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Comparison of Squamous Cell Carcinoma of the Tongue between Young and Old Patients

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Background: The worldwide incidence of squamous cell carcinoma of the tongue (SCCOT) in young patients has been increasing. We investigated clinicopathologic features of this unique population and compared them with those of SCCOT in the elderly to delineate its pathogenesis. **Methods:** We compared clinicopathological parameters between patients under and over 45 years old. Immunohistochemical assays of estrogen receptor, progesterone receptor, androgen receptor, p53, p16, mdm2, cyclin D1, and glutathione S-transferase P1 were also compared between them. **Results:** Among 189 cases, 51 patients (27.0%) were under 45 years of age. A higher proportion of women was seen in the young group, but was not statistically significant. Smoking and drinking behaviors between age groups were similar. Histopathological and immunohistochemical analysis showed no significant difference by age and sex other than higher histologic grades observed in young patients. **Conclusions:** SCCOT in young adults has similar clinicopathological features to that in the elderly, suggesting that both progress via similar pathogenetic pathways.

Key Words: Mouth neoplasms; Young adult; Smoking; Drinking; Immunohistochemistry

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Risk factors for squamous cell carcinoma of the tongue (SCCOT) are well known and include a history of smoking and alcohol consumption. Most incidences occur in older men;[1] however, several reports indicate that the global incidence of SCCOT in young, non-smoking women has been rising,[2-4] a trend also observed in the Korean population. A study by Choi et al.[5] also found that the incidence of SCCOT in younger patients, particularly women, has been increasing. SCCOT in young patients presents with similar clinical outcomes to those for older patients.[6,7] Distinct epidemiological features are apparent for these two cohorts, such as weaker association with smoking and drinking in younger patients,[4,8,9] suggesting that biology and pathogenesis in these groups might also be distinct.

This study was designated to compare a set of clinicopathological parameters between sexes (male and female) and age groups (young and old). Young was defined as adults under 45; no standard definition of young regarding SCCOT has been established; however, a cutoff of 45 is frequently used in related studies.[7,10,11]

Expression levels of several proteins were also compared through immunohistochemical (IHC) staining. Targeted proteins include p16, p53, mdm2, cyclin D1, glutathione S-transferase P1 (GSTP1), and estrogen receptor (ER), all of which were associated with tumorigenesis of SCCOT in previous studies.[12-17] p16, p53, mdm2, and cyclin D1 are essential to regulation of the cell cycle and apoptosis. Abnormal expression of these proteins in oral squamous cell carcinoma (SCC) has frequently been reported, implying an association with the pathogenesis and prognosis of SCCOT. Some reports have shown that expression levels vary by age groups.[12,15,18-20] GSTP1 is a member of the glutathione S-transferase enzyme superfamily that participates in detoxification processes. Exposure to toxic substances such as tobacco, alcohol, and betel is a major risk factor for SCCOT,[1] and impaired GSTP1 function has been associated with an increased cancer risk.[21]

The increasing proportion of women under 45 years old with SCCOT suggests potential involvement with sexual hormone receptors, and increased ER expression in SCCOT has been reported for oral SCC.[13,14] Recent studies found a relationship be-

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tween oropharyngeal SCC and hormone receptor expression.[22,23] We performed IHC stains for ER, progesterone receptor (PR), and androgen receptor (AR) to evaluate previous results and further analyze other clinicopathological features associated with hormone receptor expression.

MATERIALS AND METHODS

Study population

After searching an anonymized research database at Asan Medical Center, 295 cases of histologically confirmed SCCOT were found from 2005 to 2012. Ninety-six cases were excluded due to the lack of an available tissue block or clinical data, and 189 cases were finally retrieved for this retrospective study.

Clinical data collection

Clinical parameters collected from electronic medical records included age at onset, sex, smoking status, alcohol consumption level, treatment history, recurrence, and survival. Patients under 45 years old were classified as young patients. Smoking status was classified as non- or ex-smoker (no smoking for at least 1 year), or current smoker. Smokers were further categorized into light (< 1 pack of cigarettes/day) and heavy smokers (≥ 1 pack of cigarettes/day). Drinkers with a history of more than seven drinks per week were considered heavy drinkers. A drink was defined as roughly 14 g of pure alcohol regardless of beverage type, equivalent to approximately 12 ounces of regular beer. Cutoff values of heavy smoking and drinking were set by as described in previous work[24-26] wherein patients over these cutoff values showed a significantly increased risk of oral epithelial dysplasia or cancer. Disease-free survival (DFS) was calculated from the date of initial pathological diagnosis to the date of radiological or clinical recurrence, while overall survival (OS) was calculated to the date of patient death.

Pathological review and tissue microarray construction

All available hematoxylin and eosin slides were reviewed to obtain pathological parameters, such as tumor size, histologic grade, depth of invasion (DOI), stromal tumor-infiltrating lymphocytes (TIL) group, lymphovascular invasion (LVI), perineural invasion (PNI), and TNM stage. TNM stage for each case was revised based on the American Joint Committee on Cancer (AJCC), 8th edition.[27] TIL assessment criteria followed the International Immuno-Oncology Biomarker Working Group guidelines.[28,29] Each tumor was assigned to low, intermediate, or high TIL group according to the percentage of stromal area occupied by lymphocytic infiltrate (low, < 20%; intermediate, 20%–50%; high, > 50%). For the construction of tissue microarray (TMA) blocks, two tissue cores of approximately 2 mm were excised from the central area of the SCCOT and from tumor margins with normal mucosa.

Immunohistochemistry

IHC staining for p16 (1:6, clone E6H4, mouse mAb, Ventana Medical Systems, Tucson, AZ, USA), p53 (1:1,500, clone M7001, mouse mAb, Dako, Glostrup, Denmark), ER (1:200, clone 6F11, mouse mAb, Novocastra, Newcastle upon Tyne, UK), PR (1:200, clone 16, mouse mAb, Novocastra), AR (1:100, clone SP107, rabbit mAb, Cell Marque, Rocklin, CA, USA), mdm2 (1:50, clone SMP14, mouse mAb, Zeta, Arcadia, CA, USA), cyclin D1 (1:100, clone SP4, mouse mAb, Cell Marque), and GSTP1 (1:6,000, clone 3F2, mouse mAb, Cell Signaling, Danvers, MA, USA) was conducted in accordance with the manufacturer's manual on a Ventana BenchMark XT Autostainer (Ventana Medical Systems). Serially cut 4-μm sections of the TMA block were deparaffinized, and antigen retrieval was carried out with EDTA buffer (cell conditioner #1) for 32 minutes (p16, p53, PR, AR, cyclin D1, and GSTP1) or 64 minutes (mdm2 and ER). After inactivation of endogenous peroxidase and rinsing with Tris buffer (reaction buffer), diluted primary antibodies were added and incubated for 16 minutes (p16, p53, PR, AR, cyclin D1, and GSTP1) or 32 minutes (mdm2 and ER) at 37°C.

Expression levels of p53, p16, mdm2, and cyclin D1 were analyzed by a semiquantitative score based on the proportion of stained area (%) using criteria outlined in Table 1 and in reference to previous reports.[19,30,31] GSTP1 was interpreted as weak or strong by cytoplasmic staining intensity. IHC stains for ER,

Table 1. Interpretation criteria for immunohistochemical analysis of p53, mdm2, cyclin D1, and p16

Marker	Score	Cells stained (%)
p53	0	0
	1+	< 10
	2+	10 to < 50
	3+	≥ 50
mdm2	0	0
	1+	< 10
	2+	10 to < 50
	3+	≥ 50
Cyclin D1	Low	< 50
	High	≥ 50
p16	0	0
	1+	< 5
	2+	5 to < 25
	3+	≥ 25

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PR, and AR were considered as positive when nuclear expression was noted regardless of cellular proportion.

Statistical analysis

Statistical analysis was performed using SPSS ver. 25.0 (IBM Corp., Armonk, NY, USA). Results for age, tumor size, and DOI were described with mean and 95% confidence interval. The Mann-Whitney U test was used to compare tumor size and DOI between age and sex groups. The Kruskal-Wallis test was used for comparisons among the four groups (young men, young women, old men, and old women). Other clinicopathological parameters and IHC results were compared using Pearson's chi-square test and the Fisher's exact test. OS and DFS were analyzed according to the Kaplan-Meier method with univariate analysis (log-rank test). All calculated p-values were 2-sided, and values less than 0.05 were considered statistically significant.

Ethics statement

All procedures performed for the current study were approved by the Institutional Review Board (IRB) of Asan Medical Center (approval No. 2018-0395) in accordance with the 1964 Helsinki declaration and its later amendments. Formal written informed consent was waived by the IRB.

RESULTS

Patients were divided into four groups according to age and sex; young men, young women, old men, and old women. Clinical characteristics are listed in Table 2, and histopathological data are listed in Table 3. We also compared patients between age and sex groups. Clinicopathological data therein are included in the Supplementary Materials (Supplementary Tables S1–4).

Age at diagnosis ranged from 20.7 to 88.0 years (median, 56.1 years; 95% confidence interval, 52.88 to 57.26). Young patients accounted for 27.0% (51/189). Smoking and drinking status were markedly different between sexes, but not age groups (Tables 2, Supplementary Tables S1, 2). Most smokers (61/69, 88.4%) and regular drinkers (86/99, 86.9%) were men. Proportions of heavy smokers and drinkers were slightly lower in young patients, but these differences were not statistically significant. Women comprised 40.2% (72 of 189) of all patients. More women were in the young patient group (25 of 51, 49%), and more likely to be young non- or ex-smokers (21 of 30, 70%). OS and DFS were not significantly different by age (young or old) or sex (Fig. 1). Young women presented with relatively lower mortality rates (28.0%) than the other groups combined (48.2%), but this difference was not statistically significant (p=.082). Relatively poor survival among young men (50.0% vs 44.8% of others) was also observed but not statistically significant (p=.678).

Histologic grade tended to be higher in young patients (p=.021) but did not vary between sexes (Supplementary Tables S3, 4). When we compared the four groups divided by sex and age, none of the histopathological parameters including tumor size,

Table 2. Demographics and clinical information

Characteristic	Young (<45 yr, n=51)		Old (≥45 yr, n=138)		p-value
	Men (n=26)	Women (n=25)	Men (n=87)	Women (n=51)	
Age (yr)					<.001
Mean (95% CI)	35.5 (32.8–38.2)	34.8 (32.0–37.6)	61.5 (59.6–63.5)	64.0 (60.8–67.2)	
Smoking status					<.001
Non- or ex-smoker	9 (34.6)	21 (84.0)	43 (49.4)	47 (92.2)	
Light smoker	7 (26.9)	3 (12.0)	10 (11.5)	1 (2.0)	
Heavy smoker	10 (38.5)	1 (4.0)	34 (39.1)	3 (5.9)	
Alcohol use					<.001
Abstain	6 (23.1)	20 (80.0)	24 (27.6)	45 (88.2)	
Light drinker	15 (57.7)	3 (12.0)	35 (40.2)	6 (11.8)	
Heavy drinker	5 (19.2)	2 (8.0)	28 (32.2)	0	
Adjuvant treatment					.550
None	9 (34.6)	11 (44.0)	42 (48.3)	25 (49.0)	
Radiotherapy	12 (46.2)	8 (32.0)	26 (29.9)	20 (39.2)	
Chemotherapy	0	0	5 (5.7)	1 (2.0)	
Chemo+radiotherapy	5 (19.2)	6 (24.0)	14 (16.1)	5 (9.8)	
Recurrence (%)	11 (42.3)	9 (36.0)	25 (28.7)	18 (35.3)	.597
Deceased (%)	13 (50.0)	7 (28.0)	42 (48.3)	24 (47.1)	.309

Values are presented as number (%) unless otherwise indicated.
CI, confidence interval.

Table 3. Histopathologic data and stage

Characteristic	Young (<45, n=51)		Old (≥45, n=138)		p-value
	Men (n=26)	Women (n=25)	Men (n=87)	Women (n=51)	
Tumor size (cm)					.868
Mean (95% CI)	2.7 (2.1–3.3)	2.6 (2.0–3.1)	2.5 (2.2–2.8)	3.0 (2.1–3.8)	
Depth of invasion (mm)					.479
Mean (95% CI)	11.9 (9.0–14.8)	11.9 (8.4–15.3)	10.1 (8.5–11.6)	10.7 (9.0–12.4)	
Histological grade					.102
Well differentiated	11 (42.3)	7 (28.0)	45 (51.7)	26 (51.0)	
Moderately differentiated	8 (30.8)	13 (52.0)	35 (40.2)	19 (37.3)	
Poorly differentiated	7 (26.9)	5 (20.0)	7 (8.0)	6 (11.8)	
Lymphovascular invasion					.625
Present	6 (23.1)	6 (24.0)	63 (72.4)	42 (82.4)	
Absent	20 (76.9)	19 (76.0)	24 (27.6)	9 (17.6)	
Perineural invasion					.661
Present	11 (42.3)	10 (40.0)	29 (33.3)	22 (43.1)	
Absent	15 (57.7)	15 (60.0)	58 (66.7)	29 (56.9)	
Tumor-infiltrating lymphocyte					.335
Low	13 (50.0)	14 (56.0)	53 (60.9)	28 (54.9)	
Intermediate	10 (38.5)	7 (28.0)	24 (27.6)	10 (19.6)	
High	3 (11.5)	4 (16.0)	10 (11.5)	13 (25.5)	
T category					.569
T1	4 (15.4)	2 (8.0)	23 (26.4)	26.4 (10.0)	
T2	9 (34.6)	13 (52.0)	30 (34.5)	34.5 (17.0)	
T3	13 (50.0)	10 (40.0)	33 (37.9)	37.9 (24.0)	
T4	0	0	1 (1.1)	0	
N category					.849
N0	11 (42.3)	13 (52.0)	47 (54.0)	29 (56.9)	
N1	5 (19.2)	4 (16.0)	12 (13.8)	9 (17.6)	
N2	6 (23.1)	3 (12.0)	18 (20.7)	9 (17.6)	
N3	4 (15.4)	5 (20.0)	10 (11.5)	4 (7.8)	

Values are presented as number (%) unless otherwise indicated.
CI, confidence interval.

DOI, histologic grade, LVI, PNI, TIL group, T and N category were significantly different for any group (Table 3). Notably, higher TIL was correlated to better OS (p=.002) and DFS (p=.017) rates (Fig. 2).

IHC stains for sex hormone receptors were positive in a small number of patients. Only a single case presented with nuclear expression of ER (51.1-year-old male, non-smoker) and two cases presented with expression of AR (case 1, 40.2-year-old female, non-smoker; case 2, 56.7-year-old, male, non-smoker). PR was not expressed in any of the study participant samples. Expression levels as determined by IHC stainings of p16, p53, mdm2, GSTP1, and cyclin D1 were compared between patients grouped by age, sex, and smoking and drinking status, and no significant differences were found for any cohort (Table 4). Among immunomarkers, cyclin D1 expression was correlated to OS (p=.009) and DFS (p=.011) (Fig. 3). Other markers (p53, mdm2, p16, and GSTP1) were not correlated to any clinicopathological parameters.

DISCUSSION

SCCOT in young and non-smoking patients has been reported since the 1980s but became a substantial issue after 2000 when epidemiologic evidence demonstrated an increasing incidence in this group. Previously, physicians thought that young SCCOT patients had poorer prognoses, and more aggressive treatments were used in this population. However, this notion has yet to be empirically substantiated.[7] Many studies attempted to find biological factors unique to young patients with SCCOT, but distinctive features were not found.[11,32,33] Indeed, SCCOT in young and old patients has not been found to exhibit relevant difference at the molecular level.[34] Pickering et al.[10] found certain genomic similarities between SCCOT in young patients and older smokers by whole-exome sequencing. However, these studies were mainly conducted by Western countries so data from Asian populations are insufficient. Recently, Sun et al.[35] reported that prognoses for young Chinese patients with oral SCC were similar to those for

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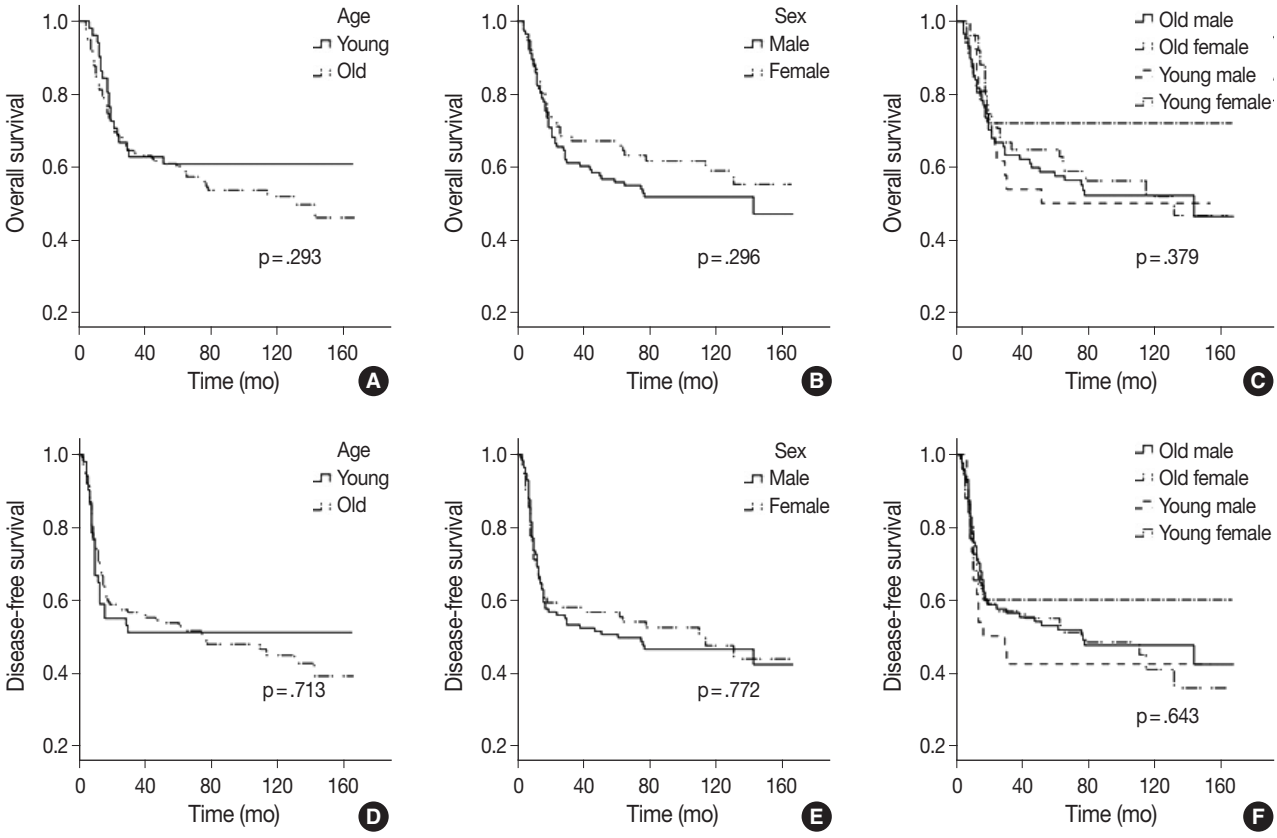

Fig. 1. Kaplan-Meier estimation of overall survival and disease-free survival curves for age and sex. Neither overall survival (A–C) nor disease-free survival (D–F) was significantly associated with age or sex.

older patients. In this study, we also found equivalent results for old and young patients from Korea.

Briefly, the only clinicopathological parameter that differed between young and old patients was histological grade (Table 3), which was found to be worse in the young group. Male-to-female ratios were also not statistically different by age, although a relatively higher proportion of women was observed in young patients. This tendency became more significant in the young, non-smoking group where women were predominant (21 of 27, 77.8%). This finding recapitulated those from previous studies reporting that young women with no history of tobacco or alcohol use were more vulnerable to SCCOT than their male counterparts.[4] This epidemiologic peculiarity aroused our interest in a potential relationship between SCCOT and sex hormone receptor expression.

Previous studies reported ER expression in 11% to 50% of SCCOT cases.[13,14,36] In this study, however, we only observed focal ER expression in a single patient, despite the use of automated IHC staining with a well-established primary antibody and staining protocol. The only ER-expressing tumor found was that of an older male with no history of smoking. Interestingly, in other reports, most patients presenting with ER-positive head and neck cancer were also older males.[13,14] These results suggest that ER involvement might be primarily related to the original patient group of older men. PR was negative in all cases; this is also consistent with a previous report.[14] Immunoreactivity with nuclear expression for AR was seen in two cases, in contrast to previous reports reporting AR positivity in up to 67% of patients, most of them expressing AR in the cytoplasm.[37] Since true positivity for AR requires nuclear expression, the importance of AR expression in young SCCOT patients seems to be limited.

Inter-individual variation in metabolic capacity for toxins could influence the carcinogenesis of SCCOT in young patients. GSTP1 is an important detoxifying enzyme, but a relationship between GSTP1 and oral carcinogenesis remains unclear. Genetic polymorphisms in the *GSTP1* gene has been reported to be associated with impaired metabolism of carcinogens, thereby elevating the risk of several tumors, including head and neck cancer.[21,38] Soares et al.[17] observed increased GSTP1 expression in non-tumor margins in both smoking and drinking patients and suggested that this result could be a reaction to carcinogen exposure. In the current study, all tumors presented with diffuse GSTP1 expres-

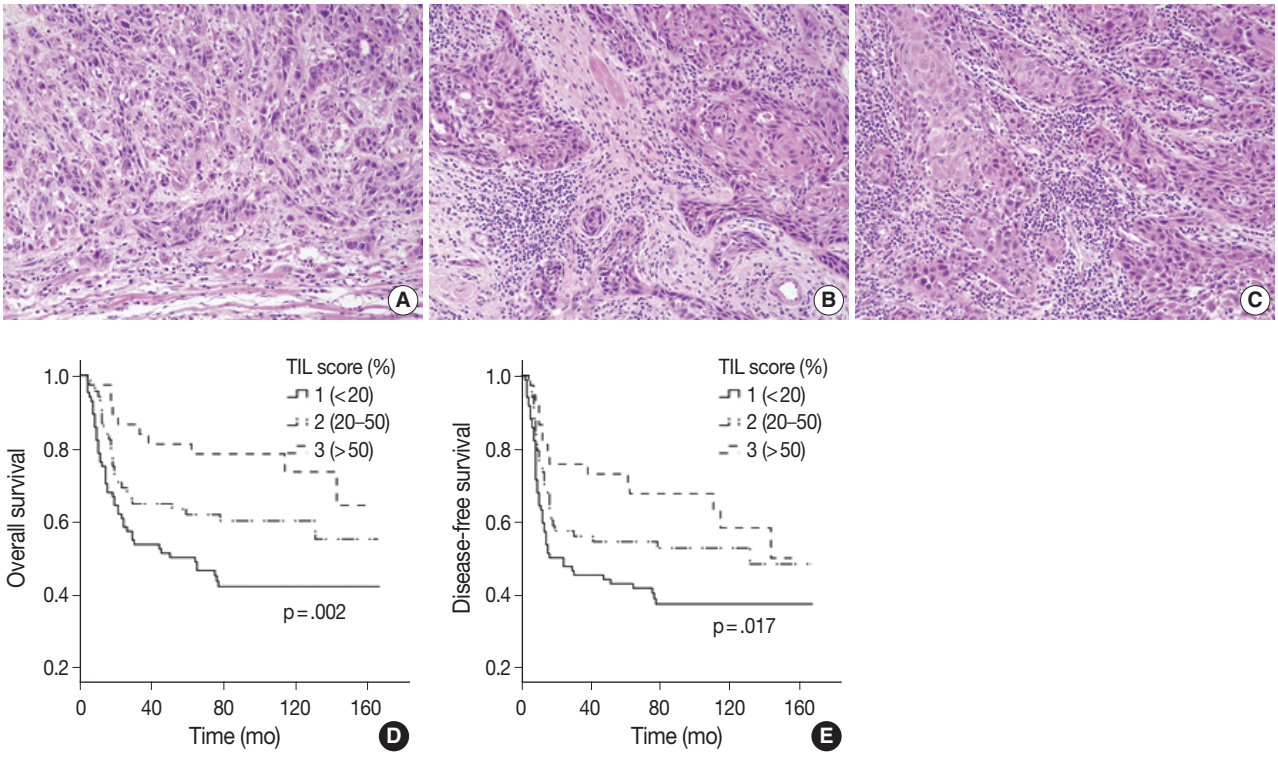

Fig. 2. Examples of tumor-infiltrating lymphocyte (TIL) scores and Kaplan-Meier survival curves. Each tumor was given a TIL group depending upon the amount of stromal lymphocytic infiltration. Images represents low (A), intermediate (B), and high (C) group. Higher TIL groups were associated with better overall survival (D) and disease-free survival (E).

Table 4. Expression profiles of immunomarkers by age, sex, smoking, and alcohol use

	Age		p-value	Sex		p-value	Smoking			p-value	Alcohol use			p-value
	Young (<45 yr)	Old (≥45 yr)		Male	Female		Non- or ex-smoker	Light smoker	Heavy smoker		Abstain	Light drinker	Heavy drinker	
p16			.072			.893				.465				.307
0	27 (52.9)	77 (55.8)		60 (53.1)	44 (57.9)		61 (50.8)	12 (57.1)	31 (64.6)		52 (54.7)	29 (49.2)	23 (65.7)	
1+	8 (15.7)	39 (28.3)		29 (25.7)	18 (23.7)		34 (28.3)	4 (19.0)	9 (18.8)		21 (22.1)	21 (35.6)	5 (14.3)	
2+	7 (13.7)	11 (8.0)		12 (10.6)	6 (7.9)		12 (10.0)	1 (4.8)	5 (10.4)		10 (10.5)	4 (6.8)	4 (11.4)	
3+	9 (17.6)	11 (8.0)		12 (10.6)	8 (10.5)		13 (10.8)	4 (19.0)	3 (6.3)		12 (12.6)	5 (8.5)	3 (8.6)	
p53			.443			.596				.739				.676
0	9 (17.6)	20 (14.5)		18 (15.9)	11 (14.5)		19 (15.8)	5 (23.8)	5 (10.4)		13 (13.7)	11 (18.6)	5 (14.3)	
1+	15 (29.4)	28 (20.3)		25 (22.1)	18 (23.7)		26 (21.7)	5 (23.8)	12 (25.0)		18 (18.9)	14 (23.7)	11 (31.4)	
2+	3 (5.9)	13 (9.4)		12 (10.6)	4 (5/3)		9 (7.5)	1 (4.8)	6 (12.5)		8 (8.4)	6 (10.2)	2 (5.7)	
3+	24 (47.1)	77 (55.8)		58 (51.3)	43 (56.6)		66 (55.0)	10 (47.6)	25 (52.1)		56 (58.9)	28 (47.5)	17 (48.6)	
mdm2			.442			.403				.464				.877
0	31 (60.8)	72 (52.2)		57 (50.4)	46 (60.5)		69 (57.5)	9 (42.9)	25 (52.1)		55 (57.9)	30 (50.8)	18 (51.4)	
1+	19 (37.3)	59 (42.8)		51 (45.1)	27 (35.5)		45 (37.5)	12 (57.1)	21 (43.8)		36 (37.9)	26 (44.1)	16 (45.7)	
2+	1 (2.0)	7 (5.1)		5 (4.4)	3 (3.9)		6 (5.0)	0	2 (4.2)		4 (4.2)	3 (5.1)	1 (2.9)	
3+	0	0		0	0		0	0	0		0	0	0	
Cyclin D1			.247			.771				.883				.662
Low	16 (31.4)	56 (40.6)		44 (38.9)	28 (36.8)		46 (38.3)	7 (33.3)	19 (39.6)		38 (40.0)	23 (39.0)	11 (31.4)	
High	35 (68.6)	82 (59.4)		69 (61.1)	48 (63.2)		74 (61.7)	14 (66.7)	29 (60.4)		57 (60.0)	36 (61.0)	24 (68.6)	
GSTP1			.082			.952				.599				.266
Weak	11 (21.6)	16 (11.6)		16 (14.2)	11 (14.5)		18 (15.0)	4 (19.0)	5 (10.4)		16 (16.8)	9 (15.3)	2 (5.7)	
Strong	40 (78.4)	122 (88.0)		97 (85.8)	65 (85.5)		102 (85.0)	17 (81.0)	43 (89.6)		79 (83.2)	50 (84.7)	33 (94.3)	

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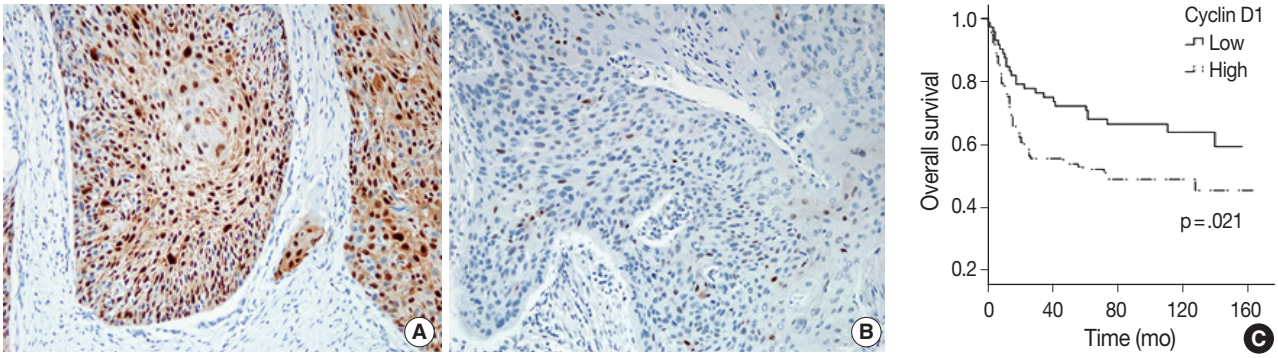

Fig. 3. Immunohistochemistry for cyclin D1 and Kaplan-Meier survival curve. Tumor cells showing strong (A) or weak (B) nuclear expression of cyclin D1. Strong expression of cyclin D1 correlated with poor overall survival (C).

sion and heterogeneous staining intensities. Overall intensity scores did not vary by age, sex or smoking or drinking status. We also found that strong GSTP1 expression was not related to prognosis. Epithelial tissues in non-tumor margins showed similar or slightly weaker intensities than those of matched tumor cells. These results suggest that expression of GSTP1 is not a suitable marker for individual cancer susceptibility or toxin exposure related to SCCOT.

Other IHC markers, p53, cyclin D1, mdm2, and p16, were variably positive in a significant proportion of cases but their expression levels were not statistically different by age, sex or smoking or drinking status. Strong expression of cyclin D1 was associated with poor prognosis, a finding that is consistent with previous reports.[15,19,39] However, the lack of standardized interpretation criteria of cyclin D1 expression parameters compromises the reliability and integrity of these results. The relationship between cyclin D1 and SCCOT is worthy of further investigation, particularly with respect to standardizing the interpretation criteria. No standardized TIL assessment guideline for oral SCC exists, either. Several reports suggest a prognostic impact of TIL on oral SCC[40,41] but they used different assessment methods and demonstrated conflicting results. We applied the TIL assessment method used in breast cancer[29] and found a significant correlation between TIL and OS, implying that this method might be a candidate for standardized assessment.

This study evaluated the clinicopathological parameters and expression profiles of several tumorigenic candidate proteins of SCCOT and found no significant difference between young and old patients nor between male and female patients. Despite epidemiologic idiosyncrasy, SCCOT in young women appears to be similar to that in older men. Previous studies reported similar data and came to similar conclusions.[6-8,10,11,42] These results, combined with epidemiological data, suggest the presence of unknown carcinogenic factors contributing to an emerging incidence of SCCOT in young women via a similar pathogenetic sequence to that associated with known risk factors. We investigated two candidates for these factors, hormone receptors and GSTP1, but significant findings were not observed. Considering that known risk factors are primarily associated with toxin exposure (tobacco, betel quid, or alcohol), extrinsic factors appear to be more important than individual factors of age, sex, or intrinsic metabolic activity in the pathogenesis of SCCOT. Possible exposure to toxic materials associated with altered lifestyles or new environmental pollutants should thereby be investigated in the young female SCCOT population.

Electronic Supplementary Material

Supplementary materials are available at Journal of Pathology and Translational Medicine (https://jpatholtm.org).

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Funding acquisition: KJC.
Investigation: GC.
Methodology: KJC.

Project administration: GC.
Resources: SHC, SYN, SYK, JLR, BKL.
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Conflicts of Interest

The authors declare that they have no potential conflicts of interest.

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