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Role of CXCR4 and MMP-9 Expressions with Regional Cervical Lymph Nodes Metastatic Status in Papillary Thyroid Carcinoma

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ABSTRACT

Background: Papillary thyroid carcinoma (PTC) that has metastasized has a higher risk because of the poor prognosis, ranging from decreased quality of life of the patient to death. There is a need for markers that can understand the image of the tumor so that it can predict earlier the level of aggressiveness and the ability of tumor cells to metastasize. Tumor cells that proliferate excessively will cause CXCR4 expression. Activation of CXCR4 will increase MMP-9 via the MAPK/ERK line. MMP-9 will degrade the extracellular matrix causing tumor cell metastasis. This study is aimed to analyze the correlation of CXCR4 and MMP-9 expressions with lymph node metastasis (LNM) in PTC.

Methods: This cross-sectional analytic observational study was conducted in 43 PTC samples taken from the Anatomical Pathology Laboratory of Dr. Soetomo Hospital from January 2011 to December 2018. The samples were divided into 2 groups based on the regional cervical LNM status. Immunohistochemical staining was used to assess the expression of CXCR4 and MMP-9. Spearman test was used to analyze the correlation of CXCR4 and MMP expression with cervical LNM status.

Results: The expression of CXCR4 and MMP-9 was higher in the papillary thyroid carcinoma group with cervical regional LNM ($p = 0.007$ and $p = 0.030$). There was a significant positive relationship between CXCR4 and MMP-9 expressions with regional LNM status with $r_s = 0.468$ and $p = 0.001$ ($p < 0.05$).

Conclusions: Expression of CXCR4 and MMP-9 was proven to correlate with regional cervical LNM status in papillary thyroid carcinoma. Both can be used as predictive markers for metastasis in papillary thyroid carcinoma.

INTRODUCTION

Thyroid cancer is the most common endocrine malignancy with increased incidence in the last 20-30 years. According to the data from Global Cancer Statistic (GLOBOCAN) 2018, thyroid carcinoma is ranked ninth as the highest incident globally with approximately 567.000 cases [1,2]. In 2013, Basic Health Research conducted by the Health Ministry of Indonesia showed that thyroid carcinoma was ranked fifth as the most common malignancy. In 2013, there were 147 new thyroid cancer cases in Indonesia [3].

Until now, it is estimated that 30–80% of papillary thyroid carcinoma (PTC) patients have regional lymph

node metastasis (LNM), causing recurrence, distant metastasis, and decreased survival rate. Paulino et al. [4] stated that lymph node metastasis is associated with a bad prognosis and occurs in > 50% of PTC cases [4–6]. A previous study shows that LNM only affects recurrence, not survival. A large-scale case-control study shows that LNM and inadequate surgical excision are the two main factors associated with morbidity [7,8].

Recently, studies showed that chemoattractant molecules secreted from target organs and specific patterns of chemoattractant receptors expressed on tumor cells can determine the goal of tumor metastasis. Therefore, the expression of these chemoattractant receptors can

promote the migration of tumor cells in response to the gradient of chemoattractant molecules originating from the lymph nodes. The identification of these molecules and their receptors can be considered as markers for PTC which is likely to undergo LNM. The chemoattractant receptor candidate is a chemokine receptor. The chemokine that plays a role in tumor cell migration is CXCL12 which has a specific receptor, namely CXCR4 [9].

Binding between CXCL12 and CXCR4 will activate AKT and mitogen-activated protein kinase (MAPK)/ERK signaling which will increase the migration ability of tumor cells including PTC cells and induce angiogenesis [10–12]. The MAPK/ERK pathway then induces MMP-9 expression via NF- κ B activity [13]. Matrix metalloproteinases-9 (MMP-9) is a group of proteolytic enzymes which plays an important role in the progression of cancer cells, increasing tumor cell invasion and metastasis. It detaches tumor cells from their place origin and then invades the blood and lymphatic system through the surrounding stroma. All these processes are related to MMP-9 which has the function of degrading type IV collagen as the main component of the basement membrane [12,14,15]. This study was aimed to analyze the correlation of CXCR4 and MMP-9 in regional cervical lymph node metastasis in PTC.

METHODS

This study was an observational analytic study with a cross-sectional approach, conducted on PTC cases obtained from Anatomical Pathology Laboratory of Dr. Soetomo General Hospital archives from January 2011 to December 2018 which met the inclusion criteria. The inclusion criteria include the use of paraffin blocks from patients with classic variant PTC obtained from hemithyroidectomy or total thyroidectomy by including lymph node (LN) specimens or radical neck dissection (RND) regardless of the level of LN. The samples were divided into two groups consisting of 32 and 11 samples with and without regional cervical LNM, respectively.

Immunohistochemical staining was used to assess the expression of CXCR4 and MMP-9. Paraffin block was sliced for 4 μ m thin, deparaffinized, and rehydrated using 96%, 80%, and 70% of ethanol. It was then processed in a decloaking chamber using citrate buffer (pH 6, temperature 95o) for 45 minutes. Primary antibody CXCR4 (sc-53534, Santa Cruz Biotechnology, Inc.) diluted 1:200 and MMP-9 (sc-13520, Santa Cruz Biotechnology, Inc.) diluted 1:200 were given overnight. A secondary antibody was then applied, and the samples were incubated for 20 minutes. Diaminobenzidine (DAB) was added, and counterstain was performed using Meyer Hematoxylin. CXCR4 and MMP-9 expressions were assessed using a percentage score. CXCR4 percentage score was divided into score 0 = stained in 0–10% tumor cells, score 1 = stained in 10–50% tumor cells, and score 2 = stained in > 50% tumor

cells. MMP-9 percentage score was divided into score 0 = not stained or stained in < 5% tumor cells, score 1 = stained in 5–25% tumor cells, and score 3 = stained in > 50% tumor cell. CXCR4 expression was observed in the cell membrane and cytoplasm of tumor cells, while MMP-9 expression was observed in the cytoplasm of tumor cells. A binocular light microscope was used to observe the expression of CXCR4 and MMP-9.

The difference in the expression of CXCR4 and MMP-9 in the two groups was carried out by the Mann-Whitney test. The results of the Mann-Whitney test were said to be significantly different if the p-value was < 0.05. Spearman correlation test was used to assess the association between regional cervical LNM status with CXCR4 and MMP-9 expressions. The results were considered significant if $p < 0.05$.

RESULTS

In this study, the mean patient age was 47.7 years. The youngest patient was 19 years old while the oldest was 75 years old, and most patients were at the age of < 55 years (67.4). In the metastatic group, most were found in the age group of < 55 years as many as 21 or 65.6% with 20 females or 62.5%. In the non-metastatic group, it was found that the age group of < 55 years was 8 or 72.7% with 9 females or 81.8%. Based on stage T, it was found that the highest group was in T3 with 16 cases or 50% in the metastatic group and 6 cases in the non-metastatic group or 54.5%. The sample characteristics are described in **Table 1**.

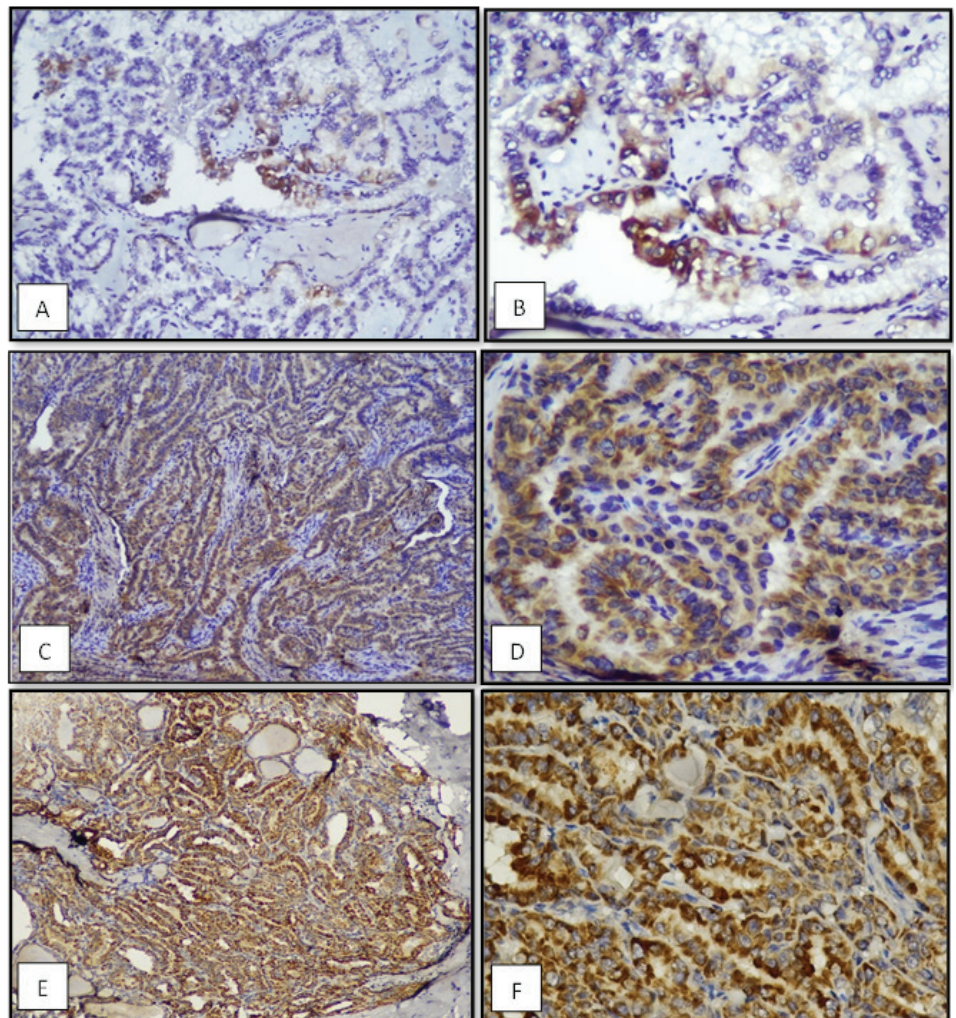
CXCR4 expression was stained in the membrane and cytoplasm of tumor cells (**Figure 1**). The results showed that CXCR4 expression with score 2 (percentage > 50%) was mostly found in patients with regional cervical lymph node metastasis (24 cases, 75%). Mann-Whitney test showed a significant CXCR4 expression difference in PTC with and without regional cervical lymph node metastasis ($p = 0.007$). Regional cervical lymph node metastasis was significantly correlated with CXCR4 expression ($p = 0.006$), with a correlation coefficient value of -0.415.

MMP-9 expression was stained in the cytoplasm of tumor cells (**Figure 2**). The results showed that MMP-9 expression with score 3 (percentage > 50%) was mostly found in patients with regional cervical lymph node metastasis (21 cases, 75%). Mann-Whitney test showed a significant MMP-9 expression difference in PTC with and without regional cervical lymph node metastasis ($p = 0.030$). Regional cervical lymph node metastasis was significantly correlated with MMP-9 expression ($p = 0.027$), with a correlation coefficient value of -0.336.

Spearman correlation test showed a significant correlation between CXCR4 and MMP9 expressions ($p = 0.001$) with a correlation coefficient of 0.468. This proved that CXCR4 expression increased along with MMP-9 expression (**Figure 3**).

Table 1.
Characteristics
of research samples

Characteristics	Metastasis	Non-Metastasis	Total	p
Age (years)				
<55	21 (65.6%)	8 (72.7%)	29 (67.4%)	0.674
≥55	11 (25.6%)	3 (27.3%)	14 (32.6%)	
Gender				
Male	12 (37.5%)	2 (18.2%)	14 (32.6%)	0.248
Female	20 (62.5%)	9 (81.8%)	29 (67.4%)	
Stadium T				
T1	4 (12.5%)	1 (9.1%)	5 (11.6%)	0.340
T2	4 (12.5%)	3 (27.3%)	7 (16.3%)	
T3	16 (50%)	6 (54.5%)	22 (51.2%)	
T4	8 (25%)	1 (9.1%)	9 (20.9%)	
CXCR4 expression				
Score 0	2 (6.2%)	2 (18.2%)	4 (9.3%)	0.006*
Score 1	6 (18.7%)	5 (45.5%)	11 (25.6%)	
Score 2	24 (75%)	4 (36.4%)	28 (65.1%)	
MMP-9 expression				
Score 0	5 (15.6%)	3 (27.3%)	8 (18.6%)	0.027*
Score 1	5 (15.6%)	3 (27.3%)	8 (18.6%)	
Score 2	1 (3.1%)	3 (27.3%)	4 (9.3%)	
Score 3	21 (65.6%)	2 (18.2%)	23 (53.5%)	

*statistically significant ($p < 0.05$)**Figure 1.**

CXCR4 expression in the membrane and or cytoplasm of tumor cells. (A) and (B) CXCR4 expression score 0, percentage 0–10% (magnification of 100x and 400x). (C) and (D) CXCR4 expression score 1, percentage 11–50% (magnification of 100x and 400x). (E) and (F) CXCR4 expression score 2, with percentage >50% (magnification of 100x and 400x).

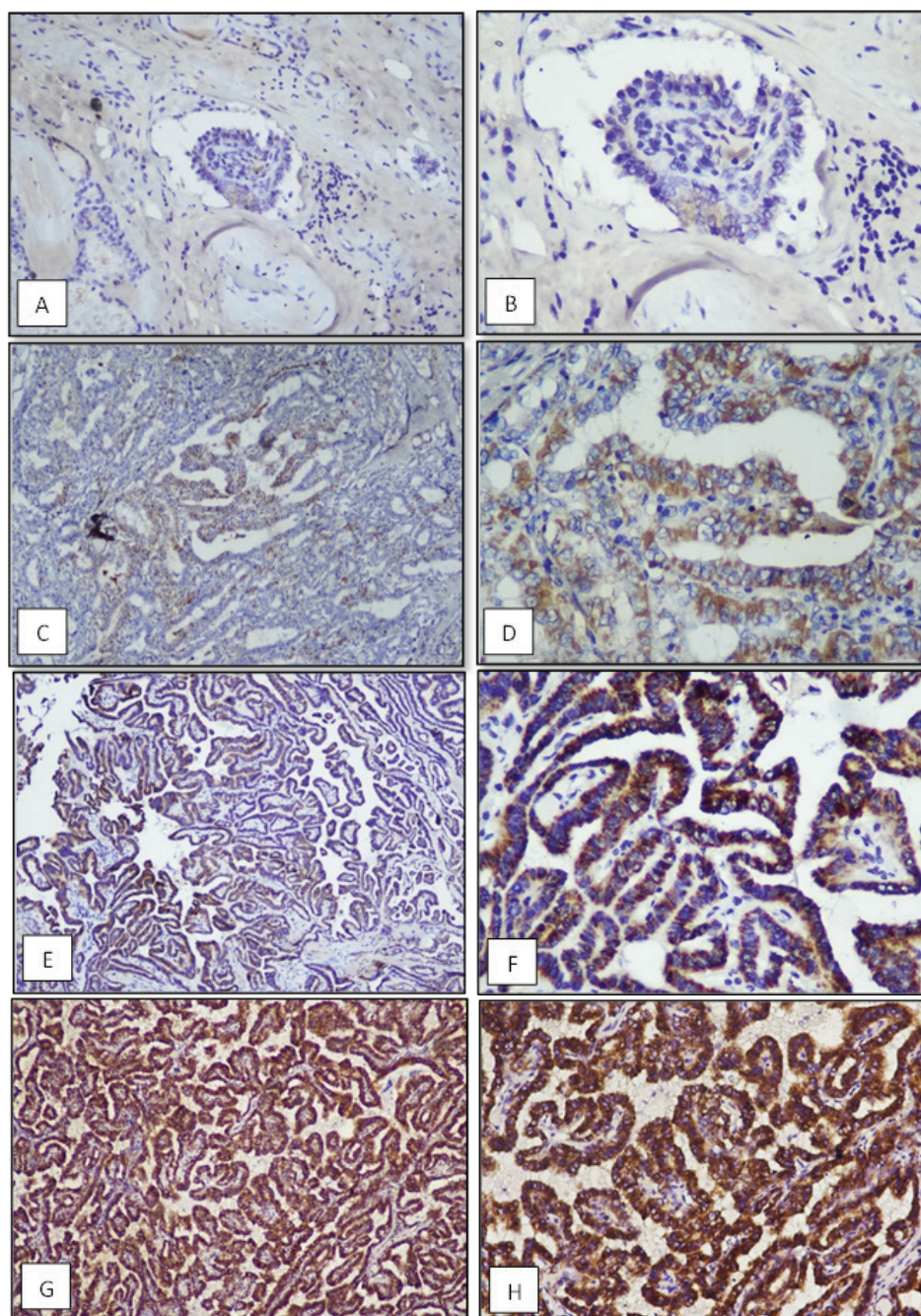


Figure 2.

MMP-9 expression in the cytoplasm of tumor cells. (A) and (B) MMP-9 expression score 0, with percentage <5% (magnification of 100x and 400x). (C) and (D) MMP-9 expression score 1, percentage 5–25% (magnification of 100x and 400x). (E) and (F) MMP-9 expression score 2, percentage 26–50% (magnification of 100x and 400x). (G) and (H) MMP-9 expression score 3, percentage >50% (magnification of 100x and 400x).

DISCUSSION

In this study, the expression of CXCR4 represented by a percentage score showed a difference with an increasing tendency in the metastatic group. The results of the Mann Whitney test showed that there were significant differences in the expressions of CXCR4 in PTC with and without metastases of neck regional lymph nodes ($p = 0.007$). The results of the Spearman correlation test showed r_s of -0.415 with $p = 0.006$ ($p < 0.05$), meaning that there was a correlation between CXCR4 expression and the status of neck regional lymph node metastases in PTC. The higher expression of CXCR4 is in

line with neck regional lymph node metastases. A negative r_s value was associated with coding where the metastatic group was in the low-value group or 1 (**Figure 3**).

The results of this study are in line with those of several previous studies. The study by Torregrossa et al. [16] assessing the expression of CXCR4 using quantitative real-time polymerase chain reaction (Q-RT-PCR) in tumor tissue of PTC patients, showed that CXCR4 expression was higher in PTC with lymph node metastasis. The study by Lin et al. [11] found the relationship between CXCR4 expression and lymph node metastasis in patients with stage III-IV PTC ($p < 0.05$). The study by Dayer et al. [17] using Q-RT-PCR to evaluate the expression of CXCR4

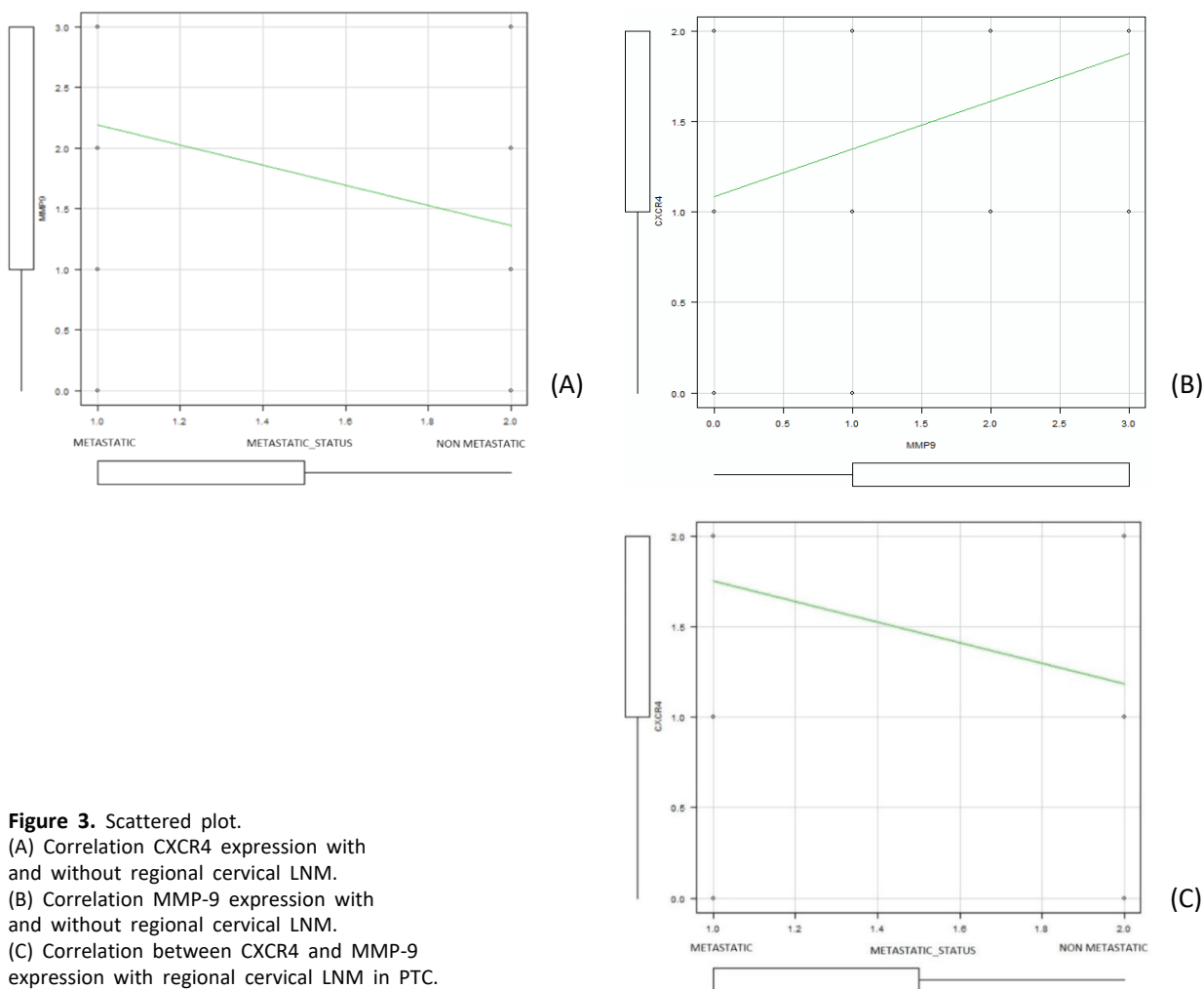


Figure 3. Scattered plot.
 (A) Correlation CXCR4 expression with and without regional cervical LNM.
 (B) Correlation MMP-9 expression with and without regional cervical LNM.
 (C) Correlation between CXCR4 and MMP-9 expression with regional cervical LNM in PTC.

in tumor tissue of patients with invasive breast cancer, showed that CXCR4 expression was higher in breast cancer with axillary lymph node metastasis.

CXC Chemokine receptor 4 is a transmembrane protein of the G-protein-coupled cell surface receptors (GPCR) family. CXC Chemokine receptor 4 is a physiological receptor of CXC chemokine stromal cell-derived factor (SDF-1, also known as CXCL12 as the ligand) [16]. CXC Chemokine Receptor 4 is expressed in various tissues, including brain tissue, lymph node, intestine, monocyte, B cell, naive T cell, and early hematopoietic progenitor cells of the immune system. Overexpression of CXCR4, caused by upregulation of Hypoxia Inducible Factor 1 Alpha (HIF-1 α), is found in > 20 types of malignancy. Tumor cells expressing CXCR4 tend to migrate to organs with increased CXCL12. Under hypoxic conditions, the von Hippel-Lindau (VHL) tumor suppressor gene, which induces the degradation of HIF-1, is inactivated; thus, elevated levels of HIF-1 stimulate CXCR4 expression via the VHL-HIF-1 pathway [17].

The binding of CXCR4 and CXCL12 activates several intracellular signaling pathways controlling cell chemotaxis, survival, proliferation, and migration of tumor cells [17].

As a G-protein-coupled receptor (GPCR), the activation of CXCR4 receptor is mediated by inner plasma membrane-related intracellular heterotrimeric G-protein. G heterodimeric protein consists of G α , G β , and G γ subunit. In a basal or inactive state, G α subunit contains guanine nucleotide diphosphate (GDP). Upon activation, G α subunit is bound to guanine nucleotide triphosphate (GTP) which will then activate PI3K. PI3K activation induces signaling cascade leading to Akt activation, which will then induce transcription factor- κ B (NF- κ B) [10,16,17].

CXCR4 protein may increase tumor cell metastasis through the angiogenesis process. The bond between CXCR4 and CXCL12 will activate endothelial progenitor cells to migrate surrounding tumor cells and create new vessels. CXCR4 is also related to VEGF expression and platelet-derived growth factor D (PDGF-D) which correspond with the formation and stabilization of new vessels around tumor cells. The formation of new vessels around tumor cells will enhance tumor cell metastasis [18,19,20]. CXCR4 might also affect the immune system around tumor cells. CXCR4 protein will attract dendritic cells to migrate around tumor cells. Dendritic cells can suppress anti-tumor immunity by inactivating cytotoxic

T-cell lymphocytes [21]. The signal from CXCR4/CXCL12 will induce epithelial to mesenchymal transition (EMT) by activating ERK1/2 pathway, enhancing MMP activation to degrade ECM around tumor cells. The basal membrane of endothelial venules is also rich in CXCL12; therefore, they will invite malignant cells which express CXCR4 to enter blood circulation resulting in distant metastasis [22]. The study by Lin et al. [11] made a comparison between the two-year survival rates of patients with osteosarcoma expressing CXCR4 and those not expressing CXCR4. They found that the two-year survival rate of CXCR4-positive patients (32.4%) was significantly lower than that of CXCR4-negative ones (78.9%). Given the negative effects of CXCR4 expression on survival, it has been suggested that CXCR4 may be used as a potential prognostic factor [13].

In this study, the expression of MMP-9 represented by a percentage score showed a difference with an increasing tendency in the metastatic group. The results of the Mann Whitney test showed that there were significant differences in the expressions of MMP-9 in PTC with and without metastases of neck regional lymph nodes ($p = 0.030$). The results of the Spearman correlation test showed $r_s = -0.336$ with $p = 0.027$ ($p < 0.05$), meaning that there was a correlation between MMP-9 expression and the status of neck regional lymph node metastases in PTC. The higher expression of MMP-9 is in line with neck regional lymph node metastases. A negative r_s value was associated with coding where the metastatic group was in the low-value group or 1 (**Figure 3**).

The result of this study was consistent with a study by Liu et al. [23] which also found a significant correlation between MMP-9 expression and central and lateral lymph node metastasis in the late-stage PTC (stage III-IV) ($p < 0.05$). A study by Hwang et al. [24] investigating MMP-9 in gastric cancer, also reported a similar result where MMP-9 was correlated with lymph node metastasis and lymphatic invasion, and bad prognosis. Liabakk et al. [25] found that MMP-2 and MMP-9 levels increased significantly with the cancer stage. Parson et al. [26] observing gelatinase enzyme (MMP-2, MMP-9), showed that overexpression in gastrointestinal malignancy was related to its role in tumor invasion and metastasis [20,26]

Matrix metalloproteinases-9 (MMP-9) is broadly divided according to substrate specificity, divided into 6 groups namely collagenases (MMP-1, 8, 13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, 10, 11), Matrilysins (MMP-7 and MMP-26), membrane-type MMP (MMP-14, 15, 16, 17, 24 and 25), and other MMPs. MMP-9 is known as 92 kDa gelatinase. MMP-9 secreted by tumor cells is related to tumor metastasis; therefore, MMP-9 can degrade collagen types IV, V, VII, and X. Type IV collagen is the main collagen supporting basal membrane and is especially abundant in vascular endothelium in the basement membrane, and it acts

as a barrier to invasion and metastasis. The high expression of MMP-9 causes degradation of the extracellular matrix making it easier for tumor cells to invade and metastasize [14,27,28]. Increased expression of MMP-9 can be a useful diagnostic marker and maybe a potential target in the treatment of thyroid carcinoma. Previous studies have shown MMP-9 to have good sensitivity and specificity for the diagnosis of PTC [23].

The correlation between CXCR4 and MMP-9 expressions in PTC with and without neck regional LNM was statistically tested using the Spearman correlation test; the result obtained was $r_s = 0.468$ with $p = 0.001$ ($p < 0.05$). This proves that there is a significantly positive correlation between the expressions of the two; the higher the expression of CXCR4 is, the higher the expression of MMP-9 will be (**Figure 3**).

A study by Liang et al. [29] also found overexpression of CXCR4 and MMP-9 in 23 out of 27 cases of PTC with lymph node metastasis, with a statistically significant difference between PTC with and without lymph node metastasis ($p < 0.05$). Hao et al. [30] proved that there was a significant correlation between CXCR4 and MMP-9 and lymph node metastasis in breast carcinoma with a correlation coefficient of 0.479 and $p < 0.001$ ($p < 0.05$). A study by Zuo et al. [31] showed that CXCR4 overexpression in lung carcinoma increased EGFR expression and MMP-9 production, which then caused migration, invasion, and metastasis of tumor cells.

CXCR4 activation will trigger various signaling pathways underneath. The activated CXCR4 will split into $G\alpha$ and $G\beta\gamma$ sub-units. The $\beta\gamma$ subunit induces phospholipase-c (PLC) which will make phosphatidylinositol 4,5-biphosphate (PIP2) produce second messengers, namely diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 will increase intracellular calcium excretion from the endoplasmic reticulum, which together with DAG will activate protein kinase C (PKC) and MAPK/ERK1/2 [10]. The MAPK/ERK 1/2 pathway will activate the $\text{I}\kappa\text{B}$ kinase (IKK) enzyme complex which will phosphorylate the inhibitor $\text{I}\kappa\text{B}$ protein. Phosphorylation will make $\text{I}\kappa\text{B}$ dissociate from $\text{NF-}\kappa\text{B}$. "Free" $\text{NF-}\kappa\text{B}$ will migrate into the cell nucleus to play a role in the protein translation process. The activity of $\text{NF-}\kappa\text{B}$ induces the expression of MMP-9 leading to cell proliferation, angiogenesis, and metastasis of tumor cells [23,32].

Another pathway that can play a role in tumor cell migration is PI3K. PI3K can be activated by both $G\beta\gamma$ and $G\alpha$ subunits and can activate the Akt and $\text{NF-}\kappa\text{B}$ pathways. The PI3K/AKT pathway plays a role in angiogenesis and tumor cell proliferation, while the $\text{NF-}\kappa\text{B}$ pathway suppresses apoptotic signals by upregulating the Bcl-2 gene. Chinni et al. [33] reported that CXCL12 induces the expression of MMP-9 which is regulated by $\text{NF-}\kappa\text{B}$ activity in prostate cancer cells by activating the PI3K-Akt- $\text{NF-}\kappa\text{B}$ and MEK pathways [13,33]. In this study, similar results were obtained showing that the

overexpression of CXCR4 can increase MMP-9 production, which ultimately results in increased cell migration and invasion.

The limitation of this study is that only two biomarkers were analyzed, whereas for all malignancies, in this case of PTC, many other pathways and biomarkers play a role in complex interactions that will ultimately affect tumor proliferation, invasion, and metastatic processes. In addition, the limitations of this study are the very small number of samples in the group without metastases and the deviation value in this study, which is quite large, namely 15%.

CONCLUSIONS

CXCR4 and MMP-9 expressions were proven to correlate with regional cervical lymph node metastasis in papillary thyroid carcinoma. Both can be used as potential prognostic factors, either concurrently or separately.

DECLARATIONS

Competing of Interest

The authors declare no competing interest in this study.

Ethical Approval

This study had been approved by the Ethical Committee of Dr. Soetomo Hospital Surabaya (0050/LOE/301.4.2/VI/2020).

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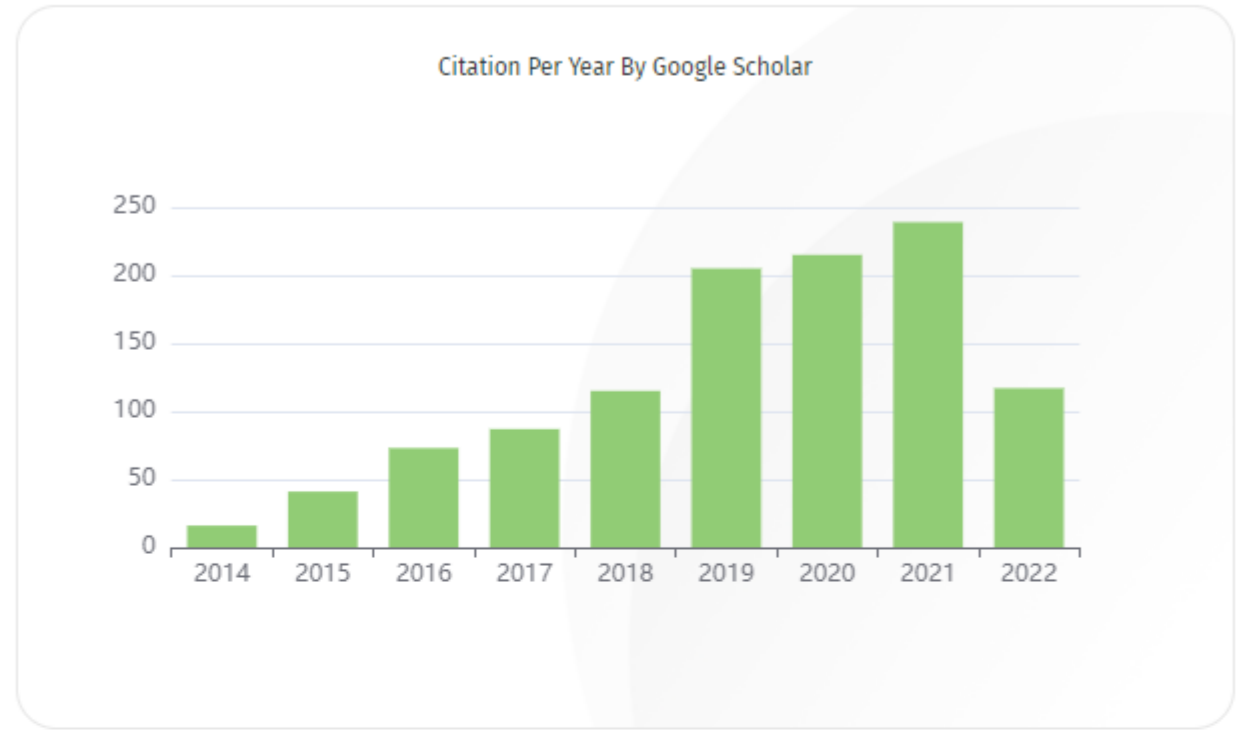
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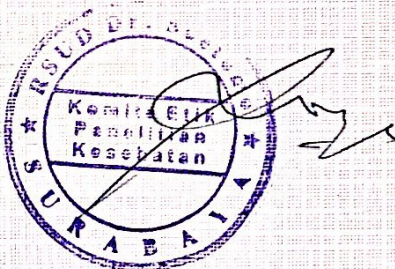
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