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## Short telomere lengths in peripheral blood leukocytes are associated with an increased risk of oral premalignant lesion and oral squamous cell carcinoma

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### Abstract

**Background**—Oral premalignant lesions (OPLs) are precursors of oral squamous cell carcinoma (OSCC). Short telomeres in peripheral blood leukocytes are associated with increased risks of several cancers. However, whether short leukocyte telomere length (LTL) predisposes to OPL and OSCC is unclear.

**Methods**—LTLs were measured in PBLs of 266 patients with OPL (N=174) or OSCC (N=92) at diagnosis and 394 age- and gender-matched control subjects. The association between LTL and OPL or OSCC risk, as well as the interaction of telomere length, cigarette smoking and alcohol drinking on OPL or OSCC risk were analyzed.

**Results**—The age-adjusted relative LTL was the shortest in OSCC (1.64±0.29), intermediate in OPL (1.75±0.43), and longest in controls (1.82±0.36) (P for trend < 0.001). When dichotomized at the median value in controls, adjusting for age, gender, smoking and alcohol drinking status, the odds ratio (OR) for OPL and OSCC risks associated with short LTL was 2.03 (95% CI = 1.29–3.21) and 3.47 (95% CI = 1.84–6.53), respectively, with significant dose-response effects for both associations. Among 174 OPL patients, 23 progressed to OSCC and the mean LTL was shorter than in progressors than non-progressors (1.66±0.35 vs. 1.77±0.44), although the difference did not reach statistical significance (P=0.258) likely due to the small number of progressors. Interaction analysis shows that short LTL, smoking, and alcohol drinking are independent risk factors for OPL and OSCC.

**Conclusion**—Short LTL is associated with increased risks of developing OPL and OSCC and likely predisposes to the malignant progression of OPL patients.

### Keywords

Telomere length; peripheral blood leukocyte; oral premalignant lesion; oral squamous cell carcinoma; smoking; alcohol drinking

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## Introduction

Oral leukoplakia, oral submucous fibrosis, and erythroplakia are the three major forms of oral premalignant lesions (OPLs) and oral leukoplakia accounts for 85% of all OPLs<sup>1,2</sup>. Individuals with these lesions are at an elevated risk for developing oral squamous cell carcinoma (OSCC)<sup>3-6</sup>. Lee et al. reported that 31.4% of OPL patients developed cancers in their upper aerodigestive tracts during a 7-year follow-up period<sup>6</sup>. The overall malignant transformation rate of dysplastic lesions depends on the length of follow-up and varies from 11% to 36%<sup>5</sup>. Tobacco chewing, tobacco smoking, and alcohol drinking have been identified as major epidemiologic risk factors for OPLs and OSCCs<sup>7</sup>. Association studies on genetic risk factors have been emerging and results suggested that genetic variants play an important role in OPL etiology<sup>8-10</sup>.

The telomeres are the extreme ends of each chromosome and protect them from degradation and end-to-end fusion<sup>11</sup>. Human telomeres are progressively shortened with each cell division, thus are varied with age and cell types with length ranging from 6 to 12 kb in somatic cells<sup>12</sup>. Oxidative damage and loss of telomere-binding proteins can also contribute to telomere shortening<sup>13,14</sup>. The shortening can be compensated by the telomerase that is constitutively expressed in germ-line cells and in most malignant cells<sup>15</sup>.

Critically short telomeres become dysfunctional, and previous studies reported that telomere dysfunction is associated with the initiation and progression of malignancies in knockout mouse models<sup>16,17</sup> and human cancers<sup>18,19</sup>. In addition, constitutive short telomere length in peripheral blood leukocytes was reported to be associated with several human cancers<sup>19-25</sup>. A recent study showed OPL tissues had significantly shorter telomeres than normal epithelium<sup>26</sup>. However, no study has evaluated the association of LTL with the risk of OPL or OSCC. In this study, we aimed to investigate the association between LTL and the risk of OPL and OSCC using a case-control study design. Furthermore, we performed an exploratory analysis of the association of LTL with the progression of OPL using a prospective study design.

## Materials and Methods

### Study population and epidemiologic data

A total of 266 OPL (N=174) or OSCC (N=92) patients were included in this study. All cases were histologically confirmed OPL (leukoplakia and/or erythroplakia) or OSCC patients aged 18 years or older, as described previously<sup>27</sup>. Patients with acute intercurrent illnesses or infections and patients with prior history of cancer (except non-melanoma skin cancer) were excluded. Detailed clinical and follow-up information were abstracted from medical charts. A self-administered questionnaire was used to collect epidemiological data, including demographic characteristics, tobacco and alcohol use history. All patients donated a blood sample in heparinized tubes for molecular analyses prior to any treatment.

Healthy controls were identified from a pool of healthy subjects recruited in an on-going case-control study<sup>27</sup>. Briefly, the controls are recruited from the Kelsey-Seybold Clinic, the largest private multispecialty group practice in the Houston metropolitan area, with 18 clinics and more than 325 physicians. The majority of control participants were healthy individuals seen at the clinic for annual physical exams. On the day of the interview, the controls visited the clinic specifically for the purpose of participating in this study but not for any treatment purposes. Epidemiological questionnaire data were collected including demographic characteristics, tobacco use history, family history of cancer, occupational and environmental exposures, alcohol drinking habits, and medical history. A blood sample was collected from each participant into a heparinized tube and sent to the lab for molecular

analysis. Controls had no prior history of cancer (except for non-melanoma skin cancer) and were frequency matched to the OPL patients on age (+5 years), sex, and ethnicity. A total of 394 healthy controls were identified and included in this analysis.

For both cases and controls, a written informed consent was obtained for participating in the study and donation of blood samples. The study was approved by Institutional Review Boards of MD Anderson Cancer Center and Kelsey Seybold Clinics. An individual who had never smoked or had smoked <100 cigarettes in his or her lifetime was defined as a never smoker. A former smoker was a person who had quit smoking at least one year prior to diagnosis (cases) or who had quit smoking at least one year prior to the interview (controls). A current smoker was someone who was currently smoking or who had stopped less than one year prior to being diagnosed (cases) or interviewed (controls). Individuals who never consumed alcohol or had no more than one drink per month were defined as never drinkers, and those who had more than one drink per month were defined as ever drinkers (one bottle or can of beer, one medium glass of wine, one straight shot or one mixed drink was defined as one drink).

### Overall leukocyte telomere length assessment by real-time PCR

Genomic DNA was extracted from whole blood using the QIAamp Maxi DNA kit (Qiagen) following the manufacturer's protocol. The relative overall LTL was measured using a modified version of the real-time quantitative PCR as described by Cawthon<sup>28</sup>. The ratio of the telomere repeat copy number (T) to the single gene (human globulin) copy number (S) was determined for each sample. The derived T/S ratio was proportional to the overall telomere length.

The PCR (15  $\mu$ L) for the telomere amplification consisted of 1 $\times$  SYBR Green Mastermix (Applied Biosystems), 200 nmol/L Tel-1, 200 nmol/L Tel-2, and 5 ng of genomic DNA. The PCR for human globulin amplification consisted of 1 $\times$  SYBR Green Mastermix, 200 nmol/L Hgb-1, 200 nmol/L Hgb-2, and 5 ng of genomic DNA. The thermal cycling conditions were 95 $^{\circ}$ C for 10 min followed by 40 cycles of 95 $^{\circ}$ C for 15 s and 56 $^{\circ}$ C (for telomere amplification) or 58 $^{\circ}$ C (for Hgb amplification) for 1 min. The telomere and Hgb PCRs were done on separate 384-well plates, with the same samples in the same well positions. In each run, negative and positive controls, a calibrator DNA, and a standard curve were included. The positive controls contained a telomere of 1.2 kb and a telomere of 3.9 kb from a commercial telomere length assay kit (Roche Applied Science). For each standard curve, one reference DNA sample (the same DNA sample for all runs) was diluted 2-fold serially to produce a six-point standard curve between 20 and 0.625 ng of DNA in each reaction. The  $R^2$  for each standard curve was 0.99, with acceptable SDs set at 0.25 (for the Ct values). If the result was found to be out of the acceptable range, the sample was repeated. The intra-assay CV was <3% and the inter-assay CV was <5% for this assay in our laboratory<sup>23–25</sup>.

### Statistical analysis

All statistical analyses were performed using STATA, 10.0 statistical software (STATA Corp. College Station, TX). Pearson chi-square test was used to test the differences in the distribution of host characteristics between cases and controls for categorical variables and the *Student's t* test was used to test the differences for continuous variables. The association between OPL risk and telomere length was assessed using unconditional multivariate logistic regression to estimate the adjusted odds ratio (aOR) and 95% confidence interval (95% CI). Analyses were adjusted for age, sex, smoking status and alcohol drinking status where appropriate. We then added an interaction term to the logistic regression models to test the interaction between telomere length and smoking (or alcohol drinking) in elevating

the risk of OPL (or OSCC). All statistical tests were two-sided, and the level of statistical significance was set at 0.05.

## Results

Cases and controls were matched in terms of ( $P=0.373$ ) and gender ( $P = 0.894$ ). The vast majority of patients and controls were whites. There were significant differences in the distribution of smoking status between OPL/OSCC cases and controls, with higher percentage of current and former smokers in cases than in controls ( $P = 0.004$ ). Also, more cases were alcohol drinkers than the controls ( $P < 0.001$ ). The age-adjusted relative LTL was the shortest in OSCC ( $1.64\pm 0.29$ ), intermediate in OPL ( $1.75\pm 0.43$ ), and longest in controls ( $1.82\pm 0.36$ ) ( $P$  for trend  $< 0.001$ ) (Table 1).

When telomere length was dichotomized at the median value in controls, the adjusted odds ratio (aOR) for OPL associated with shorter telomere length was 2.03 (95% CI = 1.29–3.21) after adjusted for age, gender, smoking and alcohol drinking status. In quartile analysis in which the cutoff points were set using quartile values in controls, compared to the 4<sup>th</sup> quartile with the longest telomere length, the aORs were 0.99 (95% CI=0.52–1.90), 1.83 (95% CI=0.95–3.53), and 2.19 (95% CI=1.18–4.06) for the 3<sup>rd</sup>, 2<sup>nd</sup>, and 1<sup>st</sup> quartiles, respectively ( $P$  for trend=0.004) (Table 2, top panel). For OSCC, short LTL was associated with a 3.47-fold increased risk of OSCC (95% CI=1.84–6.53). In quartile analysis, compared to the 4<sup>th</sup> quartile with the longest telomere length, the aORs were 1.21 (95% CI=0.45–3.25), 2.69 (95% CI=1.06–6.87), and 4.92 (95% CI=2.04–11.8) for the 3<sup>rd</sup>, 2<sup>nd</sup>, and 1<sup>st</sup> quartiles, respectively ( $P$  for trend $<0.001$ ) (Table 2, bottom panel).

We further evaluated whether there is interactions between LTL and cigarette smoking or alcohol drinking by introducing an interaction term in the logistic regression models. Compared to never smokers with long telomeres, the risks of OPL for never smokers with short LTL, ever smokers with long LTL, and ever smokers with short LTL were 2.13 (95% CI= 1.10–4.12), 2.44 (95% CI=1.25–4.80), and 4.91 (95% CI=2.57–9.36), respectively ( $P$  for interaction=0.896). The risks of OSCC for never smokers with short LTL, ever smokers with long LTL, and ever smokers with short LTL were 4.51 (95% CI= 1.84–11.08), 3.67 (95% CI=1.32–10.21), and 9.90 (95% CI=3.82–25.66), respectively ( $P$  for interaction=0.406) (Table 3). There was no significant interaction between LTL and smoking in elevating OPL or OSCC risk. Neither was there significant interaction between LTL and alcohol drinking in elevating OPL or OSCC risk (data not shown). It appeared that LTL, smoking, and alcohol drinking are independent risk factors for OPL and OSCC.

We next examined the combined effects of having multiple risk factors. Compared to individuals without any of the three risk factors, those with all the three risk factors (LTL, smoking, and alcohol drinking) exhibited a 27-fold (OR=27.37, 95% CI=10.12–73.98) increased risk of OPL and 35-fold (OR=35.24, 95% CI=10.16–122.24) increased risk of OSCC (Table 4).

Finally, we explored whether short LTL at baseline could predict future OPL progression and death in OSCC patients. Among 174 OPL patients, 23 progressed to OSCC during follow-up. The mean LTL was shorter than in progressors than non-progressors ( $1.66\pm 0.35$  vs.  $1.77\pm 0.44$ ), although the difference did not reach statistical significance ( $P=0.258$ ) likely due to the small number of progressors. The mean LTL in progressors ( $1.66\pm 0.35$ ) at baseline was similar to OSCC patients ( $1.64\pm 0.29$ ). Among 92 OSCC patients, 20 died and their baseline mean LTL was  $1.59\pm 0.24$ , compared to  $1.65\pm 0.31$  for those 72 OSCC patients who were still alive. Again, the difference did not reach statistical significance ( $P=0.393$ ) due to limited number of death event.

## Discussion

Our data demonstrated that short LTL was associated with an increased risk of developing OPL and OSCC. Our data also suggested that OPL patients with short LTL are at an increased risk of progressing to OSCC, and OSCC patients with short LTL are at an increased risk of death.

There have been many reports suggesting that LTL are associated with cancer risk in a cancer type-dependent manner: both short and long telomeres can confer increased cancer risks<sup>20–22, 24, 25</sup>. A recent large cohort study of 47,102 individuals showed that if combining all cancer types, LTL was not associated with cancer risk<sup>22</sup>. However, given that different cancers have different etiology, pooling all cancer types together may mask the significant associations of LTL with individual cancer type. There have been consistent data showing that in some cancers, for example, melanoma<sup>22, 29, 30</sup> and sarcoma<sup>22, 25</sup>, longer LTLs conferred increased cancer risks; whereas in many others, shorter LTLs conferred increased cancer risks<sup>20–22</sup>. When pooling data for all the cancer sites, the opposite associations cancel each other out, resulting in the null finding for the overall cancer risk<sup>31</sup>.

There has not been prior study that examined specifically the association of LTL with the risk of OSCCs or OPLs. However, in the aforementioned large cohort study<sup>22</sup>, cancers of “oral cavity and pharynx” were included as a specific type. With 76 cases, the observed OR (95% CI) for short LTL with this cancer was 1.17 (0.90–1.53), consistent with our current study and supporting that short telomere length in PBLs is associated with increased risks of OPLs and OSCCs.

In humans, telomeres are maintained in germline cells, but shorten as somatic cells divide owing to the down-regulation of telomerase. Telomere shortening limits the replication of somatic cells. Cancer cells invariably maintain telomeres length through the expression of telomerase. Excessive telomere shortening prior to the expression of telomerase can lead to chromosomal fusion, which has been proposed as a mechanism for chromosome instability<sup>32</sup>. It was reported that the mean cytoplasmic human telomerase reverse transcriptase increased from normal through oral epithelial dysplasia to oral squamous carcinoma<sup>33</sup>. Markedly higher expression levels of human telomerase reverse transcriptase on oral dysplasia compared with normal tissues was reported<sup>34</sup>, which suggested that telomere length shortening is an early event during oral carcinogenesis. As for the potential mechanisms underlying the association of short LTL with increased risks of OPL and OSCC, we hypothesize that shorter LTL indicates higher genetic instability, leading to elevated risks. We previously showed that LTL was inversely correlated with baseline and mutagen-induced genetic instability in lymphocytes<sup>20</sup>, supporting this hypothesis. It should be noted that regulation of the telomere length in mammalian cells may be chromosome specific<sup>35, 36</sup>, and previous studies have identified telomere shortening in specific chromosomes associated with risk of different cancers<sup>23, 37</sup>. Thus, further investigation of chromosome-specific telomere length in OPL and OSCC risks is a promising future direction.

Cigarette smoking and alcohol drinking are two major risk factors of oral cancer<sup>38–40</sup>. We did not observe significant interaction between short LTL and these two risk factors in elevating OPL and OSCC risk. It appears that LTL is an independent risk factor for OPL and OSCC. However, individuals with three risk factors (short LTL, smoking, and alcohol drinking) exhibited 27- and 35-fold increased risks of OPL and OSCC, respectively. These data suggested that adding LTL as a biomarker to environmental risk factor profile can help identify high risk individuals and also point to the importance of preventing LTL attrition and stopping smoking and alcohol drinking in reducing the risks of OPL and OSCC.

In our exploratory prospective analysis, we observed that OPL patients who later progressed to OSCC had baseline LTL similar to OSCC patients, shorter than those who did not progress. We also observed that OSCC patients who died had shorter baseline LTL than those who were still alive. Due to limited numbers of progression and death events, these two comparisons did not reach statistical significance. However, the data suggest that baseline LTL can be a valuable predictor of future malignant progression of OPL patients and worse prognosis of OSCC patients. Future large prospective studies are warranted to confirm these observations.

In conclusion, the present study is the first molecular epidemiologic study of telomere length in PBLs and OPL susceptibility and progression. Intervention strategies aimed at preventing or reversing telomere shortening together with ceasing the behaviors of smoking and alcohol drinking may be effective in lowering the oral cancer incidence.

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**Table 1**

Distributions of demographic data of OPL/OSCC patients and control subjects.

<b>Variables</b>	<b>Controls (%)</b>	<b>OPL (%)</b>	<b>OSCC (%)</b>	<b>P-value *</b>
<b>Age year (SD)</b>	58.30(11.13)	57.38(11.61)	57.22(13.85)	0.373
<b>Gender</b>				
Male	226(57.36)	99(56.90)	55(59.78)	
Female	168(42.64)	75(43.10)	37(40.22)	0.894
<b>Ethnicity</b>				
White	356(90.36)	152(87.36)	89(96.74)	
Hispanic	17(4.31)	7(4.02)	0	
Black	14(3.55)	4(2.30)	1(1.09)	
Others	7(1.78)	11(6.32)	2(2.17)	0.022
<b>Smoking status</b>				
Never	207(52.54)	61(40.40)	35(42.68)	
Former	148(37.56)	58(38.41)	36(43.90)	
Current	39(9.90)	32(21.19)	11(13.41)	0.004
<b>Alcohol drinking status</b>				
Never	251(69.72)	41(27.70)	17(22.67)	
Ever	109(30.28)	107(72.30)	58(77.33)	<0.001
<b>Telomere length (Mean, SD)</b>	1.82(0.36)	1.75(0.43)	1.64(0.30)	<0.001

\* P-values were derived from the Pearson's chi-square test for gender, ethnicity, smoking and alcohol drinking status; Student's t-test was used for age. Trend test was used for telomere length. All P-values were two-sided.

**Table 2**

Risk estimates for telomere length and OPL or OSCC.

Variable strata	Telomere length <sup>d</sup>	Control (%)	OPL (%)	Adjusted or <sup>b</sup> (95% CI)	P-value
<b>Overall</b>	Long	197(75.19)	65(24.81)	1(ref)	
	Short	197(64.38)	109(35.62)	2.03(1.29–3.21)	0.002
<b>Quartile</b>					
4 <sup>th</sup> quartile		98(72.06)	38(27.94)	1(ref)	
3 <sup>rd</sup> quartile		99(78.57)	27(21.43)	0.99(0.52–1.9)	0.983
2 <sup>nd</sup> quartile		98(68.53)	45(31.47)	1.83(0.95–3.53)	0.073
1 <sup>st</sup> quartile		99(60.74)	64(39.26)	2.19(1.18–4.06)	0.013
P for trend					<0.001
		<b>Control (%)</b>	<b>OSCC (%)</b>		
<b>Overall</b>	Long	197(88.74)	25(11.26)	1(ref)	
	Short	197(74.62)	67(25.38)	3.47(1.84–6.53)	<0.001
<b>Quartile</b>					
4 <sup>th</sup> quartile		98(90.74)	10(9.26)	1(ref)	
3 <sup>rd</sup> quartile		99(86.84)	15(13.16)	1.21(0.45–3.25)	0.705
2 <sup>nd</sup> quartile		98(79.67)	25(20.33)	2.69(1.06–6.87)	0.038
1 <sup>st</sup> quartile		99(70.21)	42(29.79)	4.92(2.04–11.8)	<0.001
P for trend					<0.001

<sup>a</sup> Long and short groups were dichotomized at the medium telomere length in the controls.

<sup>b</sup> Adjusted by age, gender, ethnicity, smoking and alcohol drinking status where appropriate.

**Table 3**

Interaction of telomere length and cigarette smoking on OPL and OSCC risk

Telomere length <sup>a</sup>	Smoking status	Control (%)	OPL (%)	Adjusted OR <sup>b</sup> (95% CI)	P-value	Interaction P-value
Long	Never	108(78.26)	30(21.74)	1(ref)		
Short	Never	99(76.15)	31(23.85)	2.13(1.10–4.12)	0.025	
Long	Ever	89(74.79)	30(25.21)	2.44(1.25–4.80)	0.009	
Short	Ever	98(62.03)	60(37.97)	4.91(2.57–9.36)	<0.001	0.896
		Control (%)	OSCC (%)			
Long	Never	108(91.53)	10(8.47)	1(ref)		
Short	Never	99(79.84)	25(20.16)	4.51(1.84–11.08)	0.001	
Long	Ever	89(88.12)	12(11.88)	3.67(1.32–10.21)	0.013	
Short	Ever	98(73.68)	35(26.32)	9.90(3.82–25.66)	<0.001	0.406

<sup>a</sup> Long and short groups were dichotomized at the median telomere length in the controls.

<sup>b</sup> Adjusted by age, gender, ethnicity, and alcohol drinking status.

**Table 4**

Cumulative effect of the telomere length, alcohol drinking and cigarette smoking on OPL and OSCC risk

Number of Risk factor <sup>a</sup>	Control (%)	OPL (%)	Adjusted OR <sup>b</sup> (95% CI)	P-value
0	48(84.21)	9(15.79)	1(ref)	
1	179(82.49)	38(17.51)	1.21(0.54–2.72)	0.636
2	121(67.98)	57(32.02)	3.08(1.38–6.85)	0.006
3	12(21.43)	44(78.57)	27.37(10.12–73.98)	<0.001
P for trend				<0.001
	Control (%)	OSCC (%)		
0	48(90.57)	5(9.43)	1(ref)	
1	179(94.21)	11(5.79)	0.68(0.22–2.12)	0.504
2	121(78.57)	33(21.43)	3.63(1.29–10.26)	0.015
3	12(31.58)	26(68.42)	35.24(10.16–122.24)	<0.001
P for trend				<0.001

<sup>a</sup>Risk factors are short telomeres, ever smoking, and ever drinking<sup>b</sup>Adjusted by age, gender, and ethnicity.