Increased Expression of Vascular Endothelial Growth Factor C in Papillary Thyroid Carcinoma Correlates with Cervical Lymph Node Metastases

Xiao-Min Yu,1 Chung-Yau Lo,1 Wai-Fan Chan,1 King-Yin Lam,2 Pauline Leung,1 and John M. Luk1

Abstract

Purpose: Despite recent studies showing that vascular endothelial growth factor C (VEGF-C) mRNA is up-regulated in papillary thyroid carcinoma (PTC), the role of VEGF-C in lymph node metastasis is still unclear. The aim of this study is to investigate the expression pattern of VEGF-C immunoreactive protein in PTC and its relationship with cervical lymph node metastasis.

Experimental Design: Tissue samples were obtained from 39 specimens of PTC (20 with and 19 without lymph node metastasis) as well as 20 benign thyroid nodules. Overexpression of the VEGF-C protein was evaluated by immunoblotting with specific anti-VEGF-C antibody in paired tumor and nontumor tissues from PTC. The data were compared with patients’ clinicopathologic features and lymph node metastasis. Immunohistochemical staining was done on selected paraffin sections to determine cellular localization of VEGF-C and to assess flt-4 (or VEGFR-3) – positive vessel density in PTC lesions.

Results: Overexpression of VEGF-C was detected in 69% of the PTC and in 5% of the benign thyroid specimens. When comparing between the metastatic and nonmetastatic groups of PTC, a higher expression level of VEGF-C was detected in both the tumor (P = 0.004) and adjacent nontumor tissues (P = 0.011). Positive immunostaining for VEGF-C was confirmed in PTC tumor tissues and metastatic lymph nodes, which correlated with flt-4-positive vessel density in tumor and peritumor tissues. The increased expression of VEGF-C protein in PTC is associated with lymph node metastasis (P = 0.004) and lymphovascular permeation (P = 0.001) but is independent of other clinicopathologic variables.

Conclusions: The VEGF-C immunoreactive protein is overexpressed in PTC lesions, which correlates with lymph node metastases. VEGF-C expression may play a role in lymphangiogenesis of PTC and further study is necessary to evaluate the clinical application of VEGF-C as a molecular marker for tumor metastases to cervical lymph nodes.

Papillary thyroid carcinoma (PTC) is the most common type of thyroid malignancy, accounting for 70% to 80% of cases. Patients with PTC have a high incidence (30-100%) of lymph node metastases at presentation (1), which is an independent risk factor for tumor recurrence (2–4). However, the clinical management of lymph node metastasis, including the extent of initial surgery and the proper indication for radioiodine therapy, is still controversial. The optimal combined treatment for lymph node metastasis requires a better understanding of the underlying molecular mechanism.

The molecular pathogenesis of lymph node metastasis has not been elucidated until recently. Evidence shows that lymphangiogenesis is the key process involved in which new lymphatic vessels sprout from preexisting ones to facilitate the shedding of tumor cells into surrounding lymphatic vessels (5). Recent studies have also emphasized that lymphangiogenesis is pivotal to tumor invasion and accounts for lymph node metastases (6, 7). During this process, neoplastic cells secrete certain kinds of lymphangiogenic factors to help the survival, invasion, and local growth in lymph nodes.

Vascular endothelial growth factor C (VEGF-C) is one of the most potent directly acting lymphangiogenic factors belonging to the VEGF family (8). It was first discovered and identified as a ligand of flt-4 (or VEGFR-3) expressed on lymphatic endothelium in adult skin and vascular tumors. By activating flt-4, VEGF-C induces proliferation of lymphatic endothelial cells in vitro (9) and lymphangiogenesis in vivo (10). Animal experimentation has shown that overexpression of VEGF-C in the skin of transgenic mice could result in proliferation and enlargement of lymphatic endothelium, but not of vascular...
endothelium. In two breast carcinoma models, VEGF-C was further shown to induce tumor lymphangiogenesis and promote lymph node metastasis (11, 12).

Clinically, up-regulation of VEGF-C has been observed in many different tumor types in humans, including colorectal (13), gastric (14), cervical (15), and breast (16) cancers. Overexpression of VEGF-C is often associated with a high prevalence of lymph node metastasis. Recent studies have also observed the overexpression of VEGF-C in thyroid cancer (17, 18). For well-differentiated thyroid carcinomas, a higher level of VEGF-C mRNA was detected in the PTC, but not in follicular thyroid carcinoma, as shown by in situ hybridization or quantitative PCR assays (19, 20). Taken together, VEGF-C is one of the key "switch-on" factors of lymphangiogenesis and possibly accounts for the high incidence of nodal metastases in PTC as well as in other solid cancer types. However, the role of VEGF-C immunoreactive protein in PTC metastasis and its relationship with flt-4-expressing tumor endothelium in lymphangiogenesis of thyroid cancer have not been addressed. Thus, the present study evaluates the VEGF-C protein expression levels in PTC in comparison with paired adjacent nontumor tissues in the contralateral lobes, as well as benign thyroid tissues, and correlates its expression with defined clinicopathologic features in PTC patients. We further investigate the flt-4-positive vessel density in tumor vasculatures to strengthen the association between VEGF-C expression and lymphatic development in nodal metastases of PTC.

Materials and Methods

Patients and tissue samples. Specimens were collected from our frozen tumor tissue bank including 39 PTC patients who underwent thyroidectomy between July 2002 and June 2004. There were 11 men and 28 women with age ranging from 14 to 80 years (median, 46 years). The size of primary tumor ranged from 0.4 to 9.0 cm (median, 2.0 cm). Total thyroidectomy was the surgical procedure of choice for patients with PTC. Selective neck dissection was routinely done for patients with clinically overt or ultrasound-detected metastatic cervical lymph nodes whereas suspicious lymph nodes were sampled and excised liberally in the central compartment during total thyroidectomy. Twenty of 39 patients were histologically confirmed to have nodal metastases. According to American Joint Committee on Cancer/Union Internationale Contre Cancer pathologic tumor-node-metastasis classification (21), there were 19 patients with stage I, 3 with stage II, 16 with stage III, and 1 with stage IV disease. Specimens were also obtained from 20 patients with benign nodular goiter after thyroidectomy. The study protocol was approved by the Research Ethics Committee of The University of Hong Kong and informed consent was obtained from all patients.

Tissue specimens from benign thyroid nodules, paired tumor and adjacent nontumor thyroid tissues from the contralateral thyroid lobe, were obtained immediately after thyroidectomy; in addition, metastatic lymph node samples were collected for patients who underwent neck dissections. One part of the tissues was snap frozen in liquid nitrogen immediately and stored at −80°C. The other part was fixed in 10% buffered formalin for paraffin embedding. Before the study, all tissue specimens were examined after H&E staining by a board-certified pathologist (K.Y.L.) for confirmation of pathologic diagnosis. Clinical data of patients' history and pathologic features were retrieved from the thyroid cancer database.

Western blot. As previously described (22), surgical tissues were homogenized in lysis buffer containing 1% NP40, 150 mmol/L sodium chloride, 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor mix (Roche Diagnostics, Mannheim, Germany) in 50 mmol/L Tris-hydrochloric acid (pH 8.0) on ice for 30 minutes. After removing cellular debris by centrifugation, protein content was measured by the Bradford assay (Bio-Rad Laboratories, Hercules, CA). The samples were fractionated on 12% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 10% skim milk at room temperature for 2 hours and then incubated with rabbit polyclonal antibodies against human VEGF-C (1:500 dilution; Abcam, Cambridge, United Kingdom; R&D Systems, Minneapolis, MN) and mouse anti-β-actin monoclonal antibody (1:5,000 dilution; Zymed Laboratories, Inc., South San Francisco, CA) at 4°C overnight. The antibody specificity of human VEGF-C antibody was previously reported not to cross-react with VEGF-D (23). After washing, the membrane was incubated with horseradish peroxidase–conjugated anti-rabbit/mouse antibodies (1:5,000 dilution; Zymed) at room temperature for 1 hour and the immunoreactive bands were detected by enhanced chemiluminescence reagents (Amersham Bioscience, Arlington Heights, IL). After image acquisition by a GS-800 Calibrated Densitometer (Bio-Rad), expression analysis was done using the software Quantity One version 4.4.1 (Bio-Rad). The β-actin band was used as internal protein loading control for normalization in each sample. Relative fold of VEGF-C expression was calculated by the ratio of tumor or nontumor tissue expression to that of normal thyroid tissue (baseline control).

Immunohistochemistry for vascular endothelial growth factor C, flt-4, and vessel counts. To examine the histopathology of thyroid tissues, 4-μm-thick sections were cut from the paraffin blocks, mounted on polylysine-coated slides, and stained with H&E for light microscopy viewing. For immunostaining, sections were treated with 0.3% hydrogen peroxide solution to quench endogenous peroxidase activity. After blocking with 3% bovine serum albumin and 3% normal rabbit serum in PBS at room temperature for 1 hour, the sections were incubated with goat polyclonal anti-VEGF-C antibody (10 μg/mL) or anti-flt-4 antibody (15 μg/mL; R&D Systems) at 4°C overnight, and followed by an incubation with peroxidase-conjugated secondary antibody (DAKO EnVision+ System, Glostrup, Denmark) at 37°C for 30 minutes. The slides were washed thoroughly with PBS between all stages of the procedures. Finally, the antibody reaction was visualized by color development using the 3,3'-diamino-benzidine tetrachloride substrate solution (DAKO EnVision+ System), and then the sections were counterstained with hematoxylin, dehydrated with xylene, and mounted in glycerol-vinyl-alcohol (GVA mount, Zymed). For the negative isotype controls, the primary antibody for VEGF-C was replaced with purified goat immunoglobulin G (1:500 dilutions; Zymed).

The immunostaining for VEGF-C was graded according to the established scoring method as previously reported (24). Specimens were counted as negative immunoreactivity when there were <5% of tumor cells having a definite positive staining. The number of flt-4-stained vessels was assessed following the scoring method of Bono et al. (25). In brief, the stained sections were scanned at a low magnification and those areas with the greatest number of microvessels were selected for further evaluation. The microvessel density was then determined by counting all immunostained vessels at ×200 magnification per 0.5-mm² microscope ocular grid and the average number in the two selected areas was then calculated as the flt-4-positive vessel density of individual specimen. Images were captured with a digital camera (DMX1200F; Nikon, Tokyo, Japan) and analyzed using MetaMorph v.3.0 software (Universal Imaging, Inc., Downingtown, PA).

Statistical analysis. The data were analyzed by the Statistical Package for Social Sciences (SPSS for Windows, version 11.0; Chicago, IL). Continuous data were presented as median value (interquartile range). Mann-Whitney U test and Wilcoxon signed-rank test were used to evaluate differences between unpaired and paired observations, respectively. Pearson χ² test was done to analyze categorical data as appropriate. Correlations between continuous variables were evaluated using the Spearman rank test. P < 0.05 was considered statistically significant.
Table 1. Frequency of VEGF-C overexpression in PTC, adjacent nontumorous, and benign thyroid tissues by Western blot analysis

<table>
<thead>
<tr>
<th>Thyroid tissues*</th>
<th>n</th>
<th>Overexpression of VEGF-C protein (% positivity)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTC tumor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN+</td>
<td>20</td>
<td>17 (85.0%)</td>
</tr>
<tr>
<td>LN−</td>
<td>19</td>
<td>10 (52.6%)</td>
</tr>
<tr>
<td>PTC adjacent nontumor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN+</td>
<td>20</td>
<td>6 (30.0%)</td>
</tr>
<tr>
<td>LN−</td>
<td>19</td>
<td>1 (5.3%)</td>
</tr>
<tr>
<td>Benign</td>
<td>20</td>
<td>1 (5.0%)</td>
</tr>
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</table>

* Tissues samples were obtained from benign nodular hyperplasia and PTC with or without lymph node (LN+, LN−) metastasis after thyroidecomy. The corresponding adjacent nontumorous tissues from contralateral lobes of PTC patients were also included.

† Intensity of VEGF-C immunoreactive band at ~43 kDa was >5-fold ratio compared with the baseline intensity (from pooled normal thyroid tissues, n = 10) as described in Materials and Methods.

Results

Increased expression of vascular endothelial growth factor-C protein in papillary thyroid carcinoma with lymph node metastasis. PTC, adjacent nontumorous tissues, and benign thyroid specimens were analyzed by Western blot with a specific VEGF-C antibody. Normal thyroid tissues did not express VEGF-C (n = 10; data not shown) and the presence of an immunoreactive band of ~43 kDa represented its overexpression. Of the 39 PTC tumor specimens examined, 27 (69%) were observed with overexpression of VEGF-C protein (Table 1). Among them, those tumor tissues from PTC with lymph node metastasis had a significantly higher proportion with VEGF-C overexpression than those without lymph node metastasis (85% versus 52.6%; P = 0.029). Similar observation was found in the corresponding nontumorous tissues (30% in metastatic group versus 5.3% in nonmetastatic group; P = 0.044). For the benign thyroid tissues, there was only one case showing positive VEGF-C overexpression.

Figure 1 showed representative immunoblots from paired tumor and the corresponding nontumorous tissues obtained from PTC patients. It was obvious that the VEGF-C protein level was notably higher in the tumor group, particularly the PTC with lymph node metastasis, than the nonmetastatic group. No apparent reactivity could be observed in the benign tissues. In addition, there was a significant increase of VEGF-C expression in the tumor compared with the paired adjacent nontumor tissues from both nonmetastatic PTC (P = 0.001) and metastatic PTC groups (P < 0.001; Fig. 2).

Semiquantitative analysis of VEGF-C protein expression by Western blot technique showed a significant difference between the three groups of surgical specimens: benign thyroid tissue, tumor tissues from PTC with, and those without lymph node metastases. The level of VEGF-C expression in PTC with lymph node metastases was 17.07-fold (interquartile range, 8.11- to 25.47-fold), which was significantly higher compared with 8.65-fold (range, 1.05- to 12.77-fold; P = 0.004) and 1.07-fold (range, 0.49- to 1.51-fold; P < 0.001) expressions in PTC without nodal metastases and in benign thyroid nodules, respectively (Fig. 3).

To determine whether or not VEGF-C expression differed between PTC and metastatic lymph nodes specimens, we analyzed five tissues of metastatic lymph nodes that were matched with corresponding primary tumor and nontumor tissues. The VEGF-C expression in metastatic lymph nodes was 11.87-fold (range, 10.89- to 22.35-fold), which was significantly higher than that in the adjacent nontumor tissues (1.41-fold; range, 1.24- to 7.88-fold; P = 0.043), whereas no significant difference was observed when compared with the primary tumor tissues (25.53-fold; range, 21.65- to 27.25-fold; P = 0.138).

Localization of vascular endothelial growth factor C and flt-4 in papillary thyroid carcinoma. Immunohistologic localization of VEGF-C immunoreactive protein was cytoplasmic in tumor cells of PTC and metastatic lymph nodes (Fig. 4). Positive staining for VEGF-C protein was observed in 7 of 8 (87.5%) cases of selected PTC specimens but none of the three benign thyroid nodules was positive. Lymph node metastases were present in four of these eight PTC patients and over 90% of their tumor cells yielded strong positive signals. For the other four PTC patients without nodal metastases, three showed immunoreactivity with VEGF-C and the other was negative. In addition, tumor cells that metastasized in the cervical lymph nodes also showed VEGF-C protein expression (Fig. 4D).

For the VEGFR-3 reactivity, flt-4 immunostaining decorated the endothelial cell linings in tumor vessels of all the eight PTC specimens that were tested. Similar staining was not observed.
in the normal nor benign thyroid tissues (data not shown). Flt-4 immunoreactivity was infrequently detected among the large and small vessels in the intratumoral tissues (Fig. 4E) as well as in the peritumoral region where flt-4-positive vessels were commonly seen scattered adjacent to the tumor (Fig. 4F). Occasionally, tumor cells invading into the flt-4-positive vessels could be observed. No flt-4 reactivity was detected in the stromal cells of PTC.

Correlation of vascular endothelial growth factor-C levels and flt-4 vessel density in tumor tissues. The median flt-4-positive vessel density was 15 (range, 5.5-32) in the eight PTC specimens. There was a positive correlation between VEGF-C expression levels and the flt-4 vessel density in PTC samples ($r = 0.738, P = 0.037$). In addition, the flt-4-positive vessel density tended to be higher for four PTC samples with lymph node metastasis (median, 18.75; range, 16.75-26.00) compared with another four samples without metastases (median, 11.75; range, 7.50-16.50; $n = 4$) although the difference did not reach statistical significance ($P = 0.083$).

Correlation of vascular endothelial growth factor-C levels in tumor tissues with clinical variables. Because overexpression of angiogenic factor is often associated with cancer progression, neoplasia, and metastasis, whether or not the increased VEGF-C level in PTC could be related to any designated clinicopathologic variables, such as age, sex, tumor size, cancer stage, and the presence of nodal or distant metastasis, was analyzed (Table 2). VEGF-C tumor expression level was significantly associated with the occurrence of lymph node metastases ($P = 0.004$) and the presence of lymphovascular permeation in histologic examination ($P = 0.001$). The only patient with scintigraphic evidence of lung metastases associated with elevated thyroglobulin had a remarkably high level of VEGF-C expression although the number of patients with distant metastasis was too small to have any statistical significance. In brief, patients with increased tumor VEGF-C level had a higher incidence of tumor metastasis and potentially shorter survival.

Discussion

PTC spreads predominantly to the regional cervical lymph nodes. Recent works on VEGF-C and its signaling axis have begun to elucidate the underlying molecular mechanisms involved in lymphangiogenesis. In the present study, we attempted to elucidate the expression patterns of PTC and, by correlating its expression with clinicopathologic features, the role of VEGF-C protein expression in PTC. The level of VEGF-C protein expression in the primary tumors correlated positively with the presence of lymph node metastases and an increased VEGF-C expression was associated with the presence of lymphovascular permeation. In addition, VEGF-C overexpression was also unexpectedly observed in the adjacent, apparently normal, nontumor tissues in the contralateral thyroid lobe and an increased expression level was associated with those PTC patients with lymph node metastases.

![Fig. 2. Paired comparison of VEGF-C expression between adjacent nontumor tissue and PTC tumor tissue from the same patient. A, PTC with lymph node metastases (LN+; $n = 20$). B, PTC without lymph node metastases (LN−; $n = 19$). * $P < 0.05$, Wilcoxon signed-rank test.](image-url)

![Fig. 3. Dot plot analysis of VEGF-C expression in PTC and benign thyroid tissues. VEGF-C level was significantly higher in the tumorous tissues of PTC from patients with lymph node metastasis ($n = 20$) compared with that of PTC without lymph node metastasis ($n = 19$; $P = 0.004$) as well as that of nodular hyperplasia (Benign; $n = 20$; $P < 0.001$). In addition, VEGF-C level in PTC tumor without lymph node metastasis was significantly higher than that in benign tissue ($P = 0.002$). Mann-Whitney U test was used for comparison between each individual group. The horizontal line in dot clusters of each individual group indicates the median values in the corresponding groups.](image-url)
The major function of VEGF-C in facilitating lymphatic endothelial proliferation has already been established using a transgenic mouse model (10). However, the role of lymphangiogenesis in enhancing nodal metastases and its clinical relevance remain controversial. Previous studies had documented a significant correlation between VEGF-C expression in primary tumor tissues and the presence of lymph node metastases in breast (16), lung (26), and colorectal carcinomas (13) but such finding was not invariably confirmed by other studies (27–29). According to the data from our study, the presence of VEGF-C overexpression as well as the level of VEGF-C protein expression in the primary tumor of PTC significantly correlated with the presence of lymph node metastases. Our findings concur with those from two previous studies on thyroid carcinoma (17, 30) suggesting that VEGF-C can enhance the invasive ability of malignant cells. Furthermore, the flt-4-positive vessel density correlates with VEGF-C expression levels. This provides further evidence that VEGF-C, by activating its receptor, is able to increase cancer cell dissemination via lymphatic vasculature. In addition, the level of VEGF-C expression also significantly correlates with the presence of lymphovascular permeation. Similar findings have been reported in other malignancies (13, 26). In vivo study using a breast carcinoma cell line also showed that VEGF-C overexpression was associated with intralymphatic growth of tumor cells (31). These results suggest that VEGF-C would enhance the invasion ability and provide the intervening routes for tumor cells to spread in the early phase of lymph node metastases. Besides, recent study has also suggested that VEGF-D expression and increased lymph vessel density may have an important role for lymph node metastasis in PTC (32). The interplay between VEGF-C and VEGF-D on flt-4-mediated lymphatic development in tumor nodal metastasis warrants further characterization.

Furthermore, a higher incidence of VEGF-C expression as well as an increased VEGF-C expression level could be shown in the contralateral nontumor tissues of PTC patients with lymph node metastases compared with those without nodal metastasis. Finding of additional microscopic tumor foci in adjacent nontumor tissue is frequent in PTC but the origin of these tumors is controversial. Although it has been shown that individual tumor foci in patients with multifocal PTC often arise as independent clonal origins (33), whether or not noncontiguous tumor foci represent multifocality or intraglandular
metastases is uncertain. VEGF-C overexpression in the contralateral nontumorous tissues can be explained by the presence of either multifocal tumors or micrometastases but analysis of VEGF-C expression levels and multifocal tumors did not show any quantitative correlation. On the other hand, thyrotropin or VEGF-C expression levels and multifocal tumors did not show an increase in VEGF-C expression level was also observed in the contralateral nontumor tissues. In fact, the finding of VEGF-C mRNA expression in the microvascular endothelium in adjacent normal thyroid tissue did support this assumption (19) although we could not localize the site of VEGF-C protein in the adjacent nontumor tissues by immunohistochemistry. Besides, several in vivo studies also showed the presence of lymphangiogenesis in the periphery of, but not inside, the tumors, suggesting that lymphangiogenic factors like VEGF-C may bind to the receptors of lymphatic vessels beyond the primary tumors (11, 35).

VEGF-C overexpression has been found to be associated with tumor progression and poor disease-free survival in various carcinomas (36–38). On the other hand, the prognosis of patients with PTC has been shown by retrospective cohort studies to be predicted by commonly used risk group stratification schemes, staging classifications, or prognostic scoring systems. In contrast to other malignancies, the presence of lymph node metastasis in PTC is not commonly included as a poor prognostic factor in these risk group stratification schemes, possibly due to a difference in tumor biology. In similarity to the prognostic role of nodal metastases in PTC, VEGF-C overexpression may not be an important prognostic factor for PTC. However, patients with nodal metastases are at increased risk of locoregional recurrence unless a routine central nodal dissection is adopted and/or postoperative radioiodine therapy is administered. Therefore, the ability of VEGF-C expression to predict nodal metastases in PTC patients may facilitate the identification of patients with occult nodal metastases and enhance clinical management. Further studies to evaluate its role and clinical application are necessary.

In conclusion, overexpression of VEGF-C protein was confirmed in PTC and the incidence of VEGF-C protein expression was higher in PTC with lymph node metastases. Moreover, a higher VEGF-C expression level was associated with the presence of lymphovascular permeation and nodal metastases. Such increase in VEGF-C expression level was also observed in the adjacent nontumor tissues when comparing PTC patients with and without nodal metastases. VEGF-C expression may play a role in lymphangiogenesis of PTC and further study is necessary to evaluate the clinical application of VEGF-C to cervical lymph nodes as a molecular marker for tumor metastases. Finally, it is also of great interest to investigate the quantitative correlation of tumor expression and serum levels of VEGF-C that may have a prognostic value in patients with PTC.

Table 2. Relationship between tumor VEGF-C protein expression and clinicopathologic features of PTC

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*Multifocality is defined as the presence of additional tumor foci noncontiguous to the primary tumors in the resected thyroid specimens.
†Extrathyroidal invasion as the extension of tumor beyond the thyroid capsule to the perithyroidal tissue.
‡The presence of tumor cells within the vascular or lymphatic endothelium.
\[ P < 0.05.\]

References
10. Veikkola T, Jussila L, Makinen T, et al. Signalling via...


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