VEGF-C is associated with lymphatic status and invasion in oral cancer

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ABSTRACT

Background: Nodal metastasis is a major prognostic indicator for oral squamous cell carcinoma (OSCC) progression. Recently, it has been revealed that lymphangiogenic growth factor VEGF-C and its receptor Flt-4 play an important role for invasion and metastasis in cancer cells.

Aim: To examine VEGF-C expression and its correlation with lymphatic status, including the number of lymph vessels and lymphatic invasion, tumour invasion and metastasis in OSCC.

Methods: Intratumoural and peritumoural lymphatic vessels were examined using D2-40 in 54 OSCC cases and correlated with VEGF-C expression and clinicopathological findings. The histological pattern of invasion and pathological findings were compared.

Results: High expression of VEGF-C was frequently observed in OSCC and was associated with increased number of lymph vessels and lymphatic invasion. VEGF-C was well correlated with invasion pattern and metastasis.

Conclusions: Results suggest that VEGF-C may play an important role for lymphangiogenesis and invasion in the metastatic process and can be a strong predicting factor for metastasis of OSCC.

Millions of people die every year from the metastatic spread of cancer that occurs via the blood vessels, lymph vessels or directly into tissues and body cavities.[1] It was previously thought that lymphatic metastasis involved passage of malignant cells along pre-existing lymph vessels near a tumour. However, recent studies using animal models suggest that lymphangiogenesis can be induced by solid tumours and may promote tumour spread.[2] Moreover, it has been revealed that lymphangiogenesis can occur adjacent to or within cancers and correlates with lymph node metastasis.[3 4] It was difficult in the past to analyse lymphatics, due to the lack of lymphatic-specific markers that could be used to discriminate between lymphatics and blood vessels. The recently developed monoclonal antibody D2-40 detects a fixation-resistant epitope on podoplanin.[5 6] It has been shown to be a selective marker for lymphatic endothelium, allowing the specific identification of lymphatic vessels in formalin fixed, paraffin-embedded tissue and the study of lymph vessel density in solid tumours.[6–9]

VEGF-C is involved in lymphangiogenesis via effects of activated Flt-4 on lymphatic endothelial cells.[10 11] Examination of VEGF-C function in a number of assays has also shown an angiogenic activity, presumably via activation of VEGFR-2.[12] Su et al suggest that the Flt-4 ligand, VEGF-C, may affect cancer development or progression by direct effect on tumour cells.[13] In order to determine the roles of VEGF-C for lymphangiogenesis in OSCC, we examined VEGF-C expression and its correlation with lymphatic status.

MATERIALS AND METHODS

All OSCC samples were used after approval by the ethical committees of our institutions and informed consent by the patients.

Patients and tumour samples

Archived paraffin-embedded tissue specimens from 54 previously untreated patients with OSCC were obtained from the Department of Oral Pathology, Faculty of Dental Sciences, University of Peradeniya, Sri Lanka. A further 18 OSCC specimens were obtained from the same hospital and were immediately frozen and stored at −80°C. All had surgery as their first line of management. Twenty-four patients had histologically confirmed metastatic spread to the neck nodes.

Reverse-transcriptase PCR

Total RNA was isolated from tumour tissues using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Preparations were quantified and their purity was determined by standard spectrophotometric methods. cDNA was synthesised from 1 μg total RNA according to the ReverTra Dash (Toyobo Biochemicals, Tokyo, Japan). Two pairs of primer sequences were: VEGF-C, 5′-GGA AAG AAG TTC CAC CAC CA-3′ (forward) and 5′-TTT GTT AGC ATG GAC CCA CA-3′ (reverse); human GAPDH, 5′-TCC ACC ACC CTG TTG CTG TA-3′ (forward) and 5′-ACC ACA GTC CAT GCC ATC AC-3′ (reverse). Aliquots of total cDNA were amplified with 1.25 U of rTaq-DNA polymerase (Qiagen); amplifications were performed in a PC701 thermal cycler (Astec, Fukuoka, Japan) for 30 cycles after an initial 20 second denaturation at 94°C, annealed for 30 s at 60°C, and extended for 1 min at 72°C in all primers.

Immunohistochemical analyses

Formalin fixed, paraffin embedded tumour tissues were cut into 4 μm thick sections and stained with H&E for histological examination. Invasive margin of the tumour was graded as types I, II, III and IV according to the Bryne's invasive front grading.[14] For immunohistochemical examination, the EnVision system was used. The tissue sections were deparaffinised and rehydrated in a graded series of alcohols. Endogenous peroxidase activity
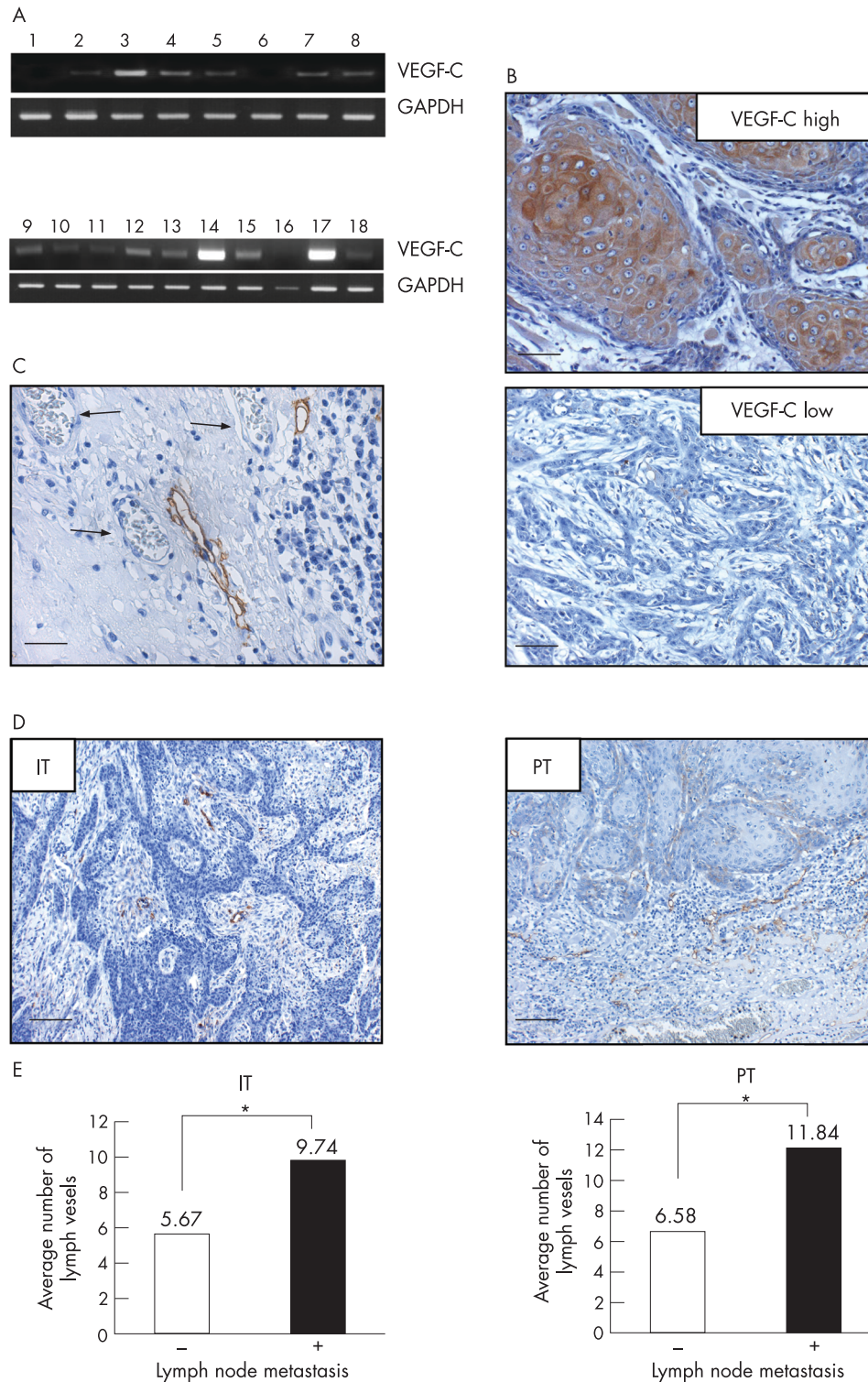
Figure 1 High expression of VEGF-C and lymphatic status in oral squamous cell carcinoma (OSCC). (A) Expression of VEGF-C mRNA by RT-PCR. GAPDH expression was used as a control. (B) Representative cases of high and low VEGF-C expression in OSCC. Bar = 50 μm. VEGF-C expression is observed in cytoplasm of OSCC cells. (C) Immunoreactivity for D2-40. Vascular endothelial cells show no immunoreactivity (arrows). Bar = 25 μm. (D) Left: intratumoural (IT) lymph vessels show immunoreactivity for D2-40 (bar = 100 μm). Right: lymph vessels located at the invasive edge of tumours (PT, peritumoural area) are observed (bar = 100 μm). (E) A comparison between number of lymph vessels in IT and PT area and nodal metastasis in 54 OSCC. Average number of IT lymph vessels in OSCC cases without nodal metastasis is 5.7 (SD 4.8) and that in OSCC cases with nodal metastasis is 9.7 (SD 7.6). Average number of lymph vessels in PT area showed highly significant results with lymph node metastasis. Average number of lymph vessels in OSCC cases without nodal metastasis is 6.6 (SD 3.5) and that in OSCC cases with nodal metastasis is 11.8 (SD 7.0). *p<0.05.

was blocked with 0.3% H_2O_2 for 30 min. The sections were microwaved three times for 5 min each in citrate phosphate buffer (pH 6.0) for antigen retrieval. The sections were then incubated with protein block serum free medium for 10 min to

block non-specific binding. Monoclonal antibody of D2-40 (Signet, Dedham, Massachusetts, USA; dilution 1:40) and VEGF-C (polyclonal antibody, Santa Cruz Biotechnology Inc., Santa Cruz, California, USA; dilution 1:25) were applied as primary antibodies and incubated at 4°C overnight. After washing with phosphate buffered saline, secondary antibodies were applied to the sections, which were then incubated for one hour at room temperature. Primary antibody was visualised with diaminobenzidine. Sections were counterstained with Mayer's haematoxylin, dehydrated and mounted. VEGF-C expression was graded as high (>10% of tumour cells showed strong or diffuse immunopositivity) or low (<10% of tumour cells showed weak or focal immunopositivity or no staining).

Evaluation of intratumoural and peritumoural lymphangiogenesis

Two distinct sets of measurements were performed in each tumour section; five fields with the highest lymphatic vascular density were identified in all 54 cases, (i) within the tumour and (ii) within an area of 1 mm from the tumour border (along the invasive front). In each, selected fields were microphotographed and the positively stained lymph vessels traced using Adobe Photoshop software. Average values of vessel counts in the total of 10 high power fields ($\times$100) were obtained from each tumour. Whole tumour area was scanned to determine tumour invasion into lymph vessels. The results were then compared with other parameters. The three pathologists (SS, YK and IO) made all the assessments.

In vitro invasion assay

MSCC-1 and MSCC-Inv1 cells were previously established in our laboratory. Using these cells, in vitro invasion assay was performed as described previously.[15]

Statistical analysis

Possible correlation between variables of the analysed tumour samples and association between lymphatic invasion and lymph node metastasis, invasion pattern, and VEGF-C expression were tested by the χ^2 test. For the correlation between number of lymph vessels and lymph node metastasis, lymphatic invasion and pattern of invasion, statistical significance was measured by the Welch test. For VEGF-C expression, statistical significance with IT and PT was also measured by the Welch test. A p value <0.05 was required for significance.

RESULTS

VEGF-C expression and lymphatic status in OSCC

It has been reported that promotion of tumour metastasis by VEGF-C is due to the induction of lymphangiogenesis via effects of activated Flt-4 on lymphatic endothelial cells.[10] [11] To determine the role of VEGF-C for lymphangiogenesis in OSCC, we examined VEGF-C expression and lymph vessels status. VEGF-C mRNA was expressed in 15 of 18 (83%) OSCC tissues by reverse transcriptase (RT)-PCR (fig 1A). By immunohistochemical analysis, high expression of VEGF-C was observed in 37 of 54 (68.5%) OSCC cases (fig 1B). Next, we examined the lymphatic status by immunohistochemistry using the D2-40 antibody in OSCC. The D2-40 antibody specifically recognised lymphatic endothelial cells; in contrast, blood vessels showed no immunostaining (fig 1C). The D2-40 lymph vessels were unevenly distributed throughout the tumours. We counted the number of lymph vessels in the IT area (within the tumour area) and PT area (within an area of 1 mm from the invasive front). The number of lymph vessels located in the PT area was higher than that in IT area (fig 1D). Moreover, OSCC cases with nodal metastasis showed a significantly higher number of lymph vessels in both the IT (p = 0.028) and PT (p = 0.002) areas than those without nodal metastasis (fig 1E).

It is widely accepted that the invasion of tumour cells into lymph vessels is one of the critical steps for the establishment of metastasis. As expected, D2-40 immunostaining also highlighted the presence of lymphatic invasion (fig 2A). Interestingly, lymphatic invasion was mostly present in the

Figure 2 Lymphatic invasion in oral squamous cell carcinoma. (A) Representative case of lymphatic invasion by tumour cells. Lymph vessels are stained by D2-40. Arrow shows tumour cells, which invaded into the lumen of a lymph vessel. Bar = 25 μm. (B) Invasion of tumour cells into lymph vessels was significantly correlated with intratumoural (IT) lymph vessels (p = 0.008) compared to peritumoural (PT) lymph vessels (p = 0.171). (C) Correlation between lymphatic invasion and nodal metastasis. Tumours with higher lymphatic invasion showed higher metastatic rate.
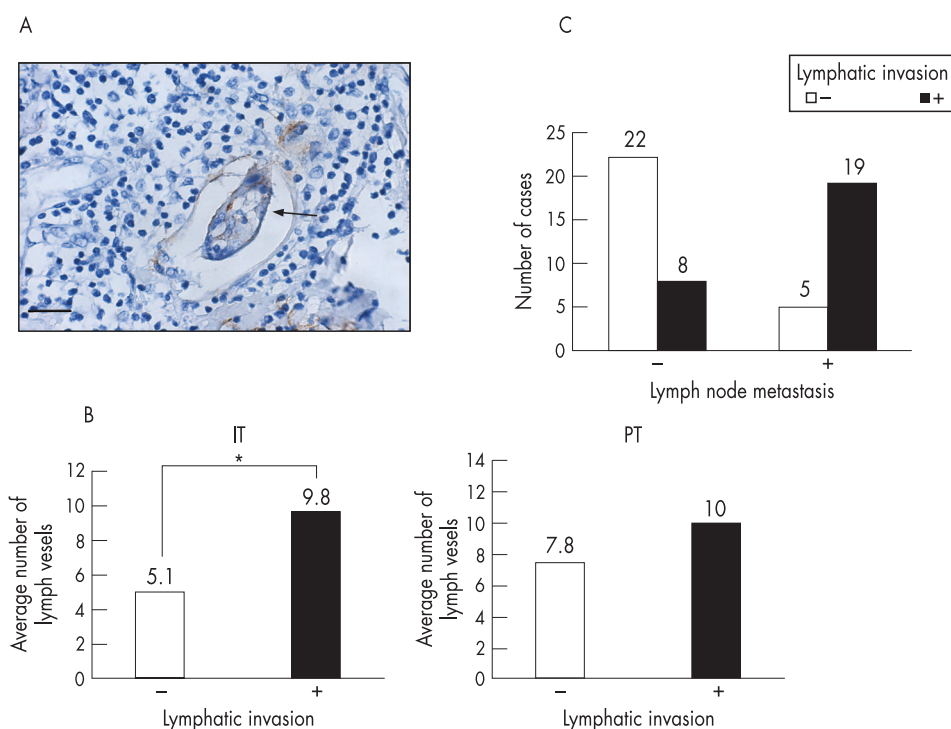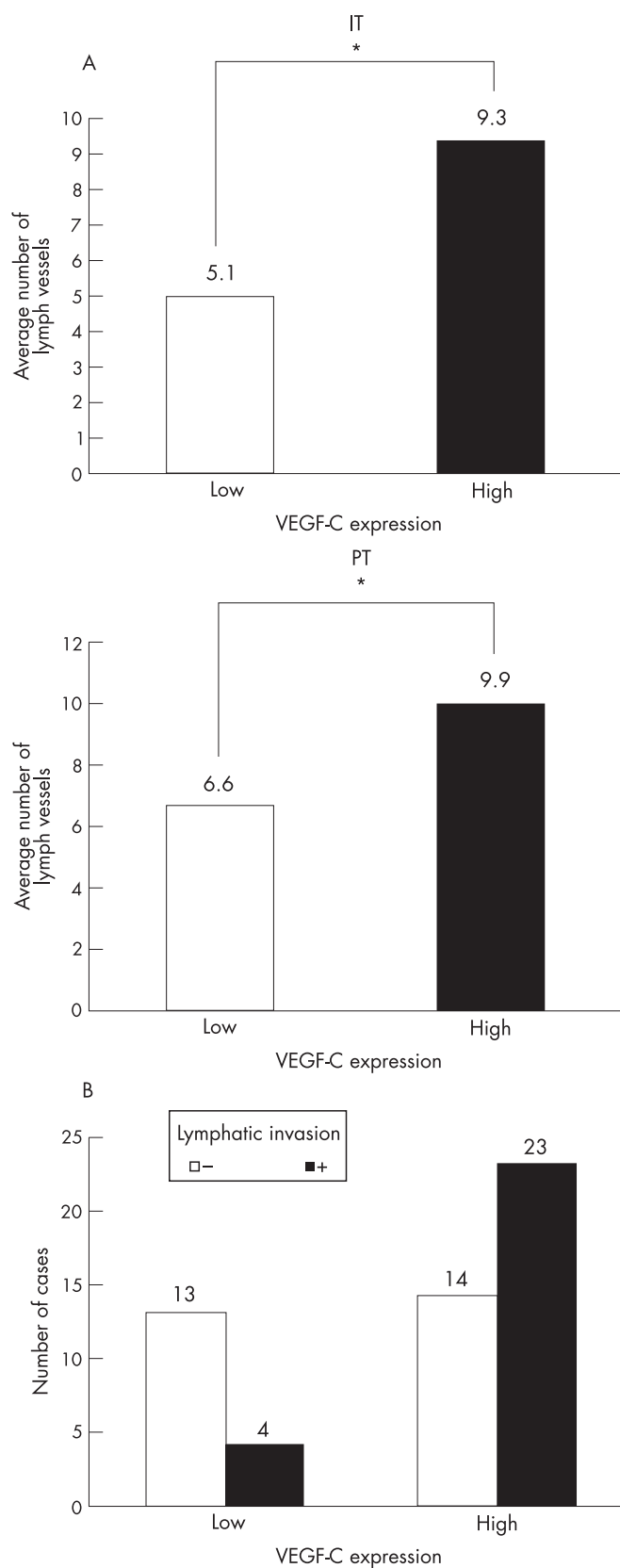
Figure 3 A possible correlation between VEGF-C expression and lymphatic status. (A) Correlation between VEGF-C expression and number of intratumoural (IT) and peritumoural (PT) lymph vessels. *p<0.05. (B) Correlation between VEGF-C expression and lymphatic invasion.

PT area. Of 54 OSCC cases, 27 (50%) showed lymphatic invasion. Lymphatic invasion was significantly correlated with IT lymphatics (p = 0.008) compared to PT lymphatics (p = 0.171) (fig 2B). Of 27 cases with lymphatic invasion, 19 (70.4%) had nodal metastasis. On the other hand, only 5/27 (18.5%) cases without lymphatic invasion had nodal metastasis. Thus, correlation between lymphatic invasion and nodal metastasis was highly significant (p<0.001) (fig 2C).

Correlation between VEGF-C expression and lymphatic status

We compared the expression of VEGF-C with lymphatic status, including the number of lymph vessels and lymphatic invasion by tumour cells. Interestingly, the number of lymph vessels in both IT and PT areas was significantly associated with VEGF-C expression (IT, p = 0.033; PT, p = 0.021) (fig 3A and table 1). Furthermore, 62% of cases with high expression of VEGF-C showed lymphatic invasion, whereas only 23.5% of cases with low expression of VEGF-C showed lymphatic invasion, indicating that the tumours with lymphatic invasion showed significantly higher expression of VEGF-C (p = 0.008) (fig 3B and table 1).

Relationship of VEGF-C expression with invasion and metastasis

VEGF-C and ligand Flt-4 play an important role in invasion and metastasis in malignant tumour cells.[10] [11] We previously isolated a highly invasive clone (MSCC-Inv1) from parent OSCC cells (MSCC-1) by using an in vitro invasion assay method.[15] [16] Interestingly, MSCC-Inv1 with higher expression of VEGF-C mRNA showed higher invasive activity, in comparison with MSCC-1 with lower expression of VEGF-C mRNA (fig 4A). Moreover, VEGF-C expression was correlated with pattern of invasion (p<0.001) in OSCC, where 95% of pattern IV cases expressed VEGF-C in contrast with pattern I cases which showed no VEGF-C expression (fig 4B and table 1). These results indicate that VEGF-C may play an important role in invasion of OSCC. Furthermore, VEGF-C expression was correlated with metastasis (p = 0.007) (fig 4C and table 1).

DISCUSSION

OSCC is one of the most common cancers; the overall 5-year survival rate is low, largely because of the propensity of some tumours to disseminate via the lymphatics. Indeed, the finding of lymph node involvement is one of the strongest indicators of poor prognosis. Hence, there is an urgent need to identify characteristics of the primary tumours that might predict nodal metastasis. Here, we examined lymphatic status, including the number of lymph vessels and lymphatic invasion and correlated them with metastasis in OSCC. We found that IT and PT lymphatics were associated with increased tendency for nodal metastasis. In support of this, the retrospective analyses of head and neck and breast cancer samples have shown a positive correlation between tumoural lymphatics and lymphatic metastasis.[3] [17] The main significance of tumour invasion to lymph vessels is that they could provide a possible route for the spread of tumour cells to regional lymph nodes. Interestingly, we found a significant correlation between lymphatic invasion and metastasis. Lymphatic invasion was mostly found in the PT area, not in IT lymphatics. This finding is consistent with a previous report that metastases were discovered despite no detectable IT lymphatics.[18] Padera *et al* proposed that IT lymphatics might not be completely functional because of collapse of IT vessels under high intratumoural pressure.[18] We found that both IT and PT lymphatics were associated with

Table 1 Relationship between VEGF-C expression and pattern of invasion and lymph node metastasis

	Total	VEGF-C expression*		p Value
		Low	High	
Oral squamous cell carcinoma	54	17	37	
Lymphatic invasion				
−	27	13	14	0.008†
+	27	4	23	
Average number of lymph vessels				
IT		5.1	9.3	0.033‡
PT		6.6	9.9	0.021‡
Invasion pattern				
I	6	6	0	
II	5	4	1	<0.001†
III	24	6	18	
IV	19	1	18	
Lymph node metastasis				
−	30	14	16	0.007†
+	24	3	21	

*VEGF-C expression was graded as high (>10% of tumour cells showed strong or diffuse immunopositivity) and low (<10% of tumour cells showed weak or focal immunopositivity or no staining).
†χ^2 test, ‡Welch test.

nodal metastasis. It has been reported that PT lymphatics are associated with lymphatic metastasis in head and neck, prostate and cervical carcinoma.[3 19 20] However, other studies showed that IT lymphatics are vital for lymphatic metastasis.[3 21] As lymphatic invasion was mostly found in PT lymphatics, we speculate that local lymph vessels at the tumour margin (PT area) are more important for spreading tumour cells. However, to clarify whether IT and/or PT vessels contribute to lymphatic metastasis, further studies will be required.

VEGF-C was found to have a specific function in lymphangiogenesis. Several groups have reported a possible correlation between VEGF-C expression and metastasis in OSCC. However, there has been no report on the correlation between VEGF-C expression and lymphatic status. Here, we showed that high expression of VEGF-C mRNA and protein was frequently observed in OSCC, and VEGF-C expression was correlated with increased number of lymph vessels and lymphatic invasion. In addition to lymphangiogenesis, it has recently been shown that the VEGF-C/Flt-4 axis enhances cancer cell mobility and invasiveness and contributes to the promotion of metastasis.[13] Here we observed that VEGF-C expression was significantly correlated with invasion pattern and nodal metastasis. Interestingly, a highly invasive clone isolated by in vitro invasion assay showed a higher expression of VEGF-C, in comparison with the parent OSCC cell line. We suggest that VEGF-C plays an important role for tumour invasion as well as lymphangiogenesis. VEGF-C produced by cancer cells may enhance lymphangiogenesis and tumour invasion, leading to nodal metastasis.

In summary, our findings suggest that VEGF-C expression may be involved in lymphangiogenesis and invasion of OSCC cells in the metastatic process, and that determination of VEGF-C expression can be a strong predicting factor for metastasis of OSCC. We conclude that there might be an option for future therapeutic intervention by obstructing lymphatic invasion or lymphangiogenesis.

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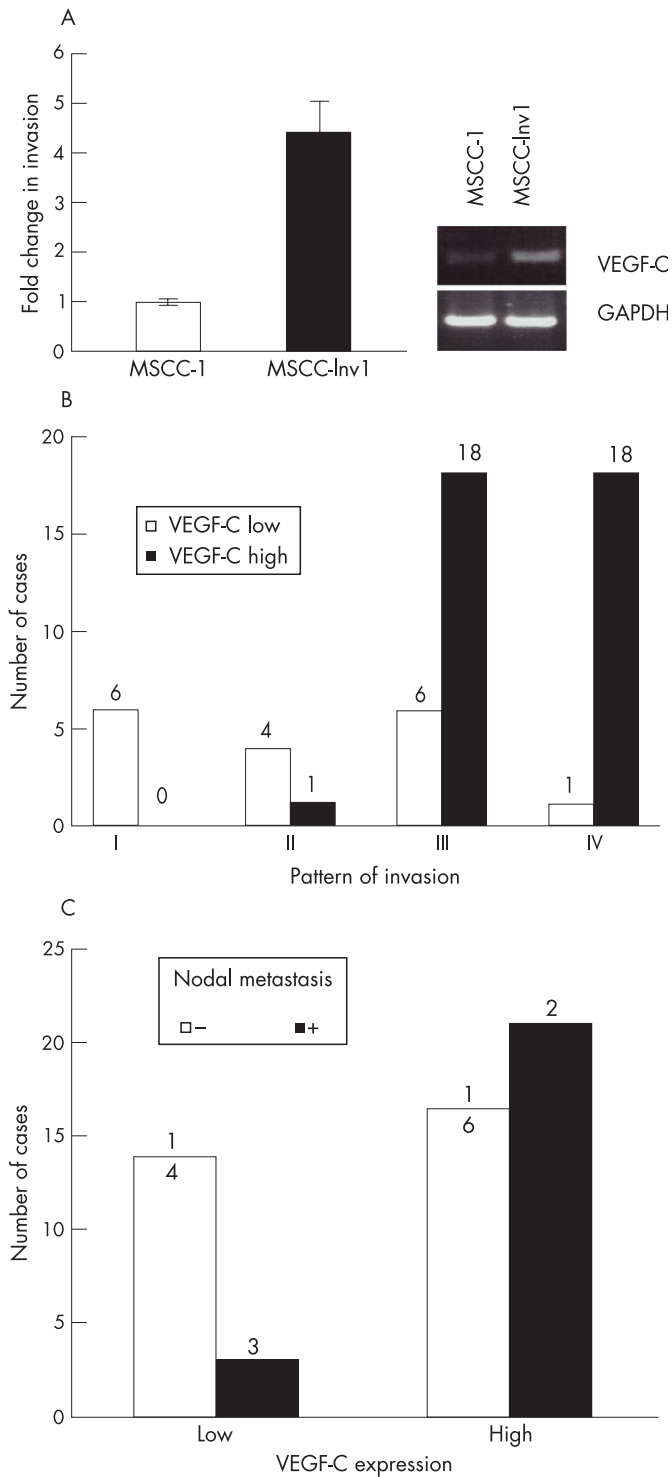
Figure 4 VEGF-C expression is correlated with invasion and metastasis. (A) Left panel shows invasive activity of parent cells (MSCC-1) and a highly invasive clone (MSCC-Inv1). Invasion was determined by in vitro invasion assay. Right panel shows VEGF-C mRNA expression in parent cells and a highly invasive clone. VEGF-C mRNA expression was examined by RT-PCR. GAPDH expression was used as a control. (B) Correlation between VEGF-C expression and pattern of invasion. (C) Correlation between VEGF-C expression and nodal metastasis.

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