Analysis for predictor of cervical lymph node metastasis in oral squamous cell carcinoma

Sakurako Yamaguchi

Departments of Oral Surgery, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan

Correspondence to:
Sakurako Yamaguchi
Telephone no: +81-47-360-9406
Fax no: +81-47-360-9405
E-mail: maki.sakurako@nihon-u.ac.jp

Abstract

There are reports in the literature that among squamous cell carcinomas (SCCs) that occur in the oral cavity, most are often observed in the tongue, which then metastasize to cervical lymph nodes at a relatively early stage. Prediction of cervical lymph node metastasis is, thus, considered to improve treatment results. The present study was to explore factors that may be predictors of cervical lymph node metastasis, while focusing on the invasive front of the tumor. The subjects of this study were 13 patients who underwent partial tongue resection and neck dissection as first-line treatment from among all patients who were histopathologically diagnosed with tongue SCC at Department of Oral Surgery, Nihon University Hospital, School of Dentistry at Matsudo over the course of 13 years between 2003 and 2016. The subjects were divided cases of tongue SCC into two groups, a cervical lymph node metastasis group and a non-metastasis group, searched clinicopathologically and immunohistologically. Budding and SOX2 showed a strong correlation with cervical lymph node metastasis. Ki-67, the depth of invasion (DOI), E-cadherin, and macroscopic type were also shown to contribute to cervical lymph node metastasis. Furthermore, it was suggested that cases which are endophytic, as observed macroscopically with a DOI of $\geq$ 4 mm, and in which the tumor cells have proliferating capability with enhanced epithelial-mesenchymal transition (EMT) are likely to progress to lymphatic metastasis. Therefore, budding, SOX2, macroscopic type, DOI, Ki-67, and E-cadherin are effective as factors for predicting the prognosis of cervical lymph node metastasis.

Keywords

Oral tongue squamous cell carcinoma, Predictor of cervical lymph node metastasis, Statistical analysis

Introduction

There are some reports in the literature that among squamous cell carcinomas (SCCs) that occur in the oral cavity, most are often observed in the tongue, which then metastasize to cervical lymph nodes at a relatively early stage (1-6). Cervical lymph node metastasis is an important factor that affects patient outcome (1-7). Prediction of cervical lymph node metastasis is, thus, considered to improve treatment results.

It has also been reported that the proliferative activity and biological properties of the tumor cells in the invasive front of the tumor are important predictors of cervical lymph node metastasis in oral squamous cell carcinoma (OSCC) (8, 9). It has also been suggested that budding, defined as the presence of single cancer cell or cluster of less than 5 cancer cells at the invasive front, which has been shown to be a predictor of lymph node metastasis in colon cancer (10), may also have a similar role in cervical lymph node metastasis in OSCC (11, 12). Furthermore, an association between the expression of stem-cell-associated factors and prognosis of SCC (hypopharynx (13), esophagus (14), lung (15), etc.) has been reported; additionally, the expression of stem-cell-associated factors also appears to be a predictor of prognosis in OSCC. The involvement of stromal lymphatic vessel density in the invasive front of the tumor in lymph node metastasis

has also been suggested to be a predictor of prognosis (16) ; furthermore, a similar study on esophageal SCC also showed a similar involvement of stromal lymphatic vessel density (17).

Currently, the predictors of OSCC cervical lymph node metastasis have yet to be elucidated. In the present study, we divided cases of tongue SCC into two groups, a cervical lymph node metastasis group and a non-metastasis group, to explore factors of tumor cells and vessel density in the tumor stroma that may be predictors of cervical lymph node metastasis, while focusing on the invasive front of the tumor.

Subjects and methods

1. Subjects and clinical investigation

The present study was conducted with the approval of the Ethics Committee of Nihon University School of Dentistry at Matsudo (Approval number: EC16-15-034-1).

The subjects of this study were 13 patients who underwent partial tongue resection and neck dissection as first-line treatment from among all patients who were histopathologically diagnosed with tongue SCC at Department of Oral Surgery, Nihon University Hospital, School of Dentistry at Matsudo over the course of 13 years between 2003 and 2016. The 13 cases were divided into two groups: one in which cervical lymph node metastasis was observed histopathologically (7 cases) and the second in which there was no cervical lymph node metastasis (6 cases). Gender, age, and tumor characteristics (site of invasion, primary tumor size, macroscopic type, and cTNM and cStage) in accordance with the Oral Cancer Handling Rules (18) were obtained from the medical records.

2. Histopathological investigation

Sections measuring 4 μm in thick were prepared by conventional paraffin embedding after fixation of the resected specimens with 10% formalin solution. Each section was stained with hematoxylin and eosin (HE staining) and histopathologically evaluated. The factors assessed and evaluated included histological malignancy (grade), mode of invasion (YK), vascular invasion (lymphatic invasion: Ly, venous invasion: V), and perineuronal infiltration (Pn), and these were selected according to the Oral Cancer Handling Rules (18) and were evaluated by four oral pathologists. In addition, we measured the depth of invasion (DOI) of the tumors by optical microscopy.

3. Immunohistochemical investigation

1) Staining method and antibodies

The sliced sections were subjected to activation processing for each antigen after deparaffinization and hydrophilization. Endogenous peroxidase was blocked using 0.3 % hydrogen peroxide in methanol. Each primary antibody was added and incubated for one hour to provide sufficient time for reaction. Primary antibodies used included Podoplanin (D2-40) , CD34, Actin (smooth muscle), Cytokeratin (AE1/AE3), E-cadherin, Ki-67 (MIB-1) , and SOX2. Further details regarding this are shown in Table 1.

Purpose	Antibody	Clone	Dilution	Company	Retrieval methods
Lymphatic vessel endothelium marker	Anti-Podoplanin	D2-40	1:50	Dako	-
Identification of the blood vessel (Vascular endothelial cell)	Anti-CD34	QBEnd 10	1:50	Dako	Autoclave,5 min (10 mM Tris-EDTA buffer pH 9.0)
Identification of the blood vessel (Vascular smooth muscle)	Anti-Actin (Smooth Muscle)	1A4	1:50	Dako	Autoclave,5 min (10 mM Tris-EDTA buffer pH 9.0)
Epithelium marker	Anti-Cytokeratin	AE1/AE3	Diluted	Dako	Autoclave,5 min (10 mM Tris-EDTA buffer pH 9.0)
Epithelial mesenchymal transition	Anti-E-cadhelin	NCH-38	1:50	Dako	Autoclave,5 min (10 mM Citric acid buffer pH 6.0)
Cell proliferation marker	Anti-Ki-67	MIB-1	1:50	Dako	Autoclave,5 min (10 mM Tris-EDTA buffer pH 9.0)
Stem cell marker (Transcription factor)	Anti-SOX2	GT1352	1:100	Abcam	Microwave,15 min (10 mM Citric acid buffer pH 6.0)

Table 1. Antibodies and retrieval methods used in the present study

After reaction with the primary antibody, the secondary antibody (Dako Chem Mate ENVISION kit) was added and incubated for one hour. 3,3-diaminobenzidine (DAB) was added for staining after secondary antibody reaction, and Mayer's hematoxylin was used for counter-staining.

2) Measurement of vessel density

Lymphatic vessels were observed on slides stained with Podoplanin, while blood vessels were observed on CD34-stained slides. The hotspot at the invasive front of the tumor was observed and selected by two fields optical microscopy under 20x magnification for each case. Quantification was performed using the imaging software Image J (NIH, Bethesda, MD). For evaluation of vessel density, tumor parenchymal areas adjacent to the tumor stroma were removed from each image. These images were separated into Podoplanin-positive lymph vessels, the lumen region of CD34-positive blood vessels, and tumor stroma using manual tracking, and they were converted to binary data using manual tracing. Vessel density was

calculated for each image as the ratio of lymph/blood vessel area to the area of tumor stroma, and the two fields average values were compared.

3) Evaluation of Actin-positive tumor cells

The invasive front of the tumor on the actin-stained slides was observed by optical microscopy under 20x magnification. The slides in which no portion of the tumor was stained by actin were defined as 0, while those in which actin-positive tumor cells were observed were defined as 1 (Fig. 1).

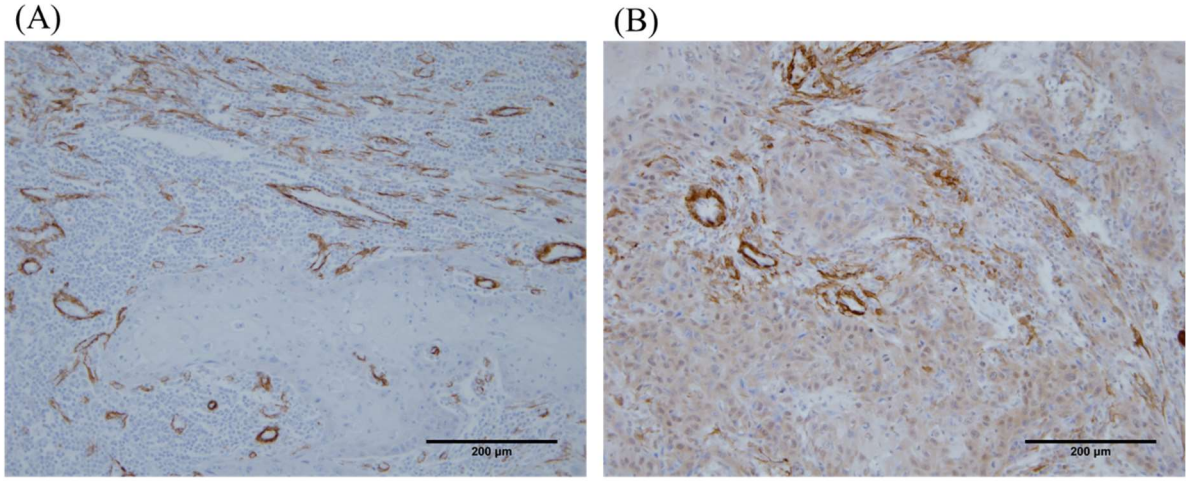

Fig. 1. Expression of Actin
Immunohistochemical staining of Actin shows different expression levels in tongue squamous cell carcinomas. The positive expression site of Actin was tumor cell stromal and vascular smooth muscle cells.
(A) Tumor cells shows no positive staining for Actin are score 0.
(B) Tumor cells with positive staining for Actin are referred to score 1.

4) Evaluation of budding

The invasive front of the tumor on the cytokeratin (AE1/AE3)-stained slides was observed by optical microscopy under 20x magnification. This portion was divided based on a 3-step grading system (grade 1:

0 to 4 buds, grade 2: 5 to 9 buds, and grade 3: 10 or more buds) according to the Colon Cancer Handling Rules (19).

5) Evaluation of E-cadherin-positive tumor cells

Slides stained with E-cadherin were used. An optical microscope at 20x magnification was used to determine the percentage of E-cadherin-positive cells in the entire tumor. The slides that had < 25 % positive cells were defined as 0, while those with ≥ 25 % positive cells were defined as 1 (Fig. 2).

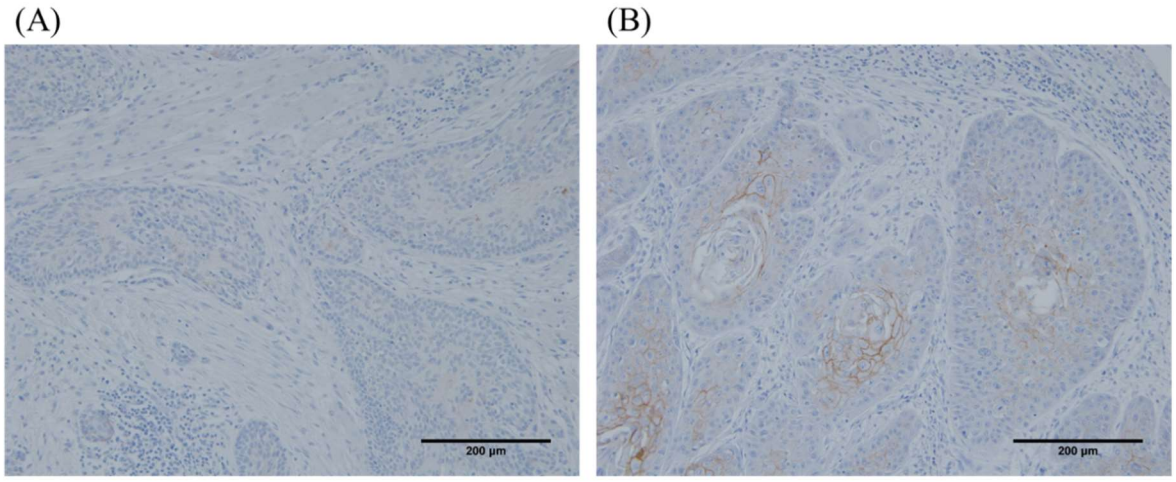

Fig. 2. Expression of E-cadherin
E-cadherin was expressed on the limbus of the cell membrane of tongue squamous cell carcinoma.
(A) Tumor with < 25 % positive cells are referred to score 0.
(B)Score 1 show 25 % ≤ positive stained cells.

6) Evaluation of proliferating cells in tumor cells

Slides stained with Ki-67 Antigen (MIB-1) were observed by optical microscopy at 20x magnification. We counted the number of Ki-67-positive cells to calculate the percentage of Ki-67-positive cells among all the tumor cells. The Ki-67 positive rate was defined as the two fields average value.

7) Evaluation of SOX2-expressing tumor cells

Anti-SOX2 stained slides were observed with an optical microscope at 20x magnification. The slides with no stained tumor cells were defined as 0, while those with SOX2-positive tumor cell(s) were defined as 1 (Fig. 3).

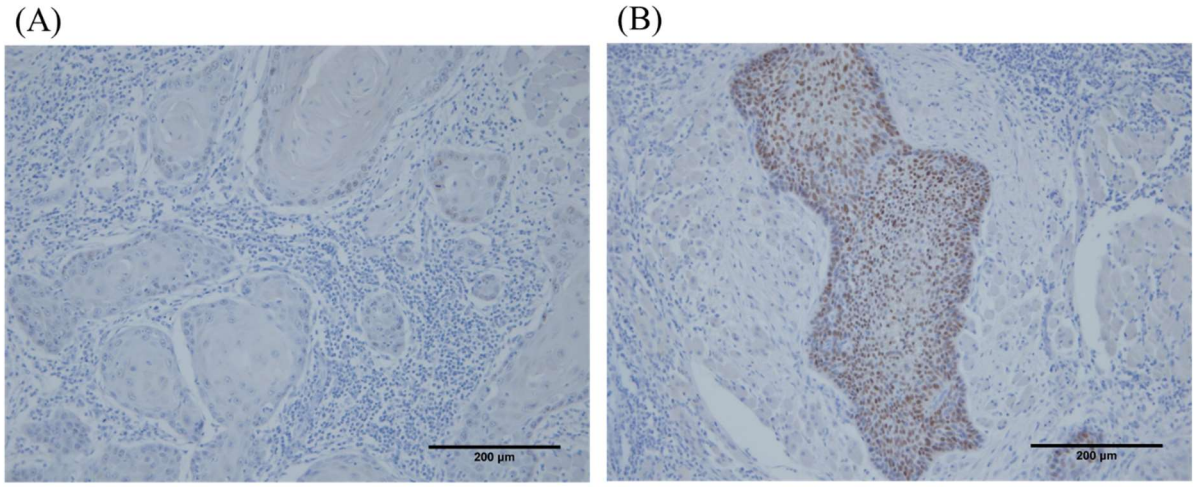

Fig. 3. Expression of SOX2
Immunohistochemical staining of SOX2 shows different expression levels in tongue squamous cell carcinomas. The positive expression site of SOX2 was mainly localized in the nucleus of tumor cells.
(A) Tongue tumor tissue that shows no positive staining for SOX2 are score 0.
(B) Tumor cells with positive staining for SOX2 are referred to score 1.

4. Statistical analysis

The DOI, vessel density, and Ki-67 positive rates were compared between the two patient groups using the Mann-Whitney U Test. Then, we calculated Cramér's V coefficient of association for macroscopic type, grade, YK, budding, SOX2, E-Cadherin, and actin with the presence of cervical lymph node metastasis. We also performed multivariate analysis (extended quantification type II) with DOI and Ki-67 positive rate, which showed significance, as well as macroscopic type and E-cadherin, which demonstrated a strong association as explanatory variables, and cervical lymph node metastasis as an independent variable, to

examine factors that affect the lymph node metastasis in the group with metastasis and the one without. The statistical significance level was set to less than 5%. SPSS version 20.0 (IBM, Tokyo, Japan) and multivariate software (Istat, Tokyo, Japan) were used for statistical processing.

Results

1) Clinical background

The patients' clinical factors are summarized in Table 2. Four male patients and three female patients were in the metastasis group, while the non-metastasis group had four males and two females. The age of the subjects ranged between 56 and 74 years (mean: 68 ± 6.6 years) in the metastasis group and between 52 to 71 years (mean: 62.5 ± 6.3 years) in the non-metastasis group. The site of tumor invasion included the tongue margins and under the tongue in both the metastasis group (six cases and one case, respectively) and the non-metastasis group (five cases and one case, respectively). With regard to tumor size, the major axis length was between 14 and 40 mm (mean: 24.1 ± 10.0 mm) and the minor axis length was between 13 and 30 mm (mean: 18.4 ± 6.5 mm) in the metastasis group; in the non-metastasis group, these values were between 10 and 50 mm (mean: 24.5 ± 13.7 mm) and between 10 and 30 mm (mean: 16 ± 8.0 mm). On the basis of the macroscopic type, in the metastasis group, two cases were classified as having superficial type, one had exophytic type, and four had endophytic type, while in the non-metastasis group one case was classified as having superficial type, three had exophytic type, and two had endophytic type. The TNM classification and staging details of each patient are shown in Table 3. The clinical classification is modified

to the postoperative pathological classification. This factor is often due to the cervical lymph node metastasis or the non-metastasis.

			LN(+)*	LN(-)**
Clinical findings	Sex	Male	4	4
		Female	3	2
	Age		56-74	52-71
		mean ± SD	68 ± 6.6	62.5 ± 6.3
	Location of the lesion	Dorsum of tongue	0	0
		Margin of tongue	6	5
		Sublingual aspect	1	1
	Size	Major length (mm)	Max:40.0 Min:14.0	Max:50.0 Min:10.0
		mean ± SD	24.1 ± 10.0	24.5 ± 13.7
		Minor length (mm)	Max:30.0 Min:13.0	Max:30.0 Min:10.0
		mean ± SD	18.4 ± 6.5	16 ± 8.0
	Macroscopic type	Superficial type	2	1
		Exophytic type	1	3
		Endophytic type	4	2
	cTNM		T1(2) T2(4) T3(1) N0(4) N1(1) N2b(1) N2c(1) M0(7)	T1(2) T2(3) T3(1) N0(2) N1(3) N2b(1) M0(6)
	cStage		Ⅰ(1) Ⅱ(3) Ⅲ(1) ⅣA(2)	Ⅱ(2) Ⅲ(3) ⅣA(1)

*lymph node metastasis:LN(+)
**lymph node non-metastasis:LN(-)

Table 2. Clinical data

	cTNM	cStage	pTNM	pStage
LN(+)* (n = 7)	T1N0M0	Ⅰ	T1N2bM0	ⅣA
	T2N0M0	Ⅱ	T4aN2bM0	ⅣA
	T2N0M0	Ⅱ	T2N2aM0	ⅣA
	T2N0M0	Ⅱ	T2N2bM0	ⅣA
	T1N1M0	Ⅲ	T1N1M0	Ⅲ
	T2N2cM0	ⅣA	T4aN2cM0	ⅣA
	T3N2bM0	ⅣA	T4aN2bM0	ⅣA
LN(-)** (n = 6)	T2N0M0	Ⅱ	T1N0M0	Ⅰ
	T2N0M0	Ⅱ	T2N0M0	Ⅱ
	T1N1M0	Ⅲ	T1N0M0	Ⅰ
	T1N1M0	Ⅲ	T2N0M0	Ⅱ
	T2N1M0	Ⅲ	T2N0M0	Ⅱ
	T3N2bM0	ⅣA	T3N0M0	Ⅲ

*lymph node metastasis:LN(+)
**lymph node non-metastasis:LN(-)

Table 3. TNM of each case

2) Histopathological background

The patients' histopathological factors are summarized in Table 4.There were two cases in which the invasive front of the tumor was graded as G1, three cases as G2, and two cases as G3 in the metastasis group; meanwhile, there were five cases in which the front portion of the invasion was graded as G1 and one case as G2 in the non-metastasis group. According to the YK classification, four cases were at YK-3 and three were at YK-4C in the metastasis group, while five cases were at YK-3 and one case was at YK-4C in the non-metastasis group. In terms of lymphatic vessel invasion, one case was Ly0, four were Ly1, and two were Ly2 in the metastatic group, while all six cases were Ly0 in the non-metastasis group. Regarding venous invasion, four cases were V0, one was V1, and two were V2 in the metastasis group; meanwhile, five cases were V0 and one case was V1 in the non-metastatic group. In terms of perineural invasion, six cases were Pn0 and one was Pn2 in the metastasis group, while all six cases were Pn0 in the non-metastasis group. Furthermore, DOI ranged between 2.9 and 16 .0 mm (mean: 7.7 ± 4.5 mm) in the metastasis group and between 1.5 and 5.3 mm (mean: 3.6 ± 1. 5 mm) in the non-metastasis group.

			LN(+)*	LN(-)**
Pathological findings	pTNM		T1(2) T2(2) T3(3) N1(1) N2a(1) N2b(4) N2c(1) M0(7)	T1(2) T2(3) T3(1) N0(6) M0(6)
	pStage		III(1) IVA(6)	I(2) II(3) III(1)
	Grade	Surface	1(6) 2(1)	1(6)
		Invasive	1(2) 2(3) 3(2)	1(5) 2(1)
	YK		3(4) 4C(3)	3(5) 4C(1)
	DOI (mm)		2.9-16	1.5-5.3
		mean + SD	7.7 ± 4.5	3.6 ± 1.5
	Lymph vessel invasion	(Podoplanin)	0(1) 1(4) 2(2)	0(6) 1(0) 2(0)
	Vascular invasion	(CD34)	0(4) 1(1) 2(2)	0(5) 1(1) 2(0)
	Neural invasion		0(6) 1(0) 2(1)	0(6) 1(0) 2(0)

*lymph node metastasis : LN(+)
**lymph node non-metastasis : LN(-)
***Depth of Invasion : DOI

Table 4. Pathological data

3) Immunohistological background

Immunohistological results of the each cases' are summarized in Table 5. The median lymphatic vessel density was 5.9 % in the metastasis group and 2.7 % in the non-metastasis group. The median blood vessel density was 13.8 % in the metastasis group and 8.3 % in the non-metastasis group. Regarding actin- positive tumor cells, one case was defined as 0 and six were defined as 1 in the metastasis group, while four cases were defined as 0 and two were defined as 1 in the non-metastasis group. In terms of budding, three cases were Grade 2 and four were Grade 3 in the metastasis group, while five cases were Grade 1 and one was Grade 3 in the non-metastasis group. Regarding E-cadherin-positive tumor cells, four cases were classified as 0 and three as 1 in the metastasis group, while one case was classified as 0 and five as 1 in the non-metastasis group. The median Ki-67 positive rate was 34.5 % in the metastatic group

and 12.9 % in the non-metastasis group. In terms of SOX2 -positive tumor cells, all seven cases were defined as 1 in the metastasis group, while four cases were 0 and two were 1 in the non-metastasis group.

	LN(+) * (n = 7)							LN(-)** (n = 6)					
Case No.	1	2	3	4	5	10	11	6	7	8	9	12	13
Podoplanin Lymph vessel density (%)	7.9	4.1	5.9	3.7	6.3	5.2	8.5	1.2	6.2	3.1	2.2	1.7	3.4
CD34 Vasucular density (%)	10.2	23.0	18.7	10.2	12.1	13.8	19.5	8.4	4.4	10.3	18.8	8.2	5.1
Actin Evaluation of positive tumor cells	0	1	1	1	1	1	1	0	0	1	1	0	0
Cytokeratin Budding grade	2	2	2	3	3	3	3	1	1	1	1	1	3
E-cadherin Evaluation of positive tumor cells	0	1	0	1	0	1	0	1	0	1	1	1	1
Ki-67 Positivity (%)	19.2	23.3	11.8	44.2	34.5	41.9	41.0	7.6	12.7	12.5	22.9	14.0	13.0
SOX2 Evaluation of positive tumor cells	1	1	1	1	1	1	1	0	0	0	1	0	1

*lymph node metastasis : LN(+)

**lymph node non-metastasis : LN(-)

Table 5. Results of immunohistochemical studies

4) Statistical analysis

There were significant differences in the following three factors: DOI ($p = 0.035$), lymph vessel density ($p = 0.003$), and Ki-67 positive rate ($p = 0.035$). Meanwhile, there was no significant difference in vessel density ($p = 0.051$) (Table 6).

Item	LN(+)* (n=7)	LN(-)** (n=6)	P value
DOI***(mm)	Max : 16.0 Min : 2.9 5.5	Max : 5.3 Min : 1.5 3.9	0.035
Lymphatic vessel density(LVD) (%)	Max : 8.5 Min : 3.7 5.9	Max : 6.2 Min : 1.2 2.7	0.003
Vascular vessel density(VVD) (%)	Max : 23.0 Min : 10.2 13.8	Max : 18.8 Min : 4.4 8.3	0.051
Ki-67 positive rate(%)	Max : 44.2 Min : 11.8 34.5	Max : 22.9 Min : 7.6 12.9	0.035

*lymph node metastasis : LN(+)

**lymph node non-metastasis : LN(-)

***Depth of Invasion : DOI

Table 6. Average of each measured value

Factors with a strong correlation to cervical lymph node metastasis were budding (V = 0.87), SOX2 (V = 0.85), grade (V = 0.55), actin (V = 0.54), E-cadherin (V = 0.41), macroscopic type (V = 0.39), and YK (V = 0.28), in descending order (Fig. 4).

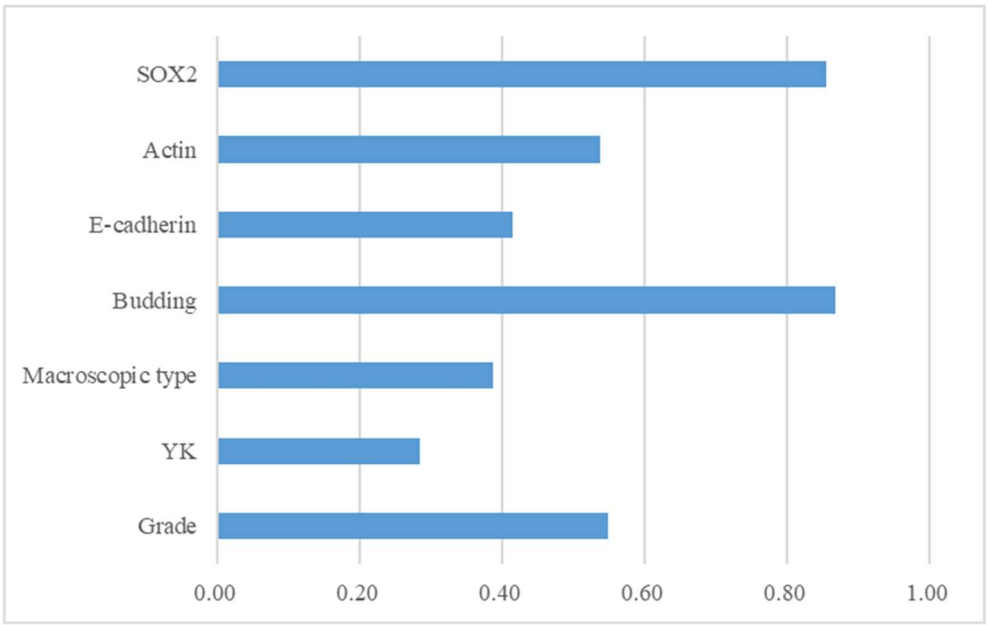

Fig. 4. Cramer's V coefficient of association to cervical lymph node metastasis Correlation with cervical lymph node metastasis was defined as greater than 0.25, and a strong correlation was defined as greater than 0.5.

Examination by extended quantification type II of the effect on classification showed that Ki-67, E-cadherin, DOI, and macroscopic type, in descending order, contributed to cervical lymph node metastasis. The correlation ratio was as high as 0.871, while the percentage of correct classification was 100 % (Table 7).

Item	Category	n	Category score	Contribution rate	P value
Macroscopic type	Superficial type	3	0.895	5.102	0.043
	Exophytic type	4	0.244		
	Endophytic type	6	-0.610		
E-cadherin	Negative	5	-0.733	8.026	0.025
	Positive	8	0.458		
DOI*		13	-0.128	7.482	0.029
Ki-67 positivity		13	-0.043	12.220	0.010
LN	(+)**	7	Correlation ratio = 0.871 (P<0.001) Discriminative predictive value = 100%		
	(-)***	6			

*Depth of Invasion: DOI

**lymph node metastasis: LN(+)

***lymph node non-metastasis: LN(-)

Table 7. Multivariate analysis (Extended quantification type II)

Discussion

Among cancers that occur in the oral region, tongue cancer leads to cervical lymph node metastasis at an early stage and survival rate is low following metastasis compared to those with no metastases (7, 20). In some hospitals, elective neck dissection (END) is proactively performed even for early-stage tongue cancer if a strong invasive tendency is observed. However, in many hospitals, a “wait and see” approach is used instead of performing END if the tongue cancer does not show obvious signs of cervical lymph node metastasis at the time of diagnosis (1-5). A study showed that subsequent cervical lymph

node metastasis was observed in 70 % of T1 and T2 cases that demonstrated a strong invasive tendency (21). Another study reported that delayed cervical lymph node metastasis was the poor prognosis (6). These studies indicate the need for END. However, as a result of surgical invasion, a decline in function is observed. Thus, the goal of treatment is to achieve improved survival rate while keeping functional decrease to a minimum so as to increase the QOL of the patient with cervical lymph node metastasis. To achieve this goal, diagnosis of potential cervical lymph node metastasis and examination of predictors strongly associated with cervical lymph node metastasis are required.

Clinically, it has been reported that a large tumor size is associated with increased metastasis frequency and that the metastasis rate is high in tongue cancer when the invasion site is at the posterior tongue (22, 23). Furthermore, the DOI was added to the TNM classification as a criterion by the Union for International Cancer Control in 2018. DOI is divided into three stages: ≤ 5 mm, 5 to 10 mm and > 10 mm. It is considered that DOI rather than tumor size is associated with metastasis rate. There have been pathological studies that have demonstrated a significant association of histological degree of malignancy and neurovascular invasion with lymph node metastasis rate (23-25). In particular, a close relationship between the invasion mode of the invasive front of the tumor and lymph node metastasis has been reported (26, 27). We compared factors reported as being associated with lymph node metastasis. We also quantified the categories of each factor and investigated the correlations to create better predictors by combining relative factors.

In the present study, we performed discriminant analysis on measurable DOI, vessel density, lymph vessel density, and Ki-67 positive rate. Regarding DOI, the median was 5.5 mm in the lymph node metastasis

group and 3.9 mm in the non-metastasis group. The Oral Cancer Clinical Guidelines (28) indicate that END is to be considered for N0 oral cancer with a DOI of ≥ 4 mm. This suggests that a DOI of ≥ 4 mm indicates potential lymph node metastasis, which is in line with the results of the present study. Vessel density did not show a significant difference regarding lymph node metastasis; however, lymphatic vessel density showed a significant difference. Sugiura et al. (16) examined vessel density in 160 cases of OSCC and reported that, the lymphatic vessel density was significantly associated with lymph node metastasis. Ki-67 is a suitable marker for the evaluation of the proliferative capacity of tumor cells as it is expressed throughout the cell cycle, with the exception of the G0 phase (resting phase) (29). Tumuluri et al. (30) report that the positive index of Ki-67 was significantly higher at depth of 5 mm and above, and for cervical lymph node metastasis. Suresh et al. (31) also reported that Ki-67 positive index was correlated with histological grade and cervical lymph node metastasis. In the present study, the Ki-67 positive rate in the invasive front of the tumor was also significantly higher in the lymph node metastasis group, suggesting a high degree of association between these two factors. Quantification and comparison of other related factors showed that budding in the invasive front of the tumor had the strongest correlation. Budding evaluated the initial stages of lymph node metastasis caused by solid carcinoma. They reported that budding have observed as tumor cells in the process of EMT and that the presence of these cells were superior as a prognostic predictor (11, 12). In the present study, although we were unable to reach a conclusion due to the small number of samples, the results were similar to those reported by them (11, 12), with budding showing a strong correlation with lymph node metastasis. SOX2 is a stem cell marker that plays an important role in maintaining pluripotency.

It has been found to be expressed in cells of malignant tissues. Du et al. (32) have shown that the expression of SOX2 in tongue SCC with no lymph node metastasis is involved in tumor progression. Furthermore, Michifuri et al. (33) have reported that there was a significant correlation between the staining pattern of SOX2 and lymph node metastasis. SOX2 showed a strong correlation with lymph node metastasis, similar to budding, in the present study as well. These results are similar to those reported in previous studies (13, 14, 15, 32, 33). However, budding and SOX2 each have a strong correlation independently, but the combination of the two was not involved in cervical lymph node metastasis. Additionally, actin is a smooth muscle marker, and Yamaguchi et al. (34) described invadopodia in cancer cells and elucidated that invadopodia, whose central structure is actin filaments, destroy the extracellular matrix, thereby leading to invasion and metastasis. The results of the present study also showed a correlation between actin and cervical lymph node metastasis. Furthermore, Kojc et al. (35) reported that actin-positive myofibroblasts were not observed in normal laryngeal mucosa or in the stroma of intraepithelial lesions (SIL) and were only found in the stroma of SCC. This suggests that SIL is associated with the transformation to SCC. It is believed that actin is involved in microenvironmental changes associated with tumor progression, as actin-positive cells are observed in the stroma of the invasive front of the tumor. Although it showed a rather weak correlation, E-cadherin is closely involved in the biding of epithelial cells and in maintaining tissue morphology; indeed, it is a factor associated with EMT. Suresh et al. (31) and Lim et al. (36) also suggested the possibility that E-cadherin is an effective predictor of lymph node metastasis. This study examined the relationship of E-

cadherin, DOI, Ki-67 positive rate, and macroscopic type with cervical lymph node metastasis and found that they were all involved.

The present study focused on the biological characteristics of the invasive front of the tumor in tongue SCC and investigated factors related to lymph node metastasis. Similar to SCC occurring in other areas, budding and SOX2 were also effective prognostic predictors with a strong association in the tongue. Furthermore, it was suggested that cases which are endophytic, as observed macroscopically with a DOI of ≥4 mm, and in which the tumor cells have proliferating capability with enhanced EMT are likely to progress to lymphatic metastasis. Therefore, budding, SOX2, macroscopic type, DOI, Ki-67, and E-cadherin are effective as factors for predicting the prognosis of cervical lymph node metastasis.

Conclusion

This study divided tongue SCC patients into a cervical lymph node metastasis group and a non-metastasis group and focused on the characteristics of tumor cells in the invasive front of the tumor and the vessel density of tumor stroma in order to investigate predictive factors strongly associated with cervical lymph node metastasis. There was a significant difference between the cervical lymph node metastasis group and the non-metastasis group in lymph vessel density in the tumor stroma.

Budding and SOX2 showed a strong correlation with cervical lymph node metastasis.

Ki-67, DOI, E-cadherin, and macroscopic type were also shown to contribute to cervical lymph node metastasis.

Reference

(1) O-charoenrat P, Pillai G, Patel S, Fisher C, Archer D, Eccles S, Rhys-Evans P: Tumour thickness predicts cervical nodal metastases and survival in early oral tongue cancer. Oral Oncol, 39: 386-390, 2003.

(2) Keski-Säntti H, Atula T, Törnwall J, Koivunen P, Mäkitie A: Elective neck treatment versus observation in patients with T1/T2 N0 squamous cell carcinoma of oral tongue. Oral Oncol, 42: 96-101, 2006.

(3) Kaneko S, Yoshimura T, Ikemura K, Shirasuna K, Kusukawa J, Ohishi M, Shiba R, Sunakawa H, Tominaga K, Sugihara K, Shinohara M, Katsuki T, Yanagisawa S, Kurokawa H, Mimura T, Ikeda H, Yamabe S, Ozeki S: Primary neck management among patients with cancer of the oral cavity without clinical nodal metastases: A decision and sensitivity analysis. Head Neck, 24: 582-590, 2002.

(4) Yanamoto S, Yamada S, Takahashi H, Kawasaki G, Ikeda H, Shiraishi T, Fujita S, Ikeda T, Asahina I, Umeda M: Predictors of locoregional recurrence in T1-2N0 tongue cancer patients. Pathol Oncol Res, 19: 795-803, 2013.

(5) Okura M, Aikawa T, Sawai YN, Iida S, Kogo M: Decision analysis and treatment threshold in a management for the N0 neck of the oral cavity carcinoma. Oral Oncol, 45: 908-911, 2009.

(6) Teichgraeber JF, Clairmont AA: The incidence of occult metastases for cancer of the oral

tongue and floor of the mouth. Treatment rationale. Head Neck Surg, 7: 15-21, 1984.

(7) Ebrahimi A, Zhang WJ, Gao K, Clark JR: Nodal yield and survival in oral squamous cancer. Cancer, 117: 2917-2925, 2011.

(8) Tumuluri V, Thomas GA, Fraser IS: The relationship of proliferating cell density at the invasive tumour front with prognostic and risk factors in human oral squamous cell carcinoma. J Oral Pathol Med, 33 : 204-208, 2004.

(9) Bryne M, Koppang HS, Lilleng R, Kjærheim Å: Malignancy grading of the deep invasive margins of oral squamous cell carcinomas has high prognostic value. J Pathol, 166: 375-381, 1992.

(10) Ueno H, Murphy J, Jass JR, Mochizuki H, Talbot IC: Tumour 'budding' as an index to estimate the potential of aggressiveness in rectal cancer. Histopathology, 40: 127-132, 2002.

(11) Almangush A, Pirinen M, Heikkinen I, Mäkitie AA, Salo T, Leivo I: Tumour budding in oral squamous cell carcinoma: a meta-analysis. Br J Cancer, 118: 577-586, 2018.

(12) Wang C, Huang H, Huang Z, Wang A, Chen X, Huang L, Zhou X, Liu X: Tumor budding correlates with poor prognosis and epithelial-mesenchymal transition in tongue squamous cell carcinoma. J Oral Pathol Med, 40: 545-551, 2011.

(13) Ge N, Lin HX, Xiao XS, Guo L, Xu HM, Wang X, Jin T, Cai XY, Liang Y, Hu WH, Kang T: Prognostic significance of Oct4 and Sox2 expression in hypopharyngeal

squamous cell carcinoma. J Transl Med, 8: 94, 2010.

(14) Wang Q, He W, Lu C, Wang Z, Wang J, Giercksky KE, Nesland JM, Suo Z: Oct3/4 and Sox2 are significantly associated with an unfavorable clinical outcome in human esophageal squamous cell carcinoma. Anticancer Res, 29: 1233-1242, 2009.

(15) Lu Y, Futtner C, Rock JR, Xu X, Whitworth W, Hogan BLM, Onaitis MW: Evidence that SOX2 overexpression is oncogenic in the lung. PLOS ONE, 5: e11022, 2010.

(16) Sugiura T, Inoue Y, Matsuki R, Ishii K, Takahashi M, Abe M, Shirasuna K: VEGF-C and VEGF-D expression is correlated with lymphatic vessel density and lymph node metastasis in oral squamous cell carcinoma: Implications for use as a prognostic marker. Int J Oncol, 34: 673-680, 2009.

(17) Chen B, Fang WK, Wu ZY, Xu XE, Wu JY, Fu JH, Yao XD, Huang JH, Chen JX, Shen JH, Zheng CP, Wang SH, Li EM, Xu LY: The prognostic implications of microvascular density and lymphatic vessel density in esophageal squamous cell carcinoma: Comparative analysis between the traditional whole sections and the tissue microarray. Acta Histochem, 116: 646-653, 2014.

(18) Japanese Society of Oral Oncology: General Rules for Clinical and Pathological Studies on Oral Cancer. The 2nd Edition, 2019, Kanahara publisher, Tokyo, Japan.

(19) Japanese Society for Cancer of the Colon and Rectum: Japanese Classification of Colorectal, Appendiceal, and Anal Carcinoma. Ninth Edition, 2018, Kanahara

publisher, Tokyo, Japan.

(20) Shingaki S, Takada M, Sasai K, Bibi R, Kobayashi Y, Nomura T, Saito C: Impact of lymph node metastasis on the pattern of failure and survival in oral carcinoma. Am J Surg, 185: 278-284, 2003.

(21) Asakage T, Yokose T, Mukai K, Tsugane S, Tsubono Y, Asai M, Ebihara S: Tumor thickness predicts cervical metastasis in patients with stage Ⅰ/Ⅱ carcinoma of the tongue. Cancer, 82: 1443-1448, 1998.

(22) Shear M, Hawkins DM, Farr HW: The prediction of lymph node metastases from oral squamous carcinoma. Cancer, 37: 1901-1907, 1976.

(23) Woolgar JA, Scott J: Prediction of cervical lymph node metastasis in squamous cell carcinoma of the tongue/floor of mouth. Head Neck, 17: 463-472, 1995.

(24) Wu K, Yang X, Li L, Ruan M, Liu W, Lu W, Zhang C, Li S: Neurovascular invasion and histological and grade serve as the risk factor of cervical lymph node metastases in early tongue squamous cell carcinoma. Mol Neurobiol, 53: 2920-2926, 2016.

(25) Okada Y: Relationships of cervical lymph node metastasis to histopathological malignancy grade, tumor angiogenesis, and lymphatic invasion in tongue cancer. Odontology, 98: 153-159, 2010.

(26) Kurokawa H, Yamashita Y, Takeda S, Zhang M, Fukuyama H, Takahashi T: Risk factors for late cervical lymph node metastases in patients with stage Ⅰor Ⅱ carcinoma

of the tongue. Head Neck, 24:731-736, 2002.

(27) Osaki T, Yoneda K, Yamamoto T, Ueta E: Clinical and histopathologic characteristics of tongue and gingiva carcinomas with occult and clinically evident cervical lymph-node metastasis. Int J Oral Maxillofac Surg, 25: 274-278, 1996.

(28) Byers RM, El-Naggar AK, Lee YY, Rao B, Fornage B, Terry NHA, Sample D, Hankins P, Smith TL, Wolf PJ: Can we detect or predict the presence of occult nodal metastases in patients with squamous carcinoma of the oral tongue? Head Neck, 20: 138-144, 1998.

(29) Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H: Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol, 133: 1710-1715, 1984.

(30) Tumuluri V, Thomas GA, Fraser IS: The relationship of proliferating cell density at the invasive tumour front with prognostic and risk factors in human oral squamous cell carcinoma. J Oral Pathol Med, 33: 204-208, 2004.

(31) Suresh TN, Hemalatha A, Kumar MLH, Mohiyuddin SMA: Evaliation of histomorphological and immunohistochemical parameters as biomarkers of cervical lymph node metastasis in squamous cell carcinoma of oral cavity: A retrospective study. J Oral Maxillofac Pathol, 19: 18-24, 2015.

(32) Du L, Yang Y, Xiao X, Wang C, Zhang X, Wang L, Zhang X, Li W, Zheng G, Wang S,

Dong Z: Sox2 nuclear expression is closely associated with poor prognosis in patients with histologically node-negative oral tongue squamous cell carcinoma. Oral Oncol, 47: 709-713, 2011.

(33) Michifuri Y, Hirohashi Y, Torigoe T, Miyazaki A, Kobayashi J, Sasaki T, Fujino J, Asanuma H, Tamura Y, Nakamori K, Hasegawa T, Hiratsuka H, Sato N: High expression of ALDH1 and SOX2 diffuse staining pattern of oral squamous cell carcinomas correlates to lymph node metastasis. Pathol Int, 62: 684-689, 2012.

(34) Yamaguchi H, Wyckoff J, Condeelis J: Cell migration in tumors. Curr Opin Cell Biol, 17: 559-564, 2005.

(35) Kojc N, Zidar N, Vodopivec B, Gale N: Expression of CD34, α-smooth muscle actin, and transforming growth factor β1 in squamous intraepithelial lesions and squamous cell carcinoma of the larynx hypopharynx. Hum Pathol, 36: 16-21, 2005.

(36) Lim SC, Zhang S, Ishii G, Endoh Y, Kodama K, Miyamoto S, Hayashi R, Ebihara S, Cho JS, Ochiai A: Predictive markers for late cervical metastasis in stage Iand II invasive squamous cell carcinoma of the oral tongue. Clin Cancer Res, 10: 166-172, 2004.