Journal of
Oral Pathology & Medicine

doi: 10.1111/jop.12167

J Oral Pathol Med
© 2014 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd
wileyonlinelibrary.com/journal/jop

Prognostic value of vascular endothelial growth factors A and C in oral squamous cell carcinoma

Mizuki Yanase, Koroku Kato, Kunio Yoshizawa, Natsuyo Noguchi, Hiroko Kitahara, Hiroyuki Nakamura

Division of Cancer Medicine, Department of Oral and Maxillofacial Surgery, Kanazawa University Graduate School of Medical Science, Ishikawa, Japan

BACKGROUND: Vascular endothelial growth factor (VEGF) family members play a major role in angiogenesis and vascularization. VEGF-A promotes tumor angiogenesis by stimulating the growth of tumor vascular endothelial cells. In addition, VEGF-C has been identified as a potent inducer of lymphangiogenesis in tumor and lymph node metastasis. Previous studies have investigated the association between clinicopathological factors and the expression of VEGF-A and VEGF-C in oral squamous cell carcinoma cancer (OSCC), but the results are contradictory. In this study, we investigated the relationship between VEGF-A and VEGF-C expression and OSCC clinicopathological factors and prognosis.

METHODS: Expression of VEGF-A and VEGF-C was evaluated in surgical specimens from 61 patients with OSCC and three human oral cancer cell lines (OSC-19, OSC-20 and HOC313) by immunohistochemical staining and enzyme-linked immunosorbent assay, respectively. We also determined the relationship between the 5-year survival rate and clinicopathological factors, such as TNM classification (Union for International Cancer Control, UICC), lymph node metastasis, recurrence, histological differentiation, location, and mode of invasion.

RESULTS: VEGF-A expression correlated significantly with lymph node metastasis. VEGF-C expression was associated with lymph node metastasis, recurrence, and a poorer 5-year survival rate. A multivariate analysis demonstrated that VEGF-C is an independent prognostic factor for patients with OSCC. VEGF-C expression was significantly up-regulated in HOC313 cells compared to OSC-19 and OSC-20 cells.

CONCLUSIONS: These results indicate that VEGF-C may be a predictive factor for OSCC outcome, lymph node metastasis, and recurrence. Moreover, VEGF-C may be an important factor in the development of new therapies for OSCC patients.

Correspondence: Koroku Kato, Department of Oral and Maxillofacial Surgery, Kanazawa University Graduate School of Medical Science, 13-1 Takara-machi, Kanazawa City 920-8640, Ishikawa, Japan. Tel: +81 76 265 2444, Fax: +81 76 234 4268, E-mail: k-koroku@oral.m.kanazawa-u.ac.jp
Accepted for publication January 7, 2014

J Oral Pathol Med (2014)

Keywords: immunohistochemistry; oral squamous cell carcinoma; prognosis; VEGF-A; VEGF-C

Introduction

Oral squamous cell carcinoma (OSCC) is a malignant tumor found most often in the head and neck region (1, 2). The presence or absence of lymph node metastasis and the mode of invasion influence the outcomes of OSCC patients. Current treatment regimens are guided by traditional clinicopathologic factors such as TNM stage, histological grade, and patient age. Despite efforts over the past two decades, treatment approaches such as surgery, radiotherapy, chemotherapy, or combinations of these have had little effect on the improvement in the survival rate (3); for example, a reported 5-year survival rate of OSCC patients was poor (approximately 56%) (2). To improve the prognoses of OSCC patients, the mechanisms underlying this cancer's pathogenesis and progression must be identified.

Tumor angiogenesis and lymphangiogenesis play an essential role in the growth, invasion, and metastatic spread of solid neoplasms (4). Various angiogenesis factors and the group of their related factors have significant effects on angiogenesis and lymphangiogenesis. Vascular endothelial growth factor (VEGF), platelet-derived endothelial cell growth factor (PD-ECGF), and basic fibroblast growth factor (bFGF) are the main factors involved in angiogenesis. The VEGF family members that have been identified include VEGF-A, -B, -C, -D, and -E, and the receptors VEGFR-1, -2, and -3. Among them, VEGF-A promotes tumor angiogenesis by strongly stimulating the growth of tumor vascular endothelial cells and chemotactic factor (5), and it is reported that expression of VEGF-A is related to tumor progression or poor prognosis in several human malignancies, including breast cancer (6), lung cancer (7), gastric cancer (8), pancreatic cancer (9), and prostate cancer (10). Overexpression of VEGF-A has been reported to be significantly associated with lymph node metastasis, histo-

logical differentiation, recurrence, and poor prognosis in head and neck cancer (11). In addition, VEGF-C has been identified as a potent inducer of the lymphangiogenesis of tumor and lymph node metastasis (2, 12, 13). There are previous studies of the association between clinicopathological factors and the expressions of VEGF-A and VEGF-C in oral SCC, but the outcome is controversial. In this study, we evaluated the expressions of VEGF-A and VEGF-C in oral SCC and investigated their relationship to clinicopathological factors and prognosis.

Materials and methods

Tissue samples

The subjects were 61 patients with primary OSCC who underwent surgical resection at the Department of Oral and Maxillofacial Surgery at Kanazawa University Hospital between 1989 and 2007. The tumors' TNM categories were classified according to the Union for International Cancer Control (UICC) system. The grade of tumor differentiation was determined according to the criteria proposed by the World Health Organization. The mode of tumor invasion was assessed according to the classification by Yamamoto et al. (14). Details of the patient and tumor characteristics are given in Table 1.

Cell lines

Three human oral squamous cell carcinoma cell lines with different invasive activities were used: OSC-20, OSC-19 (lower invasive type), and HOC313 (higher invasive type). OSC-20 is a cell line derived a 58-year-old female with metastatic tongue cancer to the cervical lymph nodes (15). OSC-19 was derived from a 61-year-old male with metastatic tongue cancer to the cervical lymph node (16). HOC313 was derived from a 51-year-old female with metastatic squamous cell carcinoma (involving the mandibular gingival and oral floor) to the cervical lymph nodes (17). In addition, the normal human dermal fibroblast line (NHDF) was used as control.

Immunohistochemical staining

Tissue specimens were fixed in 10% neutral buffered formalin and embedded in paraffin; then, 4-mm-thick sections were cut. Immunohistochemical detection of VEGF-A and VEGF-C was performed using an anti-VEGF-A rabbit polyclonal antibody at 1:200 dilution (LifeSpan BioScience, Seattle, WA, USA) and anti-VEGF-C rabbit polyclonal antibody at 1:100 dilution (Life Technologies, Carlsbad, CA, USA), respectively. Tissue sections were deparaffinized with xylene and rehydrated in graded alcohol. Endogenous peroxidase was blocked by treatment with 0.3% hydrogen peroxide in methanol for 30 min and incubated with the primary antibodies at 4°C overnight. Bound antibody was detected using the Envision system (Dako, Carpinteria, CA, USA). Diaminobenzidine (1 mg/ml) in the presence of 0.03% hydrogen peroxidase was used to visualize any bound peroxidase, and sections were counterstained with hematoxylin. The specificities of the staining were confirmed by using non-immune serum instead of the primary antibody as a negative control. The expressions of VEGF-A and VEGF-C were evaluated with immunohistochemically stained preparations using anti-VEGF-A antibody and anti-VEGF-C antibody. Immunohistochemical evaluation was carried out in accord with the report of Takanashi (18). The staining intensity of tumor cells in the leading invasion front regions was classified into four groups (0: none, 1: mild, 2: moderate, 3: strong), and cases were considered positive if their staining intensity was 2 or 3.

Enzyme-linked immunosorbent assay

For enzyme-linked immunosorbent assay analysis of VEGF-A and VEGF-C, supernatant of cultured several human oral cancer cell lines (OSC-19, OSC-20 and HOC313) and human normal dermal fibroblast cell lines (NHDF) were used. The supernatant was collected from cultured each cell line for 24 h. The concentrations of VEGF-A and VEGF-C were determined using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols.

Statistical analysis

JMP® 9 (SAS Institute Inc., Cary, NC, USA) was used for data analysis. The relationships between the expression of these proteins and clinicopathological parameters were examined by the chi-square test. We calculated the 5-year

Table 1 Clinicopathologic characteristics

Variable	*No. of patients*
Sex	
Male	32
Female	29
Age	
Median	64.3
T status	
T1	16
T2	37
T3	1
T4	7
Lymph node metastasis	
Present	16
Absent	45
Clinical stage	
1	16
2	29
3	11
4	5
Histologic differentiation	
Well	42
Moderate	12
Poorly	7
Lolaction	
Buccal mucosa	6
Gingiva	19
Tongue	30
Oral floor	5
Paratal mucosa	1
Mode of invasion (Y–K)	
1	4
2	13
3	19
4C	18
4D	7
Recurrence	
Yes	18
No	43

survival rates by the Kaplan–Meier method and compared them using the log-rank test. Factors found to be significant were then chosen for Cox's multivariate proportional hazard model in order to ascertain their prognostic values. The significance level was set at 5% for each analysis. The data of ELISA are presented as the mean values ±SEM. The differences between groups were tested for statistical significance using the two-tailed Mann–Whitney *U* test. *P*-values <0.05 were considered statistically significant.

Results

Expression of VEGF-A and VEGF-C in oral SCC

VEGF-A and VEGF-C were detected in the cytoplasm of tumor cells (Figs 1 and 2). According to the criteria for VEGF immunohistochemical evaluation, 46 cases (75.4%) were positive for VEGF-A, and 24 cases (39.3%) were positive for VEGF-C.

Association between VEGF-A or VEGF-C expression and clinicopathological features

Table 2 displays the correlations between VEGF-A and VEGF-C expression and the patients' clinicopathological factors. VEGF-A expression was significantly correlated with lymph node metastasis ($P < 0.05$) but was not correlated with tumor size, local recurrence, histological differentiation, or mode of invasion. VEGF-C expression was significantly correlated with lymph node metastasis and local recurrence ($P < 0.05$) but was not correlated with tumor size, histological differentiation, location, or mode of invasion.

Correlations between VEGF-A or VEGF-C expression and survival time

Figure 3 shows the Kaplan–Meier survival curves of the VEGF-A-positive and VEGF-C-positive groups compared to their negative group counterparts. The 5-year survival rate was 57.6% for the VEGF-A-positive group and 72.7% for the VEGF-A-negative group (Fig. 3A). The VEGF-A-positive group showed low survival rates, but there was no significant difference in survival compared with the negative group (Fig. 3A). The 5-year survival rate was 41.2% for the VEGF-C-positive group and 81.2% for the VEGF-C-negative group (Fig. 3B). The VEGF-C-positive group showed significantly poorer prognoses compared to the VEGF-C-negative group (Fig. 3B) ($P < 0.05$).

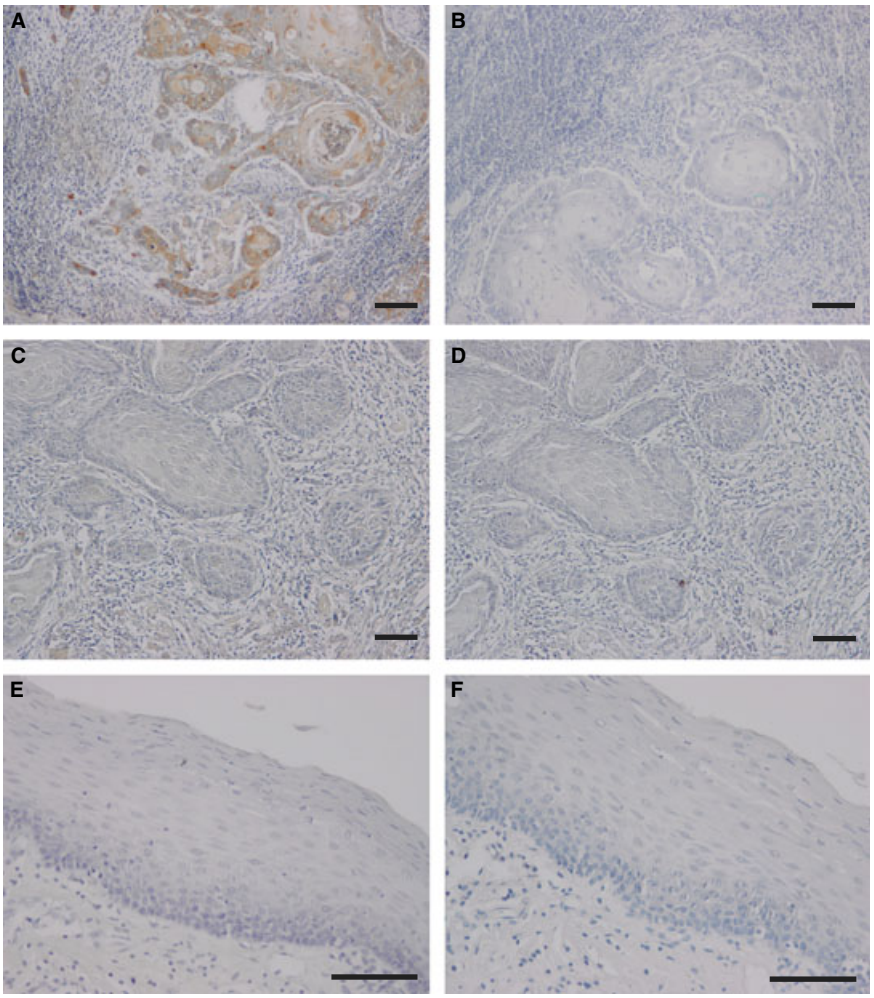

Figure 1 Immunohistochemical stain of Vascular endothelial growth factor-A (VEGF-A) in squamous cell carcinoma (A–D) and normal oral tissue (E, F). According to the expression intensity of VEGF-A, the samples are differentiated as positive (A) and negative (C) groups. The specificities of the staining were confirmed by using non-immune serum instead of the primary antibody as a negative control (B, D and F). Scale bar, 100 μm.

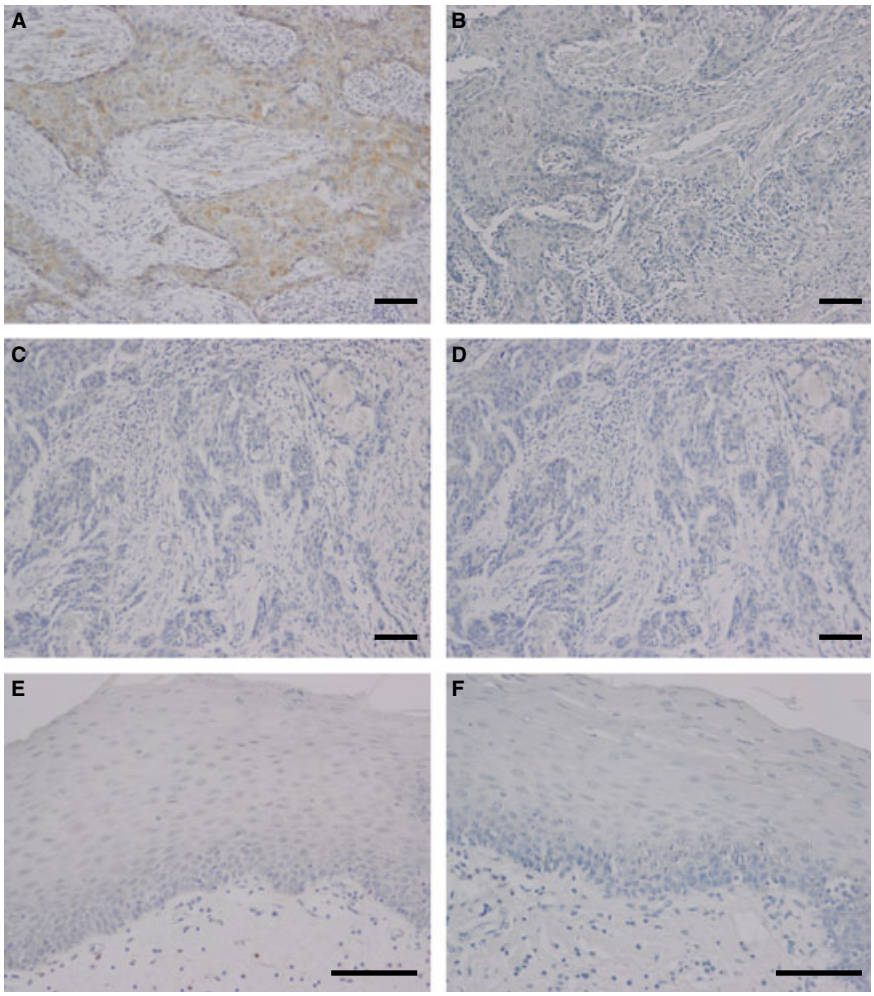

Figure 2 Immunohistochemical stain of Vascular endothelial growth factor-C (VEGF-C) in squamous cell carcinoma (A–D) and normal oral tissue (E, F). According to the expression intensity of VEGF-C, the samples are differentiated as positive (A) and negative (C) groups. The specificities of the staining were confirmed by using non-immune serum instead of the primary antibody as a negative control (B, D and F). Scale bar, 100 μm.

Cox proportional hazards regression analysis of the predictive factors for prognosis

We performed a Cox proportional hazards regression analysis to examine the significance of the predictive factors. A univariate analysis revealed that the expression of VEGF-C and the mode of invasion were significant prognostic indicators, but not VEGF-A. In addition, a multivariate analysis showed that VEGF-C expression and the mode of invasion were independent prognostic factors (Table 3).

Expression of VEGF-A or VEGF-C in human oral cancer cell lines

As demonstrated by the immunohistochemical analysis, the number of cancer cells expressed VEGF-A and VEGF-C. Therefore, we further examined several human oral SCC lines (OSC-19, OSC-20, and HOC313) with different invasive activities by measuring the concentration of the expressed VEGF-A and VEGF-C. The VEGF-A expression of OSC-19 (126.1 ± 20.92 pg/mg protein), OSC-20 (121.5 ± 4.66 pg/mg protein), and HOC313 (153.1 ± 19.7 pg/mg protein) was significantly up-regulated compare to NHDF (0.2 ± 0.68 pg/mg protein) (mean ± SEM, Fig. 4). However, there was no significant difference between OSC-19, OSC-20, and NHDF. On the other hand, the VEGF-C expression of HOC313 (337.8 ± 23.8 pg/mg protein) was significantly up-regulated compare to OSC-19 and OSC-20 (210.8 ± 64.2, 196.6 ± 56.7 pg/mg protein) (mean ± SEM, Fig. 4). There was no significant difference between OSC-19 and OSC-20. The VEGF-C expression levels of three oral cancer cells were significantly higher compared with NHDF (66.7 ± 8.84 pg/mg protein) (mean ± SEM, Fig. 4).

Discussion

VEGF-A is a key tumor-derived growth factor that promotes the switch to an angiogenic phenotype in many tumors and other tissues. Interestingly, our study shows that the expression of VEGF-A is correlated with lymph node metastasis. This is most likely because VEGF-A can bind to VEGFR-1 and VEGFR-2, but not to VEGFR-3, which is expressed in lymphatic endothelial cells. Several studies have shown that VEGF-A can induce lymphangiogenesis

Table 2 Correlations between the expressions of vascaular endothelial growth factor-A, -C (VEGF-A, VEGF-C), and the oral squamous cell carcinoma (OSCC) patients' clinicopathologic factors

	VEGF-A			*VEGF-C*		
Factors	*Positive (n = 46)*	*Negative (n = 15)*	*P-value*	*Positive (n = 24)*	*Negative (n = 37)*	P-*value*
T status						
T1	12	4		5	11	
T2	29	8		15	22	
T3	1	0	0.623	1	0	0.564
T4	4	3		3	4	
Lymph node metastasis						
Present	9	7	0.038	10	6	0.027
Absent	37	8		14	31	
Recurrence						
Yes	16	2	0.113	13	5	0.0006
No	30	13		11	32	
Histologic differentiation						
Well	33	9	0.222	16	26	0.997
Moderate	8	4		5	7	
Poorly	5	2		3	4	
Lolaction						
Buccal mucosa	6	0	0.221	2	4	0.627
Gingiva	14	5		8	11	
Tongue	23	7		13	17	
Oral floor	3	2		1	4	
Paratal mucosa	0	1		0	1	
Mode of invasion (Y-K)						
1	2	2		1	3	
2	12	1		6	7	
3	15	4	0.206	6	13	0.734
4C	11	7		7	11	
4D	6	1		4	3	

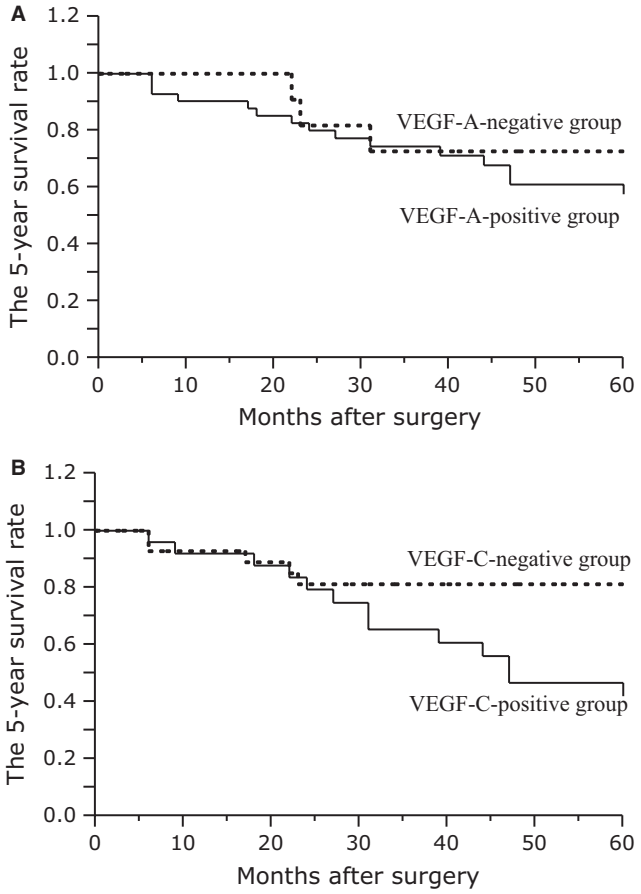

Figure 3 (A) The Kaplan–Meier survival rate of the VEGF-A-positive group shows low survival, but there was no significant difference in survival compared with the VEGF-A-negative group ($P = 0.6918$). (B) The VEGF-C-positive group showed significantly poorer prognoses compared to the VEGF-C-negative group ($P = 0.029$).

(19, 20). Other groups have demonstrated that VEGF-A expression is associated with lymph node metastasis (11, 21, 22). A recent study reported that the VEGFR-2 receptor is occasionally expressed on lymphatic endothelial cells (23). Unlike VEGFR-2, the affinity of VEGFR-1 for its ligand, VEGF-A, is approximately one order of magnitude higher than that of VEGFR-2. However, the kinase activity of VEGFR-1 is lower than VEGFR-2 (about one-tenth that of VEGFR-2), so VEGF-A is thought to act mainly through the activation of VEGFR-2 (3, 23–25). These data suggest that VEGF-A may have a direct stimulatory effect on lymphatic vessels in OSCC.

VEGF-C expression has been detected in a variety of tumors (26–29). VEGF-C, which binds to VEGFR-3, is known to be a major factor in lymphangiogenesis (30, 31). VEGF-C binds to VEGFR-3 on the surface of lymphatic endothelial cells, inducing the proliferation and growth of new lymphatic vessels and leading to tumor cell dissemination and lymph node metastasis (2, 11, 32). VEGF-C expression is associated with lymphatic vessel density (31, 33, 34) and lymph node metastases (30, 31, 35) in oral cancer. Pentheroudakis et al. (36) also reported an association between local recurrence and VEGF-C expression in head and neck cancer. In this study, we show that VEGF-C expression is associated with lymph node metastasis in OSCC. We also observed an association between VEGF-C expression and local recurrence. The data in this study are consistent with previous reports. However, further studies are needed to understand the precise role of VEGF-C in local recurrence.

There was no significant difference in the 5-year survival rate between patients from the VEGF-A-positive group and the VEGF-A-negative group. In contrast, the survival rate of the VEGF-C-positive group was significantly decreased compared to the VEGF-C-negative group. The results of our analysis using a Cox proportional hazards model show that the mode of invasion and VEGF-C expression are independent prognostic factors. Our ELISA data show that VEGF-C expression is significantly higher in a highly invasive cell line (HOC313) than in cell lines with lower invasiveness (OSC-19 and OSC-20). Highly invasive OSCC is closely related to the prognosis (14). These results suggest that VEGF-C may affect OSCC prognosis. The clinicopathological factors currently used to predict metastasis and prognosis include TNM classification, degree of differentiation, and mode of invasion. We propose including a molecular biological factor for comprehensive diagnoses in the future. More complete diagnostic methods not only improve the accuracy of diagnosing metastases, but could also help determine the prognosis and facilitate decision-

Table 3 Results of the univariate and multivariate analyses with Cox's proportional hazards mode

	Univariate			*Multivariate*		
Factors	*Hazard ratio*	*95% Confidence interval*	P-*value*	*Hazard ratio*	*95% Confidence interval*	P-*value*
VEGF-A (positive/negative)	1.541	0.508–6.657	0.4739			
VEGF-C (positive/negative)	3.122	1.176–9.734	0.0214	2.771	1.041–8.661	0.041
Lymph node metastasis (present/absent)	1.616	0.560–4.181	0.3531			
Histologic differentiation (moderate-poorly/well)	2.114	0.729–5.516	0.158			
Mode of invasion (Y-K) (4C, 4D/1, 2, 3)	4.435	1.716–12.77	0.002	4.066	1.569–11.73	0.0037

VEGF-A, vascular endothelial growth factor-A; VEGF-C, vascular endothelial growth factor-C.

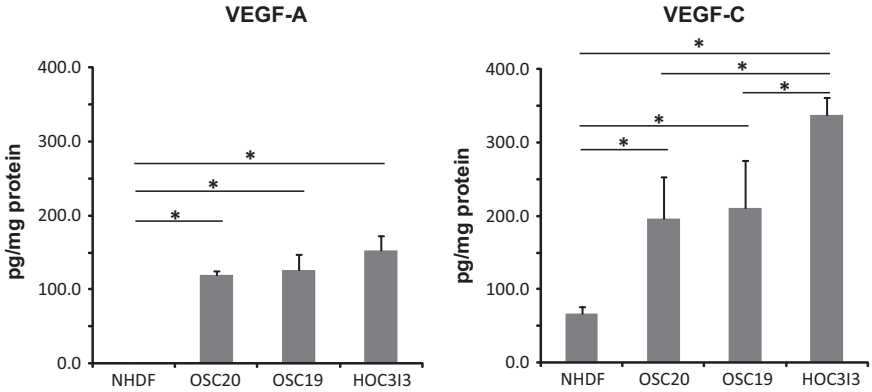

Figure 4 Expression of (vascular endothelial growth factor-A) VEGF-A or vascular endothelial growth factor-C (VEGF-C) in supernatant of cultured human oral cancer cell lines (OSC-19, OSC-20 and HOC313) and human normal dermal fibroblast cell lines (NHDF). The average concentration (pg/mg protein) of VEGF-A (left panel) and VEGF-C (right panel) is indicated. Error bars, ±SEM, $*P < 0.05$.

making regarding the necessity of prophylactic neck dissection. Our results suggest that VEGF-C may be a predictive factor for OSCC outcome.

Recently, new therapies have been developed to target VEGF-A. However, resistance to anti-angiogenic therapies limits the clinical benefit of these agents in cancer patients. The single-agent response rate to anti-angiogenic drugs such as bevacizumab (a monoclonal antibody to VEGF-A) is <10%, and even in patients who do respond, the duration of the response is typically <3 months (37–39). Similar response rates are observed in head and neck squamous cell carcinomas (40), for which bevacizumab is being evaluated in phase III clinical trials. Our results suggest that therapies targeting VEGF-C may be expected to play a major role in the treatment of bevacizumab-resistant OSCC in the future.

References

1. Oliveira-Neto HH, Gleber-Netto FO, De Sousa SF, et al. A comparative study of microvessel density in squamous cell carcinoma of the oral cavity and lip. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2012; **113**: 391–8.
2. Liang X, Zhou H, Liu X, et al. Effect of local hyperthermia on lymphangiogenic factors VEGF-C and -D in a nude mouse xenograft model of tongue squamous cell carcinoma. *Oral Oncol* 2010; **46**: 111–5.
3. Johnstone S, Logan RM. The role of vascular endothelial growth factor (VEGF) in oral dysplasia and oral squamous cell carcinoma. *Oral Oncol* 2006; **42**: 337–42.
4. Villarejo-Campos P, Padilla-Valverde D, Martin RM, et al. Serum VEGF and VEGF-C values before surgery and after postoperative treatment in gastric cancer. *Clin Transl Oncol* 2013; **15**: 265–70.
5. Kishimoto K, Yoshihama Y, Mandai T. Expression of VEGF, PD-ECGF, and VEGF-C and its clinicopathologic significance in oral squamous cell carcinoma. *Nihon Koku Geka Gakkai Zasshi* 2005; **51**: 218–25.
6. Berns EM, Klijn JG, Look MP, et al. Combined vascular endothelial growth factor and TP53 status predicts poor response to tamoxifen therapy in estrogen receptor-positive advanced breast cancer. *Clin Cancer Res* 2003; **9**: 1253–8.
7. Fontanini G, Lucchi M, Vignati S, et al. Angiogenesis as a prognostic indicator of survival in non-small-cell lung carcinoma: a prospective study. *J Natl Cancer Inst* 1997; **89**: 881–6.
8. Takahashi Y, Cleary KR, Mai M, et al. Significance of vessel count and vascular endothelial growth factor and its receptor (KDR) in intestinal-type gastric cancer. *Clin Cancer Res* 1996; **2**: 1679–84.
9. Ikeda N, Adachi M, Taki T, et al. Prognostic significance of angiogenesis in human pancreatic cancer. *Br J Cancer* 1999; **79**: 1553–63.
10. George DJ, Halabi S, Shepard TF, et al. Prognostic significance of plasma vascular endothelial growth factor levels in patients with hormone-refractory prostate cancer treated on Cancer and Leukemia Group B 9480. *Clin Cancer Res* 2001; **7**: 1932–6.
11. Chen SC, Shih CM, Tseng GC, et al. Vascular endothelial growth factor C as a predictor of early recurrence and poor prognosis of resected stage I non-small cell lung cancer. *Ann Acad Med Singapore* 2011; **40**: 319–24.

12. Kondo K, Kaneko T, Baba M, et al. VEGF-C and VEGF-A synergistically enhance lymph node metastasis of gastric cancer. *Biol Pharm Bull* 2007; **30**: 633–7.
13. Noda E, Maeda K, Inoue T, et al. Predictive value of vascular endothelial growth factor-C expression for local recurrence of rectal carcinoma. *Oncol Rep* 2007; **17**: 1327–31.
14. Yamamoto E, Kohama G, Sunakawa H, et al. Mode of invasion, bleomycin sensitivity, and clinical course in squamous cell carcinoma of the oral cavity. *Cancer* 1983; **51**: 2175–80.
15. Yokoi T, Hirata S, Nishimura F, et al. Some properties of a newly established human cell line derived from an oral squamous carcinoma. *Tumor Res* 1990; **25**: 93–103.
16. Yokoi T, Homma H, Odajima T. Establishment and characterization of OSC-19 cell line in serum and protein free culture. *Tumor Res* 1988; **24**: 1–17.
17. Ishisaki A, Oida S, Momose F, et al. Identification and characterization of autocrine-motility-factor-like activity in oral squamous-cell-carcinoma cells. *Int J Cancer* 1994; **59**: 783–8.
18. Takanashi S, Takimoto M, Ota H. Correlation with cyclooxygenase-2, vasculogenetic factor and lymph node metastasis in esophageal squamous cell carcinoma. *Showa Igakkai Zasshi* 2006; **66**: 331–9.
19. Nagy JA, Vasile E, Feng D, et al. Vascular permeability factor/vascular endothelial growth factor induces lymphangiogenesis as well as angiogenesis. *J Exp Med* 2002; **196**: 1497–506.
20. Hirakawa S, Kodama S, Kunstfeld R, et al. VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. *J Exp Med* 2005; **201**: 1089–99.
21. Sasahira T, Kirita T, Kurihara M, et al. MIA-dependent angiogenesis and lymphangiogenesis are closely associated with progression, nodal metastasis and poor prognosis in tongue squamous cell carcinoma. *Eur J Cancer* 2010; **46**: 2285–94.
22. Mineta H, Miura K, Ogino T, et al. Prognostic value of vascular endothelial growth factor (VEGF) in head and neck squamous cell carcinomas. *Br J Cancer* 2000; **83**: 775–81.
23. Björndahl MA, Cao R, Burton JB, et al. Vascular endothelial growth factor-a promotes peritumoral lymphangiogenesis and lymphatic metastasis. *Cancer Res* 2005; **65**: 9261–8.
24. Takahashi S. Vascular endothelial growth factor (VEGF), VEGF receptors and their inhibitors for antiangiogenic tumor therapy. *Biol Pharm Bull* 2011; **34**: 1785–8.
25. Shibuya M. Vascular endothelial growth factor and its receptor system: physiological functions in angiogenesis and pathological roles in various diseases. *J Biochem* 2013; **153**: 13–9.
26. Niki T, Iba S, Tokunou M, et al. 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.
27. Gunningham SP, Currie MJ, Han C, et al. The short form of the alternatively spliced flt-4 but not its ligand vascular endothelial growth factor C is related to lymph node metastasis in human breast cancers. *Clin Cancer Res* 2000; **6**: 4278–86.
28. Akagi K, Ikeda Y, Miyazaki M, et al. Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues. *Br J Cancer* 2000; **83**: 887–91.
29. Hashimoto I, Kodama J, Seki N, et al. Vascular endothelial growth factor-C expression and its relationship to pelvic lymph node status in invasive cervical cancer. *Br J Cancer* 2001; **85**: 93–7.
30. Kishimoto K, Sasaki A, Yoshihama Y, et al. Expression of vascular endothelial growth factor-C predicts regional lymph node metastasis in early oral squamous cell carcinoma. *Oral Oncol* 2003; **39**: 391–6.
31. Sugiura T, Inoue Y, Matsuki R, et al. VEGF-C and VEGF-D expression is correlated with lymphatic vessel density and lymph node metastasis in oral squamous cell carcinoma: Implications for use as a prognostic marker. *Int J Oncol* 2009; **34**: 673–80.
32. Ishii H, Chikamatsu K, Sakakura K, et al. Primary tumor induces sentinel lymph node lymphangiogenesis in oral squamous cell carcinoma. *Oral Oncol* 2010; **46**: 373–8.
33. Liang X, Yang D, Hu J, et al. Hypoxia inducible factor-alpha expression correlates with vascular endothelial growth factor-C expression and lymphangiogenesis/angiogenesis in oral squamous cell carcinoma. *Anticancer Res* 2008; **28**: 1659–66.
34. Watanabe S, Kato M, Kotani I, et al. Lymphatic vessel density and vascular endothelial growth factor expression in squamous cell carcinomas of lip and oral cavity: a clinicopathological analysis with immunohistochemistry using antibodies to D2-40, VEGF-C and VEGF-D. *Yonago Acta Med* 2013; **56**: 29–37.
35. Nakazato T, Shingaki S, Kitamura N, et al. Expression level of vascular endothelial growth factor-C and -A in cultured human oral squamous cell carcinoma correlates respectively with lymphatic metastasis and angiogenesis when transplanted into nude mouse oral cavity. *Oncol Rep* 2006; **15**: 825–30.
36. Pentheroudakis G, Angouridakis N, Wirtz R, et al. Transcriptional activity of human epidermal growth factor receptor family and angiogenesis effectors in locoregionally recurrent head and neck squamous cell carcinoma and correlation with patient outcome. *J Oncol* 2009; **2009**: 854127.
37. Yang JC, Haworth L, Sherry RM, et al. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. *N Engl J Med* 2003; **349**: 427–34.
38. Giantonio BJ, Catalano PJ, Meropol NJ, et al. Bevacizumab in combination with oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: results from the Eastern Cooperative Oncology Group Study E3200. *J Clin Oncol* 2007; **25**: 1539–44.
39. Sandler A, Gray R, Perry MC, et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N Engl J Med* 2006; **355**: 2542–50.
40. Cohen EE, Davis DW, Karrison TG, et al. Erlotinib and bevacizumab in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck: a phase I/II study. *Lancet Oncol* 2009; **10**: 247–57.