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Influences of angiogenesis and lymphangiogenesis on cancerous invasion in experimentally induced tongue carcinoma

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BACKGROUND: Although it is clear that dissemination via the blood system involves angiogenesis, it is uncertain whether tumors also induce lymphangiogenesis or simply invade existing peritumoral vessels. The purpose of this study was to elucidate changes in tumor blood and lymph vessels in cases involving the invasion of squamous cell carcinoma in the oral cavity, and its significance. Blood and lymph vessels densities in tongue carcinomas induced in hamsters were investigated.

METHODS: Tongue cancer was induced by abrading the right margin of the tongue of each hamster with an endodontic barbed broach and subsequently applying 1.0% 9,10-dimenthl-1,2-benzanthracene (DMBA) dissolved in acetone, three times a week, at the same site. Fresh frozen sections were prepared and blood vessels stained blue by perfusion with Coomassie Brilliant Blue and lymph vessels stained brown for 5′-nucleotidase. The effects on the blood vessels and lymph vessels were observed.

RESULTS: The results showed that blood and lymph vessel densities were greater in the advanced carcinoma tissues than in normal tissue. These were compared in terms of the mode of cancer invasion. As tumor invasion progressed, the blood vessel density decreased but lymph vessel density tended to be higher in high-degree tumor invasion than in low-degree tumor invasion. The expression of vascular endothelial growth factor-C was seen more frequently as tumor invasion progressed.

CONCLUSIONS: The present findings indicated that angiogenesis and lymphangiogenesis are affected by cancerous invasion.

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Angiogenesis is essential for the growth and expansion of tumors (1); the expression of vascular endothelial growth factor (VEGF) plays a major role (2, 3) in angiogenesis, and is considered to be an independent prognostic factor in many cancers. Lymph node metastasis is closely related to the prognosis of squamous cell carcinoma (SCC) of the oral cavity, and control of lymph node metastasis is an important therapeutic strategy. For quite some time, lymph vessels were thought to be absent in tumors, and a relationship between tumor lymphangiogenesis and lymph node metastasis was excluded. The mainstream hypothesis was that the existing lymph vessels are sufficient for metastasis to the lymph nodes. However, since 1995, when Kaipainen et al. (4) reported that VEGF-C is a vascular and lymphangial endothelial cell growth factor, correlations between VEGF-C expression in tumors and lymph node metastasis have been reported in various cancers including stomach, large intestine, lung, prostate, and thyroid gland cancers (5–10). Furthermore, lymphangiogenesis has been noted in breast cancers that overexpress VEGF-C (11). Since lymph node metastasis is a pathologic state in which cancer cells migrate via lymph vessels, both angiogenesis and lymphangiogenesis may be related to invasion or metastasis in oral SCC. Yamamoto et al. (12) have graded the mode of cancer invasion in SCCs of the oral cavity (Table 1). Since grading was observed as a tumor–host relationship, a strong correlation between mode of invasion and clinical course has been reported (13). This study was conducted in order to clarify the various relationships between angiogenesis, lymphangiogenesis, and tumor invasion in cancers of the oral cavity. Using a DMBA-induced hamster tongue carcinoma model, the relationships between classification of the mode of cancer invasion and blood and lymph vessel densities was investigated,

Table 1 Histologic grading of mode of cancer invasion

Grade	*Histologic grading*
1	Well-defined borderline
2	Cords, less marked borderline
3	Groups of cells, no distinct borderline
4	Diffuse invasion
	Cord-like type invasion (4C)
	Diffuse type invasion (4D)

The mode of invasion of the oral squamous cell carcinoma (SCC) was graded by the criteria reported by Yamamoto et al. (12).

as was the relationship between lymph vessel density and VEGF-C.

Materials and methods

Experimental animal

Forty-five male Syrian golden hamsters (Awazu Experimental Animal, Settsu, Japan) at 6 weeks of age were used for the present study. The animals were maintained in a laminar flow isorack under specific pathogen-free conditions at the Institute for Experimental Animals of Kanazawa University. The hamsters were given autoclaved food (Oriental Koubo, Tokyo, Japan) and water.

Carcinogenic methods

A 1.0% solution of DMBA (Wako Pure Chemical, Osaka, Japan) in acetone was applied to the right lateral border of the tongue three times per week after that part of the tongue was scratched with an endodontic barbed broach, as described by Fujita et al. (14). The treatment continued until the resulting tumor became carcinomatous. The hamsters were observed until they developed signs of cachexia. Five normal hamsters served as controls, and they were killed without treatment.

Blood vessel staining

Blood vessel staining was performed after 0.5 ml of 5% pentobarbital solution (Dai Nippon Seiyaku, Osaka, Japan) was intraperitoneally injected into the hamsters. After thoracotomy, a 20-gauge catheter was inserted into the ascending aorta via the left ventricle, and was fixed by ligation with 4.0-nylon thread. Simultaneously, the right and left auricles were dissected to secure the outflow pathway for blood and the infusion agent. Next, physiologic saline maintained at 40°C was infused for 10 min at 90–100 mmHg infusion pressure via the catheter for perfusion and exsanguinations. Blood vessel staining solution (pure water, 97 ml; polyvinylpyrolidone, 2 g; Coomassie Brilliant Blue R-250, 1 g) was infused via catheter for 20 min at the same pressure, until staining of the tongue and oral mucosa was confirmed. After perfusion, the hamsters were kept immobile for 15 min and then the tongues were excised together with the surrounding tissue.

Preparation of tissue samples

The excised tissue specimens were embedded in optimal cutting temperature (OCT) compound and frozen in liquid nitrogen; 4 and 10 μm fresh frozen sections were then prepared. About 4 μm sections were subjected to hematoxylin and eosin staining and immunohistochemical staining of VEGF-C, and 10 μm sections were subjected to double staining of the blood and lymph vessels.

Lymph vessel staining

About 10 μm fresh frozen sections were fixed for 40 min in 4% formaldehyde solution. Upon completion of the fixation procedure, the sections were washed thoroughly in 0.2 M Tris-maleate buffer. After washing, the sections were immersed for 60 min at 37°C in 5′-nucleotidase (Nase) substrate solution (0.2 M Tris-maleate buffer, 20 ml; distilled water, 22 ml; 0.1 M $MgSO_4$, 5 ml; 2% $Pb(NO_3)_2$, 3 ml; 5′-adenosine monophosphate, 25 mg; L-tetramisole, 15 mg; sucrose, 3.5 g), as described by Kato (15, 16). The sections were then washed in 0.2 M Tris-maleate buffer, immersed for 1-min development in 1 M ammonium sulfide, and washed in distilled water.

Using 10 μm double-stained sections, three leading invasion front regions were randomly selected, and the blood and lymph vessel densities were calculated under a microscope at ×100 magnification. Stained blood vessel sites were counted as the blood vessel density, and 5′-Nase-positive structures were counted as the lymph vessel density. Since 5′-Nase also reacts with blood vessels and lymphocytes (17), structures positive for both blood vessel staining and 5′-Nase were regarded as blood vessels. As 5′-Nase-positive structures measuring 20 μm or less may be lymphocytes, the number of these structures was subtracted from the total number of lymph vessels.

Immunohistochemical staining

After fresh frozen sections were fixed for 10 min in cold acetone solution, they were incubated with 1% periodic acid in distilled water for 5 min at room temperature in order to block endogenous peroxidase activity. Following a phosphate-buffered saline (PBS) rinse, the sections were incubated with serum-free protein block (Dako Japan, Kyoto, Japan) for 15 min at room temperature to block specific binding. Primary antibodies used were rabbit polyclonal antibody for VEGF-C (Immuno-Biological Lab, Gumma, Japan) at a 1:50 dilution. After overnight incubation with each of the primary antibodies at 4°C, ChemMate Envision antibodies (Dako Japan) to mouse immunoglobulins or to rabbit immunoglobulins were used as the secondary antibody to VEGF-C, and the slides were incubated for 1 h at room temperature. Immunohistochemical reactions were developed in 3,3′-diaminobenzidine tetrahydrochloride, and the samples counterstained with hematoxylin. Negative controls were treated with all of the above-mentioned reagents except for the primary antibody.

The expression of VEGF-C was evaluated using immunohistochemically stained preparations using anti-VEGF-C antibody. The degree of expression in tumor cells in the leading invasion front regions was classified into one of three groups, i.e. as low-, moderate-, and high-expression groups, respectively. The

low-expression group consisted of tumors with a small number of positive tumor cells. The moderate-expression group was composed of tumors with partially stained cancer nests. The high-expression group consisted of tumors with entirely stained cancer nests.

Classification of the mode of cancer invasion
The mode of cancer invasion was histologically classified according to the criteria of Yamamoto et al. (12) (Table 1).

Statistical analysis
For statistical analysis, one-way ANOVA and Spearman's correlation coefficient by rank test was used. When a significant difference was observed, multiple comparison was performed using the Fisher's protected least significant difference (PLSD) method. Differences were considered significant when the *P*-value was < 0.05.

Results

Histopathologic findings
Carcinogenesis was noted in 36 hamsters used in the experiment, i.e. excluding the four dead animals, and the histologic type was SCC in all animals. Most cancers were well differentiated, but some were only moderately differentiated, or poorly differentiated. As regard the classification of the mode of cancer invasion, eight (22.2%), eight (22.2%), 15 (41.7%), and five animals (13.9%) were classified as having grades 1, 2, 3, and 4C invasion, respectively, and no animal was classified as having grade 4D invasion.

Changes in blood vessel densities in induced tongue carcinoma
The blood vessel densities in induced carcinoma were investigated. The blood vessel density (mean ± SD) was 6.9 ± 1.4 in the normal group (Fig. 1) and 16.3 ± 6.8 in the advanced carcinoma group (Fig. 2). Furthermore, the blood vessel densities associated with each mode of invasion were investigated. The blood vessel density was

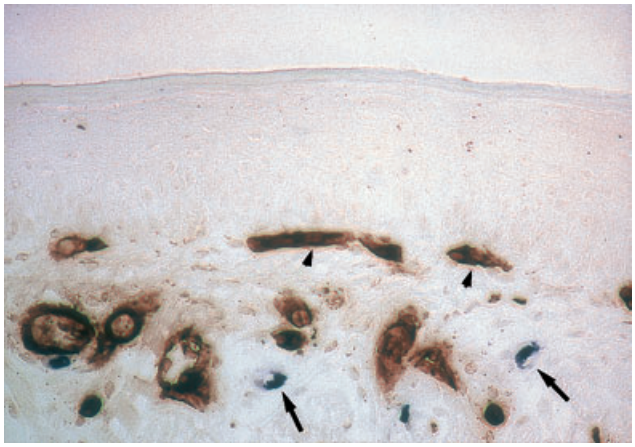

Figure 1 Photomicrographs of a normal hamster tongue. Blood and lymph vessel stains, blood vessel (blue stain, arrows) and lymph vessel (brown stain, arrowheads) in normal tissue; original magnification, ×50.

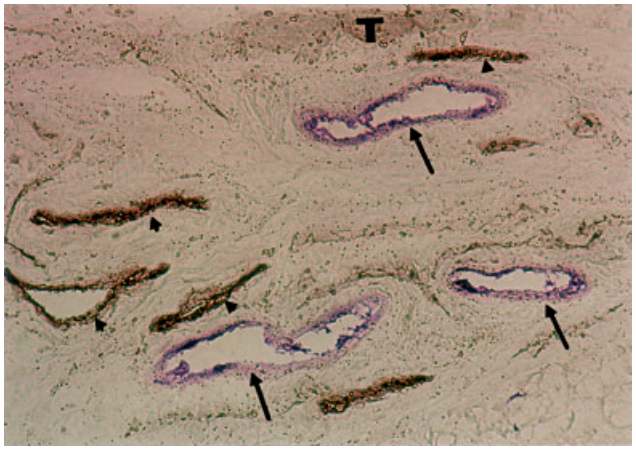

Figure 2 Photomicrographs of hamster tongue carcinomas. Blood and lymph vessel stains, blood vessel (blue stain, arrows) and lymph vessel (brown stain, arrowheads) in the tumor tissue at the invasion front (T); original magnification, ×100.

Table 2 Relationship between mode of invasion and blood vessel densities

Mode of invasion	*Number of hamster*	*Blood vessels ($\bar{X}$ ± SD)*
1	8	24.1 ± 3.0
2	8	21.1 ± 3.2*
3	15	12.0 ± 3.8**
4C	5	9.4 ± 4.7***

As tumor invasion progressed, the blood vessel density decreased.
*$P < 0.01$, compared with grade 1; **$P < 0.01$, compared with grades 1 and 2; ***$P < 0.05$, compared with grades 1, 2, and 3.

24.1 ± 3.0 in grade 1, 21.1 ± 3.2 in grade 2, 12.0 ± 3.8 in grade 3, and 9.4 ± 4.7 in grade 4C invasion, showing that the density decreased with increases in invasiveness (Table 2).

Changes in lymph vessel densities in induced tongue carcinoma
Lymph vessel density in an induced animal model of carcinoma was investigated. The lymph vessel density (mean ± SD) was 9.2 ± 1.4 in the normal group (Fig. 1) and 13.8 ± 4.8 in the case of advanced carcinoma (Fig. 2). In addition, the respective lymph vessel densities in each mode of invasion were investigated. The lymph vessel density was 15.3 ± 2.7 in the case of grade 1, 13.2 ± 5.0 in grade 2, 11.9 ± 4.3 in grade 3, and 17.6 ± 5.4 in grade 4C invasion, demonstrating

Table 3 Relationship between mode of invasion and lymph vessel densities

Mode of invasion	*Number of hamster*	*Lymph vessels ($\bar{X}$ ± SD)*
1	8	15.3 ± 2.7
2	8	13.2 ± 5.0
3	15	11.9 ± 4.3*
4C	5	17.6 ± 5.4**

Lymph vessel density was higher in grade 4C than in grades 2 and 3.
*$P < 0.01$, compared with grade 1; **$P < 0.01$, compared with grades 2 and 3.

that the density tended to be higher in grade 4C than in grades 1, 2, and 3 (Table 3). The blood vessel densities in grades 1 and 2 were significantly different from those in grades 3 and 4C, and the lymph vessel densities in grades 2 and 3 were significantly different from those in grade 4C (Fig. 3).

Expression of VEGF-C

In VEGF-C-positive cancer cells, VEGF-C was abundant in the cytoplasm (Fig. 4). A positive reaction was also detected in some vascular endothelial cells. However, VEGF-C expression was not identified in the normal epithelia. The expressions of VEGF-C and lymph vessel density were investigated. There was no statistical correlation between the expression of VEGF-C and lymph vessel density ($P > 0.05$).

The relationship between VEGF-C expression and the mode of invasion was investigated. Many cells slightly or moderately expressed VEGF-C in the less invasive cancers, i.e. grades 1 and 2, whereas numerous cells expressed high amounts of VEGF-C in the highly invasive cancers, i.e. grades 3 and 4C, showing that the expression of VEGF-C increased as invasiveness increased. Thus, it was determined that VEGF-C expression and the mode of invasion were correlated (Table 4).

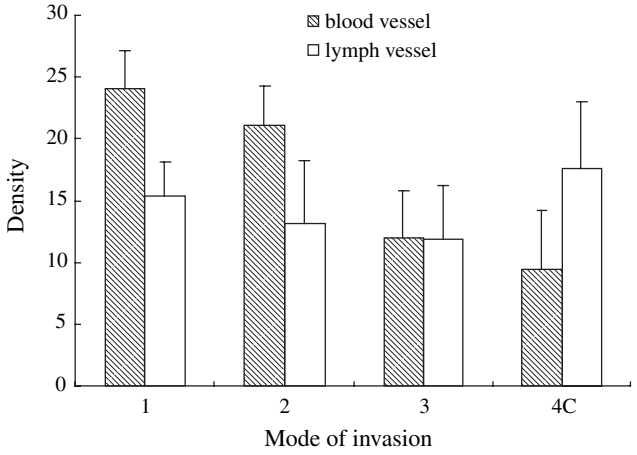

Figure 3 Relationship between the mode of invasion and blood and lymph vessel densities. Blood and lymph vessel densities: ▨, blood vessel density; □, lymph vessel density. Each value represents mean ± SD.

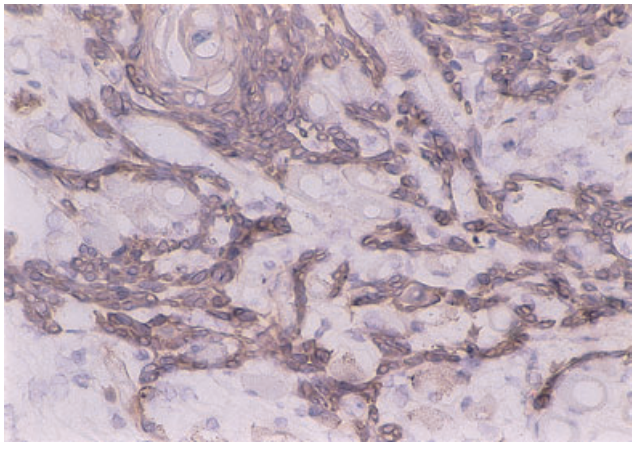

Figure 4 Immunohistochemical staining for vascular endothelial growth factor (VEGF)-C. Positive staining of VEGF-C in the cytoplasm cancer cells; original magnification, ×100.

Table 4 Relationship between mode of invasion and VEGF-C expression

Mode of invasion	*Number of hamster*	*Intensity of VEGF-C expression (%)*		
		Low	*Medium*	*High*
1	8	4 (50.0)	4 (50.0)	0
2	8	3 (37.5)	4 (50.0)	1 (12.5)
3	15	3 (20.0)	8 (53.3)	4 (27.7)
4C	5	0	1 (20.0)	4 (80.0)

The expression of vascular endothelial growth factor (VEGF)-C was higher as tumor invasion progressed, and correlated closely with the mode of invasion, $P < 0.01$, $r = 0.579$ (r, correlation coefficient).

Discussion

Past *in vivo* studies of the identification of lymph vessels have been performed using the dye needle infusion method, the hydrogen peroxide treatment method, and the India ink-supplemented silver nitrate local arterial infusion method (18), but the identification of lymph vessels has remained difficult in spite of the use of these methods. Recently, Kato (15, 16) directed their attention to the finding that 5′-Nase is very active in lymphangial endothelial cells, whereas vascular endothelial cells are strongly positive for alkaline phosphatase; these findings were then used to design a means of 5′-Nase-ALPase double staining. The usefulness of this straightforward method of lymphangial observation has previously been reported (15, 16). However, in the case of DMBA-induced tongue cancer, inflammatory cell infiltration in the interstitium is severe, and therefore a strong azo-stain results, because of the ALP reaction in inflammatory cells; this process renders it difficult to distinguish this reaction from the development of the ammonium sulfate of 5′-Nase. Thus, in the present study, blood and lymph vessels were counted separately in the same section, using the blood vessel perfusion method for the identification of blood vessels, and single 5′-Nase staining for the identification of lymph vessels.

There have been numerous reports regarding the relationship between blood vessel density and the degree of cancer invasion and/or malignancy in clinical cases. Blood vessel density has been correlated with invasion and metastasis in some of these reports (19), but not in others (20). In the present study, blood vessel density tended to decrease as invasiveness increased. In particular, blood vessel density markedly decreased in highly invasive cancers, i.e. those with grades 3 and 4C invasion, and this result was demonstrative of a significant difference. Tumor blood vessels are essential for nutrient supply in the process of tumor growth and

expansion, and angiogenesis is known to increase blood vessel density in low-invasive cancers. However, in high-invasive cancers, the tissues are extensively destroyed, and existing and new blood vessels may also be destroyed, thereby decreasing blood vessel density.

Studies of the number of lymph vessels associated with tumors have shown that tumors do not lead to an increase in the number of lymph vessels, and detected lymph vessels do not function (21, 22). However, more recently, the presence of VEGF-C as a lymphangial endothelial growth factor was reported in the literature, and the lymph vessel-specific markers desmoplakin (23) and lymphatic vessel endothelial hyaluronan receptor (LYVE-1) (24, 25) have been developed. The growth of lymph vessels has thus been considered to be tumor-associated lymphangiogenesis. Kishimoto et al. (26) reported a study of VEGF-C expression and cancer malignancy in cancers of the oral cavity that focused on the relationships between VEGF-C expression, clinicopathologic factors, and lymph node metastasis in clinical cases. A correlation between VEGF-C expression and lymph node metastasis was demonstrated, but no correlation was observed with other factors in that study. In our study, VEGF-C expression was found to increase as the mode of invasion advanced, and the lymph vessel density was decreased with advancement of the mode of invasion from grade 1 to 3. In advanced invasive carcinoma, production of matrix-degrading enzymes in cancer cells may have increased, promoting destruction of the surrounding tissue, and destruction of lymphatic vessels may have decreased the lymph vessel density. However, in the most invasive grade 4C, although tissue destruction was severe, production of VEGF-C was markedly increased and lymphangiogenesis of lymph vessels exceeded the destruction, resulting in an increase in the lymph vessels density.

The above findings suggest that blood and lymph vessels repeat destruction and neogenesis with the invasion of cancer in the oral cavity; these processes help to maintain the tumor. Cancer-induced angiogenesis and lymphangiogenesis may be essential for the invasion of such cancers, whereby inhibitors of angiogenesis and lymphangiogenesis may lead to novel therapies (27, 28).

References

1. Folkman J. Tumor angiogenesis. *Adv Cancer Res* 1985; **43**: 175–203.
2. Smith BD, Smith GL. Prognostic significance of vascular endothelial growth factor protein levels in oral and oropharyngeal squamous cell carcinoma. *J Clin Oncol* 2000; **18**: 2046–52.
3. Gasparini G. Prognostic value of vascular endothelial growth factor in breast cancer. *Oncologist* 2000; **5**: 37–44.
4. Kaipainen A, Korhonen J, Mustonen T. Expression of the FMS-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc Natl Acad Sci USA* 1995; **92**: 3566–70.
5. Yonemura Y, Endo Y, Fujita H, Fushida S. Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer. *Clin Cancer Res* 1999; **5**: 1823–9.
6. Tsurusaki T, Kanda S, Sasaki H, Kanetake H, Saito Y, Alitalo K. Vascular endothelial growth factor-C expression in human prostatic carcinoma and its relationship to lymph node metastasis. *Br J Cancer* 1999; **80**: 309–13.
7. Akagi K, Ikeda Y, Miyazaki M, Abe T, Kinoshita J, Maehara Y. Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues. *Br J Cancer* 2000; **83**: 887–91.
8. Niki T, Ibe S, Tokunou M, Yamada T, Matsuno Y, Hirohashi S. Expression of vascular endothelial growth factors A, B, C and D and their relationships to lymph node status in lung adenocarcinoma. *Clin Cancer Res* 2000; **6**: 2431–9.
9. Hashimoto I, Kodama J, Seki N, Hongo A, Yoshinouchi M, Okuda H. Vascular endothelial growth factor-C expression and its relationship to pelvic lymph node status in invasion cervical cancer. *Br J Cancer* 2001; **85**: 93–7.
10. Kitadai Y, Amioka T, Haruma K, Tanaka S, Yoshihara M, Sumii K. Clinicopathological significance of vascular endothelial growth factor (VEGF-C) in human esophageal squamous cell carcinomas. *Int J Cancer* 2001; **93**: 662–6.
11. Skobe M, Hawighorst T, Jackson DG. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med* 2001; **7**: 192–8.
12. Yamamoto E, Kohama G, Sunakawa H, Iwai M, Hiratsuka H. Mode of invasion, and lymph node metastasis in squamous cell carcinoma of oral cavity. *Cancer* 1983; **51**: 2175–80.
13. Yamamoto E, Kohama G, Hiratsuka H, Kumagai S. Role of preoperative chemotherapy for oral cancer from the viewpoint of mode of invasion. *Asian J Oral Maxillofac Surg* 1994; **6**: 113–21.
14. Fujita K, Kaku T, Sasaki M, Onoe T. Experimental production of lingual carcinoma in hamsters by local application of 9,10-dimethyl-1,2-benzanthracene. *J Dent Res* 1973; **52**: 327–32.
15. Kato S, Miyauchi R. Enzyme-histochemical visualization of lymphatic capillaries in the mouse tongue: light and electron microscopic study. *Okajimas Fol Anat Jpn* 1989; **65**: 391–404.
16. Kato S. Histochemical localization of 5′-nucleotidase in the lymphatic endothelium. *Acta Histochem Cytochem* 1990; **23**: 613–20.
17. Zimmermann H. 5′-Nucleotidase: molecular structure and functional aspects. *Biochem J* 1992; **285**: 345–65.
18. Mori K. Identification of lymphatic vessels after intra-arterial injection of dyes and other substances. *Microvasc Res* 1969; **1**: 268–74.
19. Williams JK, Calson GW, Cohen C. Tumor angiogenesis as a prognostic factor in oral cavity tumors. *Am J Surg* 1994; **168**: 373–80.
20. Gleich LL, Biddinger PW, Duperier FD. Tumor angiogenesis as a prognostic indicator in T2–T4 oral cavity squamous cell carcinoma: a clinical-pathologic correlation. *Head Neck* 1997; **19**: 276–80.
21. Dumont AE. Factor VIII-related antigen. *J Nat Cancer Inst* 1993; **85**: 674–6.
22. Folkman J. Angiogenesis and tumor growth. *N Engl J Med* 1996; **334**: 920–1.
23. Ebata N, Nodasaka Y. Desmoplakin as a specific marker of lymphatic vessels. *Microvasc Res* 2001; **61**: 40–8.
24. Banerji S, Ni J, Wang S, Clasper S, Su J. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor hyaluronan. *J Cell Biol* 1999; **144**: 789–801.

25. Maula SM, Luukkaa M, Grénman R, Jackson D. Intratumoral lymphatics are essential for the metastatic spread and prognosis in squamous cell carcinomas of the head and neck region. *Cancer Res* 2003; **63**: 1920–6.
26. Kishimoto K, Sasaki A, Yoshihama Y, Mese H. Expression of vascular endothelial growth factor-C predicts regional lymph node metastasis in early oral squamous cell carcinoma. *Oral Oncol* 2003; **39**: 391–6.
27. Noguchi N, Kawashiri S, Tanaka A, Kato K, Nakaya H. Effect of fibroblast growth inhibitor on proliferation and metastasis of oral squamous cell carcinoma. *Oral Oncol* 2003; **39**: 240–7.
28. Ueda N, Kamata N, Hayashi E, Yokoyama K, Hoteiya T, Nagayama M. Effects of an anti-angiogenic agent, TNP-470, on the growth of oral squamous cell carcinomas. *Oral Oncol* 1999; **35**: 554–60.

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