



Metastasis via Peritumoral Lymphatic Dilation in Oral Squamous Cell Carcinoma

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Abstract

Purpose: Nodal metastasis is the main prognostic factor in the patients with oral squamous cell carcinoma (OSCC). We investigated the association between tumor-associated lymphatics and OSCC characteristics.

Methods: Thirty-four specimens were used for the immunohistochemical staining with the antibody for vascular endothelial growth factor (VEGF)-C, VEGF-D, VEGF receptor (VEGFR)-3, phosphorylated VEGFR-3, D2-40, and matrix metalloproteinases (MMPs). We observed the distribution of the lymphangiogenic factors and quantified the degree of expression. We determined lymphatic vessel density (LVD) and lymphatic vessel dilatation with D2-40 immunostaining. We assessed the association of LVD or lymphatic vessel dilatation with tumor progression or tumor differentiation.

Results: OSCC cells expressed lymphangiogenic ligands. Lymphangiogenic receptor, VEGFR-3, was expressed and activated in some tumor cells as well as in tumor-associated endothelial cells. LVD was not associated with tumor size or nodal status, but lymphatic vessel dilatation was higher in tumors with nodal metastasis, and also higher in poorly differentiated tumors. In stromal area of OSCC, MMP-1 and MMP-10 were up-regulated and the basement membrane of tumor-associated endothelial cells was destroyed by these collagenases.

Conclusion: In the primary tumors with nodal metastasis, especially in poorly differentiated OSCC, tumor cells invaded the dilated lymphatic vessels via ruptured sites. MMP-1 and MMP-10 are important in the lysis of the glycocalyx inside the tumor-associated lymphatic endothelial cells.

Key words: Oral squamous cell carcinoma, Lymphangiogenic factor, Nodal metastasis, Lymphatic vessel density, Lymphatic vessel dilation

Introduction

Nodal metastasis is a critical step in the progression of malignant tumors and is one of the most important prognostic factors in many types of epithelial solid tumors. In oral squamous cell carcinoma as well as head and neck carcinoma, tumor metastasis occurs mainly via cervical

lymph nodes, and early cervical nodal involvement of the tumor results in poor survival[1].

Lymphangiogenesis, i.e., the formation of new lymphatic vessels, has a major effect on nodal metastasis of malignant tumors, regulated by the expression of lymphangiogenic factors[2,3]. The exact molecular mechanism of lymphangiogenesis and the biology of tumor-associated

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lymphatic endothelial cells however, is unclear. Specific markers for lymphatic endothelium (Prox1[4], LYVE-1[5], podoplanin[6], and vascular endothelial growth factor receptor (VEGFR)-3[7]) have recently been discovered, leading to increased research interest in tumor lymphatics.

Among several lymphangiogenic factors, the vascular endothelial growth factor (VEGF)-C, D/VEGFR-3 signaling pathway is the most powerful stimulant for the genesis of tumor-associated lymphatics[8]. Research shows an association of VEGF-C/VEGFR-3 axis with lymphatic spread of tumor cells and with clinical outcomes in solid tumors including oral squamous cell carcinoma (OSCC)[9-11].

The main hypothesis of this study is that lymphangiogenic factor levels are higher in the primary tumor with nodal metastasis than in the primary tumor without nodal metastasis in OSCC. Thus we assessed the association of tumor-associated lymphatics with tumor proliferation or nodal metastasis or tumor differentiation.

Materials and Methods

1. Patients and tumor specimens

Thirty-four surgical specimens were collected from patients diagnosed and treated in the Department of Oral and Maxillofacial Surgery, Gangneung-Wonju National University Dental Hospital and Gangneung Asan Hospital between 2006 and 2010. The mean age of the 34 patients (23 males/11 females) was 63.5, ranging from 27 to 84 years. Clinicopathological data were collected (GNAH IRB No 2011-020). Clinical and basic tumor characteristics are shown in Table 1. Tumor grading was performed by an oral pathologist using World Health Organization (WHO) classification, and staging by UICC (Union Internationale Contre le Cancer, International Union against Cancer) classification.

Table 1. Patient and tumor profiles

Patient No.	Age (yr)	Sex	Location	Tumor size*	Nodal metastasis*	Distant metastasis*	TNM stage*
1	50	Male	Left buccal mucosa	T1	N0	M0	I
2	48	Male	Left mouth floor	T1	N0	M0	I
3	27	Male	Tongue	T2	N0	M0	II
4	63	Female	Right palate	T2	N0	M0	II
5	49	Female	Retromolar area	T2	N0	M0	II
6	68	Male	Right buccal mucosa	T2	N0	M0	II
7	61	Male	Right buccal mucosa	T2	N0	M0	II
8	64	Male	Oral cavity	T2	N0	M0	II
9	68	Female	Left buccal mucosa	T2	N0	M0	II
10	51	Female	Tongue	T2	N0	M0	II
11	78	Male	Left buccal mucosa	T2	N0	M0	II
12	70	Male	Gingival mucosa	T2	N0	M0	II
13	48	Female	Tongue	T1	N0	M0	I
14	66	Male	Tongue	T2	N0	M0	II
15	74	Female	Left buccal mucosa	T2	N0	M0	I
16	59	Male	Oral cavity	T2	N1	M0	III
17	63	Male	Mouth floor	T4	N2	M0	IV
18	57	Male	Tongue	T4	N0	M0	IV
19	49	Male	Mouth floor	T4	N0	M0	IV
20	84	Female	Left buccal mucosa	T3	N2	M0	IV
21	65	Female	Buccal mucosa	T2	N2	M0	IV
22	67	Female	Right palate	T4	N2	M0	IV
23	62	Male	Left buccal mucosa	T4	N0	M0	IV
24	53	Male	Left mandible, gingiva	T4	N0	M0	IV
25	59	Male	Right buccal area	T4	N2	M0	IV
26	61	Male	Oral cavity	T2	N1	M1	IV
27	72	Male	Mouth floor	T1	N1	M0	III
28	69	Male	Tongue	T2	N2	M1	IV
29	57	Female	Mouth floor	T3	N2	M0	IV
30	64	Male	Soft palate	T2	N1	M1	IV
31	74	Male	Oral cavity	T4	N2	M0	IV
32	62	Male	Tongue	T3	N2	M0	IV
33	69	Female	Tongue	T3	N2	M0	IV
34	50	Male	Retromolar area	T3	N2	M0	IV

*Classification according to the TNM stage.

2. Antibodies

Antibodies were purchased as follows: polyclonal antibodies specific for VEGF-C (dilution 1:100), VEGF-D (dilution 1:100; Abnova, Jhongli, Taiwan), VEGFR-3 (dilution 1:400, rabbit anti-human FLT-4; Spring Bioscience, Pleasanton, CA, USA), phosphorylated VEGFR-2/3 (dilution 1:500, pVEGFR-3; Calbiochem, Darmstadt, Germany), mouse monoclonal antibody specific for D2-40 (dilution 1:40; Abcam, Cambridge, MA, USA), matrix metalloproteinase (MMP)-1, MMP-2, MMP-9, and MMP-10 (dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

3. Immunohistochemistry

Immunohistochemical staining was performed on 5 μm -thick sections. Paraffin sections were de-paraffinized in xylene, dehydrated with a graded series of alcohol, and rehydrated in phosphate buffered saline. For antigen retrieval, sections for VEGF-C and VEGFR-3 were microwaved in citrate buffer, and sections for VEGF-D and pVEGFR-3 were microwaved in EDTA buffer. Except for the staining with D2-40 and MMPs, immunohistochemical staining was performed in DakoCytomation AutostainerTM (Bond-Max; Leica Biosystems, Newcastle, UK) with envision+horseradish peroxidase diaminobenzidine.

For D2-40 staining, tissue sections treated with the primary antibodies were incubated overnight at 100% humidity in temperature chambers at 4°C. After adequate binding of the primary antibodies, biotinylated link solution as a secondary antibody was applied to the tissue sections for 30 minutes. After washing to remove any unbound secondary antibodies, streptavidin peroxidase solution was applied for 30 minutes. For colorization, diaminobenzidine solution was applied for less than five minutes. Mayer's hematoxylin was used for counter-staining. After gradual hydration with ethyl alcohol and clearing with xylene, the tissue sections were fixed by paramount solutions. MMPs staining was performed as above.

4. Quantitative immunohistochemical analysis

Tissue-slides were observed with a microscope camera (Olympus DP70 digital microscope camera; Olympus Co., Tokyo, Japan). Images were taken from two representative areas per specimen under $\times 100$ magnification. The images

were blindly assessed to prevent area bias. To quantify the expression of each protein, an image analysis program (SigmaScan Pro 5.0; SPSS Science, Chicago, IL, USA) was used. The expression level was measured by the numbers of pixels that appeared red by this program. The average percent of the ratio of red pixels to total pixels was used as a measure of protein expression for each specimen.

5. Lymphatic vessel density and lymphatic vessel dilatation

Lymphatic vessel density (LVD) and lymphatic vessel dilatation were determined with immunostaining with D2-40. To determine LVD, the sections were scanned by light microscopy at a low magnification ($\times 100$). We identified the area of the greatest number of lymph vessels each in intratumoral and peritumoral area. LVD was then determined by counting immunostained vessels that have lumina at higher magnification ($\times 200$). Counts were performed blindly by two investigators. The frequency of lymphatic vessel dilatation was determined by an oral pathologist from the same fields.

6. Statistics

The association between expressions of the lymphangiogenic factors and tumor size (T1, T2 vs. T3, T4) or nodal status (lymph node- [L/N-] vs. L/N+) was measured by the non-parametric Mann-Whitney test. The association between expressions of the lymphangiogenic factors and grade of tumor was measured by the non-parametric Kruskal-Wallis test. The associations between LVD or lymphatic vessel dilatation with tumor size or nodal metastasis were measured by the non-parametric Mann-Whitney test. The association between LVD or lymphatic vessel dilatation with grade of tumor was measured by the non-parametric Kruskal-Wallis test. Null hypotheses of no difference were rejected if *P*-values were less than 0.05. All statistical calculations used SPSS ver. 17.0.1 (SPSS Inc., Chicago, IL, USA).

Results

1. Histopathology

Classification by tumor size resulted in four OSCC as T1, 17 OSCC as T2, five OSCC as T3, and eight OSCC

as T4. Nineteen patients presented with lymph node metastasis (L/N+ group); 15 patients did not (L/N- group). By histological grading, 14 tumors were well differentiated (well group), nine tumors were moderately differentiated (moderately group), and 11 tumors were poorly differentiated (poorly group).

2. Expression of lymphangiogenic factors in oral squamous cell carcinoma

VEGF-C was expressed weakly in OSCC tumor cells in eight of 34 patients. VEGF-D expression could be detected in most tumor cells i.e., 29 of 34 patients, tumor-associated endothelial cells, and stromal cells such as mononuclear

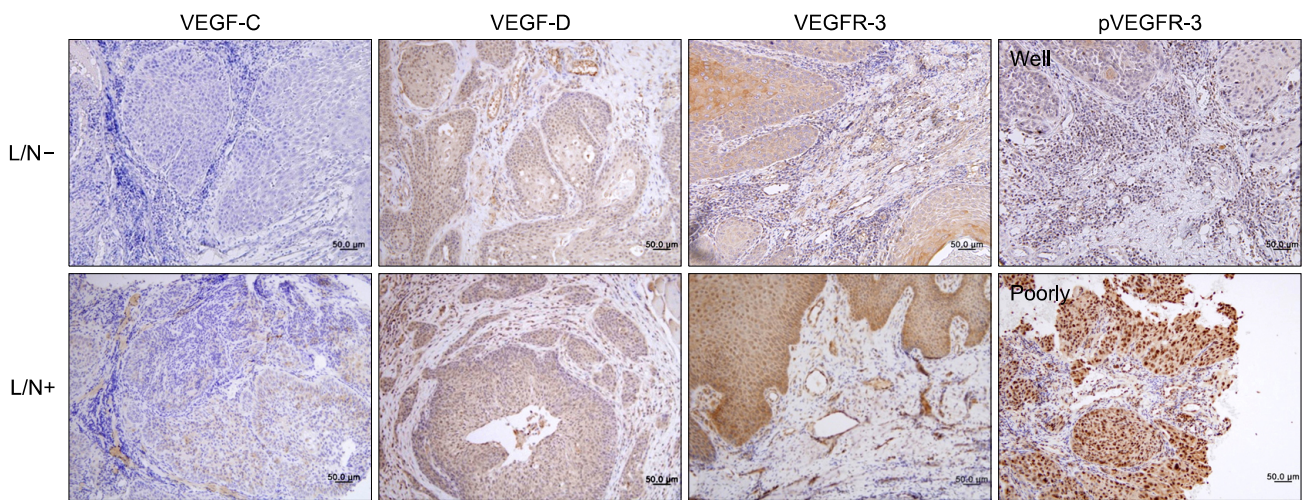


Fig. 1. Expression and distribution of lymphangiogenic factors in oral squamous cell carcinoma; VEGF-C is expressed weakly in some tumor cells. VEGF-D is expressed in most tumor cells, tumor-associated endothelial cells, and immune cells in stroma. VEGFR-3 is expressed strongly in some tumor cells, endothelial cells, and macrophages. Phosphorylated VEGFR-3 is strongly expressed in invading tumor cells in poorly differentiated tumors. Upper panel belongs to lymph node- (L/N-) group and lower panel belongs to L/N+ group. The picture of pVEGFR-3 is the poorly group. Representative slides are shown (×200) (counterstained by Mayer's hematoxyline).

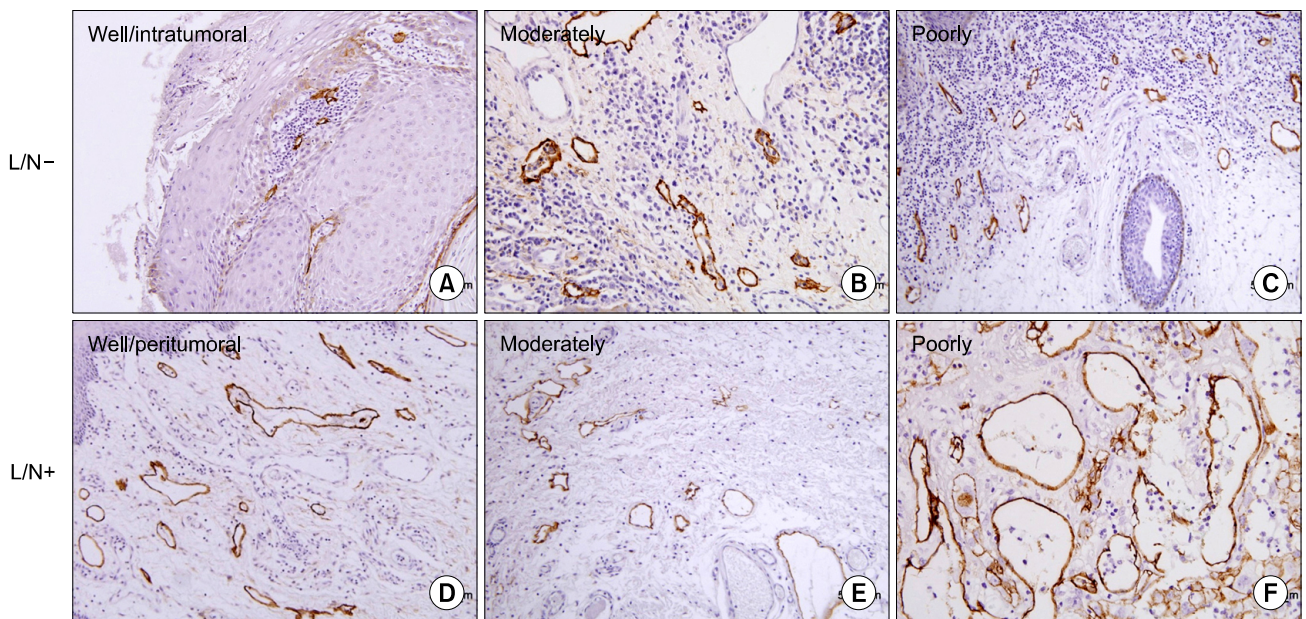


Fig. 2. Lymphatic vessel density (LVD) and lymphatic vessel dilatation; structures stained with D2-40 (brown) are compressed, apparently non-functional lymphatics (A: intratumoral LVD). Functional lymphatics are dilated and have larger surface area (D-F: peritumoral LVD), higher number in lymph node+ (L/N+) group. We can see clearly the unstained microvessels (A~D). Upper panel is L/N- group and lower panel is L/N+ group. Representative slides are shown (A~F: ×200) (counterstained by Mayer's hematoxyline).

cells and macrophages. However, the degree of VEGF-D expression on tumor cells was heterogenous. The degree of VEGF-C or VEGF-D expression was not related to the tumor size, nodal metastasis, or tumor grading.

VEGFR-3 expression in OSCC tumor tissue was found in some tumor cells, tumor-associated endothelial cells, and macrophages. VEGFR-3 was activated in invading tumor cells in primary tumor, endothelial cells, and some stromal cells of OSCC microenvironment (Fig. 1). However, the degree of VEGFR-3 or pVEGFR-3 expression was not related to the tumor size or nodal metastasis (data not shown). pVEGFR-3 expression was significantly higher in the poorly differentiated tumors (Table 2).

3. Lymphatic vessel density and lymphatic vessel dilatation

D2-40 was specifically expressed in the endothelial cells of tumor-associated lymphatic vessels. D2-40 positive lymphatic vessels were present in all OSCCs. The stained vessels were devoid of red blood cells, and D2-40 was restricted to the endothelium not only at the peritumoral lesion but also at the intratumoral lesion. Peritumoral LVD was higher than intratumoral LVD (Fig. 2). We could detect lymphatic vessel dilatation and rupture in peritumoral lymphatics, but not in intratumoral lymphatics (Fig. 3).

Table 2. Expression of lymphangiogenic factors depending on tumor differentiation

	Well (n=14)	Moderately (n=9)	Poorly (n=11)
VEGF-C	0.071±0.267	0.25±0.50	0.75±0.95
VEGF-D	2.214±0.699	2.25±0.95	2.50±1.00
VEGFR-3	2.143±0.949	1.25±0.50	2.50±0.57
pVEGFR-2/3	1.571±1.158	0.50±0.57	2.75±0.50*

Values are presented as mean±standard deviation. Well: well differentiated OSCC, Moderately: moderately differentiated OSCC, Poorly: poorly differentiated OSCC. OSCC, oral squamous cell carcinoma.

*P<0.05 compared with Moderately group (Kruskal-Wallis test).

Table 3. LVD depending on nodal status

	Group 1 (n=19)	Group 2 (n=15)	P-value
Intratumoral LVD	6.04±4.57	6.88±3.61	0.136
Peritumoral LVD	14.07±6.08	15.81±5.31	0.271

Values are presented as mean±standard deviation. Group 1: non lymph node metastasis group, Group 2: lymph node metastasis group.

LVD, lymphatic vessel density.

4. Lymphatic vessel density vs. oral squamous cell carcinoma progression or differentiation

To assess the association between LVD and OSCC progression, the tumors were divided into two groups by primary tumor size at 4 cm. LVD was not associated with tumor size or nodal status (Table 3).

5. Lymphatic vessel dilation vs. oral squamous cell carcinoma progression or differentiation

Table 4 shows that higher peritumoral lymphatic vessel dilation values were associated with significantly high scores of N-classification (P<0.05). Moreover, in peritumoral lymphatics, higher lymphatic vessel dilation values were also associated with significantly high proportions of poorly differentiated tumors (Table 5).

6. Matrix metalloproteinases expression in oral squamous cell carcinoma

We immunostained the specimens with four tissue collagenases (MMP-1, MMP-2, MMP-9, MMP-10) to evaluate their effect on the processes of OSCC progression. MMP-1 and MMP-10 were up-regulated in the stromal area of OSCC. At higher magnification (×1,000), the basement membrane of tumor-associated endothelial cells was destroyed by MMP-1 and MMP-10 (Fig. 4).

Table 4. Lymphatic vessel dilation depending on nodal status

	Group 1 (n=28)	Group 2 (n=16)	P-value
Peritumoral	0.536±0.922	1.313±1.493	0.034*
Intratumoral	1.071±2.071	1.000±1.366	0.412

Values are presented as mean±standard deviation. Group 1: non lymph node metastasis group, Group 2: lymph node metastasis group.

*P<0.05 (Mann-Whitney test).

Table 5. Lymphatic vessel dilation depending on tumor differentiation

	Well (n=14)	Moderately (n=9)	Poorly (n=11)
Peritumoral	0.464±0.881	0.500±0.926	2.375±1.302*
Intratumoral	0.714±1.117	2.250±3.327	1.000±1.604

Values are presented as mean±standard deviation. Well: well differentiated SCC, Moderately: moderate differentiated SCC, Poorly: poorly differentiated SCC.

*P<0.05 compared with Moderately and Well (Kruskal-Wallis test).

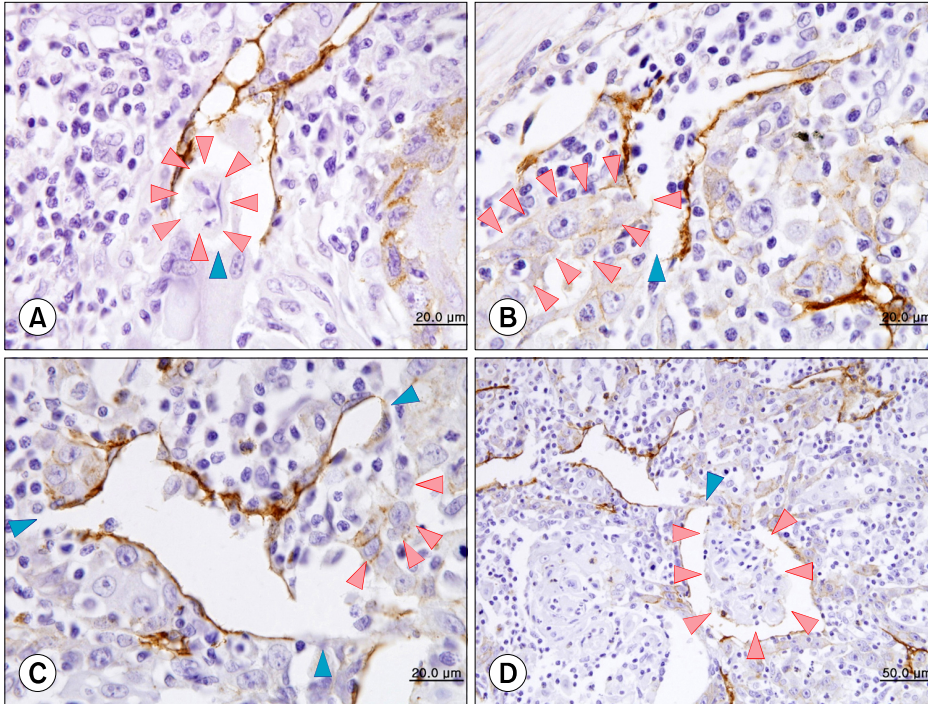


Fig. 3. Invasion of tumor cells (red arrowheads; A~C) and a tumor cluster (D) into the ruptured site (blue arrowheads) of dilated lymphatic vessels (A~C: $\times 1,000$, D: $\times 400$) (counterstained by Mayer's hematoxyline).

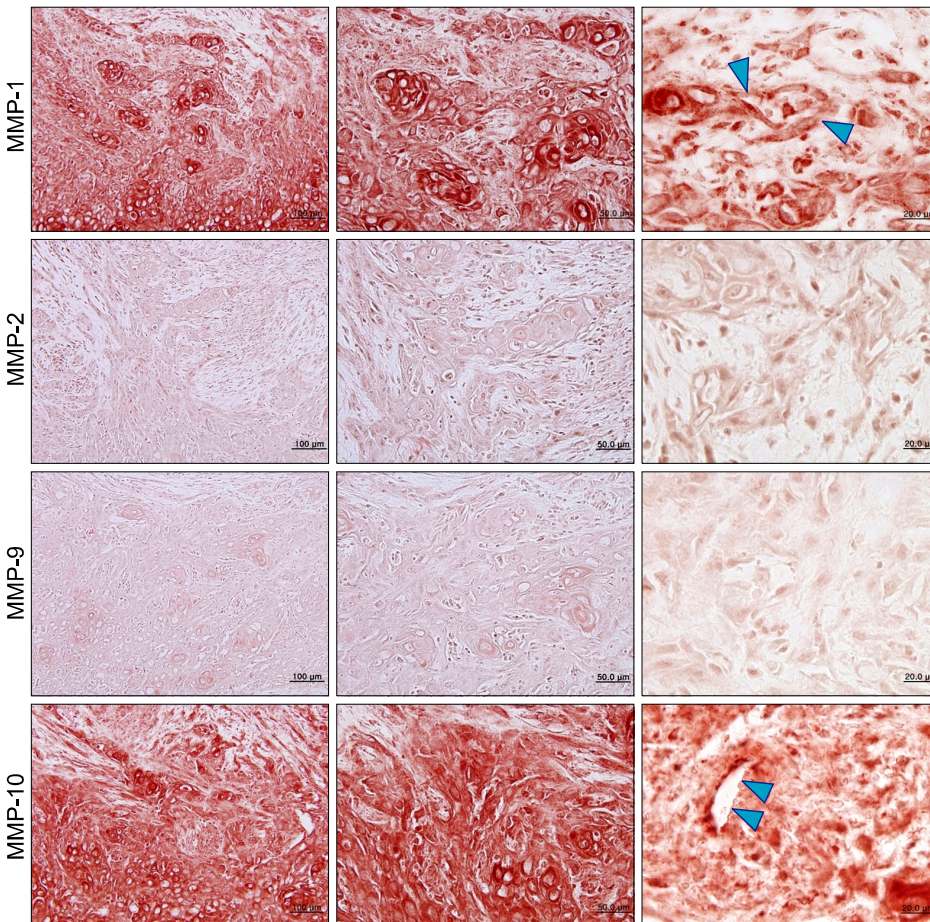


Fig. 4. Matrix metalloproteinases (MMPs) expression in oral squamous cell carcinoma; MMP-1 and MMP-10 are overexpressed in oral squamous cell carcinoma. At higher magnification ($\times 1,000$), the basement membrane of tumor-associated endothelial cells is destroyed by MMP-1 and MMP-10 (blue arrowheads).

Discussion

Oral squamous cell carcinoma preferentially metastasizes to cervical lymph nodes, but the cause is unclear. We examined 34 OSCCs on immunohistochemistry using antibodies for lymphangiogenic factors. We found a significant association of increased lymphatic vessel dilatation with nodal metastasis in patients with OSCC. For nodal metastasis, tumor cells must enter the lymphatic system. However, in OSCC as well as other malignancies, it is uncertain whether this is achieved by tumor cells invading the newly formed lymphatic vessels, or by tumor cells entering the expanded preexisting lymphatic vessels near the tumor. Our study shows OSCC tumor cells enter into the dilated lymphatics, newly formed by lymphangiogenesis, to metastasize to the lymph nodes.

OSCC is characterized by aggressive biological behavior with a low five-year survival rate, mainly due to cervical node metastasis[12]. Lymphangiogenic factors are related to cervical nodal metastasis and VEGFR-C is a main stimulator of lymphangiogenesis[13-15]. Tsirlis *et al.*[16] reported that circulating levels of VEGF-C and VEGF-D serve as predictive biomarkers of lymph node metastasis in gastrointestinal malignancies. Our previous study demonstrated an up-regulation of VEGF-C and VEGF-D in OSCC compared with normal oral mucosa[17]. In the present study, lymphangiogenic ligands are expressed heterogeneously in the tumor cells of OSCC, but their degree of expression is not related with tumor size or nodal metastasis.

VEGFR-3 is expressed in the endothelial cells of lymphatic vessels, and the VEGF-C/VEGFR-3 paracrine interaction between tumor cells and lymphatic endothelial cells is the most important system for tumor lymphangiogenesis. VEGFR-3 is also expressed not only in endothelial cells but in OSCC tumor cells[18], and VEGFR-3 expression in tumor cells can be a potential enhancer system to promote cancer progression[19]. Our results also demonstrated that VEGFR-3 is expressed and activated in some tumor cells, suggesting VEGF-C driven autocrine growth loop of VEGF/VEGFR-3 in OSCC.

Frequently, VEGF-C/VEGFR-3 expressions are associated with regional node metastasis and poor patient survival[20-22], but we could not find a relation of VEGF-C,

D/VEGFR-3 expressions to clinicopathologic parameters. The difference between our results and other reports may result from the small sample size of our study. In this study, ten SCC were located in the buccal mucosa, eight in tongue, five in the floor of mouth, three in palate, and so on. Clinically, the predilection of cervical node metastasis is quite different between tongue/mouth floor SCC and palatal/buccal mucosal SCC. The variable location of primary tumors of this study led to small numbers of homogeneous samples.

D2-40 positive structures were present in all OSCCs in our study. We found a so-called 'hot spot' of lymphatic vessels, located at the periphery of tumor islands. The area was usually involved in stromal tissue, but morphologically the lymphatic vessels seemed to be tumor-associated, newly formed lymphatics, not preexisting ones. We also observed that in most OSCCs, the hot spot of lymphatic vessels was located in other areas compared to the hot spot of microvessels. Hadler-Olsen *et al.*[23] reported peritumoral LVD was up to five times higher in tongue SCC than in skin SCC in xenograft murine model and higher in tongue SCCs compared with skin SCCs in a series of patient samples. Our analysis revealed that peritumoral LVD was about two times higher than intratumoral LVD in OSCC. Morphologically, intratumoral lymphatics are compressed, seemingly nonfunctional.

Podoplanin (D2-40) expression in OSCC may be related to its progression[24,25], but our previous study demonstrated that the number of microvessels rather than podoplanin expression was indicative of OSCC progression[26]. In the present study, LVD evaluation revealed that peritumoral LVD and intratumoral LVD are not significantly associated with tumor size or nodal metastasis. Peritumoral lymphatics are dilated in tumors with nodal metastasis or poorly differentiated SCC. Our finding suggests that the number of functional lymphatics are important in OSCC progression.

Sedivy *et al.*[27] reported that LVD is positively associated with VEGF-C expression and nodal metastasis in OSCC, but we could not reach such a conclusion. This is partly because the difference in determination method of LVD, i.e., we only count the lymphatics with clear lumina. We think morphological changes of newly-formed lymphatics, reflecting functional changes, are important in

the progression of tumor cells. The cut surface of the dilated lymphatics are polygonal or irregular in shape rather than round or ovoid. The lumina of functional lymphatics are enlarged and the lining endothelium is ruptured to transport tumor cells. In this study, we clearly showed the lymphatic dilatation, the ruptured site of lymphatics, and their relation with nodal metastasis.

Tumor progression is strongly influenced by the tumor microenvironment, i.e., tumor stroma, composed of an extracellular matrix, endothelial cells, immune and inflammatory cells. The MMPs are involved in the degradation of the extracellular matrix, and are associated with an aggressive malignant phenotype [28,29]. We immunostained four MMPs on OSCC specimens and found that MMP-1 and MMP-10 are important in the lysis of the glycocalyx inside the tumor-associated lymphatic endothelial cells.

Our findings suggest that in OSCC, nodal metastasis may be achieved mainly through the dilated lymphatic vessels in the periphery of tumor islands. The detection of lymphatic invasion of tumor cells on D2-40 immunostaining in tumor tissue is a strong predictor for nodal metastasis in OSCC. We regard our results as preliminary. The limits of this study include small sample size, the quality of the archival tissue samples, and the sensitivity of antibodies. We need more controlled further study.

Conclusion

In conclusion, this study indicates that increased dilatation of tumor-associated lymphatic vessels is significantly related to nodal metastasis. Furthermore, OSCC tumor cells invaded the ruptured site of the dilated lymphatics, apparently mediated by MMP-1 and MMP-10. In sum, peritumoral lymphatic dilatation may be an important step in the cervical nodal metastasis of oral squamous cell carcinoma. We suggest that newly formed lymphatics inside the tumor are a target for anti-metastasis therapy of OSCC.

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