Lymphatic Vessel Density and Lymphangiogenesis in Human Oral Squamous Cell Carcinoma

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Abstract: Lymphatic vessels are distributed directly under the epithelium of the oral mucosal. In the present study, we investigated whether lymphangiogenesis in tumors occurs in human oral squamous cell carcinoma and whether the density of tumor lymphangiogenesis may be related to the risk of lymph node metastasis. Moreover, these analyses identified peritumoral lymphatic vascular density as a novel prognostic indicator for the risk of lymph node metastasis in oral squamous cell carcinoma.

LYVE-1, which was an endothelial cell hyaluronic acid receptor, has been identified as a peculiar protein of endothelial cells of lymphatic vessels. LYVE-1 expression in the carcinoma tissue was divided into two types. In one it appears in contact with the cancer nest. This shows contact with basal-like cells which are located on the outer side of the cancer nest, and observation via light microscope is unable to show intervention of the fibrous connective tissues. The other case is that in which there is intervention of the clear fibrous connective tissues between the cancer nest and LYVE-1-positive cells. The cancer cells showed a high degree of differentiation, increased by the formation of the cancer nest, and in what is called highly-differentiated squamous cell carcinoma whose invasion pattern showed INF alpha, the cells staining positive to the LYVE-1 antibody could be recognized only negligibly in the connective tissue. Positive staining of the LYVE-1 antibody was seen in the endothelial cells presenting in the lumen formed between each small cancer nest in the case of undifferentiated squamous cell carcinoma in which the cancer nest was very small and composed of undifferentiated cancer cells. In this case the cancer invasion pattern shows INFy. In addition, the expression of LYVE-1 was recognized in cells which had not formed into the lumen between the cancer nests.

In the meantime, VEGF-C was expressed in endothelial cells which formed the lumen and cancer cells. In the cancer cells which formed the large cancer nest, the expression of VEGF-C was recognized in basal-like cells which located at the periphery of the nest. VEGF-C was expressed in the cancer cells which formed the small nest. The results of this study show that the incidence of VEGF-C expression in the cancer cell is low. However, lymphatic vessels form in carcinoma tissue at a high rate when VEGF-C is expressed. It was shown that in this study, the close proximity of the lymphatic vessel to the cancer nest shows correlation to lymph node metastasis. This should affect the determination of the operation range, and have a direct influence on prognosis. It is also indicated that LYVE-1 can act as a useful marker in oral cancer.

Key words: LYVE-1, VEGF-C, metastasis, invasion, lymphatic vessel.
the lymphatic vessels in the oral mucosa? 2. What is the characteristic difference between endothelial cells of blood vessels and lymphatic vessels, and how does this difference relate to cancer's invasion of the lymphatic vessels? 3. What is the effect of lymph pressure on the cancer cells' invasion of lymphatic vessels? 4. What is the correlation between adhesion factor and the progress of metastasis in the lymphatic vessels? 5. Do the tumor cells cause lymphangiogenesis? 6. Is the process of lymph node metastasis similar to that of vascular metastasis? 7. Does the cancer invasion of the lymphatic vessels result in the expansion of the lymphatic vessels? And, only few reports have described the genetic analysis of lymph node metastasis in the target gene."

It has been proven that lymphatic vessels are distributed directly under the epithelium of the oral mucosa. In the lymphangioma which arises from this lymphatic vessel, the epithelium shows the papilla during hyperplasia of the lymphatic vessels as they expand under the epithelium. This shows that the lymphatic vessel in oral mucosa exists directly under the epithelium. Therefore, as epithelial dysplasia in the oral epithelium progresses, the question arises as to whether the lymphatic vessels come in contact with the capillaries during the early stages of cancer invasion due to the destruction of the basement membrane as the cancer invades below the epithelium. As well, in regard to the structure of the lymphatic vessel, the endothelial cells of these vessels are weaker than the endothelial cells of the capillaries, and there exists a small gap junction and also, lymphatic vessels contain neither basement membrane nor pericytes. Such structure allowing the flow of materials by the lymphatic vessel shows similarity with blood vessels. The lymphatic pressure is slightly higher than the interstitial tissue and it rapidly reacts to ions and the pressure imposed by its surrounding environment, allowing the lymphatic fluid to flow smoothly. This adhesive property shares similarity with vascular endothelial cells. In addition, it has also been reported that endothelial cells express type C vascular endothelial growth factor (VEGF-C) that plays a role in the propagation and growth of endothelial cells."

It has also been reported that Vascular Endothelial Growth Factor (VEGF) is expressed during cancer invasion and metastasis. This VEGF correlates with the expression level and quantity of angiogenesis. Especially, VEGF-C is associated with invasion of the lymphatic vessel and hyperplasia of the lymphatic vessel in many tumors, and VEGFR-3, which is the VEGF receptor, has appeared in the endothelial cells of lymphatic vessels. Recently, LYVE-1, which was an endothelial cell hyaluronic acid receptor, has been identified as a peculiar protein of endothelial cells of lymphatic vessels. LYVE-1 also has 40% greater homology to the amino acid of hyaluronic acid than does CD44. The expression of CD44 has been confirmed in many metastatic tumors, and the increase of its expression has become an index of tumor invasion and potential metastasis.

In the present study, we investigated whether lymphoangiogenesis in tumors occurs in human oral squamous cell carcinoma and whether the density of tumor lymphangiogenesis may be related to the risk of lymph node metastasis. Moreover, these analyses identified peritumoral lymphatic vascular density as a novel prognostic indicator for the risk of lymph node metastasis in oral squamous cell carcinoma.

Materials and Methods

Tissues

Human oral squamous cell carcinoma specimens were obtained from 50 surgical resections carried out at Showa University School of Dentistry, and normal oral mucous epithelium of non-tumor tissue were obtained from 14 surgical resections (Table 1). All samples were embedded in TissueTek OCT medium (Sakura Finetechanical Co., Ltd., Tokyo, Japan), and then frozen in isopentane.
cooled in liquid nitrogen and store at −80°C. The tissues were sectioned at 4 µm in a cryostat.

**Immunohistochemical staining and antibodies**

Frozen section was immunostained with anti LYVE1 and anti VEGF-C. Frozen section fixed 10 min in 4% paraformaldehyde in 17mM Tris-buffered saline (TBS). Endogenous peroxides were blocked by incubating the sections in 0.3% hydrogen peroxide in absolute methanol at room temperature for 30 min. After washing by TBS, protein block serum free (DAKO, Japan Co., Ltd., Kyoto, Japan) was applied to prevent non-specific binding of antibodies at room temperature for 5 min. The antibody was used anti LYVE1 antibody (ab10278, Abcam Ltd.) diluted 100 times and anti VEGF-C antibody (18-2255, ZYMED Laboratories Inc.) diluted 100 times at 4°C for over night. After washing by TBS, the sections were incubated with the secondary antibody using Envision+ system (DAKO, Japan Co., Ltd., Kyoto, Japan) for 15 min. Washed TBS 3X each, the sections were incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAKO, Japan Co., Kyoto, Japan) for 1 min, rinsed with tap water, counterstained with hematoxylin, and mounted.

**Statistical analysis**

Statistical analysis was carried out according to the hypothesis of oral squamous cell carcinoma that lymph vessel contact to cancer nest shows lymph node metastasis and oral squamous cell carcinoma that lymph vessel progress into cancer tissue shows lymph node metastasis using the χ-square test or the direct Fisher method. Between result of immunohistochemical finding of LYVE1 and VEGF-C, the χ-square value and P value under a level of significance of 0.01 are respectively shown, and the significance was accepted.

**Results**

1. **Expression and distribution of the lymphatic vessels by shown by immunohistochemical staining for LYVE-1 and VEGF-C antibody**

In normal mucosal tissue, LYVE-1 is expressed in endothelial cells which form the lumen under the epithelium. The cell components were not recognized and non-structured material was lightly

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stained by eosin in the lumen. It is shown that the lumen which expressed the LYVE-1 is a lymphatic vessel, while the LYVE-1 positive cells were not identified as arteries, veins, nerves, or salivary glands in the connective tissue. The immunostaining results using the VEGF-C antibody were similar to those of the LYVE-1 antibody.

LYVE-1 expression in the carcinoma tissue was divided into two types. In one it appears in contact with the cancer nest (Figure 1). This shows contact with basal-like cells which are located on the outer side of the cancer nest, and observation via light microscope is unable to show intervention of the fibrous connective tissues. The other case is that in which there is intervention of the clear fibrous connective tissues between the cancer nest and LYVE-1 positive cells. In addition, the difference in the expression of LYVE-1 was recognized by the differentiation of the cancer cells and by the invasion pattern of the squamous cell carcinoma. The cancer cells showed a high degree of differentiation, increased by the formation of the cancer nest, and in what is called highly-differentiated squamous cell carcinoma whose invasion pattern showed INF alpha, the cells staining positive to the LYVE-1 antibody could be recognized only negligibly in the connective tissue. In this case, LYVE-1 positive cells were seen in the connective tissue around the carcinoma tissue, and by their presence in the adjoining carcinoma tissue, such was considered to be an adjacent case. However, there was a case which revealed the appearance of LYVE-1 positive cells by contact to cancer nest, when the invasion pattern of INF alpha is shown even in highly-differentiated squamous cell carcinoma. In these cases, the chromatin concentrations in the nuclei of the cancer cells were high and basal-like cells located on the outer edge of the cancer nest were stratified, though the degree of the cancer cells’ differentiation is high.

Positive staining of the LYVE-1 antibody was seen in the endothelial cells presenting in the lumen formed between each small cancer nest in the case of undifferentiated squamous cell carcinoma in which the cancer nest was very small and composed of undifferentiated cancer cells. In this case the cancer invasion pattern shows INF gamma. In addition, the expression of LYVE-1 was recognized in cells which had not formed into the lumen between the cancer nests.

Figure 1  LYVE1 positive lymphatic vessel which has contacted (A and A') or non-contacted (B and B') with cancer nest.
In the differentiation and invasion patterns of either cancer cell, their distribution was recognized in the cells forming the lumen which presented positive for the LYVE-1 antibody below carcinoma tissue surface layer. As well, LYVE-1 positive cells in the lumen were concentrated in areas in the carcinoma tissue where inflammatory change was strong.

In the meantime, VEGF-C was expressed in endothelial cells which formed the lumen and cancer cells (Figure 2). In the cancer cells which formed the large cancer nest, the expression of VEGF-C was recognized in basal-like cells which located at the periphery of the nest. VEGF-C was expressed in the cancer cells which formed the small nest.

2. The locational relation between lymphatic vessel and cancer nest and its correlation with metastasis

The correlation between the location of the lymphatic vessel and the cancer nest, and metastasis was analyzed (Figure 3). The number of cases in which the LYVE-1 positive lymphatic vessel had contacted the cancer cell nest was 19 examples (38.8%) in 49 cases. The number of cases in which the lymphatic vessel was recognized in a position separate from the cancer cell nest was 30 examples (61.2%). The case with 13 example (68.4%) with
metastasis and there were 5 examples (31.6%) of clinically recognized metastasis in 19 examples in which the lymphatic vessel was connected to the cancer nest. There were 23 examples (76.8%) of metastasis in 38 cases in which the lymphatic vessel was separated from the cancer nest. Seven examples (23.3%) did not show metastasis. The level of significance was (p=0.004) when statistical analysis was performed regarding the relationship between metastasis and cases in which the cancer nest had contact with the lymphatic vessel and cases that did not. This result shows that the possibility of metastasis when the lymphatic vessel has contacted in cancer nest is high.

The locational correlation of the lymphatic vessel and carcinoma tissue was examined. The number of cases which the lymphatic vessel was recognized in the carcinoma tissue was 19 examples. Clinically determined metastasis was recognized in 14 examples (73.7%), while the number of those that did not show metastasis was 5 examples (26.3%). The number of cases in which the lymphatic vessel was recognized in nearby carcinoma connective tissue was 30 cases, these not showing LYVE-1-positive cancer cells in the lymphatic vessel. Of these 30 cases, 6 examples (20%) were recognized as metastasis and 24 examples (80%) were not considered clinically metastasis. The correlation of the presence of the lymphatic vessels in carcinoma tissue and presence of metastasis showed a statistically significant difference (p=0.0006), thus demonstrating that the formation of the lymphatic vessel in carcinoma tissue was related to metastasis.

3. The relevance of the expression of VEGF-C in the cancer cell and the distribution of the lymphatic vessel and metastasis

The expression of VEGF-C in cancer cells, the locational relation of the lymphatic vessel cancer nest, and their correlation with metastasis were analyzed (Figure 2, Figure 4 and Figure 5). The number of cases showing expression of VEGF-C in the cancer cells was 10 examples (20%) in 50 cases, and the number of cases not showing VEGF-C expression was 40 cases (80%). Of the cases expressing VEGF-C in the cancer cells, the number of cases in which the lymphatic vessel was present in the carcinoma tissue was 9 examples (18%) of the 50 cases. Within these 9 examples, 5 cases were clinically recognized as metastasis. And, though the expression of VEGF-C was recognized in the cancer cell, in 1 example (20.5%) in 50 cases the

Figure 4  The cancer cells of VEGF-C negative staining has contacted (A and A') or not contacted (B and B') with lymphatic vessel.
located lymphatic vessel was not located in the carcinoma tissue. In this example lymph node metastasis was not clinically recognized.

Ten examples (20%) out of 50 cases showed no expression of VEGF-C in cancer cells in cases in which the carcinoma tissue contained lymphatic vessels. Six examples of these 10 cases expressed clinically recognized metastasis. The number of cases in which there was no lymphatic vessel formation in the carcinoma tissue was 30 examples (60%) in the 50 cases which showed no expression of VEGF-C in the cancer cell. Metastasis was clinically recognized in 6 examples of these 30 cases. The difference was statistically significant (p=0.0006) for expression of VEGF-C and the formation of lymphatic vessels in carcinoma tissue.

In addition, the relation between the lymphatic vessel contacting the cancer nest and metastasis was analyzed. In 10 examples in which the expression of VEGF-C was recognized in the cancer cells, the lymphatic vessel had contacted the cancer nest in 8 cases (80%). Metastasis was clinically recognized in these 8 cases. Among the cases in which the lymphatic vessel did not contact the cancer cell nest, the lymphatic vessel appeared close to the cancer nest in 2 cases (20%), with these 2 cases not clinically showing metastasis. In 40 cases that did not show VEGF-C expression in the cancer cells, 11 cases (27.5%) showed the lymphatic vessel to be in contact with the cancer nest, and 5 cases of these had clinically recognized metastasis. The number of cases in which the lymphatic vessel was located close to the cancer nest was 29 examples (72.5%), and metastasis was recognized in 7 examples of these cases. The difference was statistically significant (p=0.007) for the expression of VEGF-C and the localization of the lymphatic vessel close to cancer nest.

The above findings demonstrate that very little lymphatic vessel formation takes place in carcinoma tissue in which VEGF-C expression is not recognized in the cancer cells.
Discussion

It is known that oral squamous cell carcinoma metastasizes to the cervical lymph node. However, the mechanism of this lymph node metastasis is unclear. Lymphatic vessels exist in great numbers directly under the mucosal epithelium. The reticular formation of a large number of capillaries is present in this region, thus leading to difficulty in distinguishing capillaries and lymphatic vessels. For the reason, it is difficult to determine the relationship of lymphatic vessels and cancer invasion to metastasis. In addition, the phenomenon by which angiogenesis is induced in cancer cells may also occur even in lymphatic vessels. Capillaries and lymphatic vessels are formed by morphologically equal endothelial cells. These difference between these cells is that unlike the endothelial cells of blood vessels, the endothelial cells of lymphatic vessels form neither a basement membrane and nor pericytes. However, discriminating between these kinds of cells is difficult via light microscope. Then, LYVE1 has been determined to be a marker of lymphatic vessels. LYVE-1 is new hyaluronic acid receptor that recognizes the extracellular matrix. LYVE-1 has 40% more homology than does CD44 and has much stronger adhesion than does CD44. LYVE-1 immunostaining has been used to locate lymphatic vessels in mammary cancer, pancreas cancer, and head and neck cancers. It has been reported that lymphangiogenesis occurs in head and neck cancers, and that oral cancer is resistant to lymph node metastasis. In addition, it has been reported that the five year survival rate is worse in cases in which the lymphatic vessel lies within the circumference of the cancer nest, in comparison with cases in which the lymphatic vessel exists outside the carcinoma tissue. It was shown in this study that in cases in which the lymphatic vessel exists within the carcinoma tissue, the metastasis rate is significantly higher than when the lymphatic vessel contacts the cancer nest. Therefore, determination of the location of the lymphatic vessel in tumor tissue by immunohistochemical staining using the LYVE-1 antibody may provide a promising objective method for the evaluation of lymph node metastasis.

The primary molecular mechanism of lymphangiogenesis involves VEGF-C and tyrosine receptor VEGFR-3. In the transgenic mouse which peculiarly overexpressed VEGF-C, it has been reported that VEGF-C is the factor that induces lymphangiogenesis, because of the presence of hyperplasia of the lymphatic vessels in the skin. In VEGF-C knockout mice, lethality at viviparity is reached in 15–17 days. In the present study, LYVE1, which is a lymphatic vessel marker, showed positive in the endothelial cells of lymphatic vessels, revealing the differentiation of endothelial cell. However, the process in which the lymphatic vessel buds out has been inhibited. In a previous study involving VGFR-3 knockout mice, lethality was reached at viviparity in 10 days due to the growth of lymphatic vessels and reconstruction abnormality of blood vessel. These findings demonstrate that VEGF-C is the lymphangiogenesis factor.

The cancer cell secretes VEGF-C, and it is known that the growth of lymphatic vessels is induced in intratumors or peripheral tumors. In lymphangiogenesis, the opportunity for cancer cells to invade lymphatic vessels increases, and lymph node metastasis is promoted. For example, immunohistochemical staining with LYVE-1 marked the lymphatic vessels in nude mice in which breast cancer cells which overexpressed VEGF-C were transplanted. LYVE-1 staining demonstrated the intratumor and lymphatic vessels of the tumor’s circumference. Lymph node metastasis would be recognized with high frequency when VEGF-C is overexpressed in breast cancer cells which originally did not show lymph node metastasis. In addition, expression of VEGF-C in gastric cancer, colon cancer, lung cancer, prostate cancer, and thyroid cancer has clinically correlated with the cancer metastasis.
metastasis has been recognized. The metastasis rate also rises when the lymphatic vessel is connected with cancer nest. Though it is not possible to discuss the function of the lymphatic vessel within the tumor in the present study, we found a consistent correlation between metastasis and contact of the lymph node and the cancer nest. However, it is necessary to clarify the relation between lymphangiogenesis, lymphatic vessel hyperplasia, the network of existing lymphatic vessels and lymph nodes, and tumor progress. In this study, one case which did not show metastasis nonetheless showed VEGF-C expression and did show contact between the lymphatic vessel and the cancer nest. It is considered that it originates from the difference in the ability in which the tumor cell invaded to lymphatic vessels. In short, differences of the cancer cell's MMP productivity, tumor growth factor, blood or lymphatic vessel growth factor such as VEGF-A of the cancer cell etc. should be kept in mind. It is considered that the relation with these factors must be also analyzed in order to clarify lymph node metastasis.

Finally, the close proximity of the lymphatic vessel to the cancer nest shows correlation to lymph node metastasis. This should affect the determination of the operation range, and have a direct influence on prognosis. It is also indicated that LYVE-1 can act as a useful marker in oral cancer.

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