

## **Oral squamous cell carcinoma may originate from bone marrow-derived stem cells**

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DERIVED STEM CELLS

**Abstract.** Molecules that demonstrate a clear association with the aggressiveness of oral squamous cell carcinoma (OSCC) have not yet been identified. The current study hypothesized that tumor cells in OSCC have three different origins: Epithelial stem cells, oral tissue stem cells from the salivary gland and bone marrow (BM) stem cells. It was also hypothesized that carcinomas derived from less-differentiated stem cells have a greater malignancy. In the present study, sex chromosome analysis by fluorescence *in situ* hybridization and/or microdissection PCR was performed in patients with OSCC that developed after hematopoietic stem cell transplantation (HSCT) from the opposite sex. OSCC from 3 male patients among the 6 total transplanted patients were considered to originate from donor-derived BM cells. A total of 2/3 patients had distant metastasis, resulting in a poor prognosis. In a female patient with oral potentially malignant disorder who underwent HSCT, there were 10.7% Y-containing cells in epithelial cells, suggesting that some epithelial cells were from the donor. Subsequently, gene expression patterns in patients with possible BM stem cell-derived OSCC were compared with those in patients with normally developed OSCC by microarray analysis. A total of 3 patients with BM stem cell-derived OSCC exhibited a specific pattern of gene expression. Following cluster analysis by the probes identified on BM stem cell-derived OSCC, 2 patients with normally developed OSCC were included in the cluster of BM stem cell-derived OSCC. If the genes that could discriminate the origin of OSCC were identified, OSCCs were classified into the three aforementioned categories. If diagnosis can be performed based on the origin of the cancer cells, a more specific therapeutic strategy may be implemented to improve prognosis. This would be a paradigm shift in diagnostic and therapeutic strategies for OSCC.

## **Introduction**

Oral squamous cell carcinoma (OSCC) is highly invasive locally and often leads to cervical lymph node metastasis, with distant metastasis being less frequent (1,2). Factors related to these properties of OSCC (3,4) include oncogene products (5-9), mutated tumor suppressor gene products (10-12), adhesion molecules (13-15), matrix metalloproteinases (16-18) and cell cycle proteins (19-21). However, a molecule clearly associated with the aggressiveness of OSCC has not yet been identified. Similarly, histopathological features can be linked to superficial properties of OSCC, but cannot specifically explain its invasive and metastatic potentials (22). Given this background, we hypothesized that the identification of the origin of the tumor or tumor stem cells may be important in predicting the aggressiveness of OSCC.

Following advances in regenerative medicine, several somatic stem cells have been identified and the importance in regenerating certain organs has been recognized. It has been reported that hepatic and endothelial stem cells are derived from the bone marrow (BM) and are present in circulating blood (23,24). Furthermore, BM stem cells may be involved in the regeneration of the gastrointestinal epithelium (25), and gastric cancer can develop from these cells (26). Tamai *et al* found that BM stem cells (mesenchymal stem cells) are involved in epithelial repair of skin lesions in patients with hereditary epidermolysis bullosa (27). It is well known that BM stem cells include hematopoietic and mesenchymal stem cells. Multilineage-differentiating stress-enduring (Muse) cells, a small population of BM mesenchymal stem cells in circulating blood, can also differentiate into several types of cells, including epithelial cells (28).

The present study attempted to establish a novel diagnostic criterion and a method that can determine the fundamental malignancy of the tumor cells without being confused by

masquerade images or minor genetic abnormalities. The present study was based on the hypothesis that there are three origins of tumor cells in OSCC: Epithelial stem cells, oral tissue stem cells from the salivary gland and BM stem cells. We also hypothesized that carcinoma derived from less-differentiated stem cells has a higher malignancy. It was shown that OSCC derived from stem cells in the salivary gland had a significantly higher metastatic potential and poorer prognosis among oral carcinomas histopathologically diagnosed as SCC (29). In addition, a case of a male patient who underwent hematopoietic stem cell transplantation (HSCT) from his sister for treating leukemia, and developed an oral lichenoid lesion due to chronic graft versus host disease following HSCT was reported. It was demonstrated that oral lichenoid lesion epithelial cells from the patient originated from donor BM stem cells, and these cells developed SCC on the mandibular gingiva (30).

In the present study, sex chromosome analysis was performed in patients with OSCC developed following HSCT from the opposite gender, in order to examine whether OSCC originates from BM stem cells. Gene expression patterns in patients with possibly BM stem cell-derived OSCC were compared with those in patients with normally developed OSCC by microarray analysis, in order to examine whether OSCC with a possible BM origin shows a specific pattern of the gene expression.

## **Materials and methods**

### *Patients*

*Sex chromosome analysis.* Six patients (5 males and 1 female) who developed graft versus host disease (GvHD) in the oral mucosa following HSCT from a donor of the opposite sex and then developed OSCC were examined by fluorescence in situ hybridization (FISH) and G-band staining of sex chromosomes (Tables I and II). The

patients (cases 1 to 6) all presented with a lichenoid lesion, a symptom of chronic GvHD, in the oral mucosa and subsequently developed OSCC 5 to 18 years after HSCT. All patients underwent surgery for OSCC and 4 patients (cases 1, 2, 3 and 6) died from primary disease or multiple metastases. Case 7 was a male patient with OSCC who did not undergo HSCT. Three patients (cases 8, 9, 10; 2 males, 1 female) presented with a lichenoid lesion in the oral mucosa following HSCT from an opposite-sex donor, and were also examined by sex chromosome FISH (Tables I and II). Two male patients with normal labial mucosa, who did not undergo HSCT, were also subjected to this analysis (Table I). All of the above patients were treated between 2006 and 2018 at the Dokkyo University (Mibu, Japan) or Ehime University Hospitals (Toon, Japan).

*Assessment by microarray analysis.* A total of 16 samples from 14 patients with oral SCC (11 men and 3 women) were subjected to microarray analysis. One patient (case 3) had two independent tumors in the oral cavity. Two samples from different part of 1 tumor of 1 patient (case 17) was also analyzed. Each tumor was surgically resected at the Dokkyo Medical University and Ehime University Hospitals between 2017 and 2018. None of the patients had received previous radiotherapy or chemotherapy. The clinical characteristics of these patients are shown in Table III.

*Histopathological examination.* Tissue samples were fixed in formalin and embedded in paraffin. Sections of 4- $\mu$ m were stained with hematoxylin and eosin, and reviewed by experienced pathologists for histopathological diagnosis. Tumor cell differentiation (WHO classification) (31), Anneroth grade (32), Y-K mode of invasion (22) and pathological lymph node metastasis were assessed. The histopathologic features of all patients in the study are shown in Tables I and III.

*FISH analysis.* Sections (5- $\mu$ m) were deparaffinized and treated with 0.5%

pepsin solution and 0.1 N HCl at 37°C for 12-19 min. Following neutralization with PBS, the solution was washed with distilled water and dried. Dig-labeled human X chromosome and FITC-labeled Y chromosome (both from Chromosome Science Labo, Inc., Hokkaido, Japan) probe solutions were added, and the sections and probes were simultaneously denatured on a hot plate at 90°C for 10 min and then hybridized at 37°C overnight. The sections were stringently washed with 50% formaldehyde and 2 x standard saline citrate (SSC) and 1 x SSC, and X-chromosome signals were detected using anti-Dig-Cy3 (Chromosome Science Labo, Inc.). The sections were counterstained with DAPI (Merck KGaA) and mounted with an antifade mounting medium (MountMed; Chromosome Science Labo, Inc.). A CW-4000 cytogenetic workstation (Leica Microsystems, Inc.) was used to detect the FISH signals and analyze the data. An objective lens (magnification, x40) was used for imaging. More than 100 cells were counted for both X and Y signals in one slide.

*Microdissection PCR.* Approximately 20 10- $\mu$ m frozen sections were prepared from a frozen specimen, and laser capture microdissection was performed (Fig. 1A) to collect cancer cells with as little mixed normal epithelia, hemocytes or stromal tissues as possible. DNA was extracted using a QIAamp Fast DNA Tissue Kit (Qiagen AB). Amelogenin genes in both X and Y chromosomes (AMELX and AMELY) were simultaneously examined by PCR using the following primers: 5'-CCCTGGGCTCTGTAAAGAATAGTG-3' forward and 5'-ATCAGAGCTTAAACTGGGAAGCTG-3' reverse (29). PCR products were separated in a polyacrylamide gel, and sex chromosome patterns were analyzed based on the different sizes of amplified bands. Sequences of 106 and 112 bp were amplified from X and Y chromosomes, respectively.

*Microarray analysis.* Microarray analysis was performed as follows. Total RNA was extracted by lysing the tissues with Isogen (Nippon Gene) following homogenization with a TissueLyser (Qiagen AB). Total RNA (500 ng) was used to generate biotin-labeled cRNA using a GeneChip® 3' IVT PLUS Reagent Kit (Thermo Fisher Scientific, Inc.). The biotin-labeled RNA was hybridized to Human Genome U-219 Array Strips (Thermo Fisher Scientific, Inc.). After washing and staining the strips, the signal was developed and scanned using GeneAtlas (Thermo Fisher Scientific, Inc.). Data analysis was conducted using GeneSpring GX 14.9.1 (Agilent Technologies GmbH). The robust multichip average method was used with background correction and normalization. Fold change analysis was performed to identify genes with >3-fold differences, using a moderated t-test ( $P < 0.05$ ) with Benjamini-Hochberg multiple correction. Gene expressions of KRT8, ABCC3, GCLC, RYBP, TMEM97, SLC1A3, IRS2, KYNU, CSAG2, CDA and CCL21 in head and neck SCCs (528 cases) from the cancer genome atlas were analyzed by OncoPrint from cBioPortal. Microarray data have been deposited in the Gene Expression Omnibus (GEO; experiment no., GSE153918) database using minimum information about microarray experiment guidelines. The algorithm of the clustering method for the microarray data is shown in Fig. S1.

## **Results**

*Sex chromosome analysis of OSCC following HSCT.* In three male transplanted patients (cases 1, 3 and 4), more X-only cells were identified than Y-containing cells in the tissue (Table II), suggesting the presence of donor-derived cells in OSCC (case 3-1; Fig. 1A). In a second primary cancer that developed in the gingiva of case 3 (case 3-2), a small number of cancer cells were found in the FISH specimen, but most were normal epithelial

cells. However, only ~6% were X-only cells; almost all cancer cells within the observable FISH range were X-only, again indicating donor-derived cells in OSCC. Distant metastasis was observed in 2/3 patients, resulting in a poor prognosis.

Oral cancer tissues in 1 female (case 2) and 2 males (cases 5 and 6) transplanted patients contained cells with sex chromosomes matching the sex of the recipient (Table II), suggesting that OSCC derived from recipient cells (case 5; Fig. 1B). In oral cancer tissues from 1 male patient (case 7), who did not undergo HSCT, there were 13% X-only cells and 87% Y-containing cells. In oral potentially malignant disorder (OPMD) epithelial cells from a female patient (case 8) who underwent HSCT, there were 10.7% Y-containing cells, suggesting that some epithelial cells were from the donor (Fig. 1C). Adjacent normal-appearing oral squamous tissues were compared to oral SCC or OPMD tissues in 5 cases. Some normal-appearing oral squamous cells (3.7-19.8%) were repaired by donor BM stem cells (case3-1; Fig. 1D). In normal oral mucosa tissues from two male patients (cases 11 and 12) who did not undergo HSCT, there were 99.1% (case 11; Fig. 1E) and 100% Y-containing cells, respectively. Most cells in patients with OSCC had hypertetraploid patterns, including XXXY, XXXYY and XXYYYY.

*Sex chromosome analysis in OSCC by microdissection PCR.* Blood from 1 male patient (case 1) showed an X-only pattern in G-band staining; OSCC cells also showed the same pattern, suggesting that tumor cells were derived from the donor (30). The chromosome patterns in OSCC cells and normal squamous cells were confirmed using laser-captured microdissection PCR with AMELX and AMELY gene amplification (Fig 2A and B). The results of microdissection PCR showed that OSCC tissues (lane 8) in a male patient (case 1) and cancer tissues following LCM (lane 9) had an X-dominant pattern; therefore, these



cells were considered to be derived from the donor (Fig. 2C).

*Microarray and cluster analysis.* The gene expression patterns of patients with possibly BM stem cell-derived OSCC (3 samples; cases 3 and 4) were compared to those of patients with normally developed OSCC (13 samples; cases 13-24). The expression patterns in each group were evidently different (Fig. 3). In 3 samples of possibly BM stem cell-derived OSCC, high expression levels (>3-fold on average) were found for KRT8, ABCC3, GCLC, RYBP, TMEM97, SLC1A3, and IRS2, and low expression levels (<3-fold) were found for KYNU, CSAG2///CSGA3, CDA, and CCL21. Cluster analysis based on the signal intensity detected by probes in 14 OSCC patients (16 samples) identified a cluster in 5 samples including possibly BM stem cell-derived OSCCs (3 samples, case 3-2, 3-3 and 4; Fig. 3). Two patients (cases 13 and 20) were included in the cluster in addition to 3 samples from 2 patients (case 3 and 4) used for selecting probes. These patients (case 13 and 20) had extremely aggressive tumors: Locally advanced T4b, multiple cervical lymph node metastases and extranodal extension N3b, or locally advanced T4b. The expressions of the marker genes identified in this experiment were examined in head and neck SCC (528 cases) from the cancer genome atlas (Fig. S2). The alterations of the individual gene expression were very limited in each case, and no statistical correlation was identified between the expression levels of these marker genes and prognosis.

## **Discussion**

A therapeutic strategy based on the assessment of tumor grade has previously been used on factors such as histopathological features, radiographic images, gene mutation and

gene expression. OSCC was routinely assessed by TNM staging, histopathological grade, HPV status, and analysis of p53 and relevant molecular abnormalities (11). In a recent study, it was found that some SCCs derived from salivary gland stem cells were found in patients with OSCC, significantly affecting prognosis and the true malignancy of tumor cells (29).

In the present study, it was found that OSCC could develop from BM-derived stem cells. OSCC occurring from more undifferentiated stem cells, such as BM-derived stem cells, might share the characteristics of stem cells (stemness is characterized by invasion of surrounding tissues, viability of vessels and ectopic sites, and self-replicating ability); consequently, such cases had a high malignancy and poor prognosis. A total of 2/3 patients (cases 1 and 3) who developed donor-derived OSCC following HSCT died from multiple distant metastases, and the other patient (case 4) had superficial cancer and was being carefully followed up, although only the 1-year prognosis was good at the time. The two patients (cases 13 and 20) with OSCC possibly derived from autologous BM stem cells exhibited significant cervical metastases that were controlled by extended surgery and intensive chemotherapy. These patients were also under strict follow up.

In the present study, no statistical differences in clinicopathological conditions and prognosis between OSCC from possibly BM-derived stem cells and normally developed OSCC were observed, due to the small number of cases examined (Kaplan-Meier analysis; data not shown). Therefore, an attempt was made to access the expression patterns of genes with >3-fold changes in OSCC from possibly BM-derived stem cells in 167 OSCC samples from a public database (GEO; <https://www.ncbi.nlm.nih.gov/geo>, experiment number, GSE30784), and cluster analysis was performed. However, no marked cluster could be found. An attempt was also made to extract cases from the public

database that had similar expression patterns to those of our 4 patients (5 samples; case 3-2, 3-3, 4, 13 and 20) with possibly BM stem cell-derived OSCC. However, this was also unsuccessful, due to the different probe types and analysis software used. The accumulation of more data from our microarray analysis is ongoing, in order to develop a method to identify patients with possibly BM stem cell-derived OSCC among patients with normally developed OSCC, and to analyze the clinical characteristics of the possibly BM stem cell-derived OSCC. Separately, a cluster analysis with a combination of ES cell-maintaining markers (24 genes) was performed in our OSCC patients or those from the public database; however, no cluster was found. The results showed that genes maintaining ES phenotype (general stemness-maintaining genes) were highly expressed in almost all OSCCs, indicating the reversion of cancer cells to stem cells.

As shown by XY-FISH analysis conducted in a very small population of the cells, a Y-chromosome signal was not detectable in male patients with OSCC who did not undergo HSCT. This may be due to technical errors of FISH or Y chromosome dropout during malignant transformation. However, OSCC from male patients who underwent HSCT exhibited X-only signal in the majority of the cells, which might suggest that malignant transformation occurred in the epithelial cells from female donors. Furthermore, some epithelial cells in OPMDs of female patients contained Y chromosomes, which showed that a portion of the epithelial cells in the lesion might have been derived from BM stem cells of the male donor. These results suggest that a portion of the epithelial cells in the oral mucosa were repaired using donor BM stem cells in several patients following HSCT, possibly resulting in malignant transformation to SCC. A total of 2/3 (cases 13 and 20) patients with OSCC derived from donor BM stem cells developed multiple cancer, so-called "field cancerization", in the upper digestive tract, where chronic GvHD occurred

and epithelial cells were repaired in BM-derived stem cells. Although the influence of chronic inflammation and immune-suppressants appeared to be important for oral carcinogenesis following HSCT, a chronic persistent inflammation-regeneration (by BM-derived stem cells)-metaplasia-carcinoma sequence occurs in the oral mucosa. To the best of our knowledge, this is the first report that focuses on oral squamous carcinogenesis. Kano *et al* examined patients with esophageal SCC that developed from BM-derived stem cells and found similar results (33). In order to prove a chronic persistent inflammation-regeneration by BM derived stem cells-metaplasia-carcinoma sequence in the oral SCC patients who underwent HSCT, a multi-institutional big study might be necessary.

BM-derived stem cells consist of hematopoietic and mesenchymal stem cells, the latter of which can differentiate into different types of epithelial cells. Among these, Muse cells have been widely studied and found in the blood, as well as in stromal tissue from the whole body, leading to differentiation into tridermic cells. In contrast to ES and iPS cells, Muse cells were considered as pluripotent stem cells without a potential for neoplastic transformation (28). The population of the BM-derived stem cells that lead to OSCC is unclear; however, it is likely that it is a population of non-Muse mesenchymal stem cells. We are currently conducting an animal study to identify the subpopulation of BM-derived stem cells that can repair the oral epithelium and may lead to the development of OSCC. To the best of our knowledge, any animal models of oral SCC carcinogenesis which supported our hypothesis had not been reported. We recently established the BM stem cells transplantation model in mice. We will plan to inoculate the sorted sub-population of the GFP labeled-BM cells to the recipient mice. Then, an oral carcinogenesis experiment, a chronic persistent inflammation-regeneration-metaplasia-carcinoma, will be performed in the recipient mice.

The conventional therapeutic strategy for OSCC was made according to the clinical and pathological findings, radiographic features, mutations and expressions of a limited number of genes, regardless of the origin of the tumor. OSCC might consist of the carcinomas derived from epithelial stem cells, salivary gland stem cells, and BM-derived stem cells. If the genes that can discriminate the origin are identified, OSCC could be classified into three categories based on cell origin, but not superficial phenotype. An origin-based diagnosis of cancer cells can help develop a more specific therapeutic strategy to improve prognosis. This would be a paradigm shift in the diagnostic and therapeutic strategies for OSCC.

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#### **Availability of data and material**

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

#### **Authors' contributions**

All authors contributed to the study conception and design. THa, THy, YS, MS, RK and NK collected the data. THa, CF, AF, KIN, DU and HK analyzed the data. THa drafted the manuscript. All authors reviewed the final draft of the manuscript. All authors read and approved the final manuscript.

#### **Ethics approval and consent to participate**

The design of the current study was approved by the Medical Ethical Research Committee of Dokkyo Medical University Hospital (approval ID, R-25-16J) and Ehime University (approval ID, 2007024). Written informed consent was obtained from all patients for the use of their samples for research and for publication at the start of the treatment. The patients could also opt-out, based on a research plan approved by the Medical Ethical Research Committee of our institutes. We confirm that no opt-outs were recorded.

#### **Patient consent for publication**

Not applicable.

## **Competing interests**

The authors declare that they have no competing interests.

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## Figure legends

Figure 1. Fluorescence *in situ* hybridization images of sex chromosomes. (A) A male patient who developed OSCC following HSCT from a female donor. OSCC contained of 66.5% X-only cells and 33.5% Y-containing cells, indicating that it was donor-derived. (B) A male patient who developed OSCC following HSCT from a female donor. OSCC contained of 19.8% X-only cells and 80.2% Y-containing cells, indicating a recipient-derived tumor. (C) A female patient with an oral potentially malignant disorder of the oral mucosa following HSCT from a male donor. The sample contained of 89.3% X-only cells and 10.7% Y-containing cells, indicating partial epithelia restoration by transplanted bone marrow cells. (D) A male patient who developed OSCC following HSCT from a female donor. Normal-appearing oral squamous cells contained of 3.7% X-only cells and 96.3% Y-containing cells, indicating partial epithelia restoration by transplanted bone marrow cells. (E) Normal labial mucosa in a male with 100% Y-containing cells. Original magnification for hematoxylin-eosin staining: x40, fluorescence *in situ* hybridization images: x400. OSCC, oral squamous cell carcinoma; HSCT, hematopoietic stem cell transplantation.

Figure 2. X- and Y-chromosomes in tumor tissues identified by microdissection PCR. (A) Before cancer cell processing by LCM. Original magnification x200. (B) After cancer cell processing by LCM. Original magnification x200. (C) PCR amplification of Amelogenin genes. Lanes 1 and 2, female blood; lanes 3 and 4, male blood; 5, blood of patient; 6 and 7, normal oral mucosal tissue of patients; 8, OSCC tissue of a male patient; 9, carcinoma tissue of a male patient following LCM; 10, oral tissue from a male patient following LCM; 11, oral tissue of female patient following LCM; M1, molecular weight marker 1; M2, molecular weight marker 2. OSCC (lane 8) and cancer tissues following

LCM (lane 9) exhibited an X-dominant pattern; therefore, these were considered to be donor-derived cells. LCM, laser captured microdissection; OSCC, oral squamous cell carcinoma.

Figure 3. Clustering analysis from microarray data. Clustering was performed in 16 OSCC samples (14 patients) using genes with high (KRT8, ABCC3, GCLC, RYBP, TMEM97, SLC1A3 and IRS2) and low (KYNU, CSAG2///CSGA3, CDA and CCL21) expression levels in 3 samples of donor bone marrow-derived stem cells determined by sex chromosome analysis. Red indicates high expression and blue indicates low expression. A cluster was formed in 5 samples (cases 3-2, 3-3, 4, 13 and 20). OSCC, oral squamous cell carcinoma.

Figure S1. Algorithm of the clustering method for microarray data.

Figure S2. Expression of marker genes detected in the current microarray analysis was analysed in head and neck squamous cell carcinoma (528 cases) from the cancer genome atlas database.

## Tables

Table I. Clinicopathological characteristics of patients

Case no.	Sex	Age	Smoking/Alcohol	Location	Histology	TNM classification	Tumor cell differentiation	Anneroth grade	Y-K mode of invasion	p16 (HPV status)	Prognosis
1-1	M	58	Never/No	Lower gingiva	SCC	T4aN0M0	1	II	3	Negative	Dead
1-2	M	65	Never/No	Esophageal	SCC	T1N0M0	2	II	2	Negative	Dead
2	F	16	Never/No	Tongue	SCC	T1N0M0	1	III	4D	Positive	Dead
3-1	M	61	Ex/No	Tongue	SCC	T2N0M0	2	III	3	Negative	Dead
3-2	M	62	Ex/No	Lower gingiva	SCC	T4aN0M0	1	III	4D	Negative	Dead
4	M	50	Current/Daily	Buccal mucosa	SCC	T3N0M0	2	I	3	NE	Alive
5	M	30	Never/Occasionally	Tongue	SCC	T2N0M0	2	II	3	Negative	Alive
6	M	41	Never/Occasionally	Tongue	SCC	rT0N2cM0	1	II	4D	Negative	Dead
7	M	90	Current/Daily	Upper gingiva	SCC	T3N0M0	3	III	3	Negative	Unknown
8	F	38	Never/No	Tongue	OPMD	NA	NA	NA	NA	NA	NA
9	M	17	Never/No	Buccal	OPMD	NA	NA	NA	NA	NA	NA
10-1	M	72	Never/Daily	Upper gingiva	OPMD	NA	NA	NA	NA	NA	NA
10-2	M	72	Never/Daily	Upper gingiva	OPMD	NA	NA	NA	NA	NA	NA

M, male; F, female; Never, never smoked; Ex, ex-smoker; Current, current smoker; No, does not consume Alcohol; Daily, drinks alcohol daily;

Occasionally, occasionally consumes alcohol; SCC, squamous cell carcinoma; OPMD, oral potentially malignant disorders; NA, not applicable; NE,

not examined.

Table II. Sex chromosomal analysis via XY FISH in patients with oral SCC who were receiving HSCT

Case no.	Sex of donor/recipient	Age at diagnosis for oral SCC/Age at transplantation	Type of hematological malignancy	Type of HSCT	Severity of chronic GvHD	Oral SCC or OPMD		Adjacent normal-appeared oral squamous epithelium	
						X only (%)	Y containing (%)	X only (%)	Y containing (%)
1-1	F/M	58/53	MLL	Allo-PBSCT	Mild	96.4	3	19	79
1-2	F/M	65/53	MLL	Allo-PBSCT	Mild	86	14	NE	NE
2	M/F	16/4, 9	ALL	UR-BMT	Severe	100	0	NE	NE
3-1	F/M	61/44	MDS	UR-BMT	Moderate	66.5	33.5	3.7	96.3
3-2	F/M	62/44	MDS	UR-BMT	Moderate	5.7 <sup>b</sup>	94.3	NE	NE
4	F/M	50/43	CTCL	Allo-BMT	Moderate	63.3	36.7	NE	NE
5	F/M	30/14	MDS	Allo-BMT	Mild	11.7	88.3	19.8	80.2

6 <sup>a</sup>	F/M	41/32	ALL	CBT	Moderate	5	95	NE	NE
7	NA	90/NA	NA	NA	NA	13	87	NE	NE
8	M/F	38/10, 13	AML	Allo-BMT	Moderate	89.3	10.7	90.3	9.7
9	F/M	17/16	SAA	Allo-BMT	Moderate	7.4	92.6	9	91
10-1	F/M	72/59	MDS	Allo-BMT	Moderate	5.2	94.8	NE	NE
10-2	F/M	72/59	MDS	Allo-BMT	Moderate	6.7	93.3	NE	NE
11	NA	10/NA	NA	NA	NA	NA	NA	0	100
12	NA	45/NA	NA	NA	NA	NA	NA	0.9	99.1

<sup>a</sup>Metastatic lymph nose was analyzed via FISH. <sup>b</sup>A small number of cancer cells was found in the specimen used for FISH. Most were normal epithelioma and ~6% were X-only cells. However, almost all cancer cells within the observable FISH range were X-only cells, and were considered to be donor-derived cells. F, female; M, male; NA, not applicable; SCC, squamous cell carcinoma; MLL, mixed lineage leukemia; ALL, acute lymphocytic leukemia; MDS, myelodysplastic syndrome; CTCL, cutaneous T-cell lymphoma; AML, acute myeloid leukemia; SAA, severe aplastic anemia; HSCT, hematopoietic stem cell transplantation; PBSCT, peripheral blood stem cell transplantation; Allo-BMT, allogeneic bone marrow transplantation; UR-BMT, unrelated bone marrow transplantation; CBT, cord blood transplantation; GvHD, graft versus host disease; OPMD, oral



potentially malignant disorders; NE, not examined.

Table III. Clinico-pathological characteristics of the patients with oral SCC for microarray and clustering analysis

Case no.	Sex	Age at diagnosis	Smoking/Alcohol	Location	Histology	cTNM	pN	Tumor cell differentiation	Anneroth grade	Y-K mode of invasion	p16 (HPV status)	Prognosis
3-2	M	62	Ex/No	Gingiva	SCC	T4aN0M0	NDNP	2	III	4D	Negative	Dead
3-3	M	63	Ex/No	Gingiva	SCC	T4aN0M0	NDNP	2	III	4D	Negative	Dead
4	M	50	Current/Daily	Buccal Mucosa	SCC	T3N0M0	NDNP	2	I	3	NE	Alive
13	F	55	Ex/No	Tongue	SCC	T4bN3bM0	NDNP (N3b) <sup>a</sup>	1	I	2	Negative	Alive
14	M	60	Ex/No	Tongue	SCC	T2N1M0	N1	1	I	3	Negative	Alive
15	F	83	Never/Daily	Buccal Mucosa	SCC	T4aN2bM0	N0	2	I	2	Positive	Alive
16	M	87	Ex/Occasionally	Tongue	SCC	T2N1M1	NDNP	1	II	3	Negative	Unknown
17-1	M	65	Current/Daily	Tongue	SCC	T3N3bM0	N3b	1	II	4C	Positive	Alive
17-2	M	65	Current/Daily	Tongue	SCC	T3N3bM0	N3b	1	II	4C	Positive	Alive
18	M	71	Current/Daily	Tongue	SCC	T4aN2bM0	N0	1	II	4C	Negative	Alive
19	F	86	Never/No	Gingiva	SCC	T4aN1M0	N0	1	II	4C	Negative	Alive
20	M	74	Ex/Daily	Gingiva	SCC	T4bN2bM0	N0	2	III	3	Negative	Alive

21	M	69	Current/Daily	Tongue	SCC	T2N1M0	N3b	2	III	4D	Negative	Dead
22	M	61	Ex/No	Tongue	SCC	T4aN1M0	N0	1	III	4C	Negative	Alive
23	M	60	Never/Occasionally	Buccal Mucosa	SCC	T3N1M0	N0	1	II	3	Negative	Alive
24	M	59	Ex/Daily	Tongue	SCC	T3N1M0	N1	1	II	4C	Negative	Alive

<sup>a</sup>Extranodal extension is markedly detected in images. M, male; F, female; Ex, ex-smoker; Current, current smoker; Never, never smoked; No, does not consume alcohol; Daily, consumes alcohol daily; Occasionally, occasionally consumes alcohol; SCC, squamous cell carcinoma; NDNP, neck dissection was not performed; NE, not examined.