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The potential role of MMP-9 and VEGF-C as predictors of lymph node involvement in papillary thyroid carcinoma

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ABSTRACT

Introduction: Most thyroid malignancy is papillary thyroid carcinoma (PTC). However, lymph node metastases occur in 30-40% of PTC patients. Biomarkers to predict cervical lymph node metastases have now begun to be widely varied, such as matrix metalloproteinase (MMP) and vascular endothelial growth factor (VEGF). There has not been any diagnostic agreement on MMP-9 and VEGF-C as predictors of lymph node metastasis in PTC. Hence, this study aims to determine the association of MMP-9 and VEGF-C to cervical lymph node metastases in PTC patients.
Methods: A cross-sectional study was conducted at Cipto Mangunkusumo General Hospital, Jakarta. Patients diagnosed with PTC based on histopathological examination were included in this study. Patients with distant metastases were excluded from the study. The expression of MMP-9 and VEGF-C was investigated at the Anatomical Pathology Laboratory.
Results: Sixty-two patients were included in this study, 80.6% female and 19.4% male. The MMP-9 expression was higher in the metastatic group ($p<0.001$). The same results were also found in VEGF-C expression, where the median expression of this marker in the metastatic group was higher than in the non-metastatic group ($p<0.001$). We found a significant positive correlation between the MMP-9 and VEGF-C expressions (correlation coefficient of 0.618).
Conclusions: There is a significant relationship between the expression of MMP-9 and VEGFC with cervical lymph node metastases in PTC patients. The MMP-9 and VEGF-C expression was higher in the metastatic group. The increased MMP-9 expression is also positively correlated with increased VEGF-C expression.

Keywords: lymph node metastases, MMP-9, papillary thyroid carcinoma, VEGF-C.
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INTRODUCTION

Thyroid carcinoma is the most common malignancy in the endocrine field and is presented as the etiology in 7-15% of thyroid nodules.[1] Based on the American epidemiological study, there was an incident increment of thyroid carcinoma cases from 37,200 in 2009 to 63,000 in 2014.[2] Thyroid carcinoma is the ninth most prevalent cancer in Indonesia, according to the Pathological Based Registration. As much as 80-85% of thyroid malignancy consists of papillary thyroid carcinoma (PTC).[1–3] Moreover, studies report that lymph node metastases occur in 30-40% of adult PTC patients.[1–3] This lymph node metastases increase morbidity due to its association with recurrence risk increment.[4] Lymph node metastases potentially cause vein occlusion, which causes face edema with globus sensation and dysphagia. Furthermore, internal carotid artery occlusion in the later stage could cause transient ischemic attack, migraine, and dementia.[5] Lymph node metastases in PTC are usually treated with neck dissection consisting of central and lateral compartments.[5,6] However, there is no precise predictor to determine the potential for lymph node metastases in PTC.

Currently, potential biomarkers are studied to predict lymph node metastases in PTC, including matrix metalloproteinase (MMP) dan vascular endothelial growth factor (VEGF).[7] Gelatinase enzymes consisted of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) contribute in Collagen IV degradation and basement membrane main composition. These roles are associated with tumor invasion and metastases risk of a tumor. In thyroid disease, MMP-9 is reported to increase, especially lymph node metastases in PTC.[6] Vascular endothelial growth factor (VEGF) is an angiogenic protein. VEGF-C is one of its many potentials in lymphangiogenesis, crucial progress in lymph node metastases.[8]

Although there has not been any settlement on MMP-9 and VEGF-C usages as predictors of lymph node metastases of PTC, previous studies reported that they could be correlated with lymph node metastases. Maeta H et al. reported that compared to normal thyroid tissue, the expression of VEGF-C was higher in PTC and increased rapidly in lymph node metastases occurrence.[9] Moreover, it is reported that MMP-9 immunohistochemistry scores between PTC with and without lymph node metastases differed significantly ($p =$

0.014).[3,9] A Tanaka et al. study reports that messenger-RNA (mRNA) VEGF-C is significantly higher in PTC with lymph node involvement.[10] Moreover, early detection using VEGF-C expression might be considered for starting treatment to prevent further metastases.[11,12]

MMP-9 and VEGF-C have not been investigated to determine PTC lymph node metastases in Indonesia. MMP-9 and VEGF-C might be useful to early predict the metastases and to consider conducting treatment sooner, resulting in a better prognosis. Hence, this study aims to prove the association of MMP-9 and VEGF-C with PTC lymph node metastases and the correlation between these biomarkers.

METHODS

Study Design

The study design was an analytical cross-sectional study in the Cipto Mangunkusumo General Hospital from December 2019 to March 2021.

Sample Selection

The inclusion criteria of this study were patients diagnosed with PTC based on the histopathological result and paraffin blocks of PTC patients, which were eligible for MMP-9 and VEGF-C immunohistochemistry analysis. Besides, thyroid cancer patients with distant metastases (M1) and those with incomplete data were excluded from this study.

The minimum total of subjects was calculated by using analytical categorical independent sample size formula with a 5% alpha. Hence, the minimum sample size in this study required 76 patients. Patients were categorized into 2 groups: PTC with lymph node metastases group and PTC without lymph node metastases group. The samples were collected using consecutive sampling methods in the surgical outpatient clinic. Patient's clinical data were obtained from their medical records, including age, gender, TNM stages status, lymph node involvement, and cancer stadium.

Thyroid tissue samples were obtained from thyroidectomy procedures, then stored and analyzed in the anatomical pathology department. Immunohistochemistry analysis on MMP-9 and VEGF-C expressions were performed semi-quantitatively using the H-Score. The followings are the procedure for preparing the tissue samples for MMP-9 and VEGF-C expression analysis: Tissue samples were formalin-fixed and paraffin-embedded, then cut into 4mm. Then, samples were heated at 60°C for 30 minutes. De-paraffinization was done in xylol I, II, and III for 5 minutes each. In order to block the endogenous peroxidase activity, the samples were immersed in 0.5% hydrogen peroxide in a methanol solution for 30 minutes. The slides were pretreated with Tris- Ethylenediamine Tetraacetic Acid (Tris-EDTA) in the decloaking chamber at power level 8 and power level 1 (5 minutes each) before it was cooled down for 45 minutes and washed with phosphate-buffered saline (PBS) solution (pH7.4). Blocking background sniper was done in 15 minutes. Primary antibody was added for an hour or overnight. Then, a universal link was added for 15 minutes. 3,3'-diaminobenzidine (DAB) was used for 2-5 minutes, followed by lithium carbonate (5% in saline water) for 1 minute. Samples were then gradually dehydrated in alcohol series, followed by xylol I, II, and III clearing for 5 minutes each, until they were finally mounted.

Measurement of the Sample

After the preparation procedures, samples were evaluated based on the immunoreactivity level and categorized into three levels for each of MMP-9 expression and VEGF-C expression (1+: low immunoreactivity, 2+: moderate immunoreactivity, 3+: high immunoreactivity). After that, the cell percentage of each immunoreactivity category was calculated from 500 total cells. H-score was calculated with the following formula: [1× (cell percentage (%) of 1+) + 2 × (cell percentage (%) of 2+) + 3 × (cell percentage (%) of 3+)]. The range value was 0 – 300.

Two certified senior pathologists assessed the samples, calculated the H-Score, and evaluated the Kappa score.

Statistical Analysis

Data analysis was performed using IBM SPSS 22.0. A descriptive-analytical method was used to analyze the subject's characteristics data (including age, gender, T status, PTC variant, neck lymph node involvement, tumor size, stadium, MMP-9 H-Score, and VEGF-C H-Score). Moreover, 2 independent categorical inferential analyses and correlation analysis were used to prove the correlation of the H-score of MMP-9 and VEGF-C with neck lymph node metastases. The normality test was conducted using the Shapiro-Wilk method. P-values of less than 0.05 were considered statistically significant.

RESULTS

This study was performed from December 2019 to March 2021, analyzing 62 patients. Patients were categorized into 2 groups: 31 PTC patients with lymph node metastases and 31 PTC patients without lymph node metastases; all of them met the inclusion criteria and fulfilled the minimum sample size of this study.

The majority of the patients were females (80.6%), below 55 years old (74.2%), with mixed of follicular and other variants (54.8%), T3 (31%), and stadium I (46%). The baseline characteristics of patients are provided in table 1.

Immunohistochemistry results on MMP-9 and VEGF-C showed that PTC with lymph node involvement commonly expressed both markers with strong intensity. However, PTC without lymph node metastases commonly did not show any immunohistochemistry staining. The markers expression was measured semi-quantitatively by calculating the H-Score. An example of the IHC staining result is provided in figure 1.

Based on the normality test (Kolmogorov-Smirnov and Shapiro-Wilk), MMP-9 H-Score was not distributed normally in both groups of patients. Hence, a Mann-Whitney test was used to analyze the difference in H-Score between the two groups. As shown in Table 2, there was a significant difference in MMP-9 H-Score Median between PTC with metastases and PTC without metastases ($p < 0.001$, Confidence Interval (CI) 95%). The median of H-Scores of MMP-9 expressions in PTC with lymph node metastases was higher than in PTC without metastases.

Based on the Kolmogorov-Smirnov and Shapiro-Wilk tests, the H-Score of VEGF-C was not distributed normally either. As shown in table 3, the Mann-Whitney test resulted that there was also a significant difference in VEGF-C H-Score Median between PTC with metastases and PTC without metastases ($p < 0.001$, Confidence Interval (CI) 95%). The median of H-Scores of VEGF-C expression in PTC with lymph node metastases was higher than in PTC without metastases.

Moreover, the Spearman correlation test was conducted to analyze the correlation between the H-Scores of MMP-9 and VEGF-C. There was a significant positive correlation between the H-Scores of these two markers with a 0.618 correlation coefficient ($p < 0.001$). Hence, the increment of MMP-9 H-Score was correlated to the VEGF-C increment.

DISCUSSION

Based on the result of this study, MMP-9 is associated with the lymph node involvement of PTC ($p < 0.001$), where MMP-9 is expressed higher in PTC patients with lymph node involvement. Our study supports a study by Marecko et al., which states that by analyzing immunoreactivity in immunohistochemistry staining, MMP-9 might be able to predict lymph node metastases in PTC ($p = 0.004$).[13] Moreover, Wang et al. also reported that MMP-9 in PTC tissue is associated significantly with lymph node metastases ($p<0.001$)[14] MMP-9 is a proteolytic enzyme that contributes to degrading the basal membrane and the extracellular matrix.[8] Moreover, MMP-9 is in collaboration with VEGF-C in tumor progressivity. The roles of MMP-9 also include the trimming process of the substrate into the active form in the proliferation, migration, invasion, metastases, and angiogenesis process of a tumor.[8]

Table 1. Baseline characteristics of patients.

Classification		Metastases		Non-metastases		Total	%
		Total	%	Total	%		
Gender	Male	10	16.1	2	3.2	12	19.4
	Female	21	33.9	29	46.8	50	80.6
	Total	31	50	31	50	62	100
Age	<55	20	32.3	26	41.9	46	74.2
	≥55	11	17.7	5	8.1	16	25.8
	Total	31	50	31	50	62	100
PTC Variant	Follicular	7	11.3	6	9.7	13	21
	Tall Cell	4	6.5	4	6.5	8	12.9
	Microcarcinoma	0	0	1	1.6	1	1.6
	Mixed of follicular & others	16	25.8	18	29	34	54.8
	Mixed of Tall Cell & others	4	6.5	2	3.2	6	9.7
	Total	31	50	31	100	62	100
T status	1	3	4.8	3	4.8	6	9.7
	2	7	11.3	12	19.4	19	30.6
	3	15	24.2	16	25.8	31	50
	4	6	9.7	0	0	6	9.7
	Total	31	50	31	50	62	100
Tumor	≤2 cm	3	4.8	3	4,8	6	9.7
Size	>2 cm	28	45.2	28	45.2	56	90.3
	Total	31	50	31	50	62	100
Stadium	I	19	30.6	27	43.5	46	74.2
	II	10	16.1	4	6.5	14	22.6
	III	2	3.2	0	0	2	3.2
	IV	0	0	0	0	0	0
	Total	31	50	31	50	62	100

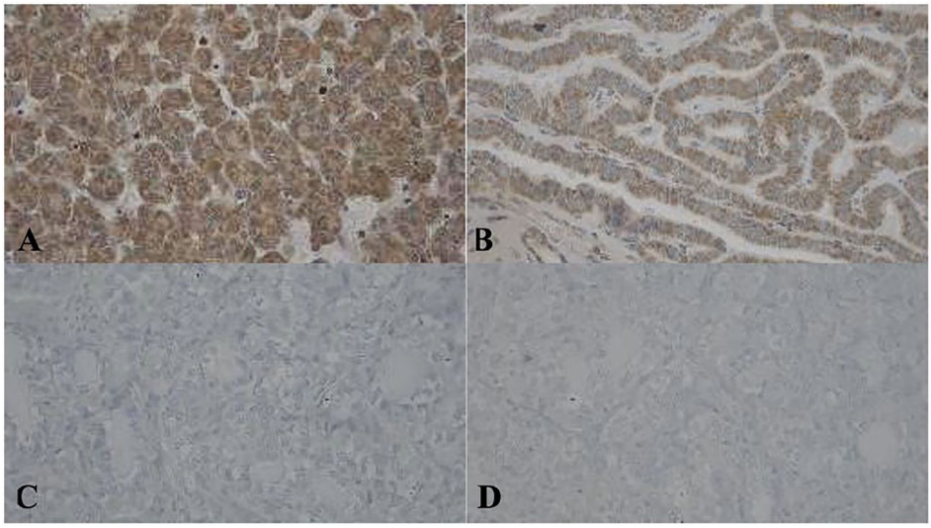

Figure 1. A) MMP-9 Immunohistochemistry (IHC) staining with high immunoreactivity (3+), B) VEGF-C IHC staining with high immunoreactivity (3+), C) Negatively stained MMP-9 IHC, D) Negatively stained VEGF-C IHC. All of the figures are in 40x magnifications.

Table 2. Analysis result on H-Scores of MMP-9 expressions between two groups.

	Median (min-max)	P value
Metastases	239.4 (48.8–300)	< 0.001*
Non-metastases	25.2 (2–184)	

Note: *significant at p<0.05 by Mann Whitney Test

Table 3. Analysis result on H-Scores of VEGF-C expression between two groups.

	Median (min-max)	P value
Metastases	267.4 (100–300)	<0.001*
Non-metastases	100 (2.6–300)	

Note: *significant at p<0.05 by Mann Whitney Test

VEGF-C is also significantly associated with the lymph node involvement of PTC in our study (p <0.001), where VEGF-C is expressed higher in PTC patients with lymph node metastases compared to non-metastases. Previously, an in vitro study by Feng W et al. reported that down-regulation of VEGF-C by using rapamycin would inhibit the progressivity of thyroid cancer cells and shows the association between VEGF-C and invasion level of thyroid cancer.[15] Tian et al. also support us by proving that there are significant VEGF-C expression differences between PTC lymph node metastases and non-metastases (p<0.01).[4] Molecular study showed VEGF-C contributes to tumor spread, especially to lymph node metastases, by enhancing the lymphangiogenesis process and facilitating its metastasis to surrounding lymph nodes.[8] Razy NH et al. also reported upregulation of the VEGF-C receptor in PTC with associated aggressiveness characteristics and lymph node metastases incidence. They stated that highly expressed VEGF-C and its receptor could be a potential treatment target for PTC patients.[16] Moreover, Plate et al. also supported this from their study result showing the positive correlation between VEGF-C expression and its receptor upregulation; hence both interactions would occur.[17] Hence, Schoenberger et al. proved that inhibiting VEGF-C tyrosine kinase receptor by using PTK787/ZK222584, a selective inhibitor of VEGFR-1 and VEGFR-2, could decrease the PTC cell and its vascular vessel density.[18]

Furthermore, MMP-9 expression also correlates positively with the VEGF-C expression in this study with a 0.618 correlation coefficient (p < 0.001). A previous study by Selemetjev et al. also concludes the correlation between MMP-9 and VEGF-C (C=0.623; p <0.001).[8] Another study by Tian et al, also proved MMP-9 and VEGF-C as predictors of lymph node metastases (C=0.517; p < 0.05.[16] MMP-9 can control the angiogenic factors. Besides, VEGF-C is activated through proteolysis, and its bioavailability is controlled by MMP-9.[8] This process has been studied bio-molecularly, where MMP-9 in peri-tumor area induced VEGF-C to facilitate cancer cell invasion with lymphangiogenesis process.[19] Hence, both MMP-9 and VEGF-C are correlated and can be predictors of lymph node metastases.[7,20]

The strength of this study is the semi-quantitively measurement of both markers using the Histo-score. This method combines the total stained cell with the staining intensity to acquire a detailed measurement result. On the other hand, previous studies still only used staining intensity to measure the MMP-9 and VEGF-C immunohistochemistry expression. Despite its strength, our study only analyzes both markers' expressions on its thyroid tissue. For example, we have not studied the level of both markers' expression in extra-thyroid, serum samples. Further studies might be necessary to study both markers' expressions from other than the thyroid without manipulating its thyroid tissue. Moreover, we also need to prove which marker, MMP-9 and VEGF-C, is more sensitive and specific to detect despite their correlation.

CONCLUSIONS

MMP-9 and VEGF-C expressions are significantly associated with lymph node metastases of PTC, where both markers are higher in PTC patients with lymph node metastases than non-metastases. Moreover, MMP-9 expression is correlated to VEGF-C expression in PTC patients with lymph node metastases. Therefore, further research regarding the factors influencing the association of MMP-9 and VEGF-C expression with lymph node metastases is needed.

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ETHICAL CONSIDERATIONS

This study has been approved by the Institutional Review Board of Dr. Cipto Mangunkusumo Hospital (KET533/UN.F1/ETIK/PPM.00.02/2020).

CONFLICT OF INTEREST

The authors affirm no conflict of interest regarding this study.

AUTHOR CONTRIBUTIONS

All Authors contribute equally in conducting research and writing manuscripts.

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