The prognostic value of lymph nodes mRNA CXCL12 expression in the breast cancer

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ABSTRACT

Prognosis of breast carcinoma is influenced by age, tumor size, histological grade and type, lymph node status, as well as metastasic status. Chemokine receptor CXCR-4 with its ligand, CXCL-12, may play an important role in metastasis of breast carcinoma. However, the role of CXCL-12 mRNA as a prognostic factor and a therapeutic target of human breast cancer remains controversial. This study aimed to investigate the level of CXCL-12 mRNA expression in lymph nodes of patients with invasive ductal breast carcinoma and the difference within the prognostic factors. Axillary lymph nodes obtained from 50 cases of invasive ductal breast carcinoma, were divided into two groups, with and without lymph node metastasis. Each group consisted of 25 cases. Total RNA was extracted from formalin-fixed paraffin-embedded. The CXCL-12 mRNA expression was examined using qRT-PCR method. The mean differences between the two groups were analyzed using Mann-Whitney test. The differences between CXCL-12 mRNA expression and each prognostic factor ware analyzed using Mann-Whitney comparison test. CXCL-12 mRNA expression was significantly higher in the lymph node of patients with metastasis of breast carcinoma compared to the non-metastasis cases (p<0.01). There were significant differences between CXCL-12 mRNA expression with poorly histological grade (p=0.003), bigger primary tumor size (p=0.005) and age of ≥45 y.o (p=0.012) in the metastatic group, but there were no significant differences between both age of <45 and ≥45 y.o.This study suggests that the higher CXCL-12 mRNA expression level are associated with bigger tumor size and poor differentiation in breast cancer patient with lymph nodes metastasis.

ABSTRAK

Prognosis karsinoma payudara dipengaruhi oleh usia, ukuran tumor, derajat dan tipe histologis, status kelenjar getah bening serta status metastasis. Reseptor kemokin CXCR-4dengan ligannya, CXCL-12 berperan penting dalam metastasis karsinoma payudara. Namun peran CXCL-12 mRNA sebagai faktor prognostik dan target terapi kanker payudara masih kontroversial. Penelitian ini bertujuan untuk mengkaji tingkat ekspresi mRNA CXCL-12 pada kelenjar getah bening pasien karsinoma payudara duktal invasif dan perbedaan faktor prognostiknya. Kelenjar getah bening aksila diperoleh dari 50 kasus karsinoma payudara duktal invasif, dibagi menjadi dua kelompok, dengan dan tanpa metastasis kelenjar getah bening. Setiap kelompok terdiri dari 25 kasus.RNA total diekstraksi dari *formalin-fixed paraffin-embedded.* Ekspresi mRNA CXCL-12 diperiksa dengan metode qRT-PCR. Perbedaan rata-rata antara kedua kelompok dianalisis menggunakan uji Mann-Whitney. Perbedaan antara ekspresi mRNA CXCL-12 dan masing-masing faktor prognostik dianalisis dengan uji perbandingan Mann-Whitney. Ekspresi mRNA CXCL-12 lebih tinggi secara nyata pada kelenjar getah bening pasien dengan metastasis karsinoma payudara dibandingkan dengan kasus non-metastasis (p <0,01). Ada perbedaannyata antara ekspresi CXCL-12 mRNA dengan derajat histologis yang buruk (p = 0,003), ukuran tumor primer yang lebih besar (p = 0,005) dan usia ≥ 45 tahun (p = 0,012) pada kelompok metastasis, tetapi tidak adaperbedaan nyata antara usia <45 dan≥ 45 tahun. Penelitian ini menunjukkan bahwa tingkat ekspresi CXCL-12 mRNA yang lebih tinggi berkaitan dengan ukuran tumor yang lebih besar dan diferensiasi yang buruk pada pasien kanker payudara dengan metastasis kelenjar getah bening.

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INTRODUCTION

The prevalence of breast cancer in Indonesia according tobasic health research (*Riset Kesehatan Dasar/Riskesdas*) in 2013 was the highest after cervical cancer (0.5 per 1000 inhabitants). In Yogyakarta Special Province, the prevalence of breast cancer was 2.4 per 1,000 inhabitants.[1] The trend of female breast cancer was increasing during 2013- 2018. Globocan, the International Agency for Research on Cancer (IARC), in2018 estimated the incidence of female breast cancer in Indonesia was 42.1 per 100,000 andthe mortality rate was 17.0 per 100,000.[2] The most common type of breast cancer is invasive ductal carcinoma non-special type (NST) as much as 59%.[3]

Metastasis is a major cause of mortality and morbidity in breast carcinoma. The metastasis has a distinctive pattern and has no correlation with blood flow patterns. However, it may show preferential homing, cell adhesion, survival and proliferation of cancer cells in specific tissues and organs, such as lymph nodes, lungs, bones, and liver. Several factors have shown to play a role in metastatic carcinoma of the breast, including the chemokine receptor CXCR4, and its ligand CXCL12.

CXCL12 or stromal cell-derived factor (SDF-1) is a member of the CXC chemokine family. Itis derived from cloned murine bone marrow and characterized as a pre-cell B growth-stimulating factor. SDF-1 is activated through CXC chemokine receptor (CXCR4), whichis a physiological receptor for SDF-1 and plays a role in chemotaxis, haematopoiesis, vasculogenesis, and metastasis. CXCR4 has been recognized to play a role in tumor cell homing to specific organs.[4] Activation of CXCR4 induces signal transduction pathways of cancer cells initiating the process of metastasis through cytoskeletal changes, actin polymerization,[5] pseudopodia formation, endothelial cell adhesion, migration, and proliferation.[6] In addition, axis CXCR4-CXCL12 is involved in repair and tissue regeneration. The repair of ischemic lesion involves selective recruitments of local or circulating progenitor cells.

Hypoxia-inducible factor-1 (HIF-1), is the main mediatorin tissue hypoxia, which induces the expression of CXCL12 in ischemic tissue. It inducesthe CXCL12 on endothelial cells by attract circulating stem cells and progenitor cells to the lesion area. During tissue regeneration, CXCL12 expression will return to normal after the oxygen tension is normalized.[7] In addition, HIF-1 also can improve the expression and function of CXCR4 in normal cells and malignant cells.[8] CXCR4 in breast carcinoma is expressed on tumor cells of primary and metastatic lesions, whereas CXCL12 is produced in many organs and tissue metastasis targets. CXCL4-CXCR12 axis affects angiogenesis, tumor cell proliferation, apoptosis, and mediates tumor cells to metastasize into the target organ. This chemokine can be examined semiquantitatively using immunohistochemical techniques or quantitatively using qRT-PCR (quantitative real time PCR).[9-12]

CXCL12 mRNA expression significantly upregulated in miofibroblast of invasive breast carcinoma, compared to that of miofibroblast in normal breast tissue. Besides direct effects on breast carcinoma cells, CXCL12 is also as a mediator to attract the circulating endothelial progenitor cells in the process of angiogenesis.[13] The role of CXCR 4-CXCL12 axis in the metastasis process also occurs in other organ malignancies, including brain tumor cells (neuronal and glial tumors),[14] neuroblastoma,[15] colorectal cancer,[16] prostate cancer,[17] melanoma,[18] and ovarian cancer.[19]

CXCR4 mRNA expression in primary tumors of colorectal cancer[20] and melanoma[18] is correlated with their recurrence, metastasis, and survival.

Research on gastric carcinoma has shown a significant difference in mRNA expression levels of SDF-1 (CXCL12) between lymph nodes with metastasis and normal lymph nodes. Positive SDF-1 mRNA expression in lymph nodes with metastatic gastric carcinoma isconsistent with the positive CXCR4 mRNA expression in carcinoma gaster.[21] Studies published that emphasize SDF-1 and CXCR4 in breast carcinoma have been performed *in vitro,* however the study of prognostic and therapeutic molecular targets CXCL12/SDF-1 in human breast carcinoma still has been very limited. This study aimedto investigate the differencesin mRNA expression levels of CXCL12 in lymph nodes of patients with breast carcinoma with and without lymph nodes metastasis, as well as its relation with histological grade, age, and size of the primary tumor, quantitatively using qRT-PCR technique.

MATERIALS AND METHODS

Patients

This research was a retrospective observational non-analytical experimental cross sectional approachconducted in the Department of Pathology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada and Dr.Sardjito General Hospital, Yogyakarta. Subjects were lymph node tissue paraffin blocks of breast carcinoma patients from 50 patients, who were selected by consecutive sampling between 2014 and 2015. The samples weregrouped into two groups lymph nodes with cancer cell infiltrationof breast carcinoma andthose without cancer cell infiltation. Each group consisted of 25 patients. The inclusion criteria of this study ware women with invasive ductal breast carcinoma of NST, modified radical mastectomy breast surgery, had complete clinicopatologic data and >2mm cancer cell in formalin-fixed paraffin embedded. The exclusion criteria were

men, with recurrent cases, incomplete supporting data and without propper formalin fixed paraffin embedded. This study was approved by the Medical and Health Research Committee, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta.

Protocol of study

Data on the clinicopathological characteristics were extracted from the medical records. Characteristic variables of patients were age using cut off 45 years old, with <45 versus ≥45 y.o, primary tumor size using ≤2 cm versus >2 cm, and histological grade or tumor differentiation. Since there was no good histological grade in this panel, the patients were classified as moderate and poor histological grade. The CXCL12 mRNA expression levels was obtained byqRT-PCR.

The extraction of RNA from paraffin embedded-samples were used in this study. The samples were prepared from the paraffin embedded-tissue that cut into 5 slices of 6-μm sections using a microtome. FavorPrep™ Tissue Total RNA Mini Kit was used for RNA extraction according to the manufacturer's instructions. Tumor samples were taken from areas of histopathologically confirmed invasive ductal carcinoma. Weutilized > 2mm in the greatest dimension or ≥80% tumor cell in lymph node tissue and scraped off the normal tissue. Paraffin was removed by extracting three times with Xylol for @ 10 min, 3 min, 3 min, followed byethanol absolute twice for 3 min. Cell or tissue was lysed using 350μL FARB buffer and 3.5μL β-mercaptoethanol, incubate at room temperature for 3 min, centrifuge for 30 sec at 1000 rpm. RNA was bound by 400μL of 70% ethanol (RNase free), centrifuge at 14000 rpm for 1 min. The RNA pellet was washed with 500 and 750μL wash buffer twice, centrifuge at 14000 rpm for 1 min, finally

for RNA elution dissolved in 50µL RNase-free water and centrifuge at 14000 rpm for 1 min. RNA concentration and purity were determined using a NanoDrop spectrophotometer. qRT-PCR was performed using the DT-Lite Real-Time PCR System (DNA Technology) with KAPA SYBR®FAST One- Step qRT- PCR Kit fast. GAPDH was used as the housekeeping gene and CXCL12 expression level was investigated using the specific primer. GAPDH sequence was *fw* 5'-GCA TCC TGG GCT ACA CTG AG-3 'and *rev* 5'-TCC ACC ACC CTG TTG CTG TA-3'. CXCL12 sequence was *fw* 5'-GAT TGT AGC CCG GCT GAA GA-3 'and *rev* 5'-TTC GGG TCA ATG CAC ACT TGT-3'. Cycling condition was reverse transcription 5 min at 42ºC, enzyme inactivation 3 min at 95ºC, followed by 40 cycles of denaturation 3 sec at 95ºC and 20 sec extension at 60ºC, according to instrument guidelines. CXCL12 expression levels were assessed according to the standard curve method with $2^{-\Delta\Delta CT}$ formula. ΔCT= PCR score of CXCL12 – PCR score GAPDH. $\Delta\Delta CT$= ΔCT of tumor sample –ΔCT normal tissue.[22]

Statistical analysis

The mean of CXCL-12 mRNA expression level between two groups and the differences in prognostic factors were analyzed using a Mann-Whitney test. A $p < 0.05$ were considered statistically significant.

RESULTS

Characteristics of patients

The characteristic features of 50 female patients with breast carcinoma are in TABLE 1. The mean age at diagnosis was 51.8 ± 11.2 y.o. (median 51 yr), 36 patients (72%) were ≥ 45 yr and 14 patients (28%) were <45 yr. The smallest tumor size was 0.80 cm and the largest size was 15 cm. Those with poor histological grade were 41 patients (82%), 9 patients (18%) with moderate grade, and there was no sample with good histological grade.The number of patients with lymph node metastatic breast carcinoma were more frequent at the age of≥45 yr, having primary tumor size >2 cm and a poor histological grade.

TABLE 1. Characteristic of patient with breast carcinoma

Variable	Frequency (%)		Total (%)
	Non lymp node metastasis	Lymph node Metastasis	
Age (yr)			
• < 45	8 (32)	6 (24)	14 (28)
• ≥ 45	17 (68)	19 (76)	36 (72)
Primary tumor size (cm)			
• ≤ 2	6 (24)	3 (12)	9 (18)
• > 2	19 (76)	22 (88)	41 (82)
Histological grade			
• 2	6 (24)	3 (12)	9 (18)
• 3	19 (76)	22 (88)	41 (82)

CXCL12 mRNA expression by groups of lymph node metastasis

The results of the qRT-PCR analysis is shown in FIGURE 1 and 2. The mean of CXCL12 mRNA expression in the metastatic group (47.70±9.01)was significantly higher (p=0.007) than that

inthe non-metastasis group (19.01±4.80). The lowest CXCL12 mRNA expression in the metastasis group was 1.23 fold and the highest was 168.89 fold, compared with the internal control. In the non-metastasis group, the lowest CXCL12 mRNA expression was 1.41 fold and the highest was 119.42 fold compared with internal control. Most of the tumor tissues had increased level, compared to normal tissue that was considered a 1-fold expression level.

The wide range might be caused by some factors, the most likely cause was the use of paraffin-embedded formalin-fixed tissue to analyzemRNA expression. Formalin fixation induces RNA molecules breakdown into small fragment, cross-linkage of nucleic acids with protein, leading to inefficient reverse transcription reaction.[23] The duration of fixation, storage,and extraction techniques impact the quality of RNA extracted from fixed and paraffi-embedded tissues.
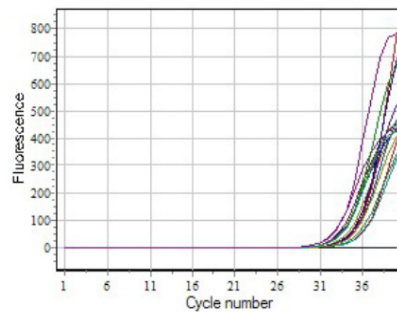

FIGURE 1. Dependence of FAM channel fluorescences on cycle number. Each line represents the expression of CXCL12 mRNA in lymph node metastasis.
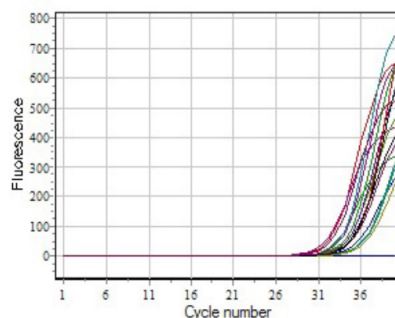

FIGURE 2. Dependence of FAM channel fluorescences on cycle number. Each line represents the expression of CXCL12 mRNA in lymph node without metastasis.
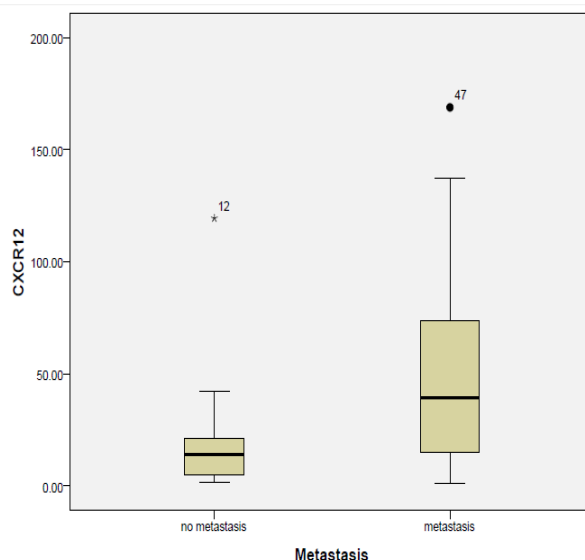
FIGURE 3. Box plot CXCL12 mRNA expression in lymph nodes with and without metastasis of breast carcinoma

The differences CXCL12 mRNA expression in both groups based on age, primary tumor size and histological grade

Mann-Whitney comparison test in TABLE 2 shows significant differences in CXCL12 mRNA expression level between lymph nodes with metastatic carcinoma of the breastand without metastasis at age≥45 yr (p = 0.012), primary tumor size >2 cm (p = 0.005) and the poor histological grade (p = 0.003). However, there was no significant difference in CXCL12 mRNA expression level in both groups at age <45 yr (p = 0.370), primary tumor size <2 cm (p = 0.80) and moderate histological grade (p = 0.360).

TABLE 2. The differences CXCL12 mRNA expression between lymph nodes with and without metastasis based on age, primary tumor size and histological grade

Characteristics	Total (%)	Non metastasis		Metastasis		p
		n (%)	Mean CXCL12	n (%)	Mean CXCL12	
Age (yr)						
• < 45	14 (28)	8 (32)	14.5±2.8	6 (24)	28.4±10.4	0.370
• ≥45	36 (72)	17 (68)	21.1±7.0	19 (76)	53.8±11.1	0.012
Primary tumor size (cm)						
• ≤ 2	9 (18)	6 (24)	14.4±6.0	3 (12)	10.3±2.6	0.800
• > 2	41 (82)	19 (76)	20.5±6.1	22 (88)	52.8±9.7	0.005
Histologic grade						
• 2	9 (18)	6 (24)	15.2±5.8	3 (12)	8.7±6.9	0.360
• 3	41 (82)	19 (76)	20.2±6.1	22 (88)	53.0±9.7	0.003

The differences between each prognostic factor of lymph nodes metastasis and without metastasis groups are presented in TABLE 3. A significant differences in CXCL12 mRNA expression between moderate and poor histological grade in the group with lymph node metastasis (p = 0.030) was observed, but not in the non-metastasis one (p = 0.850). A significant difference in CXCL12 mRNA expression level between the primary tumor size ≤2 cm and >2 cm in metastasis group (p = 0.04) was observed, but not in the non-metastasis one (p = 0.730). The tumor size >2 cm and a poor histological grade increased the mean of CXCL12 mRNA expression level by 52.8±9.7 fold in tumor size and 53.0±9.7 fold in histological grade.

TABLE 3. The difference between the ages of CXCL12 mRNA expression, pimer tumor size, histological grade in the group with lymph node metastasis and withoutmetastasis

Characteristics	Non metastasis		p	Metastasis		p
	n (%)	Mean CXCL12		n (%)	Mean CXCL12	
Age (yr)						
• < 45	8 (32)	14.5±2.8	0.82	6 (24)	28.4±10.4	0.590
• ≥ 45	17 (68)	21.1±7.0		19 (76)	53.8±11.1	
Primary tumor size(cm)						
• ≤ 2	6 (24)	14.4±6.0	0.73	3 (12)	10.3±2.6	0.040
• >2	19 (76)	20.5±6.1		22 (88)	52.8±9.7	
Histologic grade						
• 2	6 (24)	15.2±5.8	0.85	3 (12)	8.7±6.9	0.030
• 3	19 (76)	20.2±6.1		22 (88)	53.0±9.7	

DISCUSSION

CXCL12, a ligand of the chemokine receptor CXCR4, plays an important role in metastatic breast carcinoma. CXCL12-CXCR4 axis affects angiogenesis, tumor cell proliferation, apoptosis, and mediates tumor cells to metastasize into the target organ. In this study, CXCL12 mRNA expression of the axillary lymph node with invasive ductal breast carcinoma was observed by using qRT-PCR. The choice of qRT-PCR analyses was also guided by the consideration that other techniques such as immunohistochemical staining confirmed the expression of CXCL12 at the protein level, considering the staining intensity and area extent. CXCL12 appears in most tumor cells and in stromal cells, therefore more difficult to quantify. However, qRT-PCR does not reveal the cell type of expression. The increased mean level of CXCL12 mRNA expression in lymph nodes metastatic breast carcinoma was significantly higher (p=0.007) compared to that without metastasis. This supports the interaction between CXCL12 and CXCR4 as its receptor, possibly causing breast carcinoma cells to migrate to lymph nodes that have a high number of chemokines.[24] Similar findings were obtained by Kang *et al.*[25] and Shim *et al.*[9] In contrast, previous studies demonstrated that CXCL12 expression is higher in lymph nodes without metastasis of breast carcinoma. However, the study was conducted with a cohort microarray and immunohistochemical methods with a

total sample of 249 cases.[10]

Complex CXCL12-CXCR4 can significantly increase the migration speed and the invasive feature of breast carcinoma cells *in vitro*.[25] The study of CXCL12 expression in lymph node of metastatic carcinoma of extra-mammary Paget's disease showed an increased expression of CXCL12 in tumor-associated lymphatic endothelium and endothelial limphangiogenic in the subcapsular sinus tumor- draining lymph nodes, whereas CXCR4 is more expressed on the cell tumor.[26] When tumor cells or stromal tumors secrete lymphangiogenic growth factors, such as VEGF-C or VEGF-D, attracted to the lymphatic vessels expressing VEGFR-3 in the primary tumor, chemokines secreted by lymph vessels or lymph nodes will attract the migration of tumor cells expressing chemokine receptors to the lymph nodes.[27] This shows that CXCL12 and VEGF synergistically increase the angiogenic process. Other studies have shown CXCL12 can induce neovascularization *in vivo* and the formation of new blood vessels in rat aorta *ex vivo*.[28] Thus CXCL12 can be a potential marker for metastatic process in lymph node.

Vasa lymphatic preferentially facilitates metastasis by providing pathways to spread tumor cells than blood vessels due to discontinuous basement membrane, loose cell junction and low flow rate. Therefore, it can reduce pressure and increase the survival of tumor cells. Thus lymphatic metastasis in breast carcinoma can be an important predictor. Migration of tumor cells to target organs occurs through chemotactic effects of CXCL12-CXCR4 axis. The increase of CXCL12 mRNA expression in target organs is associated with prognostic factors of breast carcinoma. The prognostic factors studied were age, primary tumor size, and histological grade or tumor differentiation. CXCL12 mRNA expression in poor histological grade showed significant differences between groups of lymph nodes with metastatic breast carcinoma and without metastasis (p = 0.030). These results are similar to previous studies demonstrating high levels of SDF-1 mRNA expression in breast carcinoma with poor histological grade and moderate compared to the well-differentiated grade.[25] It showed that CXCL12 (SDF-1) increases in aggressive tumors.

The present study showedthat 88% of samples in the lymph nodes metastasis group exhibita bigger primary tumor size (> 2 cm). Of this, 80% were having poor histological grade, with CXCL12 mRNA expression higher than the non-metastasis group. In lymph nodes, metastasis group with primary tumor size >2 cm, the differences in CXCL12 mRNA expression increased significantly by 52.8±9.7 fold compared to the non-metastasis group (p = 0.005). However, there was no significantly differrence in CXCL12 mRNA expression between samples with primary tumor size <2 cm in both groups (p = 0.800). Schnitt & Guidi,[29] reported that there is a linear relationship between tumor size and axillary lymph node involvement. Patients with lymph nodes containing tumor size >2 cm have a worse prognosis compared to patients with tumor size <2cm.[30] Tumor size has been investigated as a consistent prognostic factor after lymph node status in breast carcinoma. Chang *et al.*[31] reported that large tumors (> 5 cm) with negative lymph nodes have a better prognosis than those with a tumor size of 3 to 4.9 cm. Large-sized tumors without lymph node metastasis have a lower ability to settle. However, the increase oftumor size may increases recurrence. In this study, the mean level CXCL12 mRNA expression without lymph node metastasis was lower (20.5±6.1 fold) compared to the lymph nodes with metastasis (52.8±9.7 fold).

The expression of CXCL12 mRNA in lymph nodes with metastatic breast

carcinoma tended to occur in those aged > 45 yr (TABLE 2). The difference of mean CXCL12 mRNA expression wassignificantly (p=0.012) higher in lymph nodes with metastasis (53.8±11.1 fold) than without metastasis (21.1±7.0 fold). However, no significant differences in CXCL12 mRNA expression between age ≤45 yr and > 45 yr was observed among each group (TABLE 3). This study used a wide age range, with a median age of 51 yr (32 – 79 yr). Kim, *et al.*[32] reported that there is no significant relationship between the expression of SDF-1α protein with the patient's age.

Sun *et al.*[11] reported that there is no significant correlation between CXCL12 protein expression with age and menopausal status. Women aged above 50 yr are more at risk to suffer from breast carcinoma compared with women aged under 50 yr.[33] Breast tumor at age >45 yrexpressed more ER. This may be due to decreased estrogen levels and thus upregulate the receptor.[34] The decline in blood estrogen at the time of menopause, causes the sensitized stem cells to activate the local estrogen synthesis mechanism. This would induce proliferation and genetic dysfunction.[35] CXCL12 has been investigated as estrogen-regulated genes in ovarian and breast cancer cells with positive ER through a direct path in which estrogen induces the production of CXCL12 through ER.[36]

Several limitations were present in this study. Firstly, we did not determine the level of axillary lymph nodes, which the number of involved lymph nodes and the level of involvement (level I, II or III) were independent predictive factor for survival.[37] Secondly, the different duration of sample's storage acquired from 2014 to 2015 were probably inappropriate. Thirdly, we did not distinguish between lymph nodes with sinus histiocytosis or follicular hyperplasia as the possible reaction of tumor cell metastasis. Finally, the number of sample was not sufficiently large, which could impact the robustness of the statistical result. This study provided preliminary data of CXCL12 mRNA expression of lymph node with metastasis breast cancer in the Indonesian population. Further extensive studies of chemokines in lymph node dan breast cancer especially in Indonesia are required for appropriate therapeutic strategy.

CONCLUSION

The present study suggests that the higher CXCL12 mRNA expression level is associated with bigger tumor size and poor differentiation in breast cancer patients with lymph nodes metastasis.

ACKNOWLEDGEMENTS

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