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Lymphangiogenesis in regional lymph nodes predicts nodal recurrence in pathological N0 squamous cell carcinoma of the tongue

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Title: Lymphangiogenesis in regional lymph nodes predicts nodal recurrence in pathological N0 squamous cell carcinoma of the tongue

Running Title: Lymphangiogenesis in pN0 tongue cancer

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Abstract

Aims: Cancer cells induce de novo lymphatic vessel growth within draining lymph nodes before they metastasize. Lymph node lymphangiogenesis before the establishment of nodal recurrence in squamous cell carcinoma (SCC) of the tongue was evaluated retrospectively.

Methods and Results: Surgical specimens from 28 patients with pT2-T3N0M0 SCC of the tongue after local excision with supraomohyoid neck dissection were studied by immunohistochemistry. Intranodal lymphatic endothelium was highlighted by podoplanin staining to evaluate the lymphatic vessel counts (LVC). Primary tumor sections were examined for the expressions of lymphangiogenic factors: vascular endothelial growth factor (VEGF)-C and VEGF-D. LVC in the regional LN was significantly increased in the cases with nodal recurrence ($p=0.0013$). Simultaneous increases of VEGF-C and VEGF-D protein expressions were also significantly associated with an increase of LVC in regional lymph nodes ($p=0.0001$) and a decrease in the survival rate without nodal recurrence ($p=0.0160$).

Conclusions: The status of lymphangiogenesis in the regional pN0 lymph nodes for

tongue cancer would help us predict patients developing nodal recurrence. The inclusion of a therapeutic approach to block lymphangiogenic factors, such as VEGF-C and VEGF-D, may be beneficial to suppress the lymphatic spread of tongue cancer with intense intranodal lymphangiogenesis.

Key words: squamous cell carcinoma of the tongue, intranodal lymphangiogenesis, nodal recurrence, vascular endothelial growth factor-C, vascular endothelial growth factor-D.

Introduction

The lymph node metastatic status is a predictor of a poor prognosis in patients with tongue cancer. Moreover, patients who experience neck recurrence after initial treatment frequently die of uncontrolled neck diseases [1, 2], and many candidates for prediction have been evaluated [3, 4]. More than 30% of patients with tongue cancer have cervical lymph node metastases, even in clinically node-negative disease [5]. Among patients with clinically T1/T2 or N0 tongue cancer, the regional recurrence rate of the untreated neck is 20 to 30% [6, 7]. However, the treatment planning for regional control of clinically node-negative squamous cell carcinoma (SCC) of the tongue is controversial. Some surgeons advocate elective neck dissection, while others adopt a "watchful-waiting" policy after local excision. Recently, sentinel node navigation surgery has been introduced for the detection of micrometastasis in lymph nodes [1, 2]. However, the accuracy of the prediction is not perfect, and the predictive values of negative sentinel lymph nodes are about 90% [8]. Therefore, it is vital to identify prognostic parameters that influence the occurrence of nodal recurrence.

Lymphangiogenesis, a process that generates new lymphatic vessels from

pre-existing ones with the aid of circulating lymphatic endothelial progenitor cells, is believed to underlie lymph node metastases [9]. Owing to the advent of molecular markers specific to the lymphatic endothelium, including lymphatic vessel endothelial hyaluronan receptor 1, podoplanin, and prospero homeobox protein 1, research on tumor lymphangiogenesis has been conducted over the last decade. Some clinical studies reported that the lymphatic vessel density in many types of solid cancer were associated with the status of lymph node metastases at the initial diagnosis [10-13]. Moreover, experimental studies have indicated that primary tumors induce new lymphatic vessel growth within draining lymph nodes before they metastasize [11, 14, 15].

Vascular endothelial growth factor (VEGF)-C and VEGF-D, members of the VEGF family of glycoproteins, have been identified as potent inducers of lymphangiogenesis [9]. VEGF-C and VEGF-D exert their effects on endothelial cells by stimulating VEGF receptor 3 (VEGFR-3), a tyrosine kinase receptor expressed predominantly on lymphatic endothelial cells [16]. Overexpressions of VEGF-C and VEGF-D in experimental tumor models were significantly associated with the formation of new lymphatic vessels [17, 18], and several investigators found that inhibition

of VEGF-C and VEGF-D activities reduced the level of lymphangiogenesis and lymph node metastases [19, 20]. In clinical studies, contributions of VEGF-C, VEGF-D, and VEGFR-3 to lymph node metastases, lymphatic invasion, and a poor prognosis have been observed in several malignancies [21-23].

This study was performed to evaluate the role of lymphangiogenesis in regional lymph nodes as a predictor of nodal recurrence in patients with pT2-T3N0 SCC of the tongue treated primarily by local excision with supraomohyoid neck dissection. In addition, the relationships between lymph node lymphangiogenesis and nodal recurrence in association with the expressions of VEGF-C and VEGF-D proteins were analyzed.

Materials and Methods

Patients and tissues (Table 1)

The samples studied were obtained from 28 patients who were diagnosed with pT2-T3N0M0 SCC of the tongue based on the TNM classification system of the Union Internationale Contre le Cancer after local excision and supraomohyoid neck dissection [24]. To correctly analyze the association of intranodal lymphangiogenesis with nodal recurrence, patients who experienced local relapse were not included in this study. Cases classified as pT4 were also excluded from the analyses. All patients were treated at the Division of Otolaryngology, Head and Neck surgery, Kanazawa University Hospital, between 1982 and 2006. The Institutional and Ethical Review Board at Kanazawa University approved this retrospective review of the medical records and the use of archived surgical specimens.

Twenty cases were stage II (pT2N0M0) and eight cases were stage III (pT3N0M0). The patients comprised 17 males and 11 females, and the ages ranged from 25 to 86 years old (median, 61 years old). All surviving patients had undergone a minimum of a 5-year follow-up. Six of the twenty patients developed nodal recurrence

at 14, 6, 32, 48, 7, and 9 months and recurrence sites were contralateral level I, and ipsilateral levels V, IV, II, III, and IV (patient numbers 1-6, Table 1), respectively.

The depth of invasion was measured from the surface of the mucosa to the maximum depth using an ocular micrometer. When there was exophytic tumor growth, the measurement was made from the height of the surface of the adjacent normal mucosa to the deepest-reaching front of the infiltration. The data on the depth of invasion and lymphatic invasion are also shown in Table 1.

Immunohistochemical analyses

The surgical specimens including the primary tumors and regional lymph nodes were fixed in a 10% formalin solution and embedded in paraffin. Consecutive 3-μm sections were cut from each block. Immunohistochemical stainings were performed as described previously [25]. The following antibodies were used as primary antibodies: mouse-derived monoclonal antibody for podoplanin（Dako, CA, USA）(dilution 1:100), rabbit-derived polyclonal antibody for VEGF-C (Invitrogen, CA, USA) (dilution 1:100), goat-derived polyclonal antibody for VEGF-D (R&D systems, MN, USA) (dilution

1:50), and mouse-derived monoclonal antibody for pan-cytokeratin (Dako, CA, USA) (dilution 1:100). Diaminobenzidine tetrahydrochloride was used as a chromogen, and the sections were counterstained with methyl green. The specificities of the staining were confirmed using non-immune serum instead of the primary antibody as a negative control. Two investigators (K.H. and N.W.) who had no prior knowledge of the clinicopathological findings assessed the lymphatic vessel counts (LVC) and expressions of VEGF-C and VEGF-D proteins. Each lymph node was analyzed for pan-cytokeratin to detect metastatic tumor cells, and patients with positive staining were not included in this study.

Evaluation of LVC in pN0 regional lymph nodes

To evaluate the level of lymhangiogenesis in lymph nodes, the antibody for podoplanin was used to identify the lymphatic endothelium. Lymphatic vessels were assessed under light microscopic examination of the podoplanin-positive microvessels in the biggest lymph node of the surgical specimen. The whole lymph node section was scanned at a low magnification (×40), and areas of intense lymphangiogenesis (hot

spots) were determined. After four areas with intense lymphangiogenesis were identified, lymphatic vessels were counted in a ×200 field, and the average count per field (1.1 mm^2) of the four fields was defined as LVC. Any brownish-staining endothelial cell or endothelial-cell cluster was considered a single countable lymphatic vessel.

VEGF-C and VEGF-D expressions

Primary tumor sections were examined for VEGF-C and VEGF-D protein expressions. Staining results for VEGF-C and VEGF-D in the primary tumors were classified by estimating the percentage of tumor cells showing specific immunoreactivity. Negative staining was defined when the degree of staining was < 25%, and the staining was defined as positive when the degree of staining was ≧ 25%. The threshold of 25% of cells expressing VEGFs was selected according to the median value of each expression.

For further analyses, all cases were then divided into the following two groups according to the expression levels of VEGF-C and VEGF–D in the primary tumors

(VEGF-C&D): High group, both VEGF-C and VEGF-D were positive; and Low group, both VEGF-C and VEGF-D were negative, or either VEGF-C or VEGF-D was negative.

Statistical analyses

IBM SPSS Statistics, version 19 (IBM, Armonk, USA), was used for data analyses. The development of nodal recurrence and expressions of VEGF-C and VEGF-D proteins in relation to LVC were analyzed using the Mann-Whitney *U* test. The rates of survival without nodal recurrence were analyzed with the Kaplan-Meier method, and the differences between the curves were analyzed with the log-rank test. The survival period was defined as the period between the date of surgery to that of nodal recurrence. For statistical analyses, the depth of invasion was subdivided into two groups, $\geqq$5 mm and <5 mm. p-values of < 0.05 were considered significant.

Results

Detection of lymphatic vessels in pN0 regional lymph nodes (Figure 1A, Table 1)

Immunohistochemical staining showed that antibody specific for podoplanin reacted with the endothelial cells of lymphatic vessels in pN0 regional lymph nodes, as expected according to its established role as a lymphatic marker. Most lymphatic vessels highlighted by podoplanin staining were detected as scattered in the stroma of the lymphatic tissues. LVC of each case is shown in Table 1. The median LVC in the current cohort of patients was 12, with a range from 0 to 55.

VEGF-C and VEGF-D protein expressions at the primary sites (Figure 1B and 1C, Table 1)

VEGF-C and VEGF-D proteins were observed in the cytoplasm of tumor cells. According to the criteria for immunohistochemical staining of these proteins, VEGF-C and VEGF-D expressions were evaluated as positive in 18 and 14 out of 28 patients, respectively. Eleven out of the 28 patients belonged to the High group regarding VEGF-C&D expression.

LVC in regional lymph nodes was associated with nodal recurrence, and VEGF-C and VEGF-D protein expressions in the primary tumor (Table 2)

A significant correlation was identified between the increasing number of LVC in the regional lymph nodes and development of nodal recurrence during the follow-up period ($p = 0.0013$). Although the patients who were VEGF-C-positive had a greater LVC value in the regional lymph nodes compared with their negative counterparts, the difference was not significant ($p = 0.1480$). On the other hand, VEGF-D-positive cases had a significantly higher LVC in the regional lymph nodes than VEGF-D-negative cases ($p=0.0012$). When the evaluations of each protein were combined (VEGF-C & D), the High group displayed significantly higher LVC values compared with the Low group ($p = 0.0001$). Thus, simultaneous increases of VEGF-C and VEGF-D were strongly associated with increasing lymphangiogenesis in the regional lymph nodes.

VEGF-C&D expression was associated with nodal recurrence (Figure 2)

The relationships between VEGF-C, VEGF-D, and the combination of VEGF-C

and VEGF-D (VEGF-C&D) expressions in the primary tumor, and the rates of survival without nodal recurrence were examined. The rates of survival without nodal recurrence were associated with neither VEGF-C nor VEGF-D expression (Figure 2A and 2B). However, in the combined analyses, the High group had a significantly lower survival rate without nodal recurrence compared with the Low group ($p = 0.0160$) (Figure 2C).

The relationship between the depth of invasion and lymphatic invasion, and survival rates without nodal recurrence were also examined. The rates of survival without nodal recurrence were associated with neither the depth of invasion nor lymphatic invasion (Figure 2D and 2E).

Discussion

In the present study, LVC in regional lymph nodes was associated with nodal recurrence , and could be a significant prognostic parameter in patients with pN0 tongue carcinoma. Even in pN0 lymph nodes, lymphatic vessels were increasing in number to prepare for the subsequent lymphatic spread. The high-level expressions of VEGF-C and VEGF-D proteins had a significant impact on increasing regional lymph node LVC and the development of nodal recurrence. That is, lymphangiogenic factors such as VEGF-C and VEGF-D secreted by tumor cells would facilitate lymphangiogenesis in regional lymph nodes, which increases the possibility of nodal recurrence. This result is in line with earlier animal studies on lymph node lymphangiogenesis which focused on its role in the premetastatic remodeling of lymphatic vessels at sentinel lymph nodes [14, 26].

Our study demonstrated that VEGF-C alone was less associated with lymph node LVC compared to VEGF-D. The data are not compatible with reports which suggested a significant correlation between VEGF-C expression and LVC [12, 17, 26]. The most likely reason for the above discrepancy is that the patients in our cohort were pN0, while the

patients in other studies were pN+ [12, 17, 26]. As is reported in the study of Hirakawa et al., VEGF-D is a potent inducer of lymph node lymphangiogenesis in the early phase, whereas VEGF-C would have more of an effect on lymph node lymphangiogenesis in the late phase of lymph node metastases [26]. Combination analysis of VEGF-C&D expression showed that tumors of the High group had a higher number of LVC and higher incidence of nodal recurrence compared with the Low group. Thus, when the tumor cells are positive for both VEGF-C and VEGF-D proteins, these factors would work additively to facilitate lymphangiogenesis, which results in a more agressive lymphatic spread of the tumor cells at both phases of lymph node metastases.

Recent studies on different types of cancer have highlighted the importance of lymph node lymphangiogenesis as a potential prognostic parameter [10-13]. Higher levels of lymph node lymphangiogenesis are significantly associated with lymph node metastases and poor outcomes. In rectal cancer, it was reported that intranodal lymphangiogenesis affected the decrease in disease-free survival [27]. In a B16 melanoma mouse model, it was demonstrated that lymph node lymphangiogenesis in response to tumor growth precedes tumor metastases [11]. Interestingly, the significance of sentinel

lymph node lympangiogenesis in the metastatic spread of breast cancer to non-sentinel lymph nodes has been shown in human patients [28]. These associations of regional or sentinel lymph node lymphangiogenesis with a poorer prognosis further suggest that the routine detection of lymph node lymphangiogenesis would be a useful method to predict the lymphatic spread of cancer cells.

In conclusion, our strategies to detect lymphangiogenesis in regional pN0 lymph nodes in tongue cancer would help us predict patients developing nodal recurrence. In a future study, it will be meaningful to evaluate the level of intranodal lymphangiogenesis at the metastases-free sentinel lymph node in association with the prediction of nodal recurrence in SCC of the tongue. Finally, the inclusion of a therapeutic approach to block lymphangiogenic factors, such as VEGF-C and VEGF-D, may be beneficial to prevent the lymphatic spread of tongue cancer with intense intranodal lymphangiogenesis.

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Figure legends

Figure 1. Immunohistochemical detections of podoplanin (Original magnification, 200x) (A), VEGF-C (Original magnification, 100x) (B), and VEGF-D (Original magnification, 100x). (A) Podoplanin expression was essentially restricted to vessel structures. Lymphatic vessels highlighted by podoplanin staining were detected as scattered in the stroma of the lymphatic tissue. (B, C) VEGF-C and VEGF-D proteins were localized in the tumor cytoplasms.

Figure 2. Kaplan-Meier estimates of the survival rate without nodal recurrence. The differences between the curves were analyzed with the log-rank test. The survival rate without nodal recurrence in relation to VEGF-C expression (A), VEGF-D expression (B), combination of VEGF-C and VEGF-D expressions (VDGF-C&D) (C), the depth of invasion (D), and lymphatic invasion (E).