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Analyzing the factors that influence occult metastasis in oral tongue cancer

Jung-Hyun Shin[1,2], Hye-Jung Yoon[3], Soung-Min Kim[1], Jong-Ho Lee[1], Hoon Myoung[1]

[1]*Department of Oral and Maxillofacial Surgery, School of Dentistry and Dental Research Institute, Seoul National University, Seoul,*
[2]*Department of Oral and Maxillofacial Surgery, Dankook University Jukjeon Dental Hospital, Yongin,*
[3]*Department of Oral Pathology, Dental Research Institute, School of Dentistry, Seoul National University, Seoul, Korea*

Abstract (J Korean Assoc Oral Maxillofac Surg 2020;46:99-107)

Objectives: We accessed the various clinico-histopathological factors, and their association with occult metastasis (OM) in oral tongue squamous cell carcinoma (OTSCC).

Materials and Methods: One hundred-nine patients with OTSCC were divided into the elective neck dissection (END) group and the watchful waiting (WW) group. Age, sex, T-stage, depth of invasion and differentiation were evaluated to determine the correlation between clinico-histopathological factors and OM. For immunohistochemical analysis, paraffin-embedded blocks of 41 OTSCC specimens were examined with antibodies (VEGF-c, c-Met, and ROR1).

Results: The group with tumor thickness of oral tongue cancer ≥3 mm had higher incidence of OM than those with a thickness of <3 mm. The depth of invasion was statistically correlated with OM (P=0.022). Immunohistochemical analysis showed that high expression of VEGF-c (P=0.043), c-Met (P=0.009), and ROR-1 (P=0.003) were statistically correlated with OM.

Conclusion: The analysis of these clinico-histopathological and immunohistochemical factors can help to determine neck dissection in clinically negative (cN0) patients.

Key words: Tongue cancer, Elective neck dissection, Watchful waiting, Occult metastasis, Immunohistochemistry

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I. Introduction

Tongue cancer is the most common type of oral cancer. Unlike other head and neck areas, the vascular system and the lymphatic system are well developed in the tongue. Therefore, the incidence of cervical lymph node metastasis (LNM) is high[1]. LNM is the most important prognostic factor for survival of head and neck cancer patients[2,3]. The average 5-year survival rate is >50% in patients without LNM, while that of patients with LNM is only 30%[4]. Unfortunately, this LNM often already exists when the cancer diagnosis is made. Approximately 25% of occult metastasis (OM) is too small to be detected by imaging techniques[5]. Approximately 20% to 50% of OM has been identified in oral tongue cancer patients[1,6].

The treatment of patients with clinically negative (cN0) tongue cancer remains controversial. The current treatment modalities include glossectomy followed by watchful waiting (WW), and glossectomy with elective neck dissection (END). A survey performed in the United States found that there was a lack of consensus regarding the treatment of cN0 neck[7]. A similar finding was described in a European survey in Marburg, Germany[8]. Because LNM is often identified before surgery, there is a need for significant clinicopathologic factors and a highly sensitive detection method, such as immunohistochemistry.

There is growing interest in the correlation between LNM and immunohistochemical (IHC) markers. Vascular endothelial growth factor (VEGF) is essential in angiogenesis and vasculogenesis. The increment of VEGF-c expression is related to the LNM in the human thyroid, lung, prostate, gastric,

Hoon Myoung
Department of Oral and Maxillofacial Surgery, School of Dentistry and Dental Research Institute, Seoul National University, 101 Daehak-ro, Jongno-gu, Seoul 03080, Korea
TEL: +82-2-2072-3059 FAX: +82-2-766-4948
E-mail: myoungh@snu.ac.kr
ORCID: https://orcid.org/0000-0002-9984-8479

colorectal, breast cancer, and melanoma[9-11]. c-Met is known as MET and hepatocyte growth factor receptor (HGFR). c-Met regulates the cellular processes, cell function, and tissue homeostasis in mammalian development[12]. In addition, c-Met can activate lymphangiogenesis, which can ultimately cause LNM[13,14]. ROR1 is a transmembrane protein that regulates skeletal and neuronal development, cell polarity, and cell migration[15,16]. Many studies have shown that ROR1 was overexpressed in human cancers[17-21].

The aim of this study is (1) define the relationship between clinicopathologic findings and OM; (2) identify a biomarker associated with OM by immunohistochemistry; and (3) apply a useful diagnostic method for selecting treatment.

II. Materials and Methods

1. Clinico-histological finding

Patients who visited and underwent surgery at Seoul National University Dental Hospital between 2000 and 2013 were included in this study. These patients were pathologically diagnosed with squamous cell carcinoma in the oral tongue area with no apparent cervical LNM in their pre-operative work up (clinical examination, magnetic resonance imaging, ultrasonography, and positron emission tomography). The patients were divided into the following two groups: the END group and the WW group. The patients in the END group received glossectomy with END. Patients in the WW group received glossectomy, followed by WW. In the END group, we evaluated for LNM after END. In the WW group, patients underwent surveillance for neck recurrence during the WW period. In the END group, OM was defined by the presence of LNM on the histopathological examination in the neck dissection specimen. In the WW group, OM was defined by neck recurrence without recurrence at the primary site. The total number of OM cases is defined by the sum of the patients with LNM in the END group, and the number of patients with neck recurrence in the WW group.

Patients undergoing radiation therapy or chemotherapy before surgery were excluded from this study. The age, sex, clinical manifestations, and survival analysis were obtained from the medical records. Staging of the primary site and cervical LNM in oral tongue cancer was classified by the American Joint Committee on Cancer (AJCC) 7th edition. For histopathologic review, 109 cases of H&E slides were reviewed in the Department of Oral Pathology at Seoul National University Dental Hospital from 2000 to 2013. Two oral pathologists reviewed the H&E slides and identified the depth of invasion, differentiation and T-stage. The slides were examined under an optical microscope at a final magnification of 200×. This study was reviewed by the Institutional Review Board of School of Dentistry, Seoul National University (IRB No. S-D20140041).

2. Immunohistochemistry

Paraffin-embedded blocks of 41 cases of oral tongue cancer specimens from the Department of Oral Pathology at Seoul National University Hospital from 2000 to 2013 were examined.

A tissue microarray was made using the re-location of the tissue from the paraffin blocks. The microarray blocks were sectioned to 3 μm, and were transferred to the glass. IHC staining was then performed. The slides were stained with antibodies. The slides were then incubated in the oven at 60°C for 1 hour, deparaffinized with xylene, rehydrated by serial dilutions with alcohol (72°C for 3 minutes, 3 times), and washed with tap water for 5 minutes.

The IHC analysis of VEGF-c was performed using a Bond polymer detection kit (Leica Microsystem, Seoul, Korea) with a monoclonal antibody against VEGF-c (1:500; Santa Cruz Biotechnology, Dallas, TX, USA). The IHC analysis of c-Met was performed using an Ultraview detection kit (Ventana Medical Systems, Oro Valley, AZ, USA) with a monoclonal antibody against c-Met (RTU; Ventana Medical Systems). The IHC analysis of ROR1 was performed using an Envision kit (Dako North America, Carpinteria, CA, USA) with a polyclonal antibody against ROR1 (1:200; Santa Cruz Biotechnology).

For VEGF-c, the antigen retrieval was performed at a pH of 6.0 (VEGF-c) using the Epitope Retrieval 1 solution (Leica Microsystem) for 20 minutes at 100°C. The reactions were then processed using peroxidase block solution for 5 minutes to quench the endogenous peroxidase activity. The slides were then incubated with monoclonal antibodies for 15 minutes. Next, the sections were incubated with bond polymer detection kit for 8 minutes. The slides were incubated for 10 minutes with 3, 3'-diaminobenzidine (DAB) to visualize the reaction. Finally, the slides were counterstained with Mayer's hematoxylin for one minute.

For c-Met, antigen retrieval was performed at pH 8.4 using Cell conditioning 1 (Ventana Medical Systems) for 60 minutes at 100°C. The slides were then incubated with a monoclonal antibodies for 32 minutes at 37°C. Afterwards,

an ultra-wash was performed. Finally, the slides were counterstained with Mayer's hematoxylin for 4 minutes. After the counterstain, the slides were incubated for 4 minutes in Bluing reagent.

For ROR1, the antigen retrieval was done at a pH of 9.0 in the retrieval buffer (Dako North America) overnight at 4°C. The reactions were treated with peroxidase block solution for 5 minutes to quench the endogenous peroxidase activity. The slides were then incubated with primary antibody, followed by incubation with the labelled polymer using two sequential 30-minute incubations. Next, the slides were incubated for 10 minutes using DAB to visualize the reaction. Finally, the slides were counterstained with Mayer's hematoxylin.

A final score for VEGF-C was defined as the sum of (a) and (b), as follows: (a) the intensity of the stain (0, negative; 1, weak; 2, moderate; 3, strong; and 4, very strong) and (b) the percentage of positive cancer cells was 0, 0% of immunostained cells; 1, <25% of immunostained cells; 2, 25%-50% of immunostained cells; 3, 50%-75% of immunostained cells; and 4, >75% of immunostained cells. A final score >6 was considered as high expression[22].

The final score for c-Met was defined as the sum of (a) and (b), as follows: (a) the intensity of the stain (0, none; 1, light yellow; 2, yellow brown; and 3, brown), and (b) the percent-

Table 1. Clinicopathologic characteristics

		END group (n=71)	WW group (n=38)	Total (END+WW) (n=109)
Sex	Male	58 (81.7)	23 (60.5)	81 (74.3)
	Female	13 (18.3)	15 (39.5)	28 (25.7)
Age (yr)	≥50	44 (62.0)	25 (65.8)	69 (63.3)
	<50	27 (38.0)	13 (34.2)	40 (36.7)
T stage[1]	I	32 (45.1)	30 (78.9)	62 (56.7)
	II	34 (47.9)	7 (18.4)	41 (37.6)
	III	1 (1.4)	0 (0)	1 (0.9)
	IV	4 (5.6)	1 (2.7)	5 (4.8)
	I	32 (45.0)	30 (79.0)	62 (56.9)
	II-IV	39 (55.0)	8 (21.0)	47 (43.1)
Depth of invasion (mm)	≥3	59 (83.1)	23 (60.5)	82 (75.2)
	<3	12 (16.9)	15 (39.5)	27 (24.8)
Differentiation	Well	61 (85.9)	36 (94.7)	97 (89.0)
	Moderate/poor	10 (14.1)	2 (5.3)	12 (11.0)
Area	Lateral	51 (71.8)	35 (92.1)	86 (78.9)
	Other regions (FOM, base)	20 (28.2)	3 (7.9)	23 (21.1)

(END: elective neck dissection, WW: watchful waiting, FOM: floor of mouth)
[1]Staging by the American Joint Committee on Cancer (AJCC) 7th edition.
Values are presented as number (%).
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Table 2. Relationship between clinicopathologic factors and occult metastasis

		OM in END group (n=71)	NR in WW group (n=38)	OM in total (END+WW) (n=109)	*P*-value
Sex	Male (n=81)	13/58	4/23	17/81 (21.0)	0.438
	Female (n=28)	0/13	4/15	4/28 (14.3)	
Age (yr)	≥50 (n=69)	9/44	7/25	16/69 (23.2)	0.173
	<50 (n=40)	4/27	1/13	5/40 (12.5)	
T stage[1]	1 (n=62)	2/32	6/30	8/62 (12.9)	0.053
	2-4 (n=47)	11/39	2/8	13/47 (27.7)	
Depth of invasion (mm)	≥3 (n=82)	13/59	6/23	19/82 (23.2)	0.022*
	<3 (n=27)	0/12	2/15	2/27 (7.4)	
Differentiation	Well (n=97)	11/61	7/36	18/97 (18.6)	0.698
	Moderate/poor (n=12)	2/10	1/2	3/12 (25.0)	
Area	Lateral	8/51	7/35	15/86 (17.4)	0.363
	Other regions (FOM, Base)	5/20	1/3	6/23 (26.1)	

(OM: occult metastasis, END: elective neck dissection, NR: neck recurrence, WW: watchful waiting, FOM: floor of mouth)
[1]Staging by the American Joint Committee on Cancer (AJCC) 7th edition.
*Statistically significant (P<0.05).
Values are presented as number only or number (%).
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age of positive cancer cells (0, 0%-5% of immunostained cells; 1, 6%-25% of immunostained cells; 2, 26%-50% of immunostained cells; and 3, 51%-100% of immunostained cells). A final score >4 was considered high expression[23].

The staining types of ROR1 were divided into three groups: 0, no staining; 1, low-level or low-to-moderate-level less than 50% of cancer cells; and 2, moderate-level more than 50% of cancer cells or high-level staining of the cancer cells. The "2" group was considered high expression. In contrast, "0, 1" groups were considered to have low expression[18].

3. Statistical analysis

The chi-square test and Fisher's exact test association were used to evaluate the association between OM and clinicopathologic factors, and between the IHC findings and OM. The overall survival rates (OSR) were evaluated using Kaplan–Meier method and values were compared using the log-rank test. The statistical tests were performed using IBM SPSS Statistics software (ver. 23; IBM, Armonk, NY, USA). *P*-values <0.05 were considered statistically significant.

III. Results

A total of 109 patients with squamous cell carcinoma in the tongue area were included. The distribution of clinical and pathological data (sex, age, T-stage, depth of invasion, and differentiation) in the END group and WW group are listed in Table 1. Among 71 patients who received glossectomy with END, LNM was observed in 13 patients. Neck recurrence was observed in 8 patients among the 38 patients who received glossectomy only. As a result, the incidences of OM in the END group and the WW were 18.3% and 21.1%, respectively. The incidence of total OM was 19.3%. Eighty-one of 109 patents were male and 28 were female. The mean age was 54.4±15.4 years, ranging from 23 to 91 years.

With regard to the size of the primary tumor, LNM was identified in the following patients in the END group: 2 among 32 patients of T1; 10 among 34 patients of T2; 0 among 1 patient of T3; and 1 among 4 patients of T4. In the WW group, neck recurrence was identified in: 6 among 30 patients of T1, 2 among 7 patients of T2, 0 among 0 patients of T3, and 0 among 1 patient of T4. The patients in the T2-4 group had more OM than did those in the T1 group, although this was not statistically significant (*P*=0.053).

In order to investigate the depth of invasion associated with OM, patients were divided into the following two groups: patients who had tumors with a thickness of ≥3 mm and patients with a thickness of <3 mm. The median depth of invasion in the END group was 0.77±0.56 cm (range, 0.1-3.3 cm). The median depth of invasion in the WW group was 0.46±0.34 cm (range, 0.1-1.5 cm).

The group with a thickness of ≥3 mm showed a higher incidence of OM than did the group with thickness of <3 mm. A depth of invasion ≥3 mm was statistically associated with OM (*P*=0.022).(Table 2)

In the END group, LNM was identified in 11 patients among 61 patients with well differentiation, 2 among 8 patients with moderate differentiation, and 0 among 2 patients with poor differentiation. In the WW group, neck recurrence was identified in 7 patients among 36 patients with well dif-

Table 3. Site of occult metastasis or neck recurrence

Level[1]	OM in END group (No. of patients)	NR in WW group	
		No. of patients	NR time (mo)
I	4	2	8.2
			14.4
II	2	3	4.1
			6.7
			9
III	4	0	
IV	1	0	
II+III	2	2	4.6
			8.3
I+III+IV	0	1	19.2

(OM: occult metastasis, END: elective neck dissection, NR: neck recurrence, WW: watchful waiting)
[1]Staging by the American Joint Committee on Cancer (AJCC) 7th edition.
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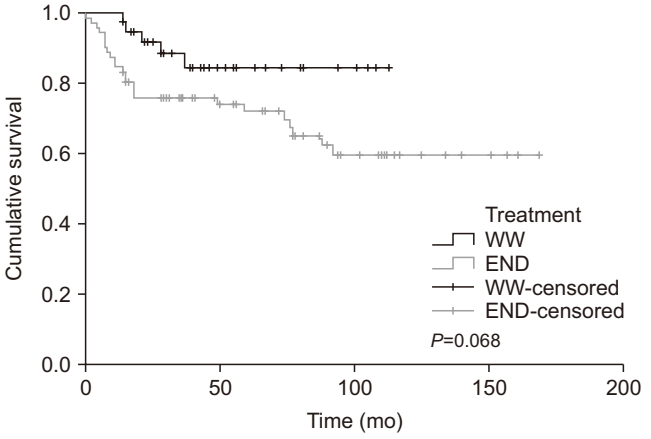

Fig. 1. Overall survival according to different treatments (Kaplan–Meier curves with univariate analysis: log-rank). (END: elective neck dissection, WW: watchful waiting)
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ferentiation, 1 among 2 patients with moderate differentiation, and 0 among 0 patients with poor differentiation. The moderate/poor differentiation group had a higher incidence of OM than did the well differentiation group, although the value was not statistically significant.(Table 2)

In the END group, the incidence of OM varied depending on the primary site of the tumor. Eight of the 51 patients displayed primary tumors on the lateral surface. Four of 18 patients had primary tumors on the floor of the mouth, and one of two patients had OM on the tongue base. In the WW group, neck recurrence was found in: 7 patients among 35 patients on the lateral surface; 1 among 3 patients on the floor of mouth; and 0 among 0 patients on the tongue base. OM and neck recurrence were identified from level I through level IV. However, the values were not statistically significant. (Tables 1, 2)

The sites of recurrence on the ipsilateral side were as follows: In the END group, OM was identified in 4 patients at level I, 2 patients at level II, 4 patients at level III, and 1 patient at level IV. Two patients displayed OM at multiple levels (level II, III). In the WW group, LNM was found in 2 patients at level I, 3 patients at level II. Three patients displayed LNM at multiple levels (level II, III: 2 patients, level I, III, IV: 1 patient).(Table 3)

The 3- and 5-year OSR in the WW group were 88.4% and 84.3%, respectively. The rates in the END group were 75.8% and 71.9%, respectively. The patients in the WW group had better survival rate than did those in the END group, although this difference was not statistically significant (P=0.068).(Fig. 1) The 3- and 5-year OSR of the pN0 patients in the END group were 80.9% and 80.9%, respectively. The OSR of the pN(+) group were 51.9% and 31.2%, respectively (P=0.001). Patients in the pN0 group demonstrated better survival rates than did those in the pN(+) group.(Fig. 2) The patients in the negative neck recurrence group also demonstrated better survival rates compared to those in the positive neck recurrence group. The 3- and 5-year OSR in the negative neck re-

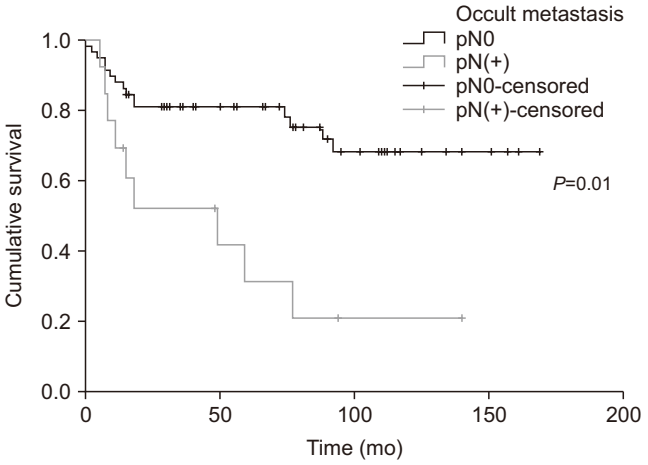

Fig. 2. Overall survival according to lymph node metastasis in elective neck dissection group (Kaplan–Meier curves with univariate analysis: log-rank).
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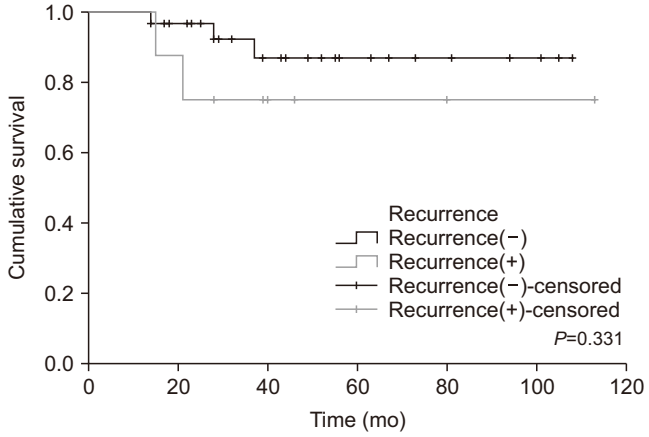

Fig. 3. Overall survival according to neck recurrence in watchful waiting group (Kaplan–Meier curves with univariate analyses: log-rank).
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Table 4. Relationship between immunohistochemical factors and occult metastasis

		END group		WW group		Total (END+WW)		P-value
		OM(–) (n=10)	OM(+) (n=13)	NR(–) (n=10)	NR(+) (n=8)	OM(–) (n=20)	OM(+) (n=21)	
VEGF-c	Low	5 (50.0)	4 (30.8)	8 (80.0)	3 (27.3)	13 (65.0)	7 (33.3)	0.043*
	High	5 (50.0)	9 (69.2)	2 (20.0)	5 (71.4)	7 (35.0)	14 (66.7)	
c-Met	Low	4 (40.0)	1 (7.7)	4 (40.0)	0 (0)	8 (40.0)	1 (4.8)	0.009*
	High	6 (60.0)	12 (92.3)	6 (60.0)	8 (100)	12 (60.0)	20 (95.2)	
ROR1	Low	7 (70.0)	2 (15.4)	6 (60.0)	2 (25.0)	13 (65.0)	4 (19.0)	0.003*
	High	3 (30.0)	11 (84.6)	4 (40.0)	6 (75.0)	7 (35.0)	17 (81.0)	

(END: elective neck dissection, WW: watchful waiting, OM: occult metastasis, NR: neck recurrence)
*Statistically significant (P<0.05) by chi-square or Fisher's exact test.
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currence group were 92.3% and 86.8%, respectively. The 3- and 5-year OSR in the positive neck recurrence group were 75.0% and 75.0%, respectively. However, the OSR according to neck recurrence was not statistically significant (*P*=0.331). (Fig. 3)

The IHC reactivity for VEGF-c, c-Met, Cox-2, ROR1 are summarized in Table 4.

Immunostaining for VEGF-c was detected in the cytoplasm. The images of IHC staining for VEFG-c are shown in Fig. 4. Positive VEGF-c expression was significantly correlated with OM (*P*=0.043). Immunostaining for c-Met was detected in the cytoplasm, and the cytoplasmic membrane. Images of IHC staining for c-Met are shown in Fig. 5. Positive c-Met expression was significantly correlated with OM (*P*=0.009). Immunostaining for ROR1 was detected in the cytoplasm, and the nucleus of cancer cells. The images of IHC staining for ROR1 are shown in Fig. 6. Positive ROR1 expression was significantly correlated with OM (*P*=0.003).

VI. Discussion

There has been a controversy among prior retrospective studies with regard to advocating for[6,24] versus opposing[25,26] END. There is no consensus regarding the use of END in cN0 tongue cancer. Unfortunately, the decision to perform an END is made at the surgeon's discretion. Our study can help to guide the use of END in cN0 tongue cancer patients.

Several papers have examined the relationship between the depth of invasion and LNM. Spiro et al.[27] and Brown et al.[28] recommend END when the tumor thickness exceeds 2 mm in

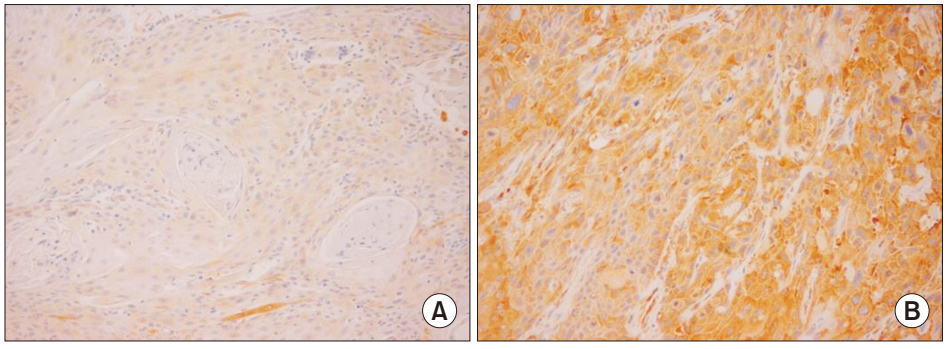

Fig. 4. Expression of VEGF-c (VEGF-c staining, ×200). A. Low expression. B. High expression.
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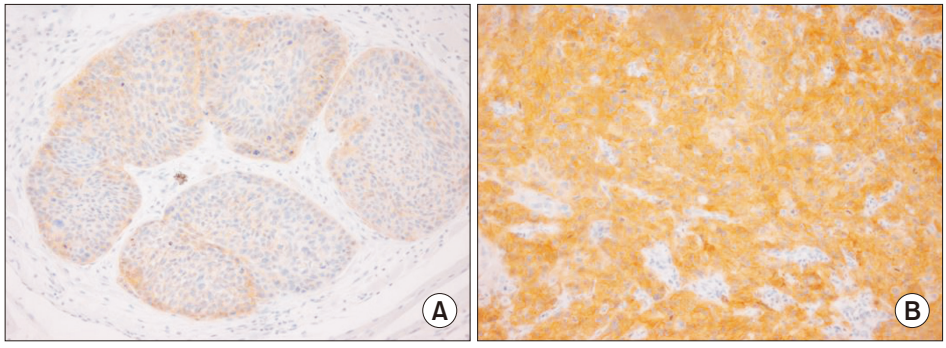

Fig. 5. Expression of c-Met (c-Met staining, ×200). A. Low expression. B. High expression.
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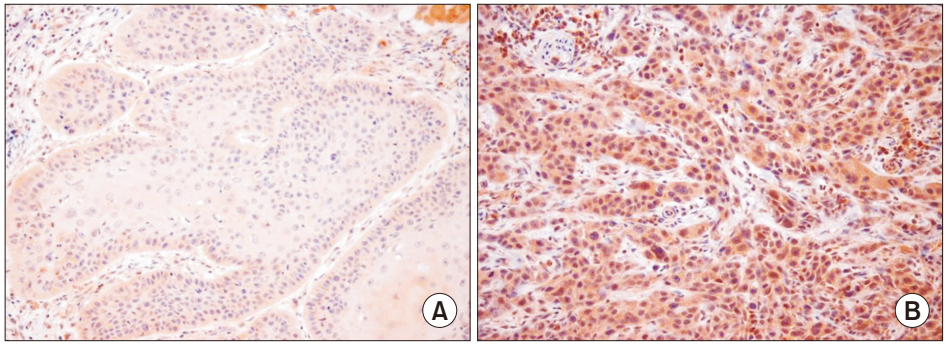

Fig. 6. Expression of ROR1 (ROR1 staining, ×200). A. Low expression. B. High expression.
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patients with oral cancer. Mohit-Tabatabai et al.[29] recommend END when the tumor thickness exceeds 1.5 mm in patients with cN0 oral cancer. Our study showed that the group with a tumor thickness of ≥3 mm had a higher incidence of OM than did those with a tumor thickness <3 mm. Patients with a tumor thickness ≥3 mm should be treated with END. However, the exact depth of invasion may not be known until after surgery. Therefore, it is difficult to use the depth of invasion to determine whether or not END should be performed.

Some authors reported a correlation between LNM and T stage[27,30], while others found no such correlation[31,32]. In our study, patients in the T2-4 group demonstrated more OM than did those in the T1 group. However, there was no correlation between the T-stage and OM (*P*=0.053).

Umeda et al.[32] and Frierson and Cooper[33] reported that patients with poorly differentiated tumors had a higher incidence of LNM than did those with well differentiated tumors. In contrast, we did not find a correlation between tumor differentiation and the incidence of OM (*P*=0.698). However, patients with moderate/poor differentiated tumors showed a higher incidence of OM than did those with well differentiated tumors.(Table 2) Therefore patients with moderate/poorly differentiated tumors must be observed carefully.

There was no association between the site of tongue cancer and OM in this study. Other authors[34,35] have identified similar results. Careful diagnosis is needed, particularly at the base of a mouth cancer, where cancer cells can spread to the lymph node of the contralateral neck.

The follow-up period of the WW group was 14-113 months (mean, 46.95±28.59 months). Neck recurrence in the WW group occurred in eight patients at 9.33±5.09 months postoperatively. Neck recurrence occurred within one year in seven patients, except for one patient in the WW group. Therefore, close follow-up is necessary for one year after surgery. Multiple lymph node metastases (level I, III, IV) occurred in one patient at 14.4 months postoperatively. Neck recurrence may not have been identified during the follow-up period in this patient. Therefore, close follow-up is an important prognostic factor in the WW group.

Some studies have failed to identify any significant differences in the OSR between the END group and the WW group[36,37]. In contrast, other studies demonstrated a survival benefit in the END group[24,38]. In this study, we found no difference between the END and WW groups. However, patients in the WW group had higher OSR than did those in the END group.(Fig. 1) There were more T1 patients than T2 patients in the WW group, while there were more T2 patients than T1 patients in the END group. It is more likely that glossectomy without neck dissection was performed in patients with T1 stage tumors than in patients with T2 tumors. Hiratsuka et al.[39] reported that the 5-year survival rates of the patients with OM and without OM were 94% and 51%, respectively. In our study, patients with pN0 had better survival rates than did those with pN(+) group in the END group.(Fig. 2)

We found that three markers that were correlated with OM were useful markers for OM detection.(Table 4)

VEGF-c expression is associated with lymphatic invasion and LNM. VEGF-c promotes lymphangiogenesis and enhances invasion via loosening of lymphatic endothelial cells[40]. In this study, positive VEGF-c expression was significantly correlated with OM (*P*=0.043).

The activation of c-Met increases cancer cell proliferation, motility, invasion, LNM, and survival rates[41-43]. In oral squamous cell carcinoma, several studies found that overexpression of c-Met was a considerable pathologic parameter for metastasis[44,45]. In this study, the positive expression of c-Met was significantly correlated with OM (*P*=0.009). c-Met might contribute to occult metastatic processes, and facilitate the invasion of cancer cells into lymphatic vessels.

ROR1-mediated signaling has been identified in various cell lines. Wnt5a (ligand of ROR1) activates NF-κB in HEK293[46]. Wnt5a involves the ROR1-dependent signaling pathway to enhance cancer cell growth[18]. In adenocarcinoma cell lines, ROR1 can phosphorylate c-SRC. EGF-induced signaling is magnified through the interaction of FZD and EGFR[47]. In gastric carcinoma and lung carcinoma cell lines, ROR1 is phosphorylated by MET. The silencing of ROR1 decreases cell growth[21]. In this study, positive ROR1 expression was significantly correlated with OM (*P*=0.003). These markers are thought to be expressed in the early stages of LNM.

V. Conclusion

Our results demonstrated a relatively high incidence of OM in the OTSCC patients with cN0 neck tumors. We also demonstrated that the depth of invasion and IHC factors (VEGF-c, c-Met, ROR-1) are important predictive factors for the detection of OM. Surgeons can decide whether or not to perform END based on the clinical, histological, and IHC factors. Initially, close follow-up is very important. Metastatic lymph nodes in the neck can act as a source of tumor cells in there. Further prospective studies are needed to substantiate our findings.

ORCID

Jung-Hyun Shin, *https://orcid.org/0000-0002-3128-5366*
Hye-Jung Yoon, *https://orcid.org/0000-0002-3534-3295*
Soung-Min Kim, *https://orcid.org/0000-0002-6916-0489*
Jong-Ho Lee, *https://orcid.org/0000-0002-8843-545X*
Hoon Myoung, *https://orcid.org/0000-0002-9984-8479*

Authors' Contributions

J.H.S. participated in data collection and wrote the manuscript. H.J.Y., S.M.K., J.H.L., and H.M. participated in the study design and performed the statistical analysis and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

This study was approved by the Institutional Review Board of School of Dentistry, Seoul National University (IRB No. S-D20140041), and the informed consent was waived.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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