Use of NRP1, a novel biomarker, along with VEGF-C, VEGFR-3, CCR7 and SEMA3E, to predict lymph node metastasis in squamous cell carcinoma of the tongue

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Received March 31, 2016; Accepted August 5, 2016

DOI: 10.3892/or.2016.5116

Abstract. Lymph node (LN) metastasis has been suggested as a major prognostic factor for oral cancer. Knockdown of the growth factors and receptors involved in these metastatic mechanisms could significantly reduce LN metastasis and improve the survival of oral cancer patients after treatment. The present study, therefore, aimed to evaluate the expression levels of the following growth factors and receptors in squamous cell carcinoma (SCC) of the tongue: the vascular endothelial growth factor (VEGF)-C and VEGF-D, which bind to the cell surface tyrosine kinase receptor VEGF receptor-3 (VEGFR-3); C-C motif chemokine receptor 7 (CCR7); neuropilin (NRP)1 and NRP2; and semaphorin 3E (SEMA3E). Furthermore, we assessed microvessel density (MVD) and lymphatic vessel density (LVD) to demonstrate the correlation between these factors and regional LN metastasis, with respect to the clinicopathological features. Finally, we analyzed the correlation between these proteins and overall or disease-free survival, in order to demonstrate their prognostic value. Univariate analysis revealed a significant association between LN metastasis and the expression levels of VEGF-C, VEGFR-3, CCR7, NRP1, and SEMA3E, as well as LVD, in SCC cells. In contrast, multivariate analysis identified associations between LN metastasis and NRP1 expression, as well as between LN metastasis and LVD; however, no correlation was found between LN metastasis and the expression levels of the other proteins. The expression levels of VEGF-C, VEGFR-3, NRP1, and SEMA3E, as well as LVD, were correlated with disease-free survival time. These results indicate that LN metastasis is associated with poor survival in SCC. This study suggests that NRP1 expression and LVD are independent factors that are likely to predict the risk of LN metastasis in SCC of the tongue, whereas the expression of VEGF-C, VEGFR-3, CCR7, and SEMA3E are non-independent predictive factors.

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Key words: squamous cell carcinoma, vascular endothelial growth factor-C, vascular endothelial growth factor-D, vascular endothelial growth factor receptor-3, C-C chemokine receptor type 7, semaphorin 3E, lymphatic vessel density

Introduction

Oral squamous cell carcinoma (SCC) is the most commonly identified cancer of the oral cavity and the head and neck region. More than 90% of the tumors occurring in the head and neck region are SCCs; these can grow in several locations of the mucosal lining, with relatively rare neoplasms arising in the minor salivary glands and soft tissues (1,2).

Lymphatic metastasis is the most significant predictive factor for the survival of patients with oral SCC. Moreover, patients who suffer from recurrence of neck cancer, after initial treatment, frequently die due to uncontrolled neck disease and many other evaluated potential prognostic factors (3).

The vascular endothelial growth factor (VEGF) family including VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF), each acting through their respective VEGF tyrosine kinase receptors (VEGFR-1, VEGFR-2, and VEGFR-3), play significant roles in angiogenesis and lymphangiogenesis (4). Many studies have reported that the overexpression of VEGF-C or VEGF-D, acting through the tyrosine kinase receptor VEGF receptor-3 (VEGFR-3), promotes the process of lymph node (LN) metastasis through the regulation of cancer lymphangiogenesis (5). Moreover, VEGF-C and VEGF-D are associated with LN metastasis through intratumoral and peritumoral lymphatics in many types of cancers, including esophageal, gastric, colon, breast, thyroid, pancreas, prostate, and lung cancers (6).

Small pro-inflammatory 'chemotactic cytokines' are secreted proteins that are involved in the migration of activated hematopoietic cells (dendritic cells) to regional LNs (7). The chemokine superfamily is generally classified into four groups based on the cysteine motifs in the protein (CXC, CC, CX3C, and C), and the involvement of these proteins in tumor

metastasis has been determined (8). Recently, several studies have shown that C-C motif chemokine receptor 7 (CCR7) plays an important role in the metastasis of head and neck SCC, esophageal carcinoma, gastric carcinoma, breast cancer, malignant melanoma, non-small cell lung carcinoma, and gastrointestinal carcinoma (9).

Neuropilin (NRP)1 and NRP2 are transmembrane glycoprotein receptors with roles in neuronal guidance, angiogenesis, and lymphangiogenesis via VEGF binding and signaling in the absence of other VEGF receptors (10). In addition, both are structural receptors of the class 3 semaphorins (11). Several studies have reported their expression and regulation in tumor cells of human breast, colon, and prostate cancers (7). NRP expression may be a prospective biomarker, and further studies may eventually lead to more efficacious treatments (7).

Semaphorin 3E (SEMA3E) is a secreted protein involved in axonal guidance and angiogenesis (12). There are eight groups of semaphorins, which bind to the cell surface receptors NRPs (NRP1 and NRP2) (13) as well as plexin-D1 for their signaling pathways (11). Casazza *et al* and other studies have reported that secreted SEMA3E and its receptor plexin-D1 inhibit tumor growth but promote the metastasis and invasiveness of cancer cells (14). They are involved in the metastasis of colon, liver, melanoma, and breast cancers; however, the mechanism remains unclear (13-15).

Although the predictive value of these biomarkers is yet to be established, it has been observed that lymphangiogenesis in cancer is not limited to the areas within or immediately adjacent to a primary tumor; however, it can also occur in the sentinel LNs (16,17). The identification of effective and innovative therapies that appear to influence cancer metastasis is critical for the improvement of SCC therapy.

Therefore, the present study aimed to evaluate the expression levels of the following growth factors and receptors in SCC of the tongue: VEGF-C, VEGF-D, VEGFR-3, CCR7, NRP1, NRP2, and SEMA3E. In order to demonstrate the prognostic value of these proteins, we analyzed the correlation between them and the overall or disease-free survival. Furthermore, we assessed the correlation between microvessel density (MVD) and LN metastasis as well as the correlation between lymphatic vessel density (LVD) and LN metastasis, with respect to their clinicopathological features.

Materials and methods

Patients and tissues. All clinical studies were approved by the Ethics Committee of Osaka University Dental Hospital. We conducted a retrospective cohort study by randomly selecting 80 patients who had been previously diagnosed with primary tongue SCC and undergone curative tumor resection at the First Department of Oral and Maxillofacial Surgery, Osaka University Dental Hospital (Osaka, Japan) between 1995 and 2008.

Information regarding the clinicopathological features of each case (including age, gender, tumor size, nodal status, and the location and status of recurrence or metastasis) was obtained from the patient histories. The study included 55 men (68.75%) and 25 women (31.25%) between the ages of 22 and 92 years, with a median age of 62 years. Forty (50%) of the patients had LN metastasis.

All patients were staged according to the 2010 Japan Society for Oral Tumors TNM Classification of Malignant Tumors (18). The histological mode of invasion was classified according to the YK classification (19,20).

Immunohistochemistry. The paraffin sections were fixed in 10% neutral buffered formalin. Sections of 5-μm thickness were cut consecutively, deparaffinized in xylene, rehydrated with graded concentrations of ethanol, and treated with citrate buffer, pH 6.0, at 98˚C for 30 min and EDTA buffer, pH 8.0, at 98˚C for 15 min for heat-induced antigen retrieval. These sections were then used in histopathological and immunohistochemical analysis. To block endogenous peroxide activity, 0.3% $H_2O_2 \cdot dH_2O$ was applied to the sections. Non-specific reactions were blocked with 1% bovine serum albumin buffer [Histofine SAB-PO (Multi) kit; Nichirei Bioscience, Tokyo, Japan]. The sections were incubated with the following primary antibodies at 4˚C, overnight: anti-human rabbit polyclonal VEGF-C antibody (dilution 1:100), anti-human rabbit polyclonal VEGF-D antibody (dilution 1:200) (both from Abcam, Tokyo, Japan), anti-human mouse 9D9F9 VEGFR-3 antibody (dilution 1:500; Millipore Corp., Billerica, MA, USA), anti-human goat polyclonal CCR7 antibody (dilution 1:250), anti-human rabbit monoclonal NRP1 antibody (dilution 1:100) (both from Abcam), anti-human rabbit polyclonal NRP2 antibody (dilution 1:100; Atlas Antibodies, Stockholm, Sweden), anti-human goat polyclonal SEMA3E antibody (dilution 1:100; Abcam), anti-human mouse monoclonal CD-34 antibody (dilution 1:100), and anti-human mouse monoclonal D2-40 antibody (pre-diluted) (both from Nichirei Bioscience). Next, the appropriate secondary antibodies and blocking agents were applied using the Histofine SAB-PO (Multi or Goat) kit (Nichirei Bioscience). Immunostaining and immunolocalization of the proteins were performed using the DAB-Peroxidase Substrate Solution Immunohistochemistry brown Histofine SAB-PO (Multi) kit (Nichirei Bioscience), according to the manufacturer's instructions. The sections were counterstained with Mayer's hematoxylin solution (Sigma-Aldrich, St. Louis, MO, USA) dehydrated with graded concentrations of ethanol, cleared with xylene, and mounted for visualization using bright-field microscopy.

A positive control (immunohistochemical staining demonstrating weakly positive tissue) and negative controls (immunohistochemical staining with the omission of the primary antibody) were included in the staining protocol. Scoring and cell counts were performed without prior clinical knowledge of the patients.

Staining evaluation. The immunohistochemical staining of these proteins in SCC cells was evaluated based on the ratio and intensity of the staining. Specimens were considered immunopositive when ≥1% of the cancer cells showed clear evidence of immunostaining. The ratio of staining for each specimen was determined as follows: specimens with no positive tumor cells, score of 0; 1-25% positive cells, score of 1; 26-50% positive cells, score of 2; 51-75% positive cells, score of 3; and 76-100% positive cells, score of 4. The intensity of immunostaining was determined as follows: specimens with no staining, score of 0; weak staining, score of 1; moderate staining, score of 2; and intense staining, score

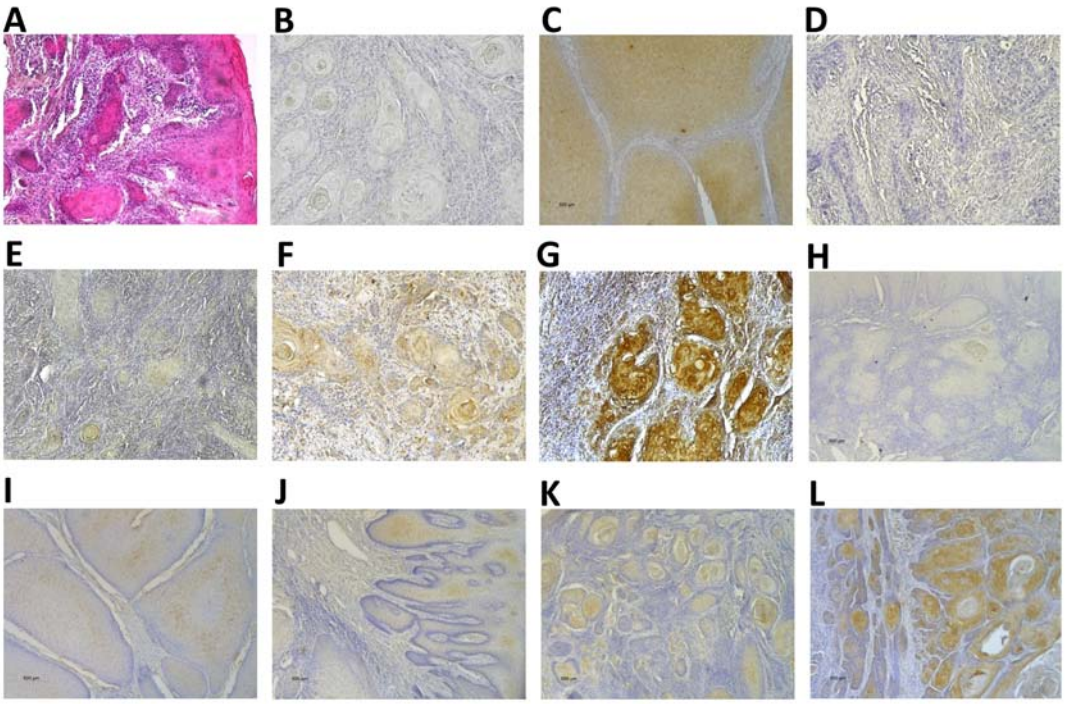

Figure 1. Immunohistochemical staining for biomarkers in SCC of the tongue. The expression of biomarkers in tongue SCC specimens, as determined by IHC staining, ranging from negative to weak, moderate, and strong. A score was given to each specimen according to the intensity of the staining and ratio of the positive tumor cells (x10 original magnification). (A) Hematoxylin and eosin staining of a SCC specimen. (B) Negative control IHC staining with omission of the primary antibody. (C) Positive control IHC staining for CCR7 demonstrates weakly positive tissue. (D) Negative IHC staining for CCR7 was given a score of 0 for intensity. (E) Weak IHC staining for CCR7 was given a score of 1 for intensity. (F) Moderate IHC staining for CCR7 was given a score of 2 for intensity. (G) Strong IHC staining for CCR7 was given a score of 3 for intensity. (H) Zero tumor cells positive for CCR7 was given a score of 0 for ratio. (I) Between 0 and 25% of the tumor cells positive for CCR7 staining was given a score of 1 for ratio. (J) Between 25 and 50% of the tumor cells positive for CCR7 staining were given a score of 2 for ratio. (K) Between 50 and 75% of the tumor cells positive for CCR7 staining was given a score of 3 for ratio. (L) Between 75 and 100% of the tumor cells positive for CCR7 staining were given a score of 4 for ratio. SCC, squamous cell carcinoma; IHC, immunohistochemical; CCR7, C-C motif chemokine receptor 7.

of 3 (21-23) (Fig. 1). The tumors in this study were sometimes heterogeneous and the staining was therefore calculated by multiplying the ratio score with the intensity score to give an overall staining score between 1 and 12, using a similar rationale to the immunoreactivity score (IRS) first described by Remmele and Stegner (24).

Using this scoring system, each component of the tumor was scored independently, and the results were calculated. The immunostaining of the specimens was evaluated independently by two authors (Dr Al-Shareef Hani and Dr Yosuke Shogen), who were blinded to patient results and other clinicopathological features, and their scores were averaged to obtain a final IRS. Subsequently, patients were divided into low and high expression groups, with the mean IRS for each protein serving as the cut-off value (21-23).

Assessment of MVD and LVD. MVD and LVD were estimated in tumor vessel 'hot spots' by immunostaining with the CD-34 and D2-40 podoplanin antibodies, respectively. Microvessel counts were assessed by light microscopy in areas of the tumor with the highest number of capillaries and small venules at the invasive edge, that is, in the areas with the most intense neovascularization. Although SCCs have a mostly heterogeneous MVD and LVD, areas of highly invasive carcinoma were identified by the presence of a higher number of discrete microvessels and lymphatic vessels that were positively stained for CD-34 and D2-40, respectively. These areas of increased neovascularization could occur anywhere in the invasive tumor; however, they were most frequently observed in the intratumoral region and at the margins of the carcinoma (3,25).

Microvessels and lymphatic vessels with positive immunostaining for CD-34 and D2-40, respectively, were counted in three hotspots per section, using a 20x objective lens, by two authors (Dr Al-Shareef Hani and Dr Sanam Bakhshishayan), who were blinded to patient results and other clinicopathological features. The mean vessel counts in each of the three hotspots were recorded and each component of the tumor was scored independently to give six hotspot scores for each specimen. After averaging the three hotspot scores of each component, to obtain a final hotspot score for LVD and MVD, the specimens were divided into two groups (low or high) based on the mean values for LVD and MVD (6.85 and 20.31 number of vessels/μm^2 of tumor-free area, respectively). In brief, the area with the highest vascular density (hotspot) was selected, and the number of microvessels was determined in each of the three microscopic fields by each investigator using the 20x objective lens. The total lymphatic vessel count was compared between the groups according to age, gender, primary tumor site, clinical stage, tumor size, grade, and the status of the lymphatic metastasis.

Statistical analysis. Statistical analysis was performed using the statistical software IBM SPSS Advanced Statistics 20.0 (IBM Corp., Armonk, NY, USA). Subjects were grouped into

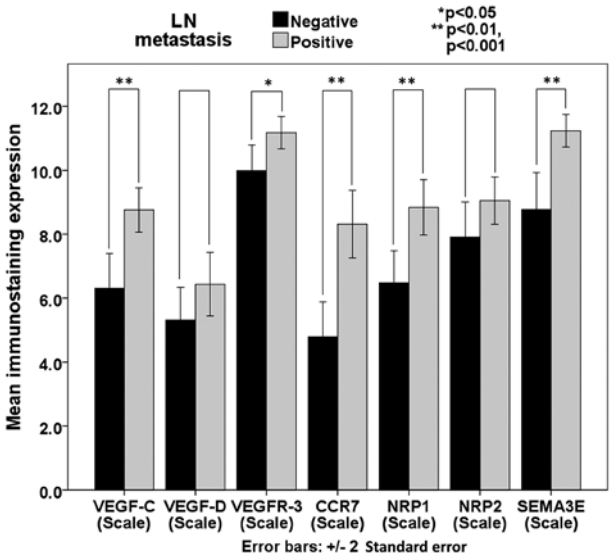

Figure 2. Relationship between the expression of proteins and LN metastasis. The relationship between protein expression and LN metastasis was analyzed by the independent samples t-test, and it is shown in this clustered bar chart. High expression levels for each of the proteins were identified in most of the specimens, and a significant association was demonstrated between LN metastasis and the expression levels of VEGF-C, VEGFR-3, CCR7, NRP1, and SEMA3E. *P<0.05; **p<0.01, p<0.001. LN, lymph node; VEGF, vascular endothelial growth factor; VEGFR-3, VEGF receptor-3; CCR7, C-C motif chemokine receptor 7; NRP, neuropilin; SEMA3E, semaphorin 3E.

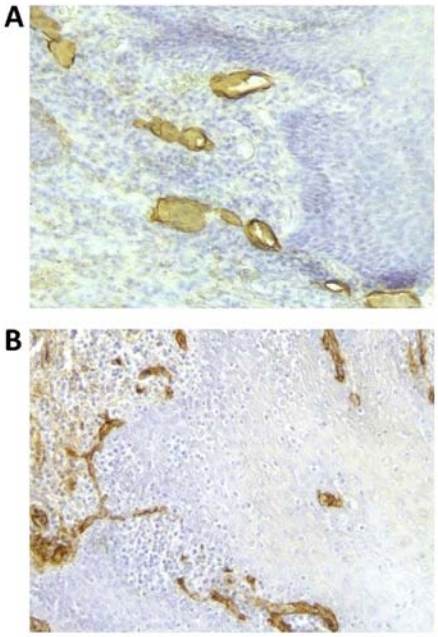

Figure 3. LVD and MVD evaluation. (A) Immunohistochemical analysis with the D2-40 podoplanin antibody was performed to evaluate the LVD. Positive staining for lymphatic vessels was detected at the invasive edge of the tumor (x20 original magnification). (B) Immunohistochemical analysis with the CD-34 antibody was performed to evaluate the blood MVD. Positive staining for microvessels was detected at the invasive edge of the tumor (x20 original magnification). LVD, lymphatic vessel density; MVD, microvessel density.

the following categories: <60 or ≥60 years of age; pathological negative (N0) or pathological positive (N1) for LN metastasis; and high or low protein expression based on the individual cut-off score for each protein. Chi-square and Fisher's exact tests were performed to assess the association between clinicopathological features and protein expression levels. An independent samples t-test was performed to determine the association between protein expression levels and LN metastasis. In addition, an independent samples t-test was performed to assess the relationship between LVD or MVD and LN metastasis or protein expression.

Univariate and multivariate logistic regression analyses were performed to assess the association between LN metastasis and clinicopathological features or protein expression as well as to assess the odds ratio (OR), and to define independent risk factors for prognosis and incidence of nodal recurrence. The overall and disease-free survival rates were estimated using the Kaplan-Meier method and analyzed using the log-rank test. For all tests, p<0.05 was accepted as statistically significant.

Results

MVD, LVD, and the expression of angiogenic and lymphangiogenic biomarkers in oral SCC. The biomarkers for LN metastasis were analyzed individually. The expression levels of VEGF-C, VEGF-D, VEGFR-3, CCR7, NRP1, NRP2, and SEMA3E in oral SCC lesions were assessed by immunohistochemistry. We found that these biomarkers were predominantly expressed in the cell layer and the cytoplasm of the SCC cells, especially at the invasive edges, and were occasionally observed in endothelial cells in the stroma around or close to carcinoma nests and tumor vessels (Fig. 1).

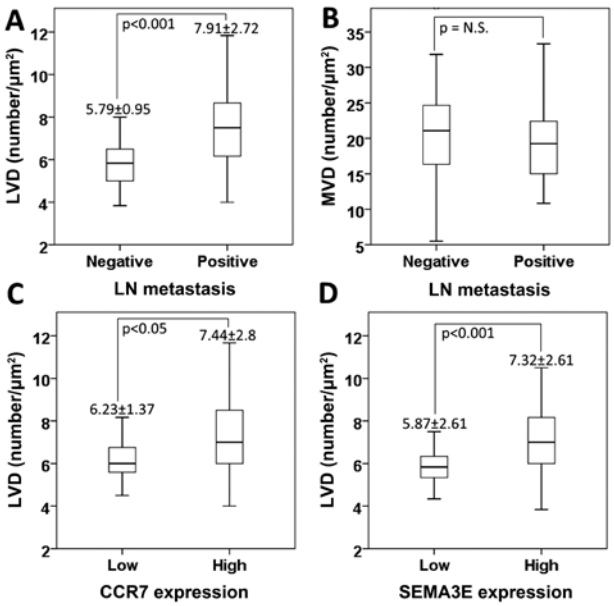

Figure 4. Relationship between predictive factors and MVD or LVD. The relationship between predictive factors and MVD or LVD was analyzed by the independent samples t-test, and it is shown in this box plot. (A) A significant correlation was shown between LVD and LN metastasis. (B) No association was observed between MVD and LN metastasis. (C) CCR7 expression showed a significant relationship with LVD. (D) SEMA3E expression showed a significant relationship with LVD. MVD, microvessel density; LVD, lymphatic vessel density; LN, lymph node; CCR7, C-C motif chemokine receptor 7; SEMA3E, semaphorin 3E; N.S., not significant.

High expression levels of VEGF-C, VEGF-D, VEGFR-3, CCR7, NRP1, NRP2, and SEMA3E were detected in 39

Table I. Relationship between clinicopathological features and the expression levels of the proteins VEGF-C, VEGF-D, VEGFR-3, CCR7, NRP1, NRP2, and SEMA3E.

		VEGF-C expression			VEGF-D expression			VEGFR-3 expression			CCR7 expression		
	Cases	Low	High	P-value	Low	High	P-value	Low	High	P-value	Low	High	P-value
Gender													
Female	25	14	11	N.S.	7	18	0.039	8	17	0.153	7	18	0.012
Male	55	27	28		29	26		27	28		32	23	
Age (years)													
<60	34	19	15	N.S.	20	14	0.033	21	13	0.005	19	15	N.S.
≥60	46	22	24		16	30		14	32		20	26	
N classification													
N0	40	26	14	0.047	20	20	N.S.	24	16	0.035	27	13	0.015
N1	13	4	9		3	10		5	8		3	10	
N2b	18	7	11		8	10		5	13		5	13	
N2c	7	2	5		4	3		1	6		3	4	
N3	2	2	0		1	1		0	2		1	1	
LN metastasis													
Negative	40	26	14	0.014	20	20	N.S.	24	16	0.003	27	13	0.001
Positive	40	15	25		16	24		11	29		12	28	

		NRP1 expression			NRP2 expression			SEMA3E expression		
	Cases	Low	High	P-value	Low	High	P-value	Low	High	P-value
Gender										
Female	25	10	15	N.S.	10	15	N.S.	7	18	N.S.
Male	55	33	22		25	30		19	36	
Age (years)										
<60	34	22	12	N.S.	17	17	N.S.	12	22	N.S.
≥60	46	21	25		18	28		14	32	
N classification										
N0	40	29	11	0.012	20	20	N.S.	20	20	0.016
N1	13	5	8		3	10		1	12	
N2b	18	7	11		8	10		4	14	
N2c	7	1	6		4	7		1	6	
N3	2	1	1		0	2		0	2	
LN metastasis										
Negative	40	29	11	0.001	20	20	N.S.	20	20	0.001
Positive	40	14	26		15	25		6	34	

p<0.05, p<0.01 and p<0.001 denote increasing levels of significance. VEGF, vascular endothelial growth factor; VEGFR-3, VEGF receptor-3; CCR7, C-C motif chemokine receptor 7; NRP, neuropilin; SEMA3E, semaphorin 3E; N.S., not significant; LN, lymph node.

(48.8%), 44 (55%), 45 (56.3%), 41 (51.3%), 37 (46.3%), 45 (56.3%), and 54 (67.5%) tumor specimens, respectively. In contrast, 41 (51.3%), 36 (45%), 35 (43.8%), 39 (48.8%), 43 (53.8%), 35 (43.8%), and 26 (32.5%) tumor specimens exhibited low expression levels of VEGF-C, VEGF-D, VEGFR-3, CCR7, NRP1, NRP2, and SEMA3E, respectively. The mean expression levels of VEGF-C, VEGF-D, VEGFR-3, CCR7, NRP1, NRP2, and SEMA3E were 7.538, 5.875, 10.581, 6.550, 7.663, 8.481, and 10.006, respectively (Fig. 2).

MVD was found to range between 5.5 and 38.5 μm^2 of blood vessels per square millimeter of the tumor (median, 19.92; mean ± standard deviation (SD), 20.31±6.26), whereas LVD ranged between 3.83 and 20.83 μm^2 of lymphatic vessels per square millimeter of the tumor (median, 6.33; mean ± SD, 6.85±2.29) (Figs. 3 and 4).

The relationship between clinicopathological features and the expression of angiogenic and lymphangiogenic biomarkers.

Chi-square and Fischer's exact tests were performed to assess the association between clinicopathological features and the expression levels of VEGF-C, VEGF-D, VEGFR-3, CCR7, NRP1, NRP2, and SEMA3E. The results showed that the expression level of VEGF-C was significantly associated with only pathological LN size ($p<0.05$) and LN metastasis ($p<0.05$), whereas the expression level of VEGF-D was significantly associated with only gender ($p<0.05$) and age ($p<0.05$). Moreover, the expression level of VEGFR-3 was significantly associated with only age ($p<0.01$), N classification (Japan Society for Oral Tumors, classification for regional LN metastasis; $p<0.05$), and LN metastasis ($p<0.01$). The expression level of CCR7 was significantly associated only with gender ($p<0.05$), N classification ($p<0.05$), and LN metastasis ($p=0.001$). In addition, the expression level of NRP1 in the SCC cells was significantly associated only with pathological LN size ($p<0.05$) and LN metastasis ($p=0.001$), and the expression level of NRP2 was not significantly associated with any of the clinicopathological features, whereas the expression level of SEMA3E was significantly associated with only N classification ($p<0.05$) and LN metastasis ($p=0.001$) (Table I).

The relationship between the expression levels of angiogenic and lymphangiogenic biomarkers and MVD or LVD. The relationship between angiogenesis and lymphangiogenesis in the primary SCC and LN metastasis was examined by assessing MVD, through immunohistochemical staining with the CD-34 antibody, and LVD, through immunohistochemical staining with the D2-40 antibody. LVD ranged between 3.83 and 20.83 μm^2 of lymphatic vessels per square millimeter of the tumor (median, 6.33; mean ± SD, 6.85±2.29). The independent samples t-test showed a significant association between LVD and LN metastasis, with the mean LVD being lower in the metastatic LN-negative group when compared to the metastatic LN-positive group [mean ± SD, 5.79±0.95 and 7.91±2.72, respectively; $p<0.001$; $t(78)=4.66$]. No significant association was detected between MVD and LN metastasis.

The independent samples t-test was also used to assess the association between biomarker expression and MVD or LVD. We found that LVD was significantly associated with the expression of CCR7 ($p<0.05$) and SEMA3E ($p<0.001$), mainly at the invasive edges. However, no such association was detected between MVD and the expression of any of the biomarkers (Fig. 4) (Table II).

Table II. The relationship between protein expression levels and MVD or LVD.

	MVD			LVD		
	Mean	SD	P-value	Mean	SD	P-value
LN metastasis						
Negative	20.79	6.72	0.497	5.79	0.95	<0.001
Positive	19.83	5.82		7.91	2.72	
VEGF-C						
Low	21.28	5.49	0.154	6.53	1.5	0.154
High	19.28	6.90		7.18	2.89	
VEGF-D						
Low	20.17	6.19	0.856	6.72	1.54	0.207
High	20.42	6.40		6.95	2.77	
VEGFR-3						
Low	19.71	6.17	0.458	6.46	1.46	0.183
High	20.77	6.37		7.15	2.75	
CCR7						
Low	20.63	6.48	0.655	6.23	1.37	0.016
High	20.00	6.11		7.44	2.8	
NRP1						
Low	20.33	6.27	0.979	6.42	1.62	0.69
High	20.29	6.35		7.35	2.83	
NRP2						
Low	20.15	6.03	0.841	6.8	1.66	0.877
High	20.43	6.50		6.89	2.7	
SEMA3E						
Low	21.49	5.60	0.245	5.87	0.86	<0.001
High	19.74	6.53		7.32	2.61	

$p<0.05$, $p<0.01$ and $p<0.001$ denote levels of significance. MVD, microvessel density; LVD, lymphatic vessel density; SD, standard deviation; LN, lymph node; VEGF, vascular endothelial growth factor; VEGFR-3, VEGF receptor-3; CCR7, C-C motif chemokine receptor 7; NRP, neuropilin; SEMA3E, semaphorin 3E.

Logistic regression analysis of predictive factors and LN metastasis. Univariate logistic regression analysis was performed to assess the association between clinicopathological features and LN metastasis. The results showed that SCC of cancer stage T4 is more likely to have LN metastasis (OR, 9.778; 95% confidence interval (CI), 1.551-61.646; $p\leq0.05$). Multivariate logistic regression analysis also showed that cancer stage T4 was significantly associated with LN metastasis (OR, 12.601; 95% CI, 0.998-159.08; $p\leq0.05$). No significant relationship was identified between LN metastasis and the mode of invasion (YK classification), the differentiation of SCC cells, or other clinical factors in any of the regression analyses (Table III).

Univariate analysis showed significant association between LN metastasis and the expression levels of VEGF-C (OR, 3.095; 95% CI, 1.243-7.706; $p<0.05$), VEGFR-3 (OR, 3.955; 95% CI, 1.546-10.114; $p<0.01$), CCR7 (OR, 4.846; 95% CI, 1.882-12.482; $p=0.001$), NRP1 (OR, 4.896; 95% CI, 1.892-12.669; $p=0.001$), and SEMA3E (OR, 5.667; 95% CI, 1.951-16.462; $p=0.001$). No correlation was identified between LN metastasis and the expression levels of NRP2 and VEGF-D. However, a significant association was observed between LN metastasis and LVD (OR, 2.832; 95% CI, 1.716-4.673; $p<0.0001$), but not MVD. By contrast, multivariate analysis showed only the association between LN metastasis and NRP1 expression level (OR, 5.905; 95% CI, 1.274-27.372; $p<0.05$) or LVD (OR, 2.527; 95% CI, 1.412-4.522; $p<0.01$). No correlation was identified between LN metastasis and the expression levels of the other proteins (Table III).

The relationship between the expression of angiogenic and lymphangiogenic biomarkers and survival time. We

Table III. Relationship between the predictive factors and LN metastasis.

	Univariate			Multivariate		
	Odds ratio	95% CI	P-value	Odds ratio	95% CI	P-value
T stage T1 ref T4	9.778	1.551-61.646	0.015	12.601	0.998-159.08	0.05
VEGF-C expression	3.095	1.243-7.706	0.015	2.06	0.462-9.178	0.343
VEGFR-3 expression	3.955	1.546-10.114	0.004	2.436	0.484-12.272	0.28
CCR7 expression	4.846	1.882-12.482	0.001	1.402	0.301-6.538	0.667
NRP1 expression	4.896	1.892-12.669	0.001	5.905	1.274-27.372	0.023
SEMA3E expression	5.667	1.951-16.462	0.001	1.979	0.389-10.065	0.411
LVD (D2-40)	2.832	1.716-4.673	<0.001	2.527	1.412-4.522	0.002

$p \leq 0.05$, $p < 0.01$ and $p < 0.001$ denote increasing levels of significance. LN, lymph node; CI, confidence interval; VEGF, vascular endothelial growth factor; VEGFR-3, VEGF receptor-3; CCR7, C-C motif chemokine receptor 7; NRP, neuropilin; SEMA3E, semaphorin 3E; LVD, lymphatic vessel density.

used the Kaplan-Meier method to determine the association between LN metastasis and prognosis in patients with oral SCC. The results showed a reduced overall survival time for patients with metastatic SCC when compared to patients with non-metastatic cancer (patients with LN metastasis were associated with overall survival time and LVD exhibited an association, log-rank $p<0.001$) (Fig. 5).

The Kaplan-Meier analysis also showed that patients with high expression levels of VEGF-C, VEGFR-3, NRP1, and SEMA3E, were more likely to have localized or regional recurrence after treatment (VEGF-C, VEGFR-3, NRP1, and SEMA3E were associated with disease-free survival time, log-rank $p<0.05$).

Patients with metastatic SCC were also more likely to have localized or regional recurrence after treatment (patients with LN metastasis were associated with disease-free survival time and LVD exhibited an association, log-rank $p<0.001$) (Fig. 5). To summarize, LN metastasis was found to be associated with poor survival in SCC.

Discussion

In the present study, several biomarkers have been proposed for SCC. The aim of this study was to evaluate the expression levels of the following growth factors and receptors in SCC of the tongue: VEGF-C, VEGF-D, VEGFR-3, CCR7, NRP1, NRP2, and SEMA3E. Furthermore, we investigated the association between the expression levels of these proteins and intratumoral MVD, using CD-34 antibody, or LVD, using D2-40 podoplanin antibody, as indicators of angiogenesis and lymphangiogenesis, respectively.

The PI3K/AKT, ERK1/2, and p38 pathways are crucial for cellular proliferation and survival (26). However, the association between disease-specific survival and the expression of VEGF-C, VEGF-D, and their receptor VEGFR-3 remains controversial. Many studies have proposed a strong association between LN metastasis and the expression levels of VEGF-C and VEGF-D in SCC. However, the association between LN metastasis and VEGF-D is controversial and has not been supported by other studies. In fact, VEGF-D has been reported to act as a suppressor in some types of cancers (27,28). A relationship between LN metastasis and the expression levels of VEGF-C or VEGFR-3 has been reported for head and neck SCC, gastric, breast, thyroid, prostate, esophageal, and colorectal carcinomas (6).

The present study showed that LN metastasis is significantly correlated with the expression levels of VEGF-C and VEGFR-3, but not VEGF-D, based on immunohistochemical staining ($p<0.05$) and univariate logistic regression analysis ($p<0.01$). Immunostaining for these proteins was extensively associated with some clinicopathological features and was strongly correlated with disease-specific survival, suggesting a role for VEGF-C and VEGFR-3 as biomarkers for oral SCC metastasis. These results are in agreement with other reports on head and neck cancer (29), which suggest that VEGF-D is less important than VEGF-C for LN metastasis. However, it is still necessary for the metastasis mechanism, although its role is presently controversial. The possibility that VEGFR-3 expression in cancer cells could be associated with increased regional LN metastasis in oral SCC is also in agreement with other recent studies, suggesting a role for VEGFR-3 expression in predicting LN metastasis of SCC.

Furthermore, we found a strong relationship between the expression levels of both VEGF-C and VEGFR-3 and lymphangiogenesis in patients with oral SCC. Many clinical and preclinical studies have suggested that clinicopathological features are usually unreliable predictors of LN metastasis, with highly variable results, particularly for oral SCC (30-32). However, in this study, we suggest that LVD could be a predictive marker for LN metastasis in oral SCC. Furthermore, VEGF-C/VEGFR-3 pathway inhibition has been shown to inhibit cancer progression (33-36). Therefore, signaling through VEGFR-3 is not necessarily a result of LN metastasis. The fact that VEGFR-3 can form heterodimers with VEGFR-2 makes this mechanism even more complicated (17).

This study also predicted a strong correlation between LVD and LN metastasis in oral SCC. Both disease-free and overall

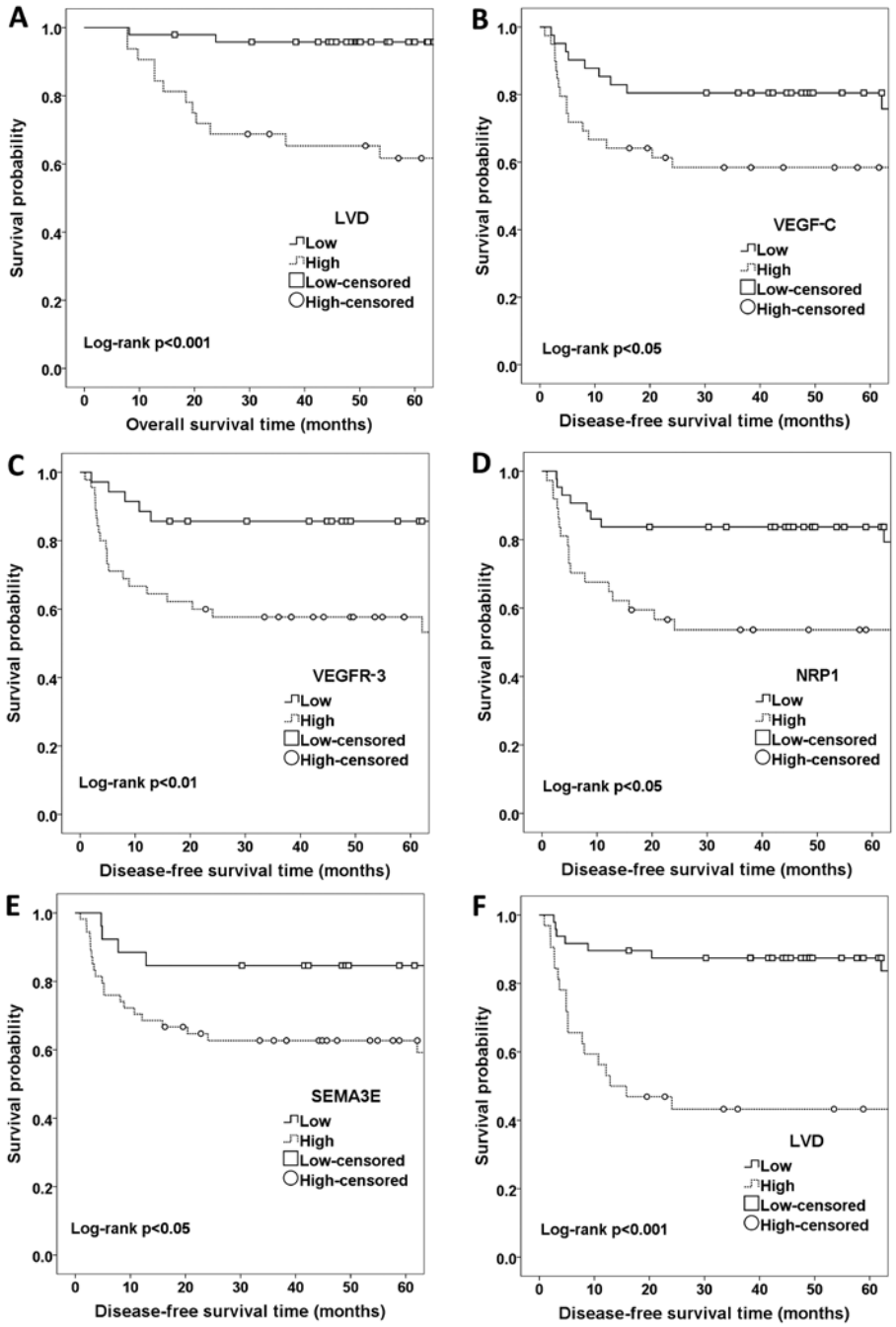

Figure 5. The association between predictive factors and patient survival or prognosis. The association between predictive factors and patient survival was measured using the Kaplan-Meier method. LVD showed an association with (A) the overall survival time as well as (F) the disease-free survival time, whereas (B) VEGF-C, (C) VEGFR-3 , (D) NRP1 and (E) SEMA3E showed an association with disease-free survival time. LVD, lymphatic vessel density; NRP, neuropilin; VEGF, vascular endothelial growth factor; VEGFR-3, VEGF receptor-3; SEMA3E, semaphorin 3E.

survival were associated with intra- and peritumoral LVD, as patients with a high LVD were revealed to have poor survival and a high possibility of recurrence. We did not detect any association between MVD and LN metastasis or prognosis. Other studies, by systematic review, have shown that MVD is unlikely to be a prognostic factor in early stage non-small cell lung cancer patients (6).

The association of angiogenic and lymphangiogenic biomarkers with prognosis in SCC patients remains controversial due to the variable outcomes reported by independent studies (17,37). Some biomarkers have been correlated with poor prognosis or prolonged survival (38-40), whereas others have failed to show any prognostic significance (37). In our study, VEGF-C and VEGFR-3 expression levels were correlated with poor prognosis and a high possibility of localized or regional recurrence after treatment.

Many studies have reported that the expression of CCR7 in tumors, such as metastatic tumor cells of gastric, esophageal, breast, renal, oropharyngeal, and head and neck cancers, is significantly correlated with poor prognosis. Many studies have also reported a relationship between CCR7 expression levels and oral as well as head and neck SCC (9,41).

In the present study, we found that CCR7 immunostaining was strong in tongue cancer tissue. Univariate logistic regression analysis showed that CCR7 expression was significantly correlated with LN metastasis (p=0.001) as well as some clinicopathological features. Furthermore, a significant association was detected between upregulated CCR7 expression and LVD; however, no association was estimated between this upregulated CCR7 expression and prognosis. The results showed a possible role for CCR7 in the progression of tongue SCC as well as in mediating signaling in metastatic SCC.

The interactions between the proteins in the VEGF pathway are complex; which includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PlGF, their tyrosine kinase receptors VEGFR-1, VEGFR-2, and VEGFR-3, and two NRP receptors NRP1 and NRP2. The VEGF receptors can form complexes with the NRP receptors and modulate the signaling outcomes through p38 MAPK pathway activation, leading to migration and survival of endothelial cells. Semaphorin family competes with VEGFs to bind to NRPs and modulate the angiogenesis mechanism (42). Many previous studies have investigated the expression of NRP1 and NRP2 in SCC of the tongue, esophagus, colon/rectum, breast, stomach, and lungs (10). Moreover, some studies have shown that NRP1 had a higher expression level compared to that of NRP2 (7). NRP1 and NRP2 expression levels have been shown to be prospective indicators of poor prognosis in colorectal cancer (7). However, other studies have found that NRP1 was neither correlated with any clinical features nor with poor prognosis in SCC of the tongue (43).

In the present study, NRP1 was shown, by immunohistochemical analysis, to be extensively expressed in cancerous tongue tissue. Univariate logistic regression analysis showed that NRP1 expression was significantly correlated with LN metastasis (p=0.001) as well as with some other clinicopathological features (such as N classification). This indicates the controversial role of NRP1 in tumor growth and progression. The upregulation of NRP1 was also significantly correlated with poor disease-free survival. However, NRP2 did not show any association with LN metastasis, prognosis, or clinicopathological features. A possible explanation may be that NRP1 inhibits apoptosis by binding to VEGF and NRP2 increases the epithelial-mesenchymal transition (7). Furthermore, NRP1 expression appears to be more involved in angiogenesis and lymphangiogenesis than NRP2 expression. In general, these data suggest that both NRP1 and NRP2 are possible targets for chemotherapy; however, agents targeting NRP1 may have a greater anti-angiogenic effects in SCC.

Semaphorins, and their receptors, are a large family of extracellular signaling proteins with important roles in angiogenesis and tumor progression. SEMA3E is a secreted protein that recognizes the plexin-D1 receptor as well as the NRP receptors (12,44). However, the functional relevance of SEMA3E in cancer is poorly understood. In addition to providing inhibitory signals for angiogenesis, SEMA3E/plexin-D1 signaling can regulate other cell types (42,45). Casazza *et al* found that 'SEMA3E inhibits tumor growth, but promotes metastasis', in an NRP-independent manner (14).

SEMA3E expression is positively correlated with metastatic progression in highly invasive and metastatic mammary carcinomas, high- vs. low-grade glioblastomas, colon and liver cancers, and melanomas (13,14). Increased expression of SEMA3E has been shown in metastatic cancers when compared to that of non-metastatic cancers. Kaplan-Meier survival analysis revealed that SEMA3E expression levels in primary tumors were significantly inversely correlated with patient survival, whereas the opposite was true for bladder carcinomas (14) as well as for primary and metastatic prostate tumors (42).

The present study is the first time where SEMA3E expression has been investigated in SCC of the tongue. Univariate logistic regression analysis revealed that SEMA3E expression was significantly correlated with LN metastasis (p=0.001). SEMA3E was shown to be highly expressed in immunohistochemical analysis, and its association with clinicopathological features was stronger than the association detected between upregulated CCR7 expression and LVD. In addition, SEMA3E is strongly correlated with disease-specific survival, suggesting its importance as a biomarker in oral SCC metastasis. This result is consistent with previous studies in colon and liver cancers, melanomas, and metastatic mammary carcinomas. The finding that SEMA3E is also important in tongue SCC suggests that its signaling is crucially involved in the metastatic mechanisms of multiple cancer cells and is therefore a promising therapeutic target for the prevention of tumor metastasis.

Due to the limitations of this retrospective cohort study, the analysis of archival documents is a better way to analyze multiple outcomes and deal with potential heterogeneity and rare cases. This may also help in obtaining sufficient data for the identification of prognostic and predictive biomarkers and may improve the study design of randomized trials. Further studies in larger cohorts would better validate our results for oral SCC patients.

In summary, we suggest that high expression levels of VEGF-C, VEGFR-3, CCR7, and SEMA3E, as detected by immunohistochemical staining, serve as non-independent predictors of LN metastasis. Furthermore, LVD and NRP1 were found to be independent predictors of LN metastasis in SCC of the tongue. Statistical regression analysis and prognostic analysis showed that these factors are more likely to predict LN metastasis; therefore, these factors would be useful in estimating the possibility of LN metastasis in SCC. The accurate assessment of LN metastasis is crucial to improve the survival of oral cancer patients after treatment.

Acknowledgements

This study was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology in Japan and by JSPS KAKENHI (grant no. 15K20519).

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