［原著］Evaluation of delayed cervical lymph node metastasis in T1/2 squamous cell carcinoma of the tongue

Evaluation of delayed cervical lymph node metastasis in T1/2 squamous cell carcinoma of the tongue

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ABSTRACT

Purpose To predict the risk of delayed cervical lymph node metastasis (delayed-CLN-met) in T1/2 squamous cell carcinoma (SCC) of the tongue, clinicopathological and immunohistochemical characteristics of cervical lymph node metastasis (CLN-met) cases were compared to those negative for cervical lymph node (neg-CLN) involvement. **Methods** Clinical characteristics, histopathological parameters, intratumoral lymphatic vessel density (LVD), and intratumoral microvessel density (MVD) were evaluated in 54 patients with T1/2 SCC of the tongue. LVD and MVD were determined by immunohistochemical staining using D2-40 and CD34 antibodies, respectively. **Results** Parameters significantly correlated with lymph node metastases included T classification (P = 0.0013) and muscle invasion (P = 0.0045). In CLN-met cases, the LVD was significantly higher (P = 0.0003) compared with neg-CLN cases. MVD was not statistically different (P = 0.39) in CLN-met cases compared with neg-CLN cases. Results of multivariate logistic regression analysis for CLN-met showed that T classification and LVD were independent risk factors for CLN-met. Although results of multivariate logistic regression analysis for delayed-CLN-met was not statistically significant, LVD had a tendency to be a risk factor for delayed-CLN-met in univariate analysis (P = 0.05). The depth of SCC invasion into the muscle layer (depth-IM) was statistically significant (P = 0.002). **Conclusions** LVD and T classification were independent risk factors for CLN-met; however, in predicting delayed-CLN-met, whether tumor invades to the muscle layer was only a risk factor in T1/2 SCC of the tongue. Although LVD was not statistically significant to predict delayed-CLN-met, LVD had a tendency to be a risk factor for delayed-CLN-met. Cases with depth-IM of $\geq$ 2 mm and high LVD are thought to be at high risk of delayed-CLN-met in stage I/II SCC of the tongue. *Ryukyu Med. J., 29(3,4)31～38, 2010*

Key words: squamous cell carcinoma of the tongue, delayed cervical lymph node metastasis, immunohistochemistry, lymphatic vessel density, receiver operating characteristic curve

INTRODUCTION

The tumor-node-metastasis (TNM) staging system is currently the most accurate prognostic parameter in patients with squamous cell carcinoma (SCC) of the oral cavity. Although it is generally accepted that overall survival of patients with SCC of the oral cavity who are clinically negative for cervical lymph node involvement (neg-CLN) is good, prognosis for patients with cervical lymph node metastasis (CLN-met) development after tumor excision or radiotherapy is poor. It is especially important to detect CLN-met in its early stages, because treatment of early lymph node metastases generally yields a better clinical outcome than treatment of late metastases[1]. Thus, a major concern in the care of patients with SCC of the oral cavity with neg-CLN is the risk of delayed cervical lymph node metastases (delayed-CLN-met).

Despite recent advances in the evaluation of CLN-met using computed tomography, magnetic resonance imaging, ultrasonography, and serum tumor markers, 20 to 50% of neg-CLN patients develop clinically evident delayed-CLN-met within 2 years of follow-up[2-4]. If a diagnostic indicator of biologic aggressiveness could be obtained at the time of incisional or excisional biopsy, it would enable accurate classification of patients in regard to their risk of delayed-CLN-met. An indicator could also enable comparison of treatment modalities and, based on the relationship between indicator and patient outcome, could ultimately facilitate changes in treatment decisions and/or determine the need for close follow-up.

Angiogenesis of tumors is widely accepted as an indicator of tumor growth and metastasis. In addition, lymphangiogenesis in tumors and its role in metastasis has become an interesting focus in cancer research. Several studies of different cancer types have found correlations between lymphatic vessel density (LVD) and metastasis[5-9]. In SCC of the oral cavity, few reports have been issued correlations between LVD and metastasis. These studies have compared metastatic groups with non-metastatic groups, but have not investigated delayed metastasis. Relationship between LVD and delayed-CLN-met has not been clear.

The present study was undertaken to predict the risk of delayed-CLN-met by comparing CLN-met cases with neg-CLN cases.

PATIENTS AND METHODS

I. Patients

This study included 173 patients with T1/2 SCC of the tongue who were surgically treated between 1988 and 2008 at the Department of Oral and Maxillofacial Surgery, University Hospital, University of the Ryukyus, Japan. The study was approved by a review committee at the University of the Ryukyus and was performed in accordance with the precepts established by the Helsinki Declaration. Tumor staging was performed according to TNM classification. In tongue cancer, stage I is T1N0M0, stage II is T2N0M0 and the others are stage III or IV. Thus, at initial examination, CLN-met cases were classified as stage III or IV and neg-CLN cases and delayed-CLN-met cases were classified stage I or II. Lymph node metastasis was confirmed by clinical and imaging examinations. Clinical and imaging characteristics of metastatic lymph node were as follows; in clinical examination, 1) elastic hard; 2) fixed; in computed tomography examination, 1) minor axis $\geq$ 10 mm; 2) round shape; 3) low density area; 4) high signal; 5) rim enhancement; in ultrasonography examination, 1) minor axis $\geq$ 10 mm; 2) round shape; 3) clear margin; 4) heterogeneous echo signal (excluded echogenic hilus)[10]. Exclusion criteria included patients who 1) were diagnosed either local recurrence, multiple cancer or distant metastasis (n = 13); 2) underwent induction therapy and did not have lymph node metastasis (n = 68); 3) underwent elective neck dissection and did not have lymph node metastases (n = 0); 4) underwent elective neck dissection and were clinically neg-CLN at initial examination but CLN-met on pathological examination (n = 7); and 5) had incisional biopsy specimens with tumor thickness less than 4 mm (n = 31). Consequently, 54 patients (neg-CLN, n = 25; CLN-met, n = 13; delayed-CLN-met, n = 16) were included in the analysis. CLN-met and delayed-CLN-met were confirmed by pathological examination. All of patients were followed up until death or for at least 24 months.

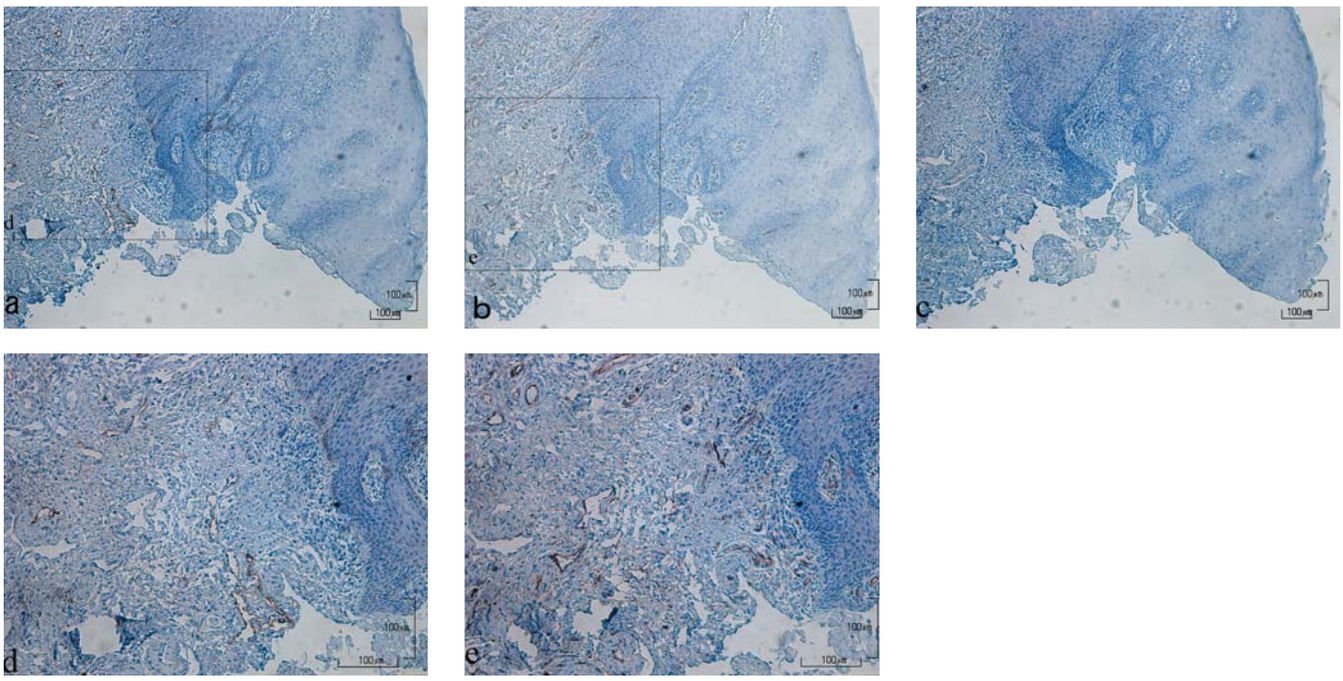

Fig. 1 **Immunohistochemical staining was performed on serial sections.** 1a: lymphatic vessels (D2-40: 100 × magnification). 1b: microvessels (CD34: 100 × magnification). 1c: negative control (PBS: 100 × magnification). 1d: lymphatic vessels (boxed area in 1a, 200 × magnification). 1e: microvessels (boxed area in 1b, 200 × magnification).

II. Pathology

All slides were made from incisional or excisional biopsy samples fixed in 10% formalin and embedded in paraffin, and were reviewed and diagnosed by pathologists. The mode of invasion (MOI) was determined according to Yamamoto's classification (modified Jacobsson's classification) by oral surgeons[11,12]. In excisional biopsy samples, the depth of SCC invasion into the muscle layer (depth-IM) was measured on serial section specimens that included the maximum tumor surface.

III. Immunohistochemistry

Sections (4 μm thick) were deparaffinized in xylene and rehydrated in graded concentration alcohol washes. Antigen retrieval was performed using sodium citrate buffer (10 mM, pH 6.0) through autoclave processing for 20 min (121 degrees Celsius, 0.12 MPa). Endogenous peroxidase was blocked by treatment with 3% hydrogen peroxide for 10 min. Sections were incubated for 1 h at room temperature with primary antibodies D2-40 (5-10 μg/ml, ready to use) for lymphatic vessels, CD34 (5-10 μg/ml, ready to use) for vascular vessels (Nichirei, Tokyo, Japan) and phosphate buffered saline (pH 7.4) for negative control. The sections were rinsed using Tris-buffered saline with 0.1% Tween-20 (pH 7.6) and incubated with Histofine simple stain MAX-PO(M) (Nichirei, Tokyo, Japan) for 1 h at room temperature. The sections were rinsed using Tris-buffered saline with 0.1% Tween-20 and visualized using 3,3'-diaminobenzidine. Mayer's hematoxylin was used for counterstaining. Figure 1 shows immunohistochemical staining for D2-40 and CD34.

IV. Calculation of lymphatic vessel density (LVD) and microvessel density (MVD) in intratumoral areas

The lymphatic vessels, which were identified as tube-like or slit-like structures, were counted. LVD and MVD were defined according to Lee's method[5]. Briefly, hotspots with the highest LVD and MVD were identified in the intratumoral area under a microscope at 40 × or 100 × magnification. Digital images of hotspots were acquired at 200 × magnification using a Nikon digital sight DS-Fi1, Nikon TV lens C-0.6x, Nikon optiphoto plan 20 lens (Nikon, Tokyo, Japan). One image field corresponded to an optical field of 0.398 mm^2.

V. Statistical Methods

All calculations were carried out using JMP version 8.0.2 software for Windows (SAS Institute

Table 1 **Clinical and histopathological parameters in neg-CLN and CLN-met cases**

Parameters	Total n = 38 (100%)	Neg-CLN n = 25 (65.8%)	CLN-met n = 13 (34.2%)	P-value
Age	24 - 99	24 - 99	27 - 90	
Sex				
Male	19 (50.0)	11 (57.9)	8 (42.1)	0.31
Female	19 (50.0)	14 (73.7)	5 (26.3)	
T classification				
T1	13 (34.2)	13 (100)	0 (0)	0.0013**
T2	25 (65.8)	12 (48.0)	13 (52.0)	
Growth type				
Exophytic	19 (50.0)	14 (73.7)	5 (26.3)	0.31
Endophytic	19 (50.0)	11 (57.9)	8 (42.1)	
Differentiation				
Well	14 (36.8)	9 (64.3)	5 (35.7)	
Moderate	21 (55.3)	14 (66.7)	7 (33.3)	0.99
Poor	3 (7.9)	2 (66.7)	1 (33.3)	
MOI				
G1	0 (0)	0 (0)	0 (0)	
G2	6 (15.8)	6 (100)	0 (0)	0.088
G3	24 (63.2)	13 (54.2)	11 (45.8)	
G4	8 (21.0)	6 (75.0)	2 (25.0)	
Muscle invasion				
Absent	11 (28.9)	11 (100)	0 (0)	0.0045**
Present	27 (71.1)	14 (51.8)	13 (48.2)	

Pearson's chi-square test, **p < 0.01

neg-CLN: negative cervical lymph node; CLN-met: cervical lymph node metastasis; delayed-CLN-met: delayed cervical lymph node metastasis; MOI: mode of invasion; G: grade; Muscle invasion: tumor invades to the muscle layer.

Japan, Tokyo, Japan). The student t-test was used to compare LVD, MVD and depth-IM according to clinicopathological variables. The receiver operating characteristic curve was used to determine the cut-off point. The Youden index (J) was taken to be the optimal cut-off point. Multivariate analysis of risk factors was performed using multivariate logistic regression analysis. The logit estimators used Haldane correction of 0.5 in every cell when a table contained a zero. $P < 0.05$ was considered statistically significant.

RESULTS

I. Clinical and histopathological parameters

The rates of CLN-met and delayed-CLN-met were 24.1% and 29.6%, respectively. Patients' clinical and histopathological parameters in neg-CLN and CLN-met cases are summarized in Table 1. A chi-square test for contingency revealed statistically significant relationships between lymph node metastasis, T classification, and muscle invasion.

II. LVD and MVD analyses in neg-CLN and CLN-met cases

Table 2 shows LVD and MVD according to lymph node status. In CLN-met cases, the LVD increased significantly ($P = 0.0003$, t-test) compared with neg-CLN cases: CLN-met, 22.23 ± 12.46; neg-CLN, 9.68 ± 7.03 (number ± S.D. / field). MVD was not statistically different ($P = 0.39$, t-test) in CLN-met cases compared with neg-CLN cases: CLN-met, 55.15 ± 23.50; neg-CLN, 45.96 ± 34.15 (number ± S.D. / field).

Table 2 **LVD and MVD according to lymph node status**

	Neg-CLN n = 25	CLN-met n = 13	P-value
LVD number / field (0.398 mm^2)	9.68±7.03	22.23±12.46	0.0003**
MVD number / field (0.398 mm^2)	45.96±34.15	55.15±23.50	0.39

t-test mean ± S.D.** p < 0.01

LVD: lymphatic vessel density; MVD: microvessel density; neg-CLN: negative cervical lymph node; CLN-met: cervical lymph node metastasis.

III. Determination of cut-off point

To determine the cut-off point for LVD, a ROC curve was drawn for LVD. The value of the area under the curve (AUC) for LVD was 0.83 (Fig. 2). LVD of 14 received a J of 0.61. Therefore, LVD ≥ 14 was classified as high LVD.

IV. Relationship between LVD and the positive predictors for CLN-met

The positive predictors for CLN-met were selected by univariate analyses. T classification and muscle invasion had a significant correlation with CLN-met (Table 1). Results for the relationship

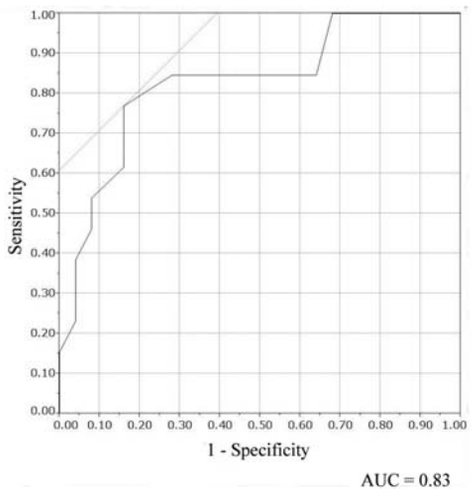

Fig. 2 **ROC curves for LVD in neg-CLN cases and CLN-met cases.** LVD: lymphatic vessel density; neg-CLN: negative cervical lymph node; CLN-met: cervical lymph node metastasis; AUC: area under the curve.

Table 3 **LVD according to positive predictors for CLN-met**

	LVD number/field (0.398 mm^2)	P-value
T classification		
T1 (n = 13)	10.38 ± 8.68	0.15
T2 (n = 25)	15.84 ± 11.61	
Muscle invasion		
Absent (n = 27)	8.00 ± 4.94	0.029*
Present (n = 11)	16.41 ± 11.76	

t-test mean ± S.D.* p < 0.05

CLN-met: cervical lymph node metastasis; LVD: lymphatic vessel density; Muscle invasion: tumor invades to the muscle layer.

between LVD and positive predictors of CLN-met cases are shown in Table 3. The LVD was not statistically different (P = 0.15, t-test) in T1 cases compared with T2 cases: T1, 10.38 ± 8.68; T2, 15.84 ± 11.61 (number ± S.D. / field). In muscle invasion cases, the LVD increased significantly (P = 0.029, t-test) compared with cases without muscle invasion: absent, 8.00 ± 4.94; present, 16.41 ± 11.76 (number ± S.D. / field).

V. Multivariate logistic regression analysis for CLN-met

T classification, muscle invasion, and LVD had significant correlations with CLN-met by univariate analyses and were selected for multivariate logistic regression analysis. LVD was classified by ROC method (high, ≥ 14; low, <14). Results of multivariate logistic regression analysis are summarized in Table 4. LVD was a risk factor for CLN-met, independent of other predictors. T classification was also an independent metastasis related factor. Muscle invasion was not an independent factor.

Table 4 **Multivariate logistic regression analysis for CLN-met**

	P-value	Odds ratio
T classification	0.0388*	7.55
Muscle invasion	0.3224	2.62
LVD	0.0066**	9.05

* P < 0.05, ** P < 0.01

Muscle invasion: tumor invades to the muscle layer; LVD: lymphatic vessel density.

VI. Evaluation of delayed-CLN-met

Multivariate logistic regression analysis for CLN-met suggested that LVD and T classification were predictors for CLN-met. Thus, to predict delayed-CLN-met, LVD and T classification were selected for multivariate logistic regression analysis for delayed-CLN-met. Although LVD had a tendency to be an independent factor, neither LVD nor T classification were significant independent factors: T classification, p = 0.98, odds ratio 0.98; LVD, p = 0.054, odds ratio 4.07. MVD was not statistically different (P = 0.71, t-test) in delayed-CLN-met cases compared with neg-CLN cases: delayed-CLN-met, 49.50 ± 19.80; neg-CLN, 45.96 ± 34.15 (number ± S.D. / field). Thus, we investigated the risk factors for delayed-CLN-met (Table 5). Muscle invasion was the only risk factor for delayed-CLN-met (p = 0.0035). LVD had a tendency to be risk factor (p = 0.050). Thus, muscle invasion and LVD were selected for multivariate logistic regression analysis for delayed-CLN-met. Neither muscle invasion nor LVD were significant independent factors: muscle invasion p = 0.050 odds ratio 4.86; LVD, p = 0.098, odds ratio 3.53. Table 6 shows the mean of depth-IM. The depth-IM was statistically significant (t-test, p = 0.002): neg-CLN, 787.40 ± 1489.74 (μm); delayed-CLN-met, 2569.69 ± 1940.30 (μm). The value of AUC for depth-IM was 0.76 (Fig. 3) and depth-IM of 2080 μm received a J of 0.63.

Table 5 **Clinical and histopathological parameters in neg-CLN and delayed-CLN-met cases**

Parameters	Total n = 41 (100%)	Neg-CLN n = 25 (61.0%)	Delayed-CLN-met n = 16 (39.0%)	P-value
Age	24 - 99	24 - 99	28 - 78	
Sex				
Male	20 (48.8)	11 (55.0)	9 (45.0)	0.44
Female	21 (51.2)	14 (66.7)	7 (33.3)	
T classification				
T1	22 (53.7)	13 (59.1)	9 (40.9)	0.79
T2	19 (46.3)	12 (63.2)	7 (36.8)	
Growth type				
Exophytic	20 (48.8)	14 (70.0)	6 (30.0)	0.25
Endophytic	21 (51.2)	11 (52.4)	10 (47.6)	
Differentiation				
Well	15 (36.6)	9 (60.0)	6 (40.0)	
Moderate	24 (58.5)	14 (58.3)	10 (41.7)	0.51
Poor	2 (4.9)	2 (100)	0 (0)	
MOI				
G1	0 (0)	0 (0)	0 (0)	
G2	7 (17.1)	6 (85.7)	1 (14.3)	0.14
G3	20 (48.8)	13 (65.0)	7 (35.0)	
G4	14 (34.1)	6 (42.9)	8 (57.1)	
Muscle invasion				
Absent	13 (31.7)	11 (84.6)	2 (15.4)	0.0035**
Present	28 (68.3)	14 (50.0)	14 (50.0)	
LVD				
Low	30 (73.2)	21 (70.0)	9 (30.0)	0.05
High	11 (26.8)	4 (36.4)	7 (63.6)	

Pearson's chi-square test, **p < 0.01

neg-CLN: negative cervical lymph node; delayed-CLN-met: delayed cervical lymph node metastasis; MOI: mode of invasion; G: grade; Muscle invasion: tumor invades to the muscle layer; LVD: lymphatic vessel density.

Table 6 **Depth-IM according to lymph node status**

	Neg-CLN n = 25	Delayed-CLN-met n = 16	P-value
Depth-IM (μm)	787.40±1489.74	2569.69±1940.30	0.002**

t-test, mean ± S.D., **P < 0.01

Depth-IM: the depth of SCC invasion into the muscle layer; neg-CLN: negative cervical lymph node; delayed-CLN-met: delayed cervical lymph node metastasis.

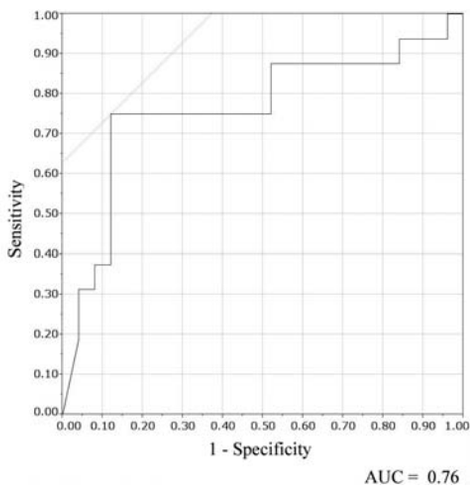

Fig. 3 **ROC curves for depth-IM in neg-CLN cases and delayed-CLN-met cases.** depth-IM: the depth of SCC invasion into the muscle layer; neg-CLN: negative cervical lymph node; delayed-CLN-met: delayed cervical lymph node metastasis; AUC: area under the curve.

DISCUSSION

There are many clinical methods for detecting cervical lymph node metastasis. Despite negative imaging findings, some patients develop delayed-CLN-met. Elective neck dissection and 'wait-and see' comprise the two methods for neck treatment in stage I/II SCC of the oral cavity[2,3,13,14]. In this study, we compared neg-CLN cases with CLN-met cases and evaluated whether any criteria could be applied to predict delayed-CLN-met cases in T1/2 SCC of the tongue.

Recently, lymphangiogenesis has become a focus in cancer research. In SCC of the oral cavity, vascular endothelial growth factor C expression in tumor cell increases intratumoral LVD[15]. There remains considerable debate regarding the evaluation of intratumoral versus peritumoral lymphatic vessels in the pathology of primary human tumors. Some studies define the peritumoral area as the junction between the tumor and normal mucosa. However, this area often represents dysplasia in SCC of the tongue. In the present study, intratumoral LVD rather than peritumoral LVD was assessed because the incisional biopsy specimens did not contain normal mucosa. Previous studies have shown that LVD is increased in the metastasis group[5-9]. Our results also showed that LVD increased

significantly in CLN-met cases rather than in neg-CLN cases. Some studies determined the cut-off point using a mean or median of LVD. However, in this study, the mean LVD in the CLN-met group was 22.23, whereas the ROC curve provided a cut-off point of 14. The mean LVD was 12.44 in delayed-CLN-met and 9.68 in neg-CLN. These findings suggest that the determination of cut-off point using mean or median values may decrease accuracy. Common, formal methods to search for a cut-off point include the maximum chi-square method, minimum P-value method, and ROC curve method[16,17]. If the median, mean, or some other prespecified cut-off point is used, there is considerable loss of information. The ROC curve allows analyses of trade-offs between sensitivity and specificity at all possible cut-off points. Thus, the ROC curve method was thought to be useful for determining the optimal cut-off point. In this study, LVD had a tendency to be a risk factor for predicting delayed-CLN-met in univariate analysis. Thus we will be adding a review on future cases.

In previous studies, depth-IM[18-21], tumor differentiation[18,21], and MOI[4,21,22] have been significantly correlated with delayed-CLN-met. In this study, muscle invasion was associated with delayed-CLN-met and neg-CLN, which is consistent with previous studies. Tumor thickness, depth of invasion, and depth-IM have also been previously reported to be predictors of lymph node metastasis[18-21,23-25]. Parameters vary between studies and not all studies specified the criteria used to determine the cut-off point. It has been commonly reported that deeper invasion, especially a depth of invasion over 4 mm, carries a higher risk of metastasis. Kurokawa et al. reported that delayed-CLN-met was found at tumor depths exceeding 3 mm. In addition, the incidence of delayed-CLN-met increased markedly when the tumor depth exceeded 4 mm in stage I/II SCC of the tongue[21]. We measured the distance from the mucosal surface to the muscle layer and depth-IM in delayed-CLN-met. The mean distance was 1300 μm and mean depth-IM was 2570 μm. The ROC curve provided a 2080 μm cut-off point for delayed-CLN-met. This result was thought to be consistent with that reported by Kurokawa et al. [21].

In conclusion, this study found that LVD and T classification were independent risk factors for CLN-met. However, in predicting delayed-CLN-met, whether tumor invades to the muscle layer was only a risk factor in stage I/II SCC of the tongue. Although LVD was not statistically significant to predict delayed-CLN-met, LVD had a tendency to be a risk factor for predicting delayed-CLN-met. Cases with depth-IM of $\geq$ 2 mm and high LVD are thought to be at high risk of delayed-CLN-met in stage I/II SCC of the tongue.

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