Determination of the origin of oral squamous cell carcinoma by microarray analysis: Squamous epithelium or minor salivary gland?

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More than 90% of oral cancers are histopathologically squamous cell carcinomas (SCCs). According to clinical behavior and histopathological features, we hypothesize that oral SCC can originate from either oral squamous epithelium or minor salivary glands. Here, we examined whether some oral SCCs originate from minor salivary glands, and investigated whether these tumors show particularly aggressive biological behavior. The mRNA expression profiles of samples obtained from six patients with oral floor SCC (five men, one woman; mean age, 62.7 years) were analyzed using a microarray containing 32,878 probes. The six samples were divided into two groups by clustering of expression levels of 845 probes differentially expressed in normal oral squamous epithelium and normal salivary glands. The expression profile in four cases was similar to that of normal oral squamous epithelium, and in two cases was similar to that of normal salivary glands. Furthermore, we identified nine genes that reveal the origin of the oral SCC. Subsequently, we examined the expression levels of these nine marker genes by reverse transcriptase-polymerase chain reaction to determine the origin of 66 oral SCCs. Twelve of the 66 oral SCCs were considered to originate from minor salivary glands, and these tumors showed high metastatic potential ($p = 0.044$, Chi-square test). Furthermore, SCC derived from minor salivary glands showed a poor event-free survival rate ($p = 0.017$, Kaplan–Meier analysis). In conclusion, determination of the origin of oral SCC is helpful in planning treatment for patients with oral SCC.

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Oral squamous cell carcinoma (SCC) is characterized by a high degree of local invasiveness and a high rate of metastasis to cervical lymph nodes, but a low rate of metastasis to distant organs.[1,2] Several investigators have attempted to

identify the factors associated with biological aggressiveness of oral SCC.[3,4] Many oncogene products,[5–9] mutated tumor suppressor gene products,[10–12] cell adhesion molecules,[13–15] matrix metalloproteinases[16–18] and cell cycle-associated molecules[19–21] have been proposed as possible factors controlling the biological behavior of oral SCC. However, no distinct factors controlling the aggressiveness of oral SCC have been identified to date. In addition, although several pathologists have attempted to identify the histological characteristics of oral SCC with metastatic potential,[22] it is difficult to predict the metastatic potential of oral SCC from its histopathological features alone. At present, therefore, the biological aggressiveness of oral SCC cannot be predicted from its superficial phenotype or the morphology of the cancer cells. It is necessary to examine the fundamental properties of the cancer cells, either determining their origin or examining stem cells, to predict biological aggressiveness.

Almost 30 years ago, we established the cell line, TYS, from well-differentiated SCC of the oral floor.[23] Interestingly, TYS cells exhibited a high level of carcinoembryonic antigen expression, which is a known tumor marker for adenocarcinoma, and the cells formed adenosquamous cell carcinoma in nude mice. Moreover, we reported that HSG cells, which were established from the irradiated submandibular gland,[24] formed adenocarcinoma in nude mice and could differentiate into several different cell types on treatment with different

What's new?

In spite of several past attempts, no distinct factors controlling the biological aggressiveness of oral squamous cell carcinoma (SCC) have been identified. Determining the origin of the cancer cells could help predict biological aggressiveness, however. Here, the authors provide evidence that oral SCC has two different origins, as mucosal or salivary SCC. Furthermore, they identify nine genes able to reveal the origin of oral SCC. Based on their expression levels, the authors determined that twelve of the 66 oral SCCs in their sample originated from minor salivary glands. Salivary SCC tumors showed high metastatic potential and poor event-free survival rate.

differentiation inducers.[25,26] During these experiments, we found that HSG-derived cells differentiated into keratinizing SCC on treatment with retinoic acid.[27] These experiments suggest that some population of salivary gland cells may be parental cells to SCC in the oral cavity.

Based on the results of our previous basic research,[23–27] we hypothesized that some oral SCCs may originate from minor salivary glands. Thus, oral SCC may consist of two different cancers: SCC derived from squamous epithelium (mucosal-SCC) and SCC derived from minor salivary glands (salivary-SCC). Salivary gland cancer is generally more resistant to radiation therapy and chemotherapy, metastasizes frequently to lymph nodes and distant organs and shows poor prognosis compared to mucosal-SCC.[28] Therefore, it is important to know the origin of SCC of the oral cavity to provide appropriate treatment.

In our study, we attempted to discriminate salivary-SCC from mucosal-SCC of the oral floor by microarray and clustering analyses, and identified nine molecular markers that allow us to identify salivary-SCC in oral SCC. Furthermore, we compared the biological aggressiveness and prognosis of salivary-SCC and mucosal-SCC, and clarified that salivary-SCC showed more aggressive biological behavior and poorer prognosis than mucosal-SCC.

Material and Methods

Patients

Samples from six patients with SCC of the oral floor (five men, one woman) were subjected to microarray analysis (Fig. 1a, Table 1). Histopathological diagnoses were made by experienced pathologists in our hospital; all were diagnosed as SCC (Fig. 1b). The mode of cancer cell invasion was assessed using the Yamamoto–Kohama (Y-K) mode of invasion classification for oral SCC[22]; most of the cancers used for microarray analysis were advanced (Stage III or Stage IVa) and highly invasive (Y-K3 or Y-K4D).

Samples from 66 patients with oral SCC (39 men, 27 women) were subjected to expression analysis by reverse transcription-polymerase chain reaction (RT-PCR). Each tumor had been surgically resected at our hospital between 1998 and 2009. The patients had not received any previous radiation therapy or chemotherapy. Gender, age, primary site, TN classification, clinical stage, tumor cell differentiation (WHO classification),[29] Anneroth's grade,[30] Y-K mode of invasion[22] and pathological lymph node metastasis of the oral SCCs are shown in Table 2. Tissue samples from the patients were fixed in formalin and embedded in paraffin, and sections 0-μm thick were stained with hematoxylin and eosin for histopathological diagnosis.

Cell lines

The human oral SCC cell lines, OSC-20 and HSC-4, were obtained from the Department of Oral and Maxillofacial Surgery, Kanazawa University, School of Medicine.[31,32] TYS cells are human oral adenosquamous carcinoma cells previously established by our research group.[23] OSC-20, HSC-4 and TYS cells were cultured in complete media consisting of Dulbecco's modified Eagle's medium (DMEM: Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS: Eqitech Bio, Kerrville, TX), 100 μg/mL streptomycin, 100 U/mL penicillin (Meiji Seika, Tokyo, Japan) and 0.25 μg/mL amphotericin B (Sigma-Aldrich, St. Louis, MO) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

To confirm the origin and identity of the cells used in this experiment, we performed short tandem repeat (STR) analysis using 10 loci (Supporting Information Table S1). As all of the cell lines used showed clear peaks with primers for human cells, these cells could be considered as being of human and not rodent origin. Furthermore, as the evaluation values (EV) among these cells were <0.7, they could be considered as independent cells (Supporting Information Table S2). The numbers of peaks in each allele from marker loci of HSC-4 cells used in this experiment (Supporting Information Table S1) were consistent with those reported in RIKEN BRC CELL BANK. On the other hand, STR analysis for OSC-20 cells and TYS cells were not reported in any database. However, we confirmed that the numbers of peaks in each allele from the marker loci of OSC-20 cells and TYS cells used in this experiment (Supporting Information Table S1) were not consistent with those of widely used SCC cells, such as HeLa and A431.

RNA extraction

All cancer tissues were snap-frozen and subjected to molecular analysis. Total RNA from the cell lines and tissues was extracted using a modified acid guanidinium–isothiocyanate–phenol–chloroform method with ISOGEN RNA extraction mixture (Nippon Gene, Tokyo, Japan) according to the manufacturer's recommendations. Tissue was resected from the central part of the tumors without necrosis; half was used for RNA preparation and half for histopathological diagnosis. The
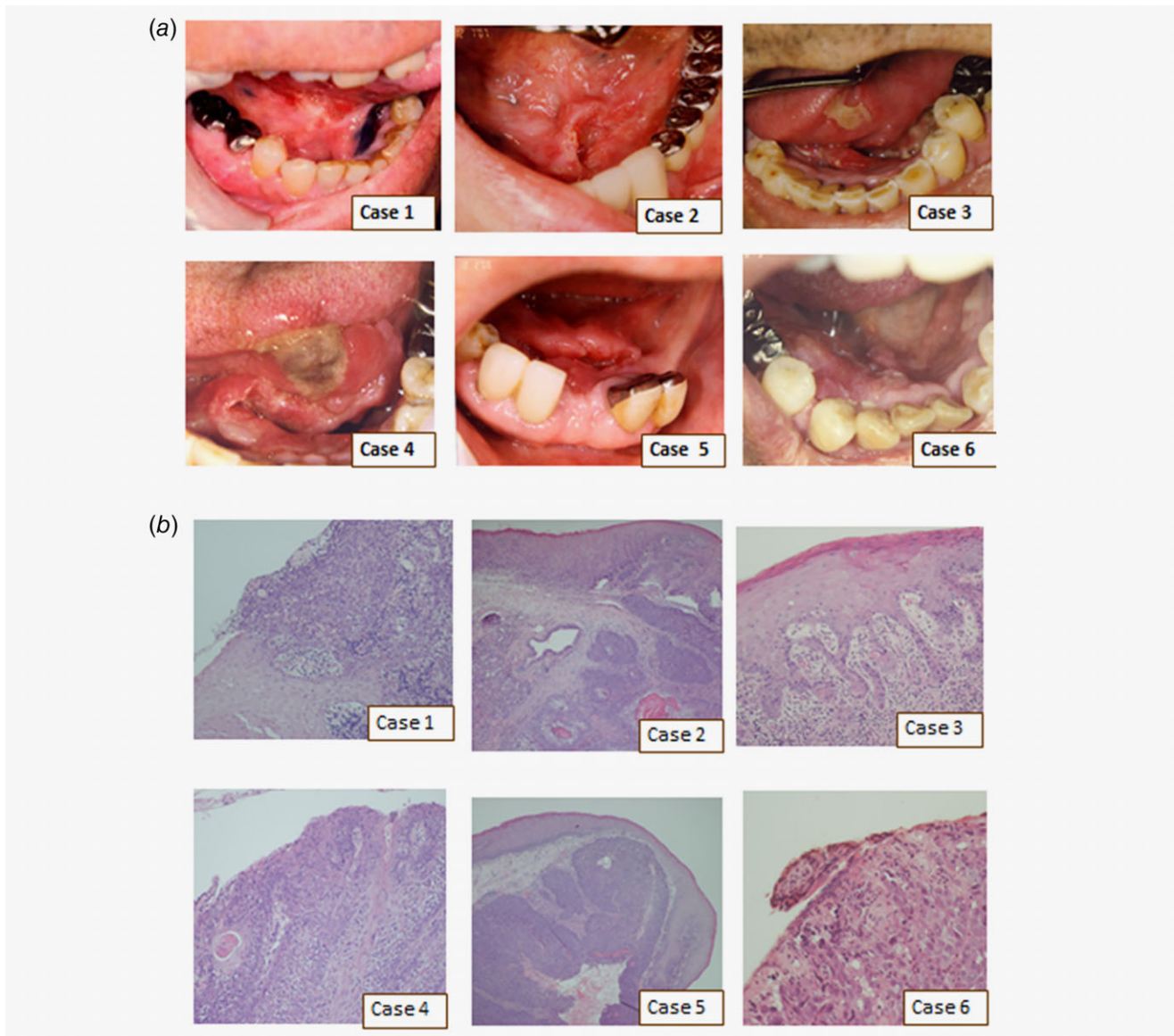
Figure 1. Clinical and histopathological appearances, and clustering analysis of six SCCs of the oral floor. (*a*) Clinical appearance of the SCCs subjected to microarray analysis. There were no clear differences in clinical appearance between the six SCCs of the oral floor. (*b*) Histopathological appearance of the SCCs subjected to microarray analysis. There were no clear differences in histopathological appearance between the six SCCs of the oral floor.

samples were confirmed to contain active cancer cells without any contamination of the minor salivary glands. The integrity of the RNA was confirmed by visualizing intact 28S and 18S rRNAs on 1% agarose gels.

Microarray analysis

Total RNA was extracted by lysing the tissues using ISOGEN (Nippon Gene), following the manufacturer's instructions, and homogenizing them in 0.5 mL of ISOGEN using Tissue Lyser (Qiagen, Valencia, CA). The integrity of the RNA was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

An Applied Biosystems Chemiluminescent RT-IVT Labeling Kit (Life Technologies, Carlsbad, CA) was used to convert total RNA to digoxigenin (DIG)-labeled cRNA. Double-stranded cDNA was generated from 1 μg of total RNA, transcribed using DIG-labeled nucleotides (Roche Diagnostics, Basel, Switzerland), fragmented and hybridized to a Human Genome Survey Array containing 32,878 probes (Life Technologies) according to the manufacturer's instructions. After washing each array, the signal was developed using a chemiluminescent detection kit (Life Technologies). Processed arrays were scanned with a 1700 chemiluminescent microarray analyzer (Life Technologies) and the results were analyzed using

Table 1. Clinical and histopathological characteristics of six cases of SCC of the oral floor

Case	Gender	Age, years	Histology	TNM classification	Y-K mode of invasion
Case 1	M	56	SCC	T3N2cM0	3
Case 2	F	58	SCC	T2N1M0	3
Case 3	M	60	SCC	T4N0M0	4D
Case 4	M	60	SCC	T2N1M0	3
Case 5	M	74	SCC	T4N2aM0	3
Case 6	M	68	SCC	T4N2cM0	3

Samples from these six patients were subjected to microarray analysis

GeneSpring GX 13.0 (Agilent Technologies). Fold change analysis was conducted to identify probes with >5-fold differences, followed by moderated t test, p-value computation (asymptotic) and multiple testing correction (Benjamini Hochberg FDR). The raw microarray data have been deposited in the Gene Expression Omnibus (GEO, http://www.ncbi. nlm.nih.-gov/geo, experiment number: GSE36090 and GSE70604), according to the minimum information about microarray experiment (MIAME) guidelines.

Reverse transcription-polymerase chain reaction
cDNA was synthesized using random primers (Takara Bio, Shiga, Japan) and M-MLV reverse transcriptase (Takara Bio) from 5 µg of total RNA. GAPDH and SCCA1 were amplified to confirm the quality of the cDNA. Subsequently, the products (1 µL) were subjected to PCR amplification under the appropriate conditions (annealing temperature, 60°C; and 25 or 30 amplification cycles) for each gene. PCR products were analyzed by agarose gel electrophoresis. The DNA was electrophoresed at 20 V for 20 min on 1.5% agarose gels, stained with 0.5 µg/mL ethidium bromide, and observed on a UV transilluminator.

Statistical analysis
Statistical analysis was carried out using SPSS software (SPSS Inc., Chicago, IL). The Chi-square test, Fisher's exact test (two-tailed), univariate analysis and multivariate analysis with Cox regression model, and univariate Kaplan–Meier analysis were used for statistical analysis.

Ethical standards
The Ethics Committee of Dokkyo Medical University School of Medicine approved this study. All patients gave their informed consent prior to inclusion in this study.

Results
Identification of genes overexpressed in normal salivary glands
Using microarray analysis, we determined the gene expression profiles in five normal salivary glands and three normal oral squamous epithelium samples (GEO, http://www.ncbi.nlm. nih.-gov/geo, experiment number: GSE36090). A total of 845 probes showed overexpressed-signals in the salivary glands as showing a change of >5-fold difference compared to the oral squamous epithelium. The 845 probes were subsequently used for clustering analyses.

Clustering analysis
Clustering analysis based on the signal strength detected by 845 probes revealed that six oral SCC cases could be divided into two groups. The expression profile of one group (Cases 1, 3, 4 and 6) was similar to that of the oral squamous epithelium, and that of the other group (Cases 2 and 5) was similar to that of normal salivary glands (Fig. 2a). Furthermore, we identified 12 of the 845 probes that can easily determine the origin of SCC of the oral floor (Fig. 2b).

We further conducted clustering analysis on 167 oral SCC samples from a public database (GEO, http://www.ncbi.nlm. nih.-gov/geo, experiment number: GSE30784). These samples could be clearly divided into mucosal-SCC and salivary-SCC (Supporting Information Fig. S1A) using 845 probes, and also with 23 probes for 13 genes including the 12 probes mentioned above (Supporting Information Fig. S1B). Different probes were produced for each exon of each gene to detect the expression of several splicing variants. Therefore, the expression patterns for each probe on each gene were different.

Characteristics of the identified marker genes
The characteristics of the identified marker genes are listed in Table 3. Probes 8 and 9 are different clones of the same gene, so 11 genes were identified as markers. Most of the identified genes are related to the function and structure of the salivary glands (Genes 1, 2, 4, 6, 7 and 10), and some of the genes are tumor-related (Genes 3 and 5). Gene 8 was reported to be stem cell-related, and gene 12 is related to the immune system. Gene 11 is currently not in any database. The mRNA length, structure of the genome and number of amino acids were also examined in several databases. Several mRNA variants were reported for the identified marker genes except Gene 1, and the number of exons and coded amino acids varied between the genes.

Table 2. Clinicopathological differences between salivary-SCC and mucosal-SCCs

Characteristic	Mucosal SCC (*n* = 54)	Salivary SCC (*n* = 12)	*p*-value[1]
Gender no.(%)			
Female	24 (44.4)	3 (25.0)	0.181
Male	30 (55.6)	9 (75.0)	
Age no (%)			
<60 years	21 (38.8)	0 (0.0)	0.03
60–69 years	9 (16.7)	5 (41.7)	
70–79 years	18 (33.4)	4 (33.3)	
>80 years	6 (11.1)	3 (25.0)	
Primary site no (%)			
Tongue	29 (53.7)	5 (41.8)	0.831
Upper gingiva	8 (14.8)	4 (33.3)	
Lower gingiva	5 (9.3)	1 (8.3)	
Oral floor	4 (7.4)	1 (8.3)	
Buccal mucosa	5 (9.3)	1 (8.3)	
Palate	2 (3.7)	0 (0.0)	
Lower lip	1 (1.8)	0 (0.0)	
UICC T stage no (%)			
T1	6 (11.1)	0 (0.0)	0.245
T2	26 (48.1)	7 (58.3)	
T3	8 (14.8)	0 (0.0)	
T4	14 (26.0)	5 (41.7)	
UICC N stage no (%)			
N0	34 (63.0)	5 (41.7)	0.327
N1	11 (20.3)	3 (25.0)	
N2	9 (16.7)	4 (33.3)	
UICC clinical stage no (%)			
I	6 (11.1)	0 (0.0)	0.635
II	19 (35.2)	5 (41.7)	
III	11 (20.4)	2 (16.6)	
IV	18 (33.3)	5 (41.7)	
Tumor cell differentiation no (%)			
High	32 (59.3)	2 (16.7)	0.025
Moderate	17 (31.5)	7 (58.3)	
Low	5 (9.2)	3 (25.0)	
Anneroth's grade no (%)			
I	1 (1.8)	0 (0.0)	0.172
II	22 (40.7)	1 (8.3)	
III	23 (42.7)	8 (66.7)	
IV	8 (14.8)	3 (25.0)	
Y-K mode of invasion no (%)			
1	8 (14.8)	1 (8.3)	0.544
2	10 (18.5)	2 (16.7)	
3	20 (37.0)	4 (33.3)	
4C	14 (26.0)	3 (25.0)	
4D	2 (3.7)	2 (16.7)	
Pathological lymph node metastasis no (%)			
Negative	43 (79.6)	6 (50.0)	0.044
Positive	11 (20.4)	6 (50.0)	

[1]Using the Chi-squared test or Fisher's exact test.
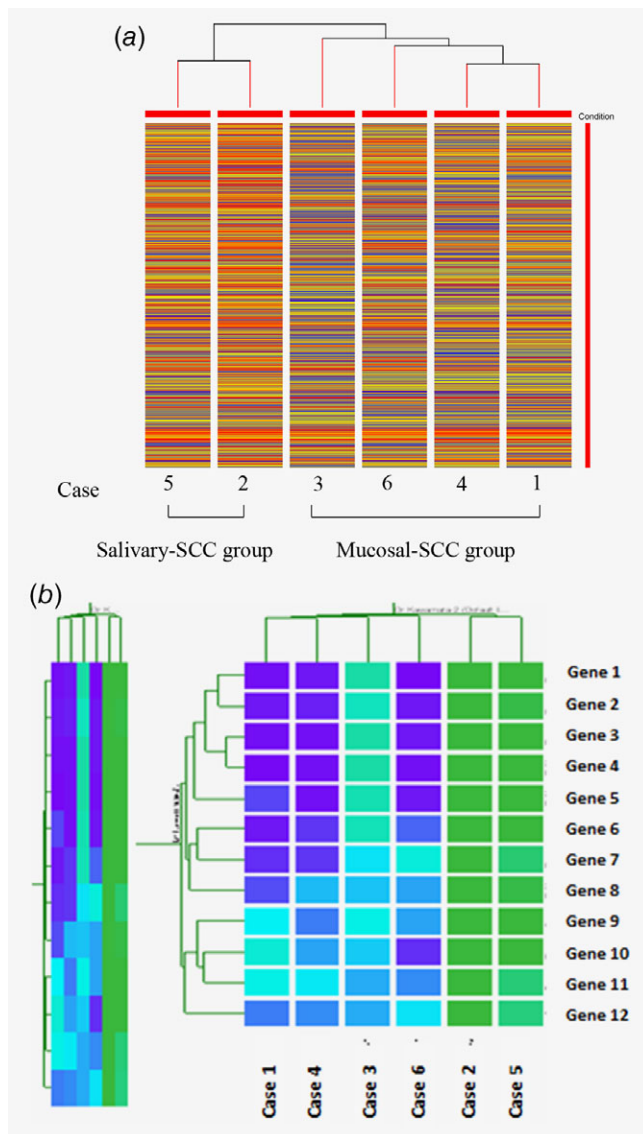
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Figure 2. Clustering analysis. (*a*) The six samples were divided into two groups based on clustering analysis by the levels of gene expression detected by 845 probes. The expression profile of one group (Cases 1, 4, 3 and 6) was similar to that of normal oral squamous epithelium, and that of the other group (Cases 2 and 5) was similar to that of normal salivary glands. (*b*) We identified 12 of the 845 probes that could identify the origin of the SCC of the oral floor by clustering analysis.

Reverse transcriptase-polymerase chain reaction

Upstream and downstream primers were designed based on the different exons to amplify the core domains of several mRNA variants (Supporting Information Table S3). Primers for Gene 11 could not be designed because the structures of its mRNA and genome have not been reported. Primers for Gene 12 cannot be designed because of its high degree of identity with several other genes. Thus, we have nine marker genes for further experiments. The

expected length of the PCR products and the cycles used are also listed in Supporting Information Table S3. To determine the appropriate conditions for amplification, we used three well-characterized cell lines, *i.e.*, OSC-20 and HSC-4 as mucosal-SCCs, and TYS as a salivary-SCC. TYS cells expressed six of the nine markers, but OSC-20 and HSC cells faintly expressed only one and two markers, respectively (Table 4). Therefore, these nine marker genes can be used to discriminate salivary-SCC from mucosal-SCC.

Identification of the origin of oral SCC

We examined the expression levels of nine marker genes in samples of 66 oral SCCs by RT-PCR and determined the origin of the SCC. Band intensity on RT-PCR was judged as very strongly positive (+++), strongly positive (++), positive (+) or negative (−) (Table 4). We considered 11 cases (Cases 1, 2, 3, 4, 6, 15, 17, 56, 64, 68 and 72) that expressed at least five marker genes to be salivary-SCCs (Table 4). Case 7, which strongly expressed four marker genes, was also considered to be salivary-SCC (Table 4). Thus, we identified 12 cases (18.2%) from 66 oral SCCs that could originate from minor salivary glands.

Clinicopathological differences between salivary-SCC and mucosal-SCCs

We examined the clinicopathological differences between salivary-SCC group and mucosal-SCC group by the Chi-squared test and Fisher's exact test (Table 2). Although there was no significant difference in gender distribution between the groups, the salivary-SCC group had more elderly patients than the mucosal-SCC group ($p = 0.031$). There were also no significant differences in primary site, UICC T stage, UICC N stage or UICC clinical stage between the groups. However, there was a significant difference in tumor cell differentiation by WHO classification between the groups ($p = 0.025$). The salivary-SCC group had more patients with low and moderate grade tumor cell differentiation than those with high grade. There was no significant difference in Anneroth's histopathological malignant grade or Y-K mode of invasion between the groups. Interestingly, the salivary-SCC group showed more pathological lymph node metastasis than the mucosal-SCC group [50.0% (6 cases/12 cases) *vs.* 20.4% (11 cases/54 cases), $p = 0.044$].

Tumor-related mortality by variable clinicopathological factors

Tumor-related mortality (TRM) was analyzed by variable factors using univariate analysis with a Cox regression model (Table 5). Although TRM was unaffected by gender, primary site, UICC N stage and UICC clinical stage, TRM was significantly increased by UICC T stage ($p = 0.048$) and age (>80 years old) ($p = 0.031$). On the histopathological aspects, patients with low and moderate grade tumor cell differentiation showed higher TRM than patients with high grade of tumor cell differentiation. However, TRM were unaffected by Anneroth's grade, Y-K mode of invasion and the presence of

Table 3. Characteristics of the identified marker genes

Probes	Name of gene	Length of mRNA (bp)	Structure of genome	No. amino acids
1	Prolactin-induced protein	591	4 exons	147
2	alpha-2-glucoprotein1, zinc-binding	1278, var[1]	4 exons	299
3	FRY-like (MLL fusion partner)	11,706, var[1]	64 exons	3014
4	Lactotransferrin	2593, var[1]	18 exons	866
5	Deleted in malignant brain tumors 1	5802, var[1]	53 exons	1786
6	WAP four-disulfide core domain 2	570, var[1]	4 exons	125
7	Mucin 7, salivary	2467, var[1]	5 exons	378
8, 9	Prominin 1	3977, var[1]	29 exons	866
10	Cholinergic receptor, muscarinic 3	2757, var[1]	5 exons	591
11	Unknown	Unknown	Unknown	Unknown
12	Leukocyte Ig-like receptor, subfamily B	2971, var[1]	16 exons	651

[1] Several mRNA variants are reported.

histological lymph node metastasis (Table 5). Surprisingly, the salivary-SCC group showed much higher TRM than the mucosal group (HR = 3.12, 95%CI, 1.15–8.49; *p* = 0.025).

We further examined TRM on age, UICC T stage, tumor cell differentiation and tumor origin by multivariate analysis (Table 3). Contrary to our expectation, only UICC T stage (T4) and tumor cell differentiation (low) showed higher TRM than the others.

Kaplan–Meier analysis
Kaplan–Meier curves were drawn and the log-rank test was used to investigate the correlation of Salivary-SCC and Mucosal-SCC with 5-year event-free survival (Fig. 3).

The Kaplan–Meier curve suggested that the salivary-SCC group was associated with poorer 5-year event-free survival compared to the mucosal-SCC group (*p* = 0.017).

Discussion
The results of the present study clearly indicated that oral SCC has two different origins, mucosal-SCC and salivary-SCC, by clustering analysis of whole gene expression using our own six oral SCC cases as well as 167 oral SCC cases in the public database (GEO). At present, patients with oral SCC, either mucosal or salivary, are treated by the same protocol. Moreover, we and other researchers have treated the two cancers the same way when investigating the biological

Table 4. Oral SCCs possibly originating from minor salivary glands

Case no.	Primary site	Gene 1	Gene 2	Gene 3	Gene 4	Gene 5	Gene 6	Gene 7	Gene 8	Gene 10
1	upper gingiva	+	+	+	−	−	−	−	+++	+
2	lower gingiva	++	+	−	+	−	−	++	++	+
3	upper gingiva	+	+	−	+	+++	−	+++	+	−
4	tongue	+	+	NE	+	++	NE	+	+	NE
6	upper gingiva	+	+++	+++	++	++	++	+++	+	+++
7	tongue	+	+++	+++	++	NE	NE	NE	NE	NE
15	buccal mucosa	−	+++	+	−	−	+	++	+	++
17	tongue	++	+	−	−	+	+	+	++	+
56	upper gingiva	+	+++	−	−	++	−	−	++	+
64	oral floor	+	+	−	−	++	+	−	++	+
68	upper gingiva	−	+	−	+++	+++	−	++	+++	−
72	tongue	−	++	−	−	−	+	+	+++	+
TYS	salivary-SCC	+++	−	++	−	++	+	++	++	−
HSC	mucosal-SCC	−	−	+	−	−	−	+	−	−
OSC	mucosal-SCC	−	−	+	−	−	−	−	−	−

+++: very strongly positive, ++: strongly positive, +: positive, −: negative, NE: not examined.
Twelve cases from 66 oral SCC (18.2%) were considered to originate from minor salivary glands.
TYS (six positive per nine markers) is a known SCC cell line derived from the minor salivary gland. HSC cells (two faint positive in nine markers) and OSC cells (one faint positive in nine markers) are SCC cells derived from oral squamous epithelium.

Table 5. Tumor-related mortality by variable clinicopathological factors with Cox regression model

	Univariate analysis			Multivariate analysis[1]		
	Crude HR	95% CI	*p*-value	Adjusted HR	95% CI	*p*-value
Gender						
Female	1.00					1.00
Male	0.95	0.36–2.49	0.910	0.73	0.244–2.18	0.572
Age						
‹60 years	1.00					1.00
60–69 years	2.28	0.51–10.21	0.280	2.29	0.42–12.4	0.336
70–79 years	1.67	0.40–6.98	0.484	1.58	0.33–7.69	0.57
›80 years	4.84	1.15–20.33	0.031	3.27	0.92–16.28	0.149
Primary site						
Tongue	1.00					
Upper gingiva	0.58	0.13–2.68	0.485			
Lower gingiva	1.47	0.32–6.81	0.622			
Oral floor	2.55	0.69–9.45	0.161			
Buccal mucosa	0.62	0.08–4.89	0.649			
Palate	NA	NA		NA		
Lower lip	NA	NA		NA		
UICC T stage						
T1–T3	1.00					1.00
T4	2.62	1.01–6.79	0.048	2.67	0.92–7.7	0.07
UICC N stage						
N0	1.00					
N1	2.66	0.73–9.67	0.138			
N2	1.96	0.61–6.25	0.256			
UICC clinical stage						
I–III	1.00					
IV	1.34	0.51–3.52	0.554			
Tumor cell differentiation						
High	1.00					1.00
Moderate	3.52	1.08–11.45	0.036	2.69	0.75–9.72	0.130
Low	5.18	1.29–20.71	0.020	5.04	1.03–24.5	0.045
Anneroth's grade						
I–III	1.00					
IV	1.76	0.57–5.39	0.325			
Y-K mode of invasion						
1	1.00					
2	1.08	0.18–6.48	0.931			
3	1.39	0.29–6.72	0.678			
4C	1.11	0.20–6.05	0.906			
4D	1.03	0.09–11.38	0.980			
Pathological						
Lymph Node metastasis						
Negative	1.00					
Positive	2.21	0.84–5.81	0.108			
Origin						
Mucosal	1.00					1.00
Salivary	3.12	1.15–8.49	0.025	1.28	0.36–4.48	0.705

[1] Variables with p ‹ 0.05 on univariate analysis, excluding gender, were entered.

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characteristics of oral SCC. In future, mucosal-SCC should be treated as conventional SCC, and salivary-SCC as salivary gland cancer.

Eighteen percent of oral SCC was considered to originate from the salivary glands in our cohort. Although there was no significant difference in gender distribution between the salivary-SCC group and mucosal-SCC group, the salivary-SCC group had more elderly patients than the mucosal-SCC group. There were also no significant differences in primary site, UICC T stage, UICC N stage or UICC clinical stage between the groups. The salivary-SCC group had more patients with low and moderate grade tumor cell differentiation than those with high grade differentiation, but there were no significant differences in the other factors. These observations indicated that there was no phenotypic difference between groups. It was noteworthy that the salivary-SCC group showed frequent metastasis to cervical lymph nodes compared to the mucosal-SCC group. Furthermore, the origin of SCC as well as UICC T stage, tumor cell differentiation was significantly correlated with TRM by univariate analysis in our cohort. However, the origin of SCC was not extracted as an explanatory variable for TRM by multivariate analysis. Only tumor cell differentiation was extracted as an explanatory variable for TRM by multivariate analysis. Tumor cell differentiation may be strongly associated with the origin of SCC and affect the origin of SCC as a confounding factor on TRM. On the other hand, the 5-year event-free survival of the salivary-SCC group was significantly lower than that of the mucosal-SCC group on Kaplan–Meier analysis.

In this experiment, we performed semi-quantitative RT-PCR to detect the expression of marker genes in the clinical materials. We initially attempted to quantify the expression levels of these genes in oral SCC tissues using GAPDH as an RNA quality control and SCC1 as a control for the ratio of epithelial cells by TaqMan PCR. However, because the clinical oral SCC samples contained cancer cells in various levels and various activities and/or the expression levels of target genes and control genes were quite different, we could not obtain clear data from quantitative RT-PCR. As the purpose of the RT-PCR experiment was qualitative detection of the expression of marker genes, semi-quantitative RT-PCR, in which three cultured cells were used as internal controls under strict manipulation, was sufficient to confirm our hypothesis.

The primary sites of salivary-SCC were the upper gingiva, lower gingiva, tongue, oral floor and buccal mucosa. There are many minor salivary glands in the oral floor and buccal mucosa, so it is possible to understand that tumors in this region may originate from minor salivary glands. However, this raises the question why salivary-SCC occurs in the gingiva and tongue. Salivary-SCC in the gingiva may occur in the non-keratinized buccal gingiva, which contains many minor salivary glands in the submucosal area, and invade into the keratinized alveolar gingival area. Salivary-SCC in the tongue may occur in the non-keratinized ventral mucosa, which again contains many minor salivary glands or sublingual glands, and invade into the keratinized side edge or dorsal area of the tongue.

Salivary gland cancer is generally recognized as being more resistant to radiation therapy and chemotherapy than mucosal-SCC—it frequently metastasizes to distant organs and shows poor prognosis.[33,34] Surgically, 10-mm safety margins are recommended for resection of oral SCC,[35,36] while safety margins of >20–25 mm are recommended for salivary gland cancer, such as adenoid cystic carcinoma.[37] Thus, salivary-SCC should probably be treated as salivary gland cancer and resected with wide safety margins. Salivary-SCC frequently metastasizes to cervical lymph nodes. Therefore, clinicians should be wary of enlarged cervical lymph nodes on CT or MRI for salivary-SCC, and suspicious lymph nodes should be removed by neck dissection.

In our study, we could not compare chemosensitivity or radiosensitivity between mucosal-SCC and salivary-SCC because the numbers of cases were limited. Generally, salivary gland tumors show chemo- and/or radiation resistance compared to mucosal-SCC.[28] Therefore, induction chemo-radiation therapy may not be suitable for resistant salivary-SCC and should not be chosen based on the histopathological diagnosis alone. If we choose induction chemo-radiation therapy for salivary-SCC, the tumor may progress to a higher stage or become inoperable after induction therapy.

To confirm the aims of this study, further large-scale studies into the origin and biological aggressiveness of oral SCC
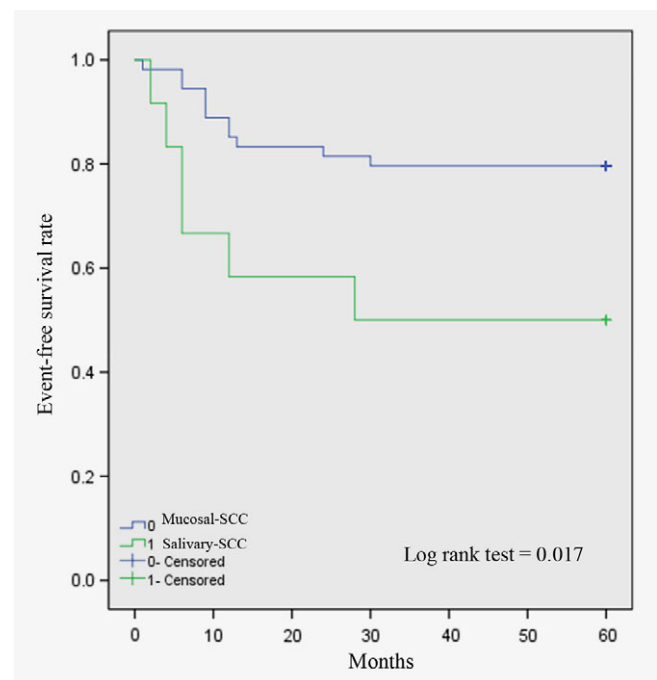

Figure 3. Kaplan–Meier analysis. Kaplan–Meier curve suggested that the salivary-SCC group had poorer 5-year event-free survival compared to the mucosal-SCC group (*p* = 0.017).

are required. We are now working to establish a high-throughput system to determine the origin of oral SCC, discriminating salivary-SCC from mucosal-SCC using immunohistochemical staining for nine markers combined with quantitative PCR. We preliminarily confirmed the expression of marker gene products in salivary-SCC tissues (Supporting Information Figs. S2A and S2B). Furthermore, an interinstitutional collaborative study is underway to accumulate larger numbers of cases. Determination of the origin of oral SCC, whether mucosal or salivary, will be helpful in designing appropriate treatment plans for patients with oral SCC.

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