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Vascular endothelial growth factor receptor-3 is independently associated with cervical lymph node metastasis in papillary thyroid carcinoma

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Summary. *Objective:* Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer. Despite excellent prognosis, the presence of cervical lymph node metastasis (cLNM) has a significant impact on tumor recurrence. However, the clinical management of cLNM remains controversial. The present study aims to shed light on the correlation between markers for hypoxia, angiogenesis and lymphangiogenesis, as well as cLNM in PTC. *Methods:* Ninety-five paraffin-embedded surgical specimens of PTC were constructed into tissue microarray. Immunohistochemical staining for markers of hypoxia (HIF-1α and CA-IX), angiogenesis (VEGF-A, VEGF-C and VEGFR-2), and lymphangiogenesis (VEGF-C, VEGF-D and VEGFR-3) was performed. A Kaplan-Meier plot of recurrence was performed according to the VEGFR-3 status. *Results:* Out of all the PTC cases, 11.6% had cLNM. CA-IX, VEGF-D and VEGFR-2 were associated with capsule invasion. Of the biomarkers, VEGF-C and VEGFR-3 were correlated with cLNM. The Kappa coefficient test showed that HIF-1α and CA-IX correlated with VEGF-A, which, together with VEGF-C, correlated with both VEGFR-2 and VEGFR-3. In contrast, VEGF-D was only associated with VEGF-C. *Conclusions:* VEGF-C and VEGFR-3 are associated with cLNM but only VEGFR-3 independently correlates with cLNM after adjustments for TMN staging. It may be useful to perform VEGFR-3 immunostaining in primary resected thyroid carcinoma specimens.

Key words: papillary thyroid carcinoma, VEGF, VEGFR, lymph node metastasis

«Il recettore 3 per il fattore di crescita vasculo-endoteliale (VEGF) è associato in modo indipendente alle metastasi del linfonodo cervicale da carcinoma papillare della tiroide»

Riassunto. *Obiettivo:* Il carcinoma papillare della tiroide (PTC) è il tipo più comune di cancro tiroideo. A dispetto di una eccellente prognosi, la presenza di metastasi al linfonodo cervicale (cLNM) ha un impatto significativo sulla recidiva del tumore. Tuttavia, l'approccio clinico alle cLNM rimane controverso. Il presente studio ha lo scopo di fare luce sulla correlazione tra marcatori dell'ipossia, angiogenesi e linfo-angiogenesi e significato della cLNM nel PTC. *Metodi:* Novantacinque campioni chirurgici di PTC inclusi in paraffina vengono allestiti per eseguire microarray. La colorazione immunoistochimica è stata eseguita per i marcatori dell'ipossia (HIF-1α e CA-IX), dell'angiogenesi (VEGF-A, VEGF-C e VEGFR-2), e della linfo-angiogenesi (VEGF-C, VEGF-D e VEGFR-3). Il grafico di Kaplan-Meier dell'incidenza di recidiva è stato costruito in accordo con lo stato del VEGFR-3. *Risultati:* Tra tutti i casi di PTC l'11,6% mostrava cLNM. I marcatori

CA-IX, VEGF-D e VEGFR-2 erano associati all'invasione della capsula tiroidea. Tra i marcatori, VEGF-C e VEGFR-3 erano correlati con cLNM. Il test coefficiente K ha mostrato che HIF-1α e CA-IX sono correlati con VEGF-A, il quale insieme al marcatore VEGF-C si correla sia con VEGFR-2 che con VEGFR-3. Al contrario il marcatore VEGF-D è associato soltanto a VEGF-C. *Conclusioni:* VEGF-C e VEGFR-3 sono associati a cLNM ma soltanto VEGFR-3 si correla in modo indipendente a cLNM, dopo aver ottimizzato la stadiazione TMN. Quindi eseguire immunocolorazione con VEGFR-3 potrebbe essere utile nei campioni ottenuti da resezione di carcinoma primario della tiroide.

Parole chiave: carcinoma papillare della tiroide, VEGF, VEGF-R, metastasi linfonodali

Introduction

Papillary thyroid carcinoma (PTC) is the most common type of thyroid malignancy, accounting for 80-85 percent of all thyroid cancers. It has a tendency to be biologically indolent and despite excellent prognosis, with 95 percent survival rate and 25 years expanded life (1), the presence of cervical lymph node metastasis (cLNM) reportedly has a significant impact on tumor recurrence in patients older than 45 years of age (2, 3). Lymph node recurrences are also distressing for patients. Moreover, the clinical management of cLNM, including the extent of initial surgery, prophylactic lymph node dissection, and the proper indication for radioiodine therapy, remains controversial. Understanding the underlying molecular mechanism in cLNM is important for providing optimal treatment. Unfortunately, there is still no accurate system of predicting cLNM.

Vascular endothelial growth factor (VEGF) is a specific mitogen for endothelial cells *in vitro* and an angiogenic factor *in vivo* (4, 5). First reported as an angiogenic factor, it has since been found to be the same protein as vascular permeability factor (4, 5). It consists of mainly four types of ligands, from VEGF-A to VEGF-D, and three types of VEGF receptors (VEGFR): type 1 (Flt-1), type 2 (Flk-1), and type 3 (Flt-4) (6). The binding between VEGF and its receptors regulates angiogenesis through a paracrine effect and hematopoiesis through an autocrine loop (7), which in turn are responsible for the angiogenesis pathway VEGF-A/VEGF-C/VEGFR-2 and the lymphangiogenesis pathway VEGF-C/VEGF-D/VEGFR-3 (6). Hypoxic induction of VEGF *in vivo* is well documented. Accordingly, insufficient vascular supply and the subsequent reduction in tissue oxygen tension leads to angiogenesis, as a response so as to meet the oxygen demands of tissues (8).

Hypoxia-inducible factor-1α (HIF-1α) is an endogenous hypoxia marker. Under normal oxygenation, it is rapidly degraded by proteosomes after being targeted for ubiquitinization (9, 10). Carbonic anhydrase-IX (CA-IX) is an enzyme expressed in all tissues and is responsible for catalyzing the reversible hydration of carbon dioxide. It is also the downstream target of HIF-1α and studies report that CA-IX is closely associated with the pattern of HIF-1α expression (11). HIF-1α and CA-IX are hypoxia markers for evaluating angiogenesis and lymphangiogenesis.

Not surprisingly, many biomarkers have been studied in PTC in relation to predicting cLNM. But most studies are not consistent with each other and all lack a thorough investigation. The present study aimed to shed light on the correlation between markers for hypoxia (HIF-1α and CA-IX), angiogenesis (VEGF-A, VEGF-C, and VEGFR-2), and lymphangiogenesis (VEGF-C, VEGF-D, and VEGFR-3), and cLNM in PTC.

Materials and methods

Patients

All the diagnosed cases of PTC (totally 103 cases) between 1995 and 2000 at Cardinal Tein Hospital

were retrospectively collected. Based on interviews at the time of admission for surgery, no one had a family history of thyroid cancer or malignancy in first-degree relatives. All of the patients had undergone routine total thyroidectomy with regional lymphadenectomy. Those who had received chemotherapy or radiotherapy before resection were excluded.

Ninety-five cases were finally enrolled. All clinical charts and histopathology reports were reviewed for data on age, sex, tumor size, bilaterality, extracapsular invasion, lymph node or distant metastasis, and TMN staging. Local regional recurrence, distant metastasis, and disease-specific survival rate were also documented. All cases were de-linked anonymously for confidentiality. The hospital's institutional review board approved the study protocol.

Construction of the tissue microarray

All tissue samples were routinely fixed in formalin and embedded in paraffin wax. Representative tissue areas were chosen at the junction of the major tumor mass and the adjacent benign part marked on standard hematoxylin and eosin (H&E) sections, taken from the paraffin block using a 2.0-mm punch, and inserted into a recipient paraffin block. Sections 4 μm thick were cut from the completed array block and transferred to silanized glass slides.

Histology, Immunohistochemistry and Scoring

The constructed tissue array of paraffin-embedded blocks were cut in 5 μm-thick sections for H&E staining. In each case, carcinoma type, cell differentiation, growth pattern, tumor cell nuclear morphology, metaplasia, calcification, necrosis, mitosis count, invasion status and other specific differentiations were rechecked by two pathologists.

Immunohistochemical (IHC) stains were performed using the Ventana BenchMark XT automated stainer (Ventana, Tucson, AZ). The primary antibody, hypoxia, markers of HIF-1α (1:100, GeneTex, San Antonio, Texas), CA-IX (1:50, Santa Cruz, Santa Cruz, California), and angiogenesis and lymphangiogenesis markers of VEGF-A (1:25, GeneTex, San Antonio, Texas), VEGF-C (1:20, Abnova, Taipei City, Taiwan), VEGF-D (1:150, Bioworld Technology, Minneapolis, Minnesota), VEGFR-2 (1:40, GeneTex, San Antonio, Texas), and VEGFR-3 (1:25, GeneTex, San Antonio, Texas) were tested by immunohistochemical stains.

Two experienced pathologists reviewed the IHC slides and immuno-staining results were graded as 0 for no staining, 1 for faint, 2 for moderate, and 3 for intense staining. Grades 0 and 1 were classified as negative, while grades 2 and 3 were classified as positive.

Statistical analyses

All statistical analyses were performed using the SPSS-16.0 software (SPSS Inc., Chicago, IL). To test for differences between positive and negative IHC expression, chi-square analysis was used for categorical variables, while the Kappa coefficient was used to observe the correlation between different parameters. Multiple logistic regression analysis was used to identify independent factors. The Kaplan-Meier plot of recurrence with log rank test was calculated. All statistical tests were two-sided and statistical significance was set at $p<0.05$.

Results

Clinical correlation

In total, there were ninety-five cases of PTC with a median age of 41 (16-79), median size of 1.9 cm (0.1-4.5), and median 3.6 years follow-up periods. Based on the clinico-pathologic characteristics of the study participants (Table 1), twelve percent of cases had cLNM. The biomarkers were also associated with the clinicopathologic characteristics (Table 2). VEGF-A and VEGF-C were higher in patients aged ≥45 years old. CA-IX, VEGF-D and VEGFR2 were associated with capsule invasion. Angioinvasion was only associated with CA-IX and VEGF-D. In addition, VEGFR-2 was higher in tumor size >1 cm and all 8 cases of follicular variant PTC were immunoreactive for VEGF-D. Of all the biomarkers, VEGF-C and VEGFR-3 were correlated with cLNM. In order to show the connection between each biomarker, a Kappa coefficient test was done (Table 3). HIF-1α and CA-IX correlated

Table 1. Demographic data of 95 study cases.

	N. (%)
Age (year)	
< 45	54 (57)
≥ 45	41 (43)
Sex	
Male	19 (20)
Female	76 (80)
Type	
Classic	86 (91)
Follicular variant	9 (9)
Tumor Size	
≤ 1 cm	20 (21)
< 1 cm and ≤ 2 cm	31 (33)
> 2 cm and ≤ 4 cm	39 (41)
> 4 cm	5 (5)
Invasion	
No invasion	52 (55)
Capsule invasion	24 (25)
Angioinvasion	8 (8)
Soft tissue invasion	11 (12)
Lymph node metastasis	
No	84 (88)
Yes	11 (12)
Stage	
I	71 (74)
II	12 (13)
III and IV	12 (13)

with VEGF-A. Moreover, VEGF-A and VEGF-C correlated with both VEGFR-2 and VEGFR-3. In contrast, VEGF-D was only associated with VEGF-C. By multivariate logistic regression, only VEGFR-3 independently correlated with cLNM after adjustments for TMN staging (p=0.038) (Table 4). However, the Kaplan-Meier plot of progression-free survival failed to show differences between the group with and without VEGFR-3 expression (p =0.147) (Figure 1).

Immunohistological expression profiles

Although the expression rates among biomarkers were different, all of them stained in the cytoplasm of cancer cells (Figure 2). Hypoxia markers of HIF-1α and CA-IX were expressed only focally within the array dot. In contrast, angiogenesis and lyamphaniogenesis markers were diffusely expressed. In addition, the

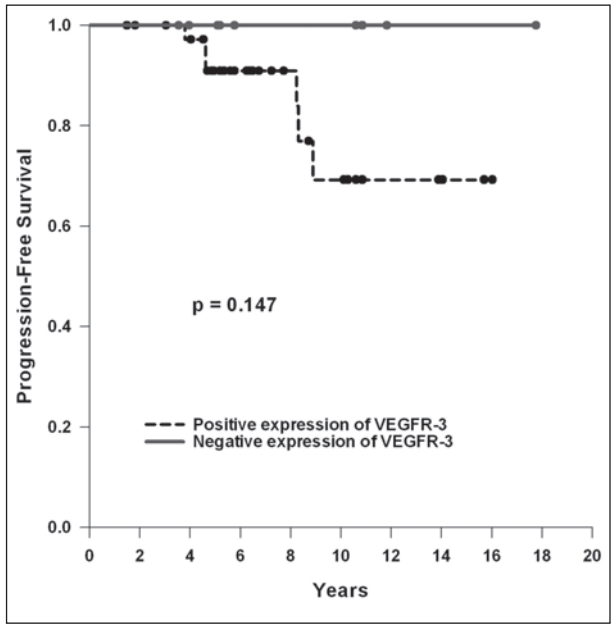

Figure 1. Kaplan-Meier plot of recurrence according to the VEGFR-3 status.

invasive part of tumor cells expressed these markers more intensly. In all of the biomarkers, VEGF-D had the strongest and most consistent expression intensity whether in the main tumor part or invasive part.

Discussion

Biomarkers and cLNM in PTC

There are many hypoxia, angiogenesis, and lymphangiogenesis markers correlated with PTC, especially in cLNM (12-15). Most of the results in the present study show similar findings. Hypoxia markers HIF-1α and CA-IX are associated with VEGF-A, and VEGFR-2, which is consistent with the literature (10, 11). However, angiogenesis and lymphangiogenesis markers of ligands and receptors have variable relationships that are consistent with angiogenesis and lymphangiogenesis pathways (Table 3). Recently, a meta-analysis revealed that patients with VEGF-A positive tumors had a 3.02-fold higher risk of cLNM than did patients with negative tumors (OR=3.02, 95 % CI=2.05-4.43, p<0.001). However, ethnic differences were present. The relationship between VEGF-

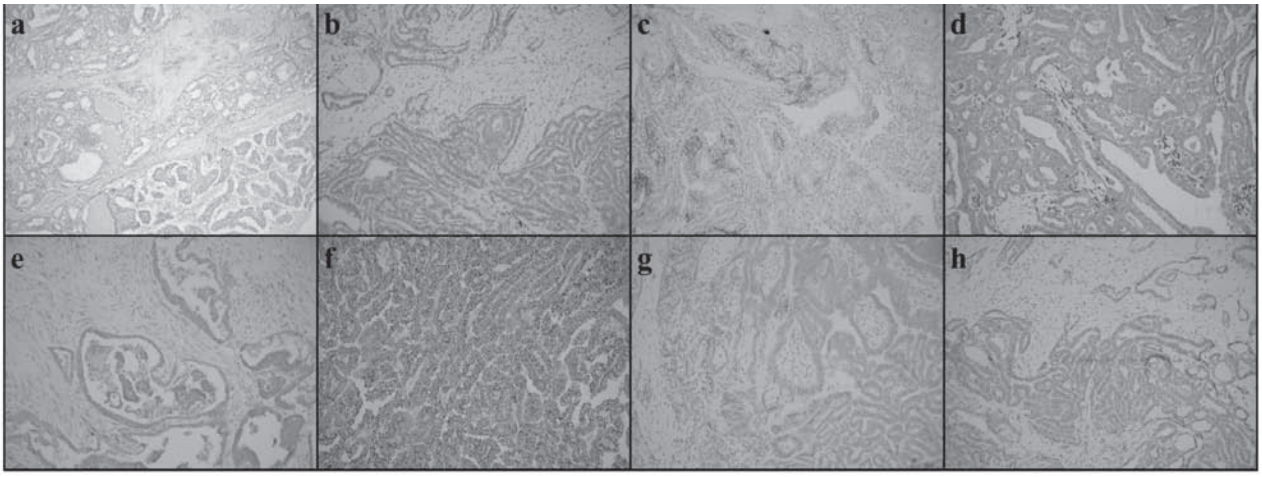

Figure 2. H&E stain of **(A)** classical papillary thyroid carcinoma and immunohistochemical stains of **(B-H)** HIF-1α, CA-IX, VEGF-A, VEGF-C, VEGF-D, VEGFR-2, and VEGFR-3, respectively.

Table 2. Chi square test of different clinico-pathologic parameters and biomarkers[a].

	HIF-1α	CA-IX	VEGF-A	VEGF-C	VEGF-D	VEGFR-2	VEGFR-3
Age (year)							
< 45	14 (29)	20 (39)	39 (74)*	8 (20)**	29 (47)	18 (33)	8 (20)
≥ 45	14 (36)	17 (41)	37 (90)	29 (55)	12 (55)	20 (49)	11 (21)
Type							
Classic	28 (35)	34 (40)	67 (79)	54 (63)	49 (59)*	36 (42)	19 (22)
FV	1 (11)	3 (33)	9 (100)	7 (78)	8 (100)	3 (33)	0 (0)
Size							
≤ 1 cm	3 (16.7)	9 (47.4)	13 (68.4)	11 (57.9)	15 (78.9)	4 (21.1)*	2 (10.0)
> 1 cm	26 (37.7)	28 (38.4)	61 (83.6)	52 (67.5)	40 (58.8)	35 (47.3)	17 (23.3)
Invasion							
No invasion	16 (32)	18 (35)*	41 (80)	33 (64)	36 (72)**	18 (35)*	11 (21)
Capsule	8 (33)	16 (67)	21 (88)	17 (71)	10 (42)	11 (46)	3 (13)
Angioinvasion	4 (50)	5 (63)	5 (63)	4 (50)	5 (63)	6 (75)	3 (38)
Soft tissue	5 (39)	3 (21)	10 (93)	9 (71)	10 (77)	10 (71)	5 (33)
LN meta							
No	24 (30.4)	31 (38.3)	69 (83.1)	51 (60.7)*	49 (61.3)	32 (38.1)	11 (13.1)**
Yes	5 (50.0)	4 (57.1)	7 (63.6)	10 (90.9)	8 (72.7)	7 (63.6)	7 (63.6)
Stage							
I, II	24 (32)	32 (40)	66 (80.5)	52 (62.7)	48 (59.3)	32 (38.6)	17 (22.1)
III, IV	2 (29)	3 (33)	10 (83.3)	9 (90.0)	8 (88.9)	7 (70.0)	4 (33.3)

[a] Data are shown as numbers and percentages FV, follicular variant; HIF-1α, hypoxia induced factor-1α; CA-IX, carbonic anhydrase-IX; VEGF-A, vascular endothelial growth factor-A; VEGF-C, vascular endothelial growth factor-C; VEGF-D, vascular endothelial growth factor-D; VEGFR-2, vascular endothelial growth factor receptor-2; VEGFR-3, vascular endothelial growth factor receptor-3
*p< 0.05, **p< 0.01

A and cLNM was seen in Chinese, but not among Korean, Turkish, and Japanese populations (16). The present study reveals that VEGFR-3 is an independent factor in predicting cLNM after adjusting for TMN staging. Furthermore, the VEGFR-3 expression in tumor cells has a ten-fold increased risk of de-

Table 3. Kappa coefficient test of the different biomarkers.

	CA-IX	VEGF-A	VEGF-C	VEGF-D	VEGFR-2	VEGFR-3
HIF-1α	NS	0.025	NS	NS	NS	NS
CA-IX	–	0.002	0.010	NS	0.001	0.005
VEGF-A	–	–	0.001	NS	0.012	0.004
VEGF-C	–	–	–	0.015	0.001	0.002
VEGF-D	–	–	–	–	NS	NS
VEGFR-2	–	–	–	–	–	0.003

HIF-1α, hypoxia induced factor-1α; CA-IX, carbonic anhydrase-IX; VEGF-A, vascular endothelial growth factor-A; VEGF-C, vascular endothelial growth factor-C; VEGF-D, vascular endothelial growth factor-D; VEGFR-2, vascular endothelial growth factor receptor-2; VEGFR-3, vascular endothelial growth factor receptor-3; NS, non-significant

Table 4. Univariate and multivariate logistic regression of different biomarkers in lymph node metastasis.

	Univariate			Multivariate		
variables	OR	95% CI	*p*	OR	95% CI	*p**
HIF-1α	2.5	0.64-9.37	0.191	11.3	0.99-129.12	0.051
CA-IX	0.9	0.25-3.36	0.884	8.1	0.71-92.91	0.093
VEGF-A	0.4	0.10-1.58	0.193	8.3	0.92-63.62	0.059
VEGF-C	6.6	0.80-54.50	0.079	1.7	0.12-24.11	0.701
VEGF-D	1.8	0.43-7.31	0.425	7.2	0.88-58.91	0.066
VEGFR-2	2.9	0.79-10.97	0.109	2.0	0.20-19.61	0.562
VEGFR-3	10.3	2.62-40.85	0.001	10.4	1.13-95.64	0.038

HIF-1α, hypoxia induced factor-1α; CA-IX, carbonic anhydrase-IX; VEGF-A, vascular endothelial growth factor-A; VEGF-C, vascular endothelial growth factor-C; VEGF-D, vascular endothelial growth factor-D; VEGFR-2, vascular endothelial growth factor receptor-2; VEGFR-3, vascular endothelial growth factor receptor-3; OR, odds ratio; CI; confidence interval
*p value is adjusted for TMN staging

veloping cLNM. However, the significantly higher expression rate of VEGF-C by chi-square test becomes non-significant after multivariate logistic regression analysis. The current study is the first to demonstrate that VEGFR-3 is not only expressed in PTC tumor cells but also significantly and independently correlates with cLNM. Thus, VEGFR-3 is a potential biomarker that can be used to determine lymph node dissection in thyroid surgery.

Hypoxia markers and cLNM

HIF-1α is reported to be absent from normal thyroid tissue but is variably expressed in primary thyroid carcinomas (17). In addition, it is associated with advanced tumor grades (17). However, in the study done by Burrows *et al.* it does not seem to be associated with any biological feature, including age, tumor size, invasiveness, and cLNM stage (18). In the present study, HIF-1α again has no correlation with any clinical feature. This may be due to the short half-life or the transient regions of hypoxia that prevent consistent detection of HIF-1α expression (18). This hypothesis is partly supported by the fact that HIF-1α expression is highest in anaplastic thyroid carcinomas but is not linked with vessel distribution (18).

CA-IX is the downstream target of HIF-1α and is closely associated with the pattern of HIF-1α expression (11). This finding is not demonstrated in the present study and may be due to the aforementioned reasons. Unlike HIF-1α, CA-IX is correlated with invasiveness. This is consistent with findings of cell line models wherein CA-IX is associated with a more aggressive disease phenotype (17). Furthermore, CA-IX expression is more intense and has a higher percentage of capsular invasion and angioinvasion.

Angiogenesis markers and cLNM

VEGF-A is one of the first factors correlated with angiogenesis, which is induced by hypoxia. However, VEGF-A has not yet been linked clinically to HIF-1α activity in thyroid carcinomas. In the present study, there tends to be association between VEGF and HIF-1α, but it is not statistically significant. Interestingly, previous studies have shown conflicting results concerning VEGF-A in cLNM. Klein *et al.* (8), Kilicarslan *et al.* (19) and Gong *et al.* (20) show that VEGF-A immunostaining scores are an independent marker for lymph node or systemic metastases during thyroidectomy. In addition, serum levels of VEGF-A were reported to be higher in PTC with distant metastasis. Every additional 100 ng/l of serum VEGF-A levels increased the risk of PTC recurrence by 20.3%, but this did not reach statistical significance. Thus, the predictive value of serum VEGF-A requires further investigation (21). Moreover, De la Torre *et al.* (22) and Hsueh *et al.* (23) did not establish a connection between VEGF-A and cLNM, just as our results also fail to demonstrate such a connection. The inconsistencies may be due to the different methods used to quantify VEGF-A expression, as well as the use of different antibodies.

For the VEGF-C, Siironen *et al.* (24) found that VEGF-C increases with age, which is similar to the findings here. This may be because COX-2 enhanced VEGF-C gene expression through the prostanoid receptor (24). As in other studies, the data here also show that VEGF-C is related to cLNM (24-26). Another study done by Salajegheh *et al.* showed that the expression of VEGF-A and VEGF-C correlated with each other at both mRNA and protein levels (both $p < 0.001$). Hence Salajegheh *et al.* suggest that the significant correlations between the expressions of these genes add weight to hypotheses concerning VEGF-A and VEGF-C interaction in cancer progression (27). However, this relationship is not shown in multivariate logistic analysis. In other words, previous data have found an association between VEGF-C and cLNM but lack multivariate data for further analysis. Thus, the relationship is weak. This may partly explain why some investigators have not found this relationship (13, 15).

It is proven that VEGFR-2 is one of the earliest markers of endothelial cell development. More importantly, VEGFR-2 directly regulates tumor angiogenesis. It is found not only in endothelial cells but also in various cancers, including lung, colon, uterus, ovary, breast, and thyroid cancers (28). In colon cancer, VEGFR-2 expression is related to disease stage, recurrence, and worse prognosis (28). However, the results here demonstrate that VEGFR-2 is associated with tumor size and invasion, but not with cLNM.

Lymphangiogenesis markers and cLNM

Activation of the VEGF-C/VEGF-D/VEGFR-3 axis through ligand over-expression induces intra-tumoral lymphangiogenesis, peri-tumoral lymphatic hyperplasia, and/or increased lymph node metastasis in animal models (29). These model systems reveal extensive paracrine effects by tumor ligand secretion on lymphatic endothelia, including increased lymphatic endothelial cell size, proliferation, and vessel permeability, which lead to lymphatic metastasis of primary tumor xenografts. In humans, up-regulation of the VEGF-C/VEGF-D/VEGFR-3 axis through ligand over-expression also promotes tumor invasion and increases lymph node metastases (29). However, the connection between VEGF-D and cLNM was not seen in the present study. Instead, our results show that VEGF-D is associated with tumor size and invasiveness. There is evidence that VEGF-D expression is up-regulated in numbers of other tumors (14, 30). Yasuoka *et al.* (31) were the first to demonstrate that increased VEGF-D expression correlates with cLNM in PTC. VEGF-D has been shown to promote filopodia formation and cancer cell migration and invasion. The invasiveness is regulated by neuropillin-2, the ligand of VEGF-D, in a major role. However, Tanaka *et al.* (32) were unable to find a significant relationship between VEGF-D expression and lymph node metastasis. The data here show a pattern of higher expression in cLNM but this is not statistically significant.

The endothelial expression of VEGFR-3 and the lymphovascular density have been well studied. VEGFR-3 is a receptor tyrosine kinase that is overexpressed in some human carcinomas, but its role in tumorigenesis has not been fully elucidated. There is

evidence that the biological effects of VEGFR-3 overexpression on cancer progression vary with tumor type. Moreover, Kurenova *et al.* (29) reported that VEGFR-3 promotes breast cancer cell proliferation, motility, as well as survival *in vitro* and tumor formation *in vivo*. To date, the present study is the first to establish that VEGFR3 is not only expressed in the tumor itself but is also independently associated with cLNM in PTC. However, VEGFR-3 expression seems uninfluential in future recurrence in our study. Due to the relative early stage of our study participants (13% were stage III and IV) and routine lymph node dissection, the recurrence rate in the study participants with median 3.6 years follow-up periods was only 5.3%. The log-rank test is insignificant between VEGFR-3 (+) and (-) groups. All the study participants underwent routine cervical lymph node dissection which is not the general concept of treatment with cervical lymph nodes. Moreover, we think this procedure would reduce the rate of local recurrence. Generally speaking, most surgeons will only dissect cervical lymph nodes with clinically suspicious or obvious lymphadenopathy. This is because the prognosis is not related to cLNM, especially in under 45 year-old female patients. We aim to find an ancillary test to evaluate the need for cervical lymph node dissection, minimizing the surgical status. The only reason not to dissect cervical lymph nodes is, as we described in the introduction, the fear of its increasing the local recurrence rate. Hence, we think it might be a good practice to perform VEGFR-3 IHC in frozen sections or do two-stage surgery (perform cervical lymph node dissection later, after thyroidectomy).

Limitations

There were several limitations in the current study. First, the protein expression level was demonstrated by immunohistochemistry alone without any other methodology for secondary confirmation. Second, this is a retrospective study: future prospective cohorts are needed. Finally, although we suggested VEGFR-3 may be a potential biomarker that can be used to determine lymph node dissection in thyroid surgery, there might be a need for re-operation in current clinical practice. However, pre-operation evaluation is another way to solve this problem. Which is to say, immunocytochemical staining by fine needle aspiration test or immunohistochemical staining with frozen sections are the better course of clinical application. Nevertheless, more studies and further evaluations are needed.

Conclusions

Although both VEGF-C and VEGFR-3 are associated with cLNM, only VEGFR-3 is independently associated with cLNM after adjustment for TMN staging. Performing VEGFR-3 immunostaining in primary resected thyroid carcinoma specimens may be useful for predicting cLNM.

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