

Microvessel and Lymphatic Vessel Density and VEGFR-3 Expression of Papillary Thyroid Carcinoma with Comparative Analysis of Clinicopathological Characteristics

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Background : This study was done to see if there were correlations between anatomic and molecular parameters such as microvessel density (MVD), lymphatic vessel density (LVD), and vascular endothelial growth factor receptor (VEGFR)-3 expression and various clinical parameters for papillary thyroid carcinomas of size > 1.0 cm (PTCs) and size \leq 1.0 cm (papillary thyroid microcarcinomas, PTMCs). PTMCs were divided into two subgroups (0-5 mm and 6-10 mm). **Methods :** We analyzed 197 thyroid carcinomas including 113 PTCs and 84 PTMCs. Tissue samples from 30 patients from each group matched for clinical characteristics were selected for immunostaining. **Results :** Although PTCs and PTMCs showed significant differences in clinical characteristics, they did not show significant difference in MVD, LVD, or VEGFR-3 expression. There was a significantly higher LVD in the PTMC subgroup with the larger tumors but no difference in clinical characteristics. LVD was higher in patients > 45 years old (more apparent in the PTC group) and LVD had suggestive correlations with multicentricity and extrathyroidal extension depending on analytic conditions. **Conclusions :** Since LVD showed variable correlations with clinical variables for papillary carcinoma of the thyroid depending on analytic conditions, the individually planned treatments based on overall clinicopathological factors are advised.

Key Words : Thyroid; Carcinoma, papillary; Neovascularization, pathologic; Lymphangiogenesis; Vascular endothelial growth factor receptor-3

Papillary carcinoma is the most common type of thyroid malignancy, representing 85-90% of all malignant tumors of the thyroid gland.^{1,2} Papillary thyroid microcarcinoma (PTMC) is defined as a papillary carcinoma \leq 1.0 cm in size. With the advent of widespread use of high-resolution sonography and fine needle aspiration cytology, thyroid malignancies have been diagnosed at early stages, which has resulted in an exponentially increased incidence of PTMC.³ Papillary carcinoma of the thyroid gland usually shows good prognosis with a mortality rate < 10% and PTMCs have a more favorable prognosis than papillary carcinomas \geq 1.1 cm in size. Many PTMCs may remain occult and become diagnosed as incidental findings during surgery for goiter or other benign thyroid lesions. However, some PTMCs may be aggressive and lethal because of locoregional tumor growth, lymph node metastasis, or distant metastasis. The identification of risk factors that are related to metastatic potential or recurrence in PTMC would be clinically important for establishing a prognosis and deciding on a treatment strategy.

The factors predictive of clinical recurrence in PTMC have not been clearly determined and trials to define reasonable therapeutic guidelines are currently underway.⁴⁻⁶

One factor, angiogenesis, is crucial for tumor growth and metastasis in many solid tumors.^{7,8} A high incidence of nodal metastasis in papillary carcinoma of the thyroid gland suggests a possible association of tumor spread and lymphangiogenesis. While there have been a large number of studies on angiogenesis of papillary carcinoma of the thyroid gland, lymphangiogenesis has not been widely studied and the mechanisms that drive metastasis are largely unknown.⁹ There have been few studies reported on lymphangiogenesis and nodal metastasis in PTMC.^{10,11}

Angiogenesis is a complex process that is regulated by the vascular endothelium, extracellular matrix, and numerous related growth factors and enzymes. The balance between proangiogenic and inhibitory stimuli is an important controller of the switch mechanism in angiogenesis. The development and maintenance of blood vessels are dependent on various molecular systems, and

roles for vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) have been documented. Unlike vascular endothelial cell-specific VEGFR-1 and VEGFR-2, VEGFR-3 expression is associated with lymphatic vessels in the adult. VEGF-C stimulates lymphatic proliferation by activating VEGFR-3. Increased expression of VEGFs and VEGFRs has been demonstrated in carcinomas of solid organs. For papillary carcinoma of the thyroid gland, several studies have demonstrated an association between lymph node metastasis and VEGF, VEGF-C, and VEGF-D expression.^{9,10,12,13} While there is an abundant literature on VEGFR-1 and VEGFR-2 expression in PTCs, studies on VEGFR-3 expression in PTCs are rare. In those studies, reverse transcription-PCR analysis of VEGFR-3 mRNA was performed or VEGFR-3 positive vessel density was counted.¹⁴⁻¹⁶

Accordingly, our study was designed to investigate possible correlations between anatomic and molecular parameters such as CD34 positive microvessel density (MVD), D2-40 positive lymphatic vessel density (LVD), and VEGFR-3 expression in tumor cells and various clinical parameters for papillary carcinoma of the thyroid. We also compared findings for papillary thyroid carcinomas larger than 1.0 cm (PTCs), PTMCs (0-5 mm in size), and PTMCs (6-10 mm in size) to see if tumor size predicts clinical variables.

MATERIALS AND METHODS

Patients

Thyroid papillary carcinoma tissues from consecutive patients of both sexes, and ages ranging from 26 to 76 years, were obtained between January 1997 and August 2005 from the archival records of the Department of Pathology, Ewha Womans University Medical Center. Most of the patients were treated with total or near-total thyroidectomy. Information on the following background parameters was included: age at histological diagnosis, sex, tumor diameter, multicentricity, primary tumor extension, lymph node metastasis, number of positive lymph nodes, recurrence, and distant metastasis at diagnosis. Multicentricity was defined as the presence of additional tumor foci noncontiguous with the primary tumor. Extrathyroidal extension was defined as the extension of the tumor beyond the capsule to the perithyroidal soft tissue. Metastatic thyroid cancer noted in regional lymph nodes prior to surgery or at the time of surgery was considered to be nodal metastasis at diagnosis, not disease recurrence.

We analyzed 197 thyroid carcinomas including 113 cases of

PTC and 84 cases of PTMC. PTMCs were subdivided into two groups: tumor size of 1-5 mm (10 cases) and 6-10 mm (74 cases).

Immunohistochemical studies

Tissue samples from 30 pairs of patients from PTMC (0-5 mm, 8 cases; 6-10 mm, 22 cases) and PTC groups were selected for immunohistochemical staining for CD34, D2-40, and VEGFR-3. To minimize the effect of factors that influence MVD, LVD, and VEGFR-3 expression, each pair was matched for clinical characteristics including age, sex, multicentricity, extrathyroidal extension of tumor, nodal status, and stage. After selection of 30 matched pairs, statistical analysis showed no significant differences between the two groups except for T classification. Since the grouping of PTMC versus PTC itself causes this difference, statistical adjustment of T stage was not done.

Hematoxylin and eosin-stained sections were used to select a representative part of the tumor. In the case of tumor heterogeneity, the most cellular area of the tumor was selected. Immunohistochemical staining was done on thin sections (4 μ m) of formalin-fixed, paraffin-embedded tissue by BondTM Automated Immunohistochemistry (Vision Biosystems, Inc., Mount Waverley, VIC, Australia) and a bond polymer detection system with counterstaining (Vision Biosystems, Inc.). Heat-induced epitope retrieval was carried out to facilitate staining by immersing the slides in citrate buffer (pH 6.0) and microwaving at 90-100°C for 10 minutes. Slides were then quenched in 3% hydrogen peroxidase for 5 minutes to inactivate endogenous peroxidase activity. Sections were incubated with primary antibodies for 60 minutes at 25°C. We used commercial antibodies against CD34 (1 : 2000, monoclonal, Leica Biosystems, Newcastle, UK), D2-40 (1 : 100, Biocare Medical, Concord, CA, USA), and VEGFR-3 (1 : 200, monoclonal, Leica Biosystems). Negative controls were obtained by omitting the primary antibodies.

Microvessel and lymphatic vessel counting

All scoring and interpretations of immunohistochemical results were done by one examiner without knowledge of the clinical data. Any discrete areas staining for CD34 or D2-40, whether they were single endothelial cells or clusters of endothelial cells (regardless of the presence or absence of a lumen that was separate from adjacent vessels, tumor cells, or connective tissue elements) were counted as individual microvessels (CD34 staining) or lymphatic vessels (D2-40 staining). We used hot spot analysis because it was reported to be the most sensitive method.¹⁷ Sec-

tions were scanned at low magnification ($\times 40$) and the areas with the highest concentration of microvessels (vascular hot spot) were selected in tumors and in peritumoral parenchyma. When the surrounding nonneoplastic thyroid tissue was examined, areas with intermediate cellularity were selected and fields with lymphocytic infiltration or fibrosis were avoided. In selected areas, five high magnification fields ($\times 400$) were evaluated with an Olympus BX51 microscope (Olympus, Tokyo, Japan) and we calculated the average of all counted microvessels or lymphatic vessels (mean microvessel density, mean MVD/field or mean lymphatic vessel density, mean LVD/field).

Assessment of VEGFR-3 staining

To determine VEGFR-3 immunostaining scores, we used a previously proposed design with slight modifications.¹²⁻¹⁴ In single representative tissue sections for each case, the area with the highest intensity of staining and the highest percentage of follicular cells that were positive for VEGFR-3 was chosen to assess the positive reactions. Stain intensity was graded as follows: 0 (negative); 1 (weakly positive); 2 (moderately positive); 3 (strong positive). The percentage of positive cells was assessed by manual counting. Each sample field was assigned a value from 0 to 3 (0, negative; 1, < 5% of the cells with positive staining; 2, between 5 to 50% of the cells with positive staining; 3, more than 50% of the cells with positive staining). The sum of scores of intensity and percentage was recorded as 0-6.

Statistical analysis

We used the SAS ver. 9.1 (SAS Institute Inc., Cary, NC, USA) for statistical analysis. The χ^2 test was used in analyzing the following correlations: correlation of clinical variables (sex, age, multicentricity, T classification, extrathyroidal extension, N stage, recurrence, distant metastasis, and stage) between PTMC and PTC groups and between the two size subgroups of PTMCs (1-5 mm vs 6-10 mm). The association of MVD, LVD and VEGFR-3 expression with clinical parameters (age, multicentricity, extrathyroidal extension, nodal metastasis, and recurrence) was analyzed by logistic regression. Differences in MVD, LVD, and VEGFR-3 expression between PTMC and PTC and between the two subgroups of PTMC were analyzed by T test or Wilcoxon Rank Sum test. Pearson's correlation coefficient was used to evaluate the relationship between MVD and LVD. To assess the relationship between MVD, LVD, and VEGFR-3, Spearman's correlation coefficient was used. A p-value of less than 0.05 was

regarded as statistically significant.

RESULTS

Clinicopathological characteristics of patients

Clinical characteristics of all 197 patients at the time of diagnosis are summarized in Table 1. Significant differences between the PTMC and PTC group were found in T classification, extrathyroidal extension, and nodal metastasis. In the PTMC group, two patients had recurrent tumors; one showed involvement of lymph nodes; the other showed involvement of the trachea. In the PTC group, three patients had recurrent tumors involving lymph nodes only, which were diagnosed 14-133 months after the diagnosis of the primary tumor. One patient showed a recurrent tumor involving larynx and lymph nodes, which was diagnosed 102 months after the diagnosis of the primary tumor. Two patients in the PTC group (1.8%) showed distant metastasis at the time of diagnosis; one involved the mediastinum, the other involved the lungs.

Table 2 shows the clinical characteristics of the two PTMC subgroups (1-5 mm and 6-10 mm). The proportion of cases with multicentric tumor, extrathyroidal extension, nodal metastasis, and recurrence was higher in the larger tumor size group, but

Table 1. Clinical characteristics of patients with PTMC and PTC

Patient characteristics	PTMC (n = 84)	PTC (n = 113)	p-value
Women	76 (90.5)	99 (87.6)	0.527
Age \geq 45 (yr)	44 (52.4)	57 (50.4)	0.787
Multicentricity	45 (53.6)	55 (48.7)	0.446
T classification			< 0.0001
T1	36 (42.9)	9 (8.0)	
T2	0 ^a	6 (5.3)	
T3	46 (54.8)	85 (75.2)	
T4	2 (2.4)	13 (11.5)	
Extrathyroidal extension	49 (58.3)	97 (85.8)	< 0.0001
Nodal metastasis	35 (41.7)	78 (69.0)	0.0001
Recurrence	2 (2.4)	4 (3.5)	0.639
Distant metastasis	0	2 (1.8)	0.220
Stage			0.154
I	53 (63.1)	58 (51.3)	
II	0 ^a	3 (2.7)	
III	25 (29.8)	37 (32.7)	
IV	6 (7.1)	15 (13.3)	

Values are presented as number (%).

^aPTMC does not fit the requirements of T2 or stage II because of its size below 1 cm.

PTMC, papillary thyroid microcarcinoma; PTC, papillary thyroid carcinoma of size > 1.0 cm.

none of these clinical variables showed a statistically significant difference.

Microvessel density

Numerous CD34 positive microvessels were detected throughout the tumor in PTMC and PTC cases (Fig. 1A). The microvessel number ranged between 32.7 and 176.4 (mean, 115.52 ± 32.66) in PTMCs; it ranged between 64.8 and 211.5 (mean, 124.65 ± 38.67) in PTCs. The difference was not statistically significant ($p = 0.327$). The larger and smaller tumor size subgroups in the PTMC group did not show a significant MVD difference (mean, 118.30 ± 36.54 vs 107.90 ± 17.95 ; $p = 0.450$). In peritumoral normal thyroid tissue, microvessels were scattered in spaces between thyroid follicles, which ranged between 17 and 61 (mean, 38.09 ± 12.31) in PTMCs and between 17 and 58 (mean, 33.48 ± 11.26) in PTCs. Compared with normal

thyroid tissues, tumor tissues had a markedly increased MVD ($p < 0.01$). The correlations of MVD with clinical parameters for the PTMC and PTC groups are summarized in Tables 3 and 4. The correlation of MVD with clinical parameters in all 60 patients revealed a strong tendency towards increased MVD in tumors with lymph node metastasis, which was not statistically significant ($p = 0.051$) (Table 3). The correlation of MVD with clinical parameters was not significant for either the PTMC group or the PTC group (Table 4).

Lymphatic vessel density

The irregular, thin walled, D2-40 positive, lymphatic vessels were clearly distinguished from blood vessels, which were round in shape and showed a positive reaction with CD34 antibody. Lymphatic vessels were mainly localized in peripheral areas of tumors and tumor infiltration into adjacent thyroid tissue or

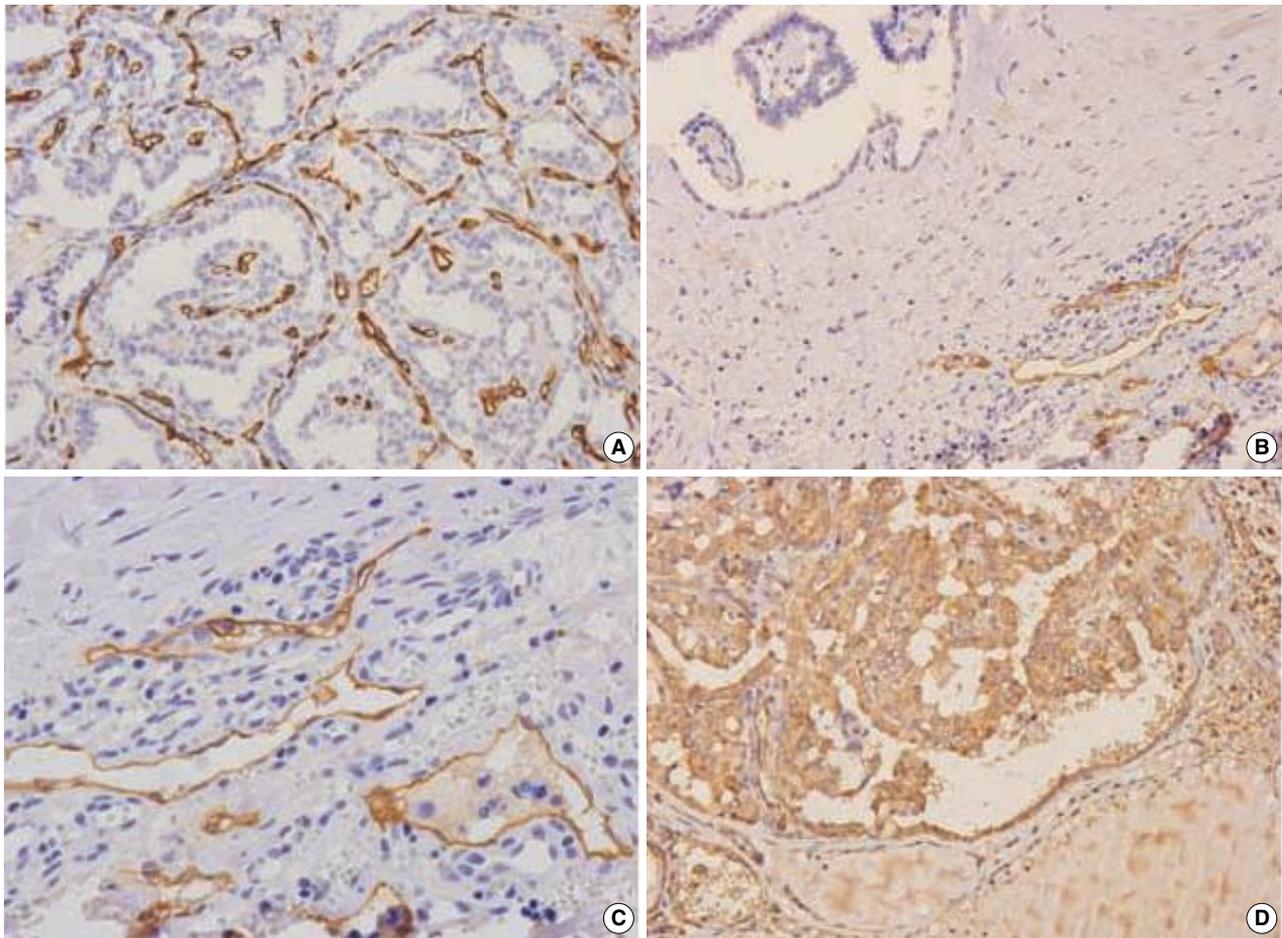


Fig. 1. Microvessel and lymphatic vessel density and vascular endothelial growth factor receptor-3 (VEGFR-3) expression in papillary thyroid carcinoma (PTC) and papillary thyroid microcarcinoma (PTMC). (A) CD34 positive microvessels in PTC, (B, C) low and high magnification of D2-40 positive lymphatic vessels in PTMC, (D) VEGFR-3 positive tumor cells in PTC.

Table 2. Clinical characteristics of patients according to tumor size in papillary thyroid microcarcinoma

Patient characteristics	0-5 mm (n = 10)	6-10 mm (n = 74)	p-value
Women	8 (80)	68 (91.9)	0.229
Age ≥ 45 (yr)	7 (70)	37 (50)	0.234
Multicentricity	4 (40)	40 (54.1)	0.403
T classification			0.174
T1	7 (70)	29 (39.2)	
T2	0	0	
T3	3 (30)	43 (58.1)	
T4	0	2 (2.7)	
Extrathyroidal extension	4 (40)	46 (62.2)	0.180
Nodal metastasis	3 (30)	32 (43.2)	0.425
Recurrence	0	2 (2.7)	0.598
Distant metastasis	0	0	0.220
Stage			0.222
I	6 (60)	47 (63.5)	
II	0	0	
III	2 (20)	23 (31.1)	
IV	2 (20)	4 (5.4)	

Values are presented as number (%).

Table 3. Correlations of MVD, LVD, and VEGFR-3 with clinical parameters in all 60 patients

	MVD	LVD	VEGFR-3
Age ≥ 45 (yr)			
Yes	118.11 ± 35.49	12.43 ± 4.18	3.45 ± 1.53
No	126.01 ± 37.22	8.87 ± 3.74	3.87 ± 1.06
p-value	0.497	0.006	0.944
Multicentricity			
Yes	122.61 ± 37.68	12.60 ± 4.50	4.11 ± 1.45
No	117.88 ± 34.88	10.61 ± 4.03	3.72 ± 1.40
p-value	0.997	0.077	0.255
Extrathyroidal extension			
Yes	122.33 ± 32.27	11.26 ± 3.53	3.83 ± 1.51
No	114.85 ± 43.48	12.19 ± 5.87	4.06 ± 1.21
p-value	0.988	0.340	0.656
Nodal metastasis			
Yes	128.90 ± 35.96	11.18 ± 4.48	4.06 ± 1.34
No	110.02 ± 33.40	11.95 ± 4.20	3.71 ± 1.51
p-value	0.051	0.474	0.576
Recurrence			
Yes	128.87 ± 40.77	12.28 ± 3.21	3.00 ± 2.45
No	119.11 ± 35.48	11.46 ± 4.46	4.00 ± 1.26
p-value	0.413	0.620	0.136

MVD, microvessel density; LVD, lymphatic vessel density; VEGFR-3, vascular endothelial growth factor receptor-3 immunostain score.

perithyroidal soft tissue (Fig. 1B, C). Papillary cores showed extremely rare lymphatic vessels compared with intratumoral fibrous septae, which showed several lymphatic vessels. They ranged between 3.3 and 19.6 vessels (mean, 11.37 ± 4.32) in PTMCs and between 4 and 20.8 (mean, 11.71 ± 4.42) in PTCs; neither difference was statistically significant (p = 0.768). The

Table 4. Correlations of MVD with clinical parameters in PTMC and PTC

	PTMC (mean ± SD)	PTC (mean ± SD)
Age ≥ 45 (yr)		
Yes	113.33 ± 32.80	123.58 ± 38.42
No	124.28 ± 33.51	127.17 ± 41.47
p-value	0.843	0.860
Multicentricity		
Yes	122.33 ± 26.16	122.98 ± 50.52
No	107.75 ± 38.31	125.77 ± 29.92
p-value	0.779	0.828
Extrathyroidal extension		
Yes	121.19 ± 26.81	123.37 ± 37.15
No	104.19 ± 41.30	128.18 ± 45.10
p-value	0.358	0.366
Nodal metastasis		
Yes	126.51 ± 34.23	131.01 ± 38.33
No	104.53 ± 27.90	116.35 ± 39.01
p-value	0.180	0.166
Recurrence		
Yes	128.20 ± 26.02	129.20 ± 50.45
No	114.62 ± 33.28	123.95 ± 37.75
p-value	0.810	0.405

MVD, microvessel density; PTMC, papillary thyroid microcarcinoma; PTC, papillary thyroid carcinoma of size > 1.0 cm.

PTMC subgroup with larger sized tumors showed significantly higher numbers of LVDs compared with the subgroup with smaller sized tumors (mean, 12.30 ± 4.06 vs 8.83 ± 4.20; p = 0.049). Peritumoral normal thyroid tissue showed a few scattered lymphatic vessels between thyroid follicles, which ranged between 1 and 6 (mean, 3.09 ± 1.62) in PTMCs and between 1 and 12 (mean, 3.95 ± 2.50) in PTCs. The correlation of LVD with clinical parameters in PTMC and PTC are summarized in Tables 3 and 5. The correlation of LVD with clinical parameters in all 60 patients revealed that patients over age 45 have significantly more LVDs (p = 0.006) (Table 3). The correlation of LVD with clinical parameters in PTMC subgroups and in the PTC group showed a statistically significant relationship between age and LVD only for the PTC group (p = 0.006) (Table 5). Multicentricity of the tumor showed a suggestive correlation with LVD only in analysis of combined PTMC and PTC cases analysis (p = 0.077) (Table 3) and extrathyroidal extension showed a suggestive correlation with LVD only in the PTC group (p = 0.076) (Table 5).

Vascular endothelial growth factor receptor-3

Positive VEGFR-3 staining was found in the cytoplasm of follicular cells of the tumors (Fig. 1D). No positive staining was observed in normal tissue surrounding the tumor. The mean score for VEGFR-3 positive reactions in tumor cells of PTMCs

Table 5. Correlations of LVD with clinical parameters in PTMC and PTC

	PTMC (mean ± SD)	PTC (mean ± SD)
Age ≥ 45 (yr)		
Yes	11.97 ± 4.13	12.95 ± 4.29
No	8.98 ± 4.60	8.80 ± 3.36
p-value	0.090	0.006
Multicentricity		
Yes	12.29 ± 4.48	13.01 ± 4.68
No	10.32 ± 4.03	10.84 ± 4.14
p-value	0.221	0.428
Extrathyroidal extension		
Yes	11.52 ± 3.63	11.03 ± 3.51
No	11.09 ± 5.67	13.56 ± 6.21
p-value	0.832	0.076
Nodal metastasis		
Yes	11.28 ± 4.94	11.10 ± 4.18
No	11.47 ± 3.77	12.52 ± 4.75
p-value	0.710	0.570
Recurrence		
Yes	13.75 ± 0.78	11.55 ± 3.85
No	11.20 ± 4.42	11.73 ± 4.57
p-value	0.472	0.955

LVD, lymphatic vessel density; PTMC, papillary thyroid microcarcinoma; PTC, papillary thyroid carcinoma of size > 1.0 cm.

and PTCs were 4.13 ± 1.22 and 3.67 ± 1.58 , respectively; the difference was not statistically significant ($p = 0.174$). Among PTMCs, the larger and smaller size groups did not show a significant difference (mean, 4.18 ± 1.18 vs 4.00 ± 1.41 ; $p = 0.862$). The correlation of VEGFR-3 staining with clinical parameters in PTMC and PTC groups are summarized in Tables 3 and 6. The correlation of VEGFR-3 with clinical parameters in all 60 patients revealed no statistically significant relationship (Table 3). The correlation of VEGFR-3 with clinical parameters in the PTMC group alone or in the PTC group alone showed a statistically significant inverse relationship between disease recurrence and VEGFR-3 expression in the PTMC group ($p = 0.008$) (Table 6).

Mutual association of MVD, LVD, and VEGFR-3

When analyzing associations between MVD, LVD, and VEGFR-3 expression, there were no significant correlations among these three variables.

DISCUSSION

Differences in clinicopathological characteristics of PTCs and in PTMCs grouped according to the size of the tumor are well

Table 6. Correlations of VEGFR-3 with clinical parameters in PTMC and PTC

	PTMC (mean ± SD)	PTC (mean ± SD)
Age ≥ 45 (yr)		
Yes	4.04 ± 1.30	3.76 ± 1.79
No	4.50 ± 0.84	3.44 ± 1.01
p-value	0.118	0.993
Multicentricity		
Yes	4.00 ± 1.37	4.25 ± 1.60
No	4.29 ± 1.07	3.28 ± 1.49
p-value	0.864	0.133
Extrathyroidal extension		
Yes	4.00 ± 1.26	3.68 ± 1.73
No	4.40 ± 1.17	3.63 ± 1.19
p-value	0.573	0.530
Nodal metastasis		
Yes	4.10 ± 1.39	4.06 ± 1.34
No	4.20 ± 1.08	3.15 ± 1.77
p-value	0.593	0.121
Recurrence		
Yes	2.00 ± 2.83	3.50 ± 2.52
No	4.29 ± 0.98	3.69 ± 1.46
p-value	0.008	0.793

VEGFR-3, vascular endothelial growth factor receptor-3 immunostain score; PTMC, papillary thyroid microcarcinoma; PTC, papillary thyroid carcinoma of size >1.0 cm.

known.^{1-3,5,6,18,19} In our study, the PTC group showed a higher incidence of extrathyroidal extension and nodal metastasis compared with the PTMC group, which could account for the difference in T classification. In contrast, PTMCs did not show any significant differences in clinicopathological variables with respect to tumor size (0-5 mm and 6-10 mm). These findings are different from findings from previous studies, in which occult papillary carcinomas of the thyroid (less than 5 mm in size) showed different features compared with larger ones and were considered as a normal finding with no need for further treatment.²⁰ In a recent large scale study of 2,678 cases of papillary carcinoma of the thyroid gland in the Korean population (50.6% were PTMCs), PTMC cases showed lymph node metastasis, recurrence, and distant metastasis in 26.9%, 0.9%, and 0.5%, respectively.² In comparison, PTMCs in the present study showed a slightly higher rate of lymph node metastasis (41.7%) and recurrence (2.4%). In their study, small carcinomas (1-2 cm) showed a higher incidence of extracapsular invasion and lateral neck node metastasis compared with PTMCs, which was similar to results from the present study in spite of the difference in size of the PTCs analyzed in each study.

To identify lymphatic vessels, D2-40 antibody was used in this study, which originally reacts with an O-linked sialoglycoprotein (MW 40K) found on lymphatic endothelium, fetal testis,

and on the surface of testicular germ cell tumors. It is a selective marker of lymphatic endothelium in normal tissues and vascular lesions such as lymphangiomas and is distinct from other vessel markers including CD34, CD31, and factor VIII in terms of responding antigen. CD34 is a single chain transmembrane glycoprotein that is selectively expressed on human lymphoid and myeloid hematopoietic progenitor cells as well as on vascular endothelium. CD31 is expressed on the surface of platelets, monocytes, granulocytes, and B cells, and at the endothelial intracellular junction. On the other hand, blood coagulation factor VIIIa is the last enzyme generated in the blood coagulation cascade, which shows mainly large blood vessels on immunostaining. The irregular, thin walled, D2-40 positive, lymphatic vessels were clearly distinguished from the round, CD34-positive, blood vessels in this study.

The present study showed markedly increased CD34 positive microvessels in tumors compared with peritumoral normal thyroid tissue. Although various antibodies for microvessels (CD34, factor VIII, VEGFR) were used in previous studies, they all had similar findings.²¹⁻²⁴ The relationship between MVD and clinical parameters for prognosis have been studied and showed controversial results, such as lower MVD and poor differentiation of tumors, increased MVD in patients with metastasis in lymph nodes or locoregional tumor recurrences, shorter recurrence and free survival in hypervascular tumors, or increased MVD and improved survival in certain histological types.^{21,23-26} These contradictory outcomes could be partly explained by different methods of assessing MVD and/or by differences in analyzing conditions. In this study, there was a suggestive relationship between MVD and lymph node metastasis in combined PTMC and PTC groups with no significant correlation in separated PTMC and PTC groups. The significance of these findings is uncertain at this point and should be confirmed with further studies.

In a large cohort study on angiogenesis and lymphangiogenesis of 191 thyroid proliferative lesions by de la Torre *et al.*,¹⁰ PTMCs had higher MVDs and LVDs than PTCs or other benign and malignant proliferative lesions. They did not find any correlation between lymph node metastasis and MVD or LVD in PTCs or PTMCs. In contrast, another study, one by Liang *et al.*,¹¹ showed lower MVDs and LVDs in PTMCs compared with PTCs. Compared with the contradictory results in those two previous studies, the present study showed no significant difference in MVD or LVD between PTMCs and PTCs. Since these findings suggest that PTMCs and PTCs have a similar tendency for MVD-associated or LVD-associated lymph node metastasis, the difference in nodal metastasis between PTMCs and PTCs noted in this study

must be due to other factors, such as size of the tumor, or to an unknown functional difference in microvessels or lymphatic vessels associated with lymph node metastasis. In addition, significantly higher LVDs in PTMCs with larger tumors compared with those with smaller tumors in this study was not correlated with any differences in clinical characteristics.

In contrast to a strong association between the presence of intratumoral lymphatics and regional lymph node metastasis in other previous studies, Padera *et al.*²⁷ emphasized the functional aspect of lymphatic vessels located at the peripheral portion of the tumor in lymphatic spread of tumor cells.^{11,28} Similar localization of lymphatic vessels in peripheral areas of the tumor and infiltrating lesions into adjacent thyroid tissue or perithyroidal soft tissue was also noted in this study.

Many studies found that old age predicted an unfavorable outcome on survival in thyroid papillary carcinoma patients.^{29,30} Siironen *et al.*¹² observed a correlation between poor prognosis with age > 45, tumor size > 4 cm, extrathyroidal extension, nodal metastasis, distant metastasis, and stage IV, and suggested a possible relationship of poor prognosis with the high VEGF-C expression noticed in patients over 45. According to previous studies that searched for prognostic factors in PTMC, age was not a significant independent predictor.^{4,5,18} In the present study, only the PTC group showed a significantly higher LVD in patients older than 45 compared with younger patients. A possible role for LVD in extrathyroidal extension was suggested in this study. In addition, although PTMC and PTC groups did not show any correlation between LVD and multicentricity, the combined PTMC and PTC groups showed suggestive correlations.

Previous studies on VEGF and VEGFR-3 showed variable results such as a correlation between nodal metastasis and increased LVD and VEGF-D expression, a positive correlation between VEGF-D immunoreactivity and increased LVD, and a positive correlation between VEGF-C expression and VEGFR-3 positive vessel density, lymph node metastasis, and lymphovascular permeation.^{9,11,15,16,22} The present study did not show any significant relationship between VEGFR-3 positive reactions of tumor cells and clinical parameters in PTCs. In contrast, PTMC cases with disease recurrence had a lower VEGFR-3 expression compared with PTMC cases without recurrence. Since this study included only two cases of PTMC with recurrence, the significance of this finding should be confirmed by further studies with more cases. The additional combined study on VEGFs and VEGFR-3 will be helpful for a better understanding of angiogenesis and lymphangiogenesis in papillary carcinoma of the thyroid gland.

In conclusion, since the correlation between clinical parameters

and anatomic parameters of papillary carcinoma of the thyroid gland showed variable results depending on factors used in the analysis, treatment of patients should be based on the combination of clinical presentation and all available tumor risk factors.

REFERENCES

- Pellegriti G, Scollo C, Lumera G, Regalbutto C, Vigneri R, Belfiore A. Clinical behavior and outcome of papillary thyroid cancers smaller than 1.5 cm in diameter: study of 299 cases. *J Clin Endocrinol Metab* 2004; 89: 3713-20.
- Lee J, Yun JS, Nam KH, Chung WY, Soh EY, Park CS. Papillary thyroid microcarcinoma: clinicopathologic characteristics and treatment strategy. *J Korean Surg Soc* 2007; 72: 276-82.
- Lee J, Rhee Y, Lee S, *et al.* Frequent, aggressive behaviors of thyroid microcarcinomas in Korean patients. *Endocr J* 2006; 53: 627-32.
- Kim TY, Hong SJ, Kim JM, *et al.* Prognostic parameters for recurrence of papillary thyroid microcarcinoma. *BMC Cancer* 2008; 8: 296.
- Roti E, Rossi R, Trasforini G, *et al.* Clinical and histological characteristics of papillary thyroid microcarcinoma: results of a retrospective study in 243 patients. *J Clin Endocrinol Metab* 2006; 91: 2171-8.
- Chow SM, Law SC, Chan JK, Au SK, Yau S, Lau WH. Papillary microcarcinoma of the thyroid: prognostic significance of lymph node metastasis and multifocality. *Cancer* 2003; 98: 31-40.
- He Y, Karpanen T, Alitalo K. Role of lymphangiogenic factors in tumor metastasis. *Biochim Biophys Acta* 2004; 1654: 3-12.
- Stacker SA, Williams RA, Achen MG. Lymphangiogenic growth factors as markers of tumor metastasis. *APMIS* 2004; 112: 539-49.
- Yasuoka H, Nakamura Y, Zuo H, *et al.* VEGF-D expression and lymph vessels play an important role for lymph node metastasis in papillary thyroid carcinoma. *Mod Pathol* 2005; 18: 1127-33.
- de la Torre NG, Buley I, Wass JA, Turner HE. Angiogenesis and lymphangiogenesis in thyroid proliferative lesions: relationship to type and tumour behaviour. *Endocr Relat Cancer* 2006; 13: 931-44.
- Liang QC, Wei QY, Fan SQ. Expression of VEGF-C and angiogenesis, and lymphangiogenesis in papillary thyroid carcinoma. *Zhong Nan Da Xue Xue Bao Yi Xue Ban* 2006; 31: 414-6, 9.
- Siironen P, Ristimäki A, Narko K, *et al.* VEGF-C and COX-2 expression in papillary thyroid cancer. *Endocr Relat Cancer* 2006; 13: 465-73.
- Klein M, Vignaud JM, Hennequin V, *et al.* Increased expression of the vascular endothelial growth factor is a pejorative prognosis marker in papillary thyroid carcinoma. *J Clin Endocrinol Metab* 2001; 86: 656-8.
- Jebrael A, England J, Bedford K, Murphy J, Karsai L, Atkin S. Vascular endothelial growth factor (VEGF), VEGF receptors expression and microvascular density in benign and malignant thyroid diseases. *Int J Exp Pathol* 2007; 88: 271-7.
- Shushanov S, Bronstein M, Adélaïde J, *et al.* VEGF_C and VEGFR3 expression in human thyroid pathologies. *Int J Cancer* 2000; 86: 47-52.
- Yu XM, Lo CY, Chan WF, Lam KY, Leung P, Luk JM. Increased expression of vascular endothelial growth factor C in papillary thyroid carcinoma correlates with cervical lymph node metastases. *Clin Cancer Res* 2005; 11: 8063-9.
- Wong NA, Willott J, Kendall MJ, Sheffield EA. Measurement of vascularity as a diagnostic and prognostic tool for well differentiated thyroid tumours: comparison of different methods of assessing vascularity. *J Clin Pathol* 1999; 52: 593-7.
- Besic N, Pilko G, Petric R, Hocevar M, Zgajnar J. Papillary thyroid microcarcinoma: prognostic factors and treatment. *J Surg Oncol* 2008; 97: 221-5.
- Siironen P, Louhimo J, Nordling S, *et al.* Prognostic factors in papillary thyroid cancer: an evaluation of 601 consecutive patients. *Tumour Biol* 2005; 26: 57-64.
- Harach HR, Franssila KO, Wasenius VM. Occult papillary carcinoma of the thyroid: a "normal" finding in Finland. A systematic autopsy study. *Cancer* 1985; 56: 531-8.
- Akslen LA, Livolsi VA. Increased angiogenesis in papillary thyroid carcinoma but lack of prognostic importance. *Hum Pathol* 2000; 31: 439-42.
- Bunone G, Vigneri P, Mariani L, *et al.* Expression of angiogenesis stimulators and inhibitors in human thyroid tumors and correlation with clinical pathological features. *Am J Pathol* 1999; 155: 1967-76.
- Dhar DK, Kubota H, Kotoh T, *et al.* Tumor vascularity predicts recurrence in differentiated thyroid carcinoma. *Am J Surg* 1998; 176: 442-7.
- Goldenberg JD, Portugal LG, Wenig BL, Ferrer K, Wu JC, Sabnani J. Well-differentiated thyroid carcinomas: p53 mutation status and microvessel density. *Head Neck* 1998; 20: 152-8.
- Fontanini G, Vignati S, Pacini F, Pollina L, Basolo F. Microvessel count: an indicator of poor outcome in medullary thyroid carcinoma but not in other types of thyroid carcinoma. *Mod Pathol* 1996; 9: 636-41.
- Herrmann G, Schumm-Draeger PM, Muller C, *et al.* T lymphocytes, CD68-positive cells and vascularisation in thyroid carcinomas. *J Cancer Res Clin Oncol* 1994; 120: 651-6.
- Padera TP, Kadambi A, di Tomaso E, *et al.* Lymphatic metastasis in the absence of functional intratumor lymphatics. *Science* 2002; 296: 1883-6.
- Hall FT, Freeman JL, Asa SL, Jackson DG, Beasley NJ. Intratumoral lymphatics and lymph node metastases in papillary thyroid carcinoma. *Arch Otolaryngol Head Neck Surg* 2003; 129: 716-9.
- Cushing SL, Palme CE, Audet N, Eski S, Walfish PG, Freeman JL.

Prognostic factors in well-differentiated thyroid carcinoma. *Laryngoscope* 2004; 114: 2110-5.

30. Jung TS, Kim TY, Kim KW, *et al.* Clinical features and prognostic

factors for survival in patients with poorly differentiated thyroid carcinoma and comparison to the patients with the aggressive variants of papillary thyroid carcinoma. *Endocr J* 2007; 54: 265-74.