Papillary thyroid carcinoma involving cervical neck lymph nodes: correlations with lymphangiogenesis and ultrasound features

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Abstract. Stratification of risk factors for cervical lymph node metastasis (LNM) in thyroid papillary carcinoma is important for providing standards for post-operative adjuvant radio-iodine therapy and for patient prognosis. We investigated pathological factors based on the lymphatic vessel system and radiological features associated with tumor with cervical neck LNM. Among patients who had undergone thyroidectomy confirmed to be papillary thyroid carcinoma, we selected 126 age-sex matched paired patients without cervical LNM (group 1) and with LNM (group 2) to evaluate risk factors. Pathological factors evaluated were size, multiplicity, and extra thyroid extension state, based on the pathological reports using stored data. The lymphatic vessel density (LVD) of each tumor was evaluated by staining for VEGFR-3 and D2-40 and correlated with cervical LNM state. Malignant ultrasound features were evaluated to compare the differences between these two groups. Larger tumor size, multiplicity, extrathyroid extension were more common in group 2 (*p*<0.05). The median percentage of VEGFR-3 for group 1 was 20 (range 0-30) and D2-40 was 13 (range 7-23) while for group 2, VEGFR-3 was 80 (70-90) and D2-40 was 78 (54-114). LVD measured by intratumoral D2-40 staining was 20.6% and 79.4% for group 1 and group 2, respectively. Intra-tumoral lymphatics measured by D2-40 stain had a strong correlation with cervical LNM (Odds 1.230, CI 1.01.-1.499 *p* value 0.040). Ultrasound (US) features had no significant differences between the two groups although calcifications tended to be higher in group 2 (84% *vs.* 76% *p*=0.264). Lymphatic vessel density and nodule echogenicity were not associated with LNM. Intratumoral lymphangiogenesis was most strongly associated with LNM and thus, could be a useful predictive marker for cervical LNM.

Key words: Lymphangiogenesis, Papillary thyroid cancer, Ultrasonography, Pathology-thyroid, Thyroid cancer

PAPILLARY THYROID CANCER (PTC) is known to have a high overall survival rate with 5-20% local or regional recurrences [1-5]. Risk stratification has been investigated as a way to minimize the extent of surgery and exposure to high dose iodine therapy [6-9]. Patient age, tumor subtype, extra thyroid extension (ETE), and cervical lymph node metastasis (LNM), are known risk factors associated with increased recurrence of PTC [8, 10-13]. Patients with cervical LNM at the time of surgery should undergo lateral neck dissection and consider high dose iodine therapy, even though it can lower a patient's quality of life. Furthermore, patients with cervical LNM have an increased chance of recurrences even after the high dose iodine therapy [10, 14]. Thus, improved prediction of cervical LNM and understanding of the factors affecting LNM would have a strong clinical impact. We designed the current

study to evaluate suspected risk factors and focused on the pathological features, especially in lymphatic vessel systems, among the others in thyroid PTC. Since the degree of tumor lymphangiogenesis may theoretically be related to the risk for LNM, we sought to evaluate the relationships between cervical LNM status and lymphatic vascular density (LVD) using Podoplamin (D2-40) intratumorally and peritumorally, and vascular endothelial growth factor receptor-3 (VEGFR-3) expression. We attempted to identify malignant ultrasound (US) feature that were associated with cervical LNM, since US is a highly sensitive and specific imaging modality used in the diagnosis of thyroid malignancy.

Materials and Methods

Patient Selection

Institutional review board approval was obtained for this retrospective study in Kang buk Samsung Hospital, Sungkyunkwan University College of Medicine in Seoul, Korea. All participants gave their written informed consent. The study samples were collected consecutively from the patient data of January 2007 to December 2010. The data consisted of 1012 patients who had undergone thyroid cancer surgery in our institution, Kangbuk Samsung Hospital (Seoul, Korea) (age range: 16-83 years; mean: 51 years). Ninety-one patients subsequently underwent total thyroidectomy with central and lateral neck dissection, 371 underwent a total thyroidectomy with central node dissection alone, 455 had a total thyroidectomy or lobectomy with ipsilateral central neck dissection, and 95 patients underwent only lobectomy, without nodal dissection. Based on the pathology of the dissection results, the patients were divided into four groups: no lymph node submitted to pathology group, no central LNM group, central LNM only group, and both central and lateral LNM group. When cases were matched to controls by age and sex, a total of 126 patients were selected to make up two groups, group 1 with no LNM (n=63) and group 2 with both central and lateral LNM (n=63). When multiple nodules were present, we selected the larger main mass to evaluate the lymphatic vessel and US features. Immunohistochemical staining for D2-40 and VEGFR-3, which specifically stains the lymphatic vessel, was done to evaluate lymphatic vessel systems for these patients. US characteristics of the main malignant mass were evaluated in the selected 126 patients. Our series was comprised of PTC of the conventional form only. All patients had their surgical resections done by the same surgeon (YJS) and the diagnoses were categorized according to World Health Organization classification by one of the authors (KDH).

Immunohistochemistry

Blinded to the final report of LNM state, 126 thyroid tissue samples were analyzed for immunohistochemical expression of D2-40 and VEGFR-3 using a mouse monoclonal D2-40 clone (Cell Marque, Rocklin, CA, USA) specific antibody and mouse monoclonal antibody for VEGFR-3 (Novocastra, Benton Lane, Newcastle, UK). Expression levels of D2-40 expression in intra- and peritumoral neoplastic areas were quantified and compared. Determination of LVD assessed by immunostaining for podoplanin was performed as suggested by Weidner *et al.* [15]. After scanning the immunostained section at low magnification (40X), the area of tissue with the greatest number of distinctly highlighted micro-vessels, or "hotspot," was selected. LVD was then determined by counting all immune-stained vessels at a total magnification of 200X from total areas for each case. LVD was defined as a single endothelial cell or a cluster of endothelial cells positive for D2-40, located near a visible lumen clearly separate from adjacent micro-vessels and other connective tissue components. The numbers of lymphatic spaces counted were divided by the area (total number of lymphatic spaces/cm^2). Additionally, as lymphatic vessels can generally appear as distorted and overlapped structures in a cancer setting, packed vessels were assumed to be one lymphatic unit. Determination of the staining reaction was strictly confined to the hotspots. Fig. 1 demonstrates an immunohistochemical reaction for D2-40 intratumorally, Fig. 2 demonstrates peritumoral reactions for D2-40 in thyroid cancer, and was considered positive when the staining was visible in the cytoplasm of the lymphatic endothelial cells (Figs. 1, 2).

The lymphatic molecular marker VEGFR-3 was analyzed in all 126 cases and instances of positive expression of VEGFR-3 were assessed. Micro-vessel counts were performed by one pathologist, blinded to the patient's pathologic and clinical status. The mean value of micro-vessel densities was calculated for each patient blindly. The following grading system was used: weak expression <30%, and moderate to strong >30%. Fig. 3 demonstrates strong diffuse cytoplasmic staining of VEGFR-3 within thyroid nodule.
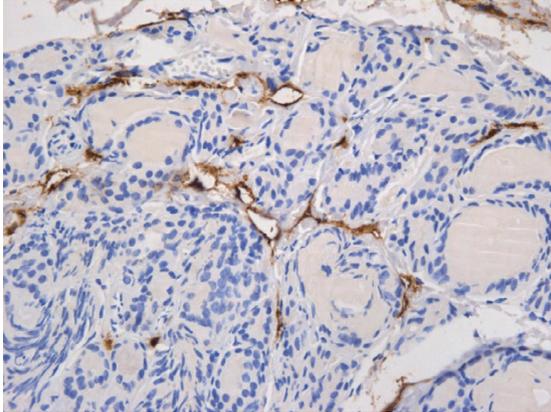
Fig. 1 D2-40 immunostain showed positive for lymphatic spaces in intratumoral area (immunohistochemical staining with DAB, ×400)
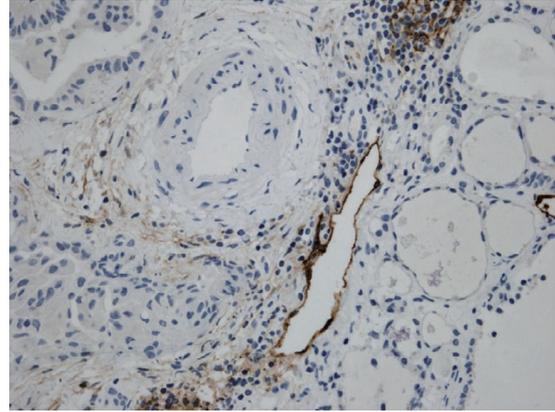

Fig. 2 D2-40 immunostain showed positive for lymphatic spaces in peritumoral area (immunohistochemical staining with DAB, ×400)
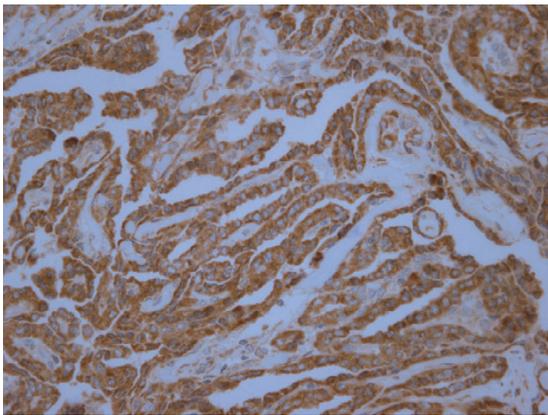

Fig. 3 VEGFR-3 immunostain showed diffuse cytoplasmic staining (immunohistochemical staining with DAB, ×400)

resentative Digital Imaging and Communications in Medicine images. Analysis of the US images included examination and comparisons of echogenicity, orientation, shape, margin, and calcifications. Echogenicity of the solid portion was classified as hypoechogenicity or marked hypoechogenicity. Marked hypoechogenicity was defined as decreased echogenicity as compared to the strap muscles [17]. Orientation was categorized as non-parallel (greater in its anteroposterior dimension than transverse dimension) or parallel. The shape of the nodule was categorized as ovoid to round or irregular (when a nodule was not ovoid to round). Margins were classified as well-defined smooth, microlobulated or spiculated, and indistinct [20]. Calcifications were noted when hyperechoic foci without acoustic shadows were present regardless of size.

Imaging and Imaging Analysis

US features suggestive of malignancy in thyroid nodules have been well established, such as the presence of microcalcifications, hypoechogenicity, irregular margins, and solid composition [16-19] but US features of thyroid malignancy associated with LNM have not been previously reported. A board-certified radiologist with 11 years of experience in thyroid US performed preoperative US examinations of the thyroid lesions and cervical lymph nodes, using a Logiq 9 (General Electric, Milwaukee, WI, USA) with a high-frequency 13 MHz linear transducer. US images were taken using the scanning protocol and in all cases included both transverse and longitudinal real-time imaging of the thyroid nodules with the use of rep-

Statistical Analysis

The statistical significance of differences between Group 1 and Group 2 were determined using *t*-tests and chi-square tests. Comparisons between the two groups were analyzed for nodule size, multiplicity, and extrathyroid extension state using chi-square tests and *t*-tests. The odds ratios (ORs) and 95% confidence intervals (CIs) of each factor and immunochemical stain were calculated using logistic regression analysis. US features of main thyroid malignant nodule were compared between the groups. Also each US feature was analyzed for infra-centimetric malignant nodules and larger sized nodules (>1 cm) and for nodules with lympho-vascular invasion (LVI) and without LVI based on the pathological report. Multivariate logistic

Table 1 Multivariate analysis of risk factors for cervical lymph node metastasis

	Odds Ratio	*p* value	95% Conf. Interval	
			Lower	Upper
Size	0.470	0.647	0.018	11.849
Multiplicity	10.101	0.062	0.886	115.107
ETE	2.977	0.380	0.260	34.069
VEGFR-3 strong	82.146	0.003	4.475	1508.055
D2-40 intra >50%	1.230	0.040	1.010	1.499
D2-40 peri >50%	1.034	0.317	0.968	1.105

VEGFR-3, vascular endothelial growth factor receptor-3; ETE, extrathyroid extension; D2-40 intra, D2-40 intra-tumoral; D2-40 peri, D2-40 peritumoral

Table 2 Distribution of clinical and pathological risk factors of patients with no cervical neck LNM (Group1) compared to patients with LNM (Group 2) in age-sex matched patients

Clinical variables		Cervical LNM status		Univariate analysis *p* value
		Group 1(-) (n=63)	Group 2(+) (n=63)	
Tumor size	<1 cm	46 (73.02)	17 (26.98)	0.000
	>1 cm	17 (26.98)	46 (73.02)	
Multiplicity	single	46 (58.23)	33 (41.77)	0.017
	multiple	17 (36.17)	30 (63.83)	
ETE	No	42 (66.67)	17 (26.98)	0.000
	Yes	21 (33.33)	46 (73.02)	
VEGFR-3	weak	52 (82.54)	5 (7.94)	0.000
	strong	11 (17.46)	58 (92.06)	
D2-40 intra	0-25%	33 (52.38)	0 (0.00)	0.000
	26-50%	26 (41.27)	5 (7.94)	
	51-75%	4 (6.35)	28 (44.44)	
	76-100%	0 (0.00)	30 (47.62)	
D2-40 peri	0-25%	32(50.79)	1(1.59)	0.000
	26-50%	26(41.27)	4(6.35)	
	51-75%	4(6.35)	28(44.44)	
	76-100%	1(1.59)	30(47.62)	

ETE, extrathyroid extension; D2-40 intra, D2-40 intra-tumoral; D2-40 peri, D2-40 peritumoral

regression analysis was done to assess the independent associations of cervical neck LNM with statistically significant predictive factors in univariate anlaysis with adjustments for various prognostic factors (Table 1). Statistical analyses were performed using Stata version 11 (StataCorp LP, College Station, TX, USA). All reported *p* values are 2-tailed, and statistical significance was set at *p*<0.05.

Results

Table 2 summarizes comparisons of the two groups for each factor. Tumor size larger than 1cm, multiple foci of malignant nodules, and extrathyroid extension were all significantly more common in group 2 (*p*<0.05) than in group 1. VEGFR-3, intratumoral D2-40 and peritumoral D2-40 showed statistical significant differences. For group 1, the median percentage of VEGFR-3 was 20 (0-30) and D2-40 was 13 (7-23), while for group 2 the median percentage of VEGFR-3 was 80 (70-90) and D2-40 was 78 (54-114). For the quantification of lymphatic vessel staining for VEGFR-3, the lower quantile was significantly more frequent in group 1 and the higher quantile was more frequently seen in group 2 (26% *vs*. 73.68% *p*<0.001). Intratumoral D2-40 staining was present at the higher

Table3 Comparisons of various ultrasound features between two groups, micropapillary carcinoma, and malignant nodules with LVI

Ultrasound features		Group1/group2 (*p* value)	PMC/PTC (*p* value)
Echogenicity	Hypoechoic	42/41 (0.851)	40/43 (0.573)
	Marked hypo	21/22	23/20
Orientation	Non-parallel	40/40 (1.000)	30/16 (0.010)
	parallel	23/23	33/47
Shape	Oval or round	18/21 (0.563)	17/22 (0.335)
	Irregular	45/42	46/41
Margin	Smooth	5/6 (0.862)	4/7 (0.515)
	Spiculated	11/8	9/10
	Microlobulated	27/30	27/30
	indistinct	20/19	23/16
Calcification	No	15/10 (0.264)	19/6 (0.004)
	yes	48/53	44/57

LVI, lympho-vascular invasion; PMC, papillary microcarcinoma; PTC, papillary thyroid carcinoma

quantile in group 2 (22% *vs*. 77.4% *p*<0.001) and similar results were found in a comparison of the 2 quantiles with peritumoral D2-40 staining in the two groups (20.6% *vs*. 79.4% *p*=0.000). The VEGFR3-positive structures higher than >30% was significantly higher (*p*<0.001) in group 2 (Table 2). Multivariate logistic regression analysis was performed to assess the independent associations of lateral LNM with statistically significant predictive factors in univariate analysis with adjustment for various established clinico-pathological prognostic factors (larger tumor size, multiplicity, and extrathyroid extension) in age-sex matched patients. Table 1 summarizes the odds ratios and confidence intervals of the factors which showed statistical significance in univariate anlaysis. The presence of intratumoral lymphatics more than 50% using D2-40 remained associated with the presence of nodal metastases at presentation (odds 1.23, CI 1.010 -1.499, *p*=0.040) and VEGFR-3 >30% remained significant with odds ratio of 82.146, Confidence interval of 4.475-1508.055, *p* value of 0.003.

There were no significant differences between groups 1 and 2 for US features including echogenicity, orientations, shape, and margin. Although calcifications were found to be higher in group 2, (84% *vs*. 76% *p*=0.264) the difference was not significant. The comparisons of various US features are summarized in Table 3. We also compared US features according to nodule size (with a cut-off of 1 cm). Orientations and calcifications were significantly different (*p*=0.001) in malignancies with size less than 1cm. Smaller nod-

ules more frequently showed a taller than wide feature whereas larger nodules more frequently showed parallel orientations (Table 3). Furthermore, calcifications were much more frequent in the larger group which had rates up to 90% with *p*=0.004. Others showed no statistically significant differences.

Discussion

Clinico-pathological factors

In an attempt to establish a reliable prognostic scoring system in PTC, most published studies have primarily considered clinical factors such as age and sex, tumor size, and extra thyroidal extensions. Younger patients less than 20 years old, patients older than 45 years, and patients with PTC subtypes such as tall-cell, columnar-cell, and diffuse sclerosing variant types are known to have higher risks for recurrences [10-12, 21]. The biological behaviors of well differentiated thyroid malignant nodules were not considered when selecting a treatment strategy.

Since PTC can metastasize *via* the lymphatic channel to a regional cervical lymph node [22-26], and increased LVD is assumed to be one of the most important parameters associated with the aggressive behavior of a malignant tumor [26], we hypothesized that nodules with more LVD would have an increased risk of LNM and it would be clinically useful if immunohistochemical staining that can predict the risk of LNM and US features that correlates with the risk of LNM. We do not know whether the existing tumor

induces lymphangiogenesis or invades peritumoral vessels so, in addition to D2-40, we also analyzed VEGFR-3. Some reports claim that there are relationships between increased intratumoral lymphatics and an association with the spread of tumors to regional lymph nodes [27-30], while, in contrast, there are also published data showing that there are no relationships between the intratumoral LVD related to LNM [31]. Our results showed that higher LVD measured by intratumoral D2-40 of PTC nodules shows significantly increased cervical LNM. Intra tumoral LVD showed high correlations with cervical LNM, making it an important factor indicating tumor aggressiveness in an age- and sex-adjusted setting. When we analyzed these factors in a multivariate setting, both intra-tumoral and VEGFR-3 were independent factors along with the tumor size, multiplicity, and extrathyroid extension. The reason for such high odds ratio of VEGFR-3 compare to the intratumoral D2-40 can be explained by the bias made when grouping the amount of stain using cutoff as 30%. Our data indicates that expression levels of VEGFR-3 and intra-tumoral D2-40 should be considered with respect to tumor aggressiveness and cervical LNM. Pathological features such as larger size, extra-thyroid extension, multiplicity and positive immunostaining for D2-40 and VEGFR-3 to evaluate the LVD can be used as strong predictive prognostic markers and have the potential to play significant roles in pathological routine workup of thyroid cancer cervical LNM.

US features related to high risk of cervical LNM

Thyroid cancer with cervical LNM, showing strong VEGFR-3 staining and increased LVD with D2-40 staining did not show any correlations with assorted US features such as echogenicity, orientation, shape, margin, and calcifications. None of these factors showed any significant differences between the two groups although in a published paper microcalcifications and contact of >25% with the adjacent capsule on US are associated US features with lateral LNM [32,

33]. Our hypothesis was that since water or vessels can show low echogenicity compared to solid mass comprised of compact cells, a tumor with higher LVD would be correlated with low or marked echogenicity, but the observed effect was not statistically significant. We also hypothesized that spiculation or ill defined margins would be seen more often with an aggressive tumor with increased peritumoral LVD invasion, but the results for this factor were also not significant although there are published report showing that ill-defined edges were positively associated with lateral LNM [32]. Our findings show that US features vary according to the size of the malignant nodule, rather than the aggressiveness of the tumor, showing more frequent "taller than wide" appearance in microcarcinoma (p=0.010) and more associated calcifications in PTCs larger than 1 cm. Size is also an important prognostic factor of thyroid cancer [34, 35] but in this study there was no association with cervical neck LNM. Multifocality was not an independent factor of cervical neck LNM although in univariate anlaysis it revealed significant results.

The limitations of this study is that we analyzed clinical and pathological data retrospectively. We used age- and sex-matched pair data in order to concentrate on pathological factors and reduce selection bias. It was impossible to perform staining procedures for all the surgical specimens. We also excluded patients with central LNM because a considerable number of cases showed micrometastasis to the central lymph nodes and such cases have little clinical impact due to the routine surgical removal of the ipsilateral central lymph nodes, but it may be considered as another form of selection bias.

In predicting the outcome, prognosis, and recurrence of thyroid papillary cancer, not only the clinical factors should be considered but also lymphangiogenesis of thyroid nodules since it shows a strong clinical relevance in cervical LNM. D2-40 or VEGFR3 staining can be a predictor of LNM and cases with high D2-40 staining should be carefully followed for lymph node recurrence.

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