoking status were used to make treatment decisions for SCCOP patients. More recently, HPV status has become an important prognostic marker for SCCOP. However, this cancer is a biologically heterogeneous disease with heterogeneous clinical outcomes. Thus, these prognostic markers may be inadequate for accurate risk stratification of SCCOP patients, leading to undertreatment or overtreatment and, subsequently, negative effects on survival and quality of life. For example, although most patients with HPV-positive SCCOP have exceptional outcomes, the outcomes of smokers with HPV-positive SCCOP are worse than those of non-smokers and similar to those of patients with HPV-negative SCCOP. In another study of patients with late-stage SCCOP, the median time to progression did not differ between HPV-positive and -negative patients (5). Thus, new biomarkers are needed to identify SCCOP patients at high risk of death and/or recurrence and to facilitate the development of more specific, less toxic and more aggressive targeted treatment strategies to improve therapeutic efficacy and outcomes.

Studies of LTL as a marker for cancer survival have been controversial. In colorectal cancer, Chen *et al.* (44) found that patients with short LTLs had significantly worse OS and relapse-free survival than did those with long LTLs, whereas Garcia-Aranda *et al.* (45) and Jia *et al.* (46) found that long LTLs predicted poor cancer prognosis. For patients with breast cancer, some

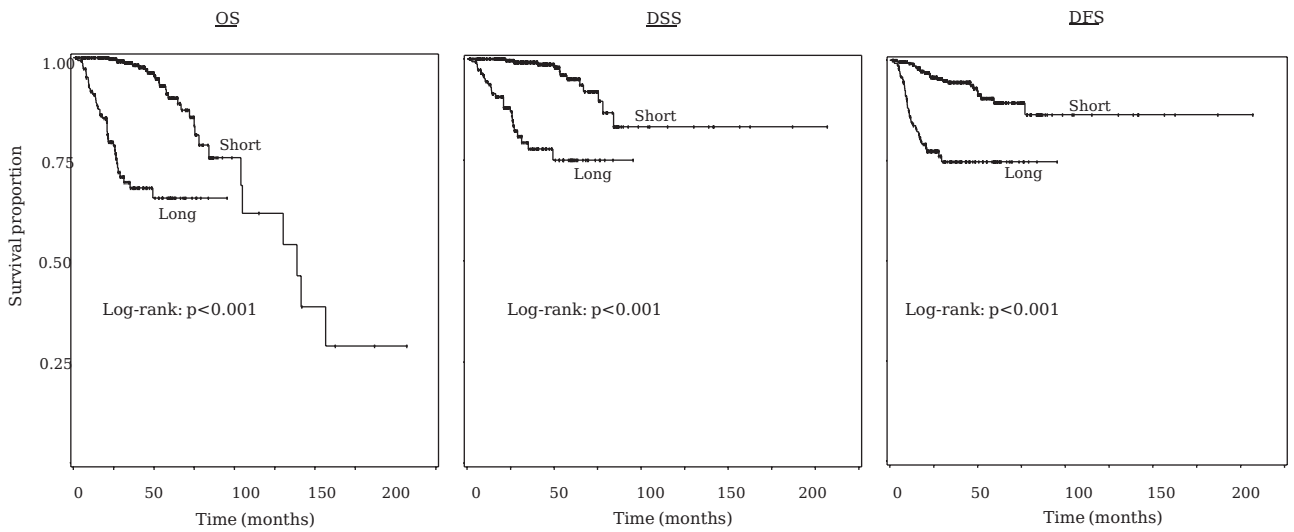

Figure 3. Survival according to LTL in HPV16-positive (*N* = 482) and HPV16-negative (*N* = 82) SCCOP patients.

Table 3. Association of LTL with OS, DSS and DFS in SCCOP patients stratified according to tumor HPV16 status

	OS			DSS			DFS		
LTL	Overall deaths/total (*N*)	Log-rank *P*	HR[a] (95% CI)	Deaths due to SCCOP/total (*N*)	Log-rank *P*	HR[a] (95% CI)	SCCOP recurrences/ total (*N*)	Log-rank *P*	HR[a] (95% CI)
HPV-positive SCCOP patients (*N* = 482)									
		<0.001			<0.001			<0.001	
Long LTL[b]	39/152		1.0	25/152		1.0	33/152		1.0
Short LTL	27/330		0.1 (0.0–0.3)	12/330		0.1 (0.0–0.3)	22/330		0.2 (0.1–0.4)
HPV-negative SCCOP patients (*N* = 82)									
		0.162			0.551			0.351	
Long LTL[b]	1/19		1.0	1/19		1.0	1/19		1.0
Short LTL	11/63		3.6 (0.5–29.0)	6/63		1.8 (0.2–15.5)	8/63		3.5 (0.4–28.4)

[a]Adjusted for age, sex, ethnicity, alcohol status, stage, comorbidities, and treatment.
[b]Reference group.

authors reported that long LTLs were associated with poor survival (47), whereas other researchers failed to observe a significant association between the two (48–50). Short telomeres are associated with shorter survival in classic and variant hairy cell leukemia (51) and non-small cell lung cancer (52) cases, whereas long LTLs are a prognostic factor for not only poor clinical outcome but also radiotherapy resistance in glioma cases (53). In a large prospective study of 47 102 individuals in the Danish general population, short LTLs were associated with reduced survival (23). Investigators have also conducted a meta-analysis of 33 published reports of 45 independent studies (22). The results of those studies indicated that LTL was an independent predictor of OS and chronic lymphocytic leukemia progression. Specifically, short LTLs were associated with a higher mortality rate in colorectal cancer and shorter OS in esophageal cancer but not other cancers (22). In this study, we found that shorter LTLs were associated with better survival in patients with HPV-associated SCCOP. In another recent study of the associations between HPV infection, LTL and prognosis in esophageal squamous cell carcinoma, Zhang *et al.* (54) proposed that long LTL was a marker of poor prognosis independent of other clinicopathological variables, which is consistent with the results of this study.

Because of these contradictory findings, a study with a larger cohort is needed to validate the results of our study. Furthermore, a more in-depth investigation is needed to explore the effects of the interaction between HPV infection and LTL on prognosis in SCCOP and to identify the mechanism(s) underlying these associations.

Radiotherapy is the primary treatment modality for SCCOP, particularly HPV-positive SCCOP. Radioresistant SCCOP cells are likely to give rise to local recurrence and distant metastatic relapse, reducing survival. Because (i) radiotherapy preferentially targets telomeres and reduces TL and (ii) shortening telomeres increases cell sensitivity to irradiation, LTL may affect SCCOP radiosensitivity. However, whether LTL regulates tumor radioresistance and can be exploited as a radiosensitizing target remains unclear. Therefore, once LTL is confirmed to be associated with SCCOP outcome, it will be important to examine whether LTL affects SCCOP radiosensitivity in a biologically relevant way, which will require more *in vitro* and *in vivo* experiments.

Although this study reveals significant associations between LTL and survival in HPV-associated SCCOP, some limitations should be addressed. First, LTLs may not reflect actual TLs in tumors, leading to some misclassification of study patients and thus biased estimates of the association. The assumption that LTL is reflected in SCCOP tissues needs further investigation, and measuring LTL at more than one time point should be considered, given its variability over time (55,56). Second, details about the chemotherapy treatments the study patients received are lacking, as we did not collect data on the dosage or duration of chemotherapy and radiotherapy. Well-designed studies with detailed treatment information and a uniform treatment plan are thus needed. Third, we recruited patients at a single cancer center, and the study population was primarily non-Hispanic white individuals, so the results may not be generalizable to other ethnic groups. Thus, prospective multicenter studies with larger sample sizes and more diverse study populations are needed to validate our findings.

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