RESEARCH ARTICLE

Clinicopathological Significance of Lymphangiogenesis and Tumor Lymphovascular Invasion in Indonesian Breast Cancers

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Abstract

Background: Lymphangiogenesis, assessed as lymphovascular density (LVD), is the initial step of generalized tumor lymphovascular invasion (LVI). It also involves VEGF-C as the most important protein family. Lymphangiogenesis among breast cancer cases correlations with several clinicopathological factors are important to determine prognosis and treatment strategies, but results have been controversial and require clarification. Aim: To define correlations between VEGF-C expression, LVD and LVI with several clinicopathological parameters from Indonesian breast cancer patients. Materials and Methods: Using a cross-sectional study, a total of 48 paraffin-embedded tissues of breast cancer from Dr. Sardjito General Hospital Indonesia were assessed for VEGF-C expression, LVD and LVI by immunohistochemistry. Correlations of these markers with clinicopathological parameters like patient age, tumor size, lymph node status, grade, ER/PR and Her-2 status, cell proliferation and p-53 expression were investigated by linear analysis. Correlations of VEGF-C expression and LVI with several clinicopathological parameters were analyzed with Coefficient Contingency Chi-Square test. Results: The mean of patients age was 53.0 year, pre and post-menopausal patients accounting for 56.3% and 43.8%, respectively. Some 10.4% were well, 41.7% moderate and 47.9% poorly differentiated. ER positivity was evident in 50% while PR and Her-2 positivity was found in 31.3% and 33.3%, respectively. Breast cancer cells with over-expression of p-53 was 64.6% and with high cell proliferation was 56.3%. Lymph node metastasis was noted in 63.5%, and LVI in 72.9%. Significant correlations were found between LVD and tumor size (p:0.037), grade (p:0.000), lymphnode status (p:0.036), LVI (p:0.003), as well as with p-53 and cell proliferation. There were also significant correlation of VEGF-C (p:0.011) and LVI (p:0.001) with tumor grade. Only ER status was found to have a correlation with tumor size (p:0.027). Conclusions: This study suggested that in Indonesian breast cancer patients, lymphangiogenesis is correlated with tumor size, grade, lymph node status and tumor lymphovascular invasion, the latter also being related with p-53 over expression and high cell proliferation.

Keywords: VEGF-C - LVD - LVI - age - tumor size - lymphnode status - grade - ER/ PR status - Her-2 status

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Introduction

Breast cancer is one of the most common types of cancer found in women in Dr. Sardjito General Hospital Jogjakarta, Indonesia. Most of those patients were in the stage IIB and high grade (Aryandono et al., 2000). Prognosis of breast cancer is determined by several clinicapathological parameters which is also can be used to predict the natural history of this cancer. The presence or absence of lymph nodes metastasis, tumor size, grade, hormonale status, Her-2 status, cancer cell proliferation rate, p-53 mutation and patient's age are well known prognostic factors for breast cancer patients. Lymphnode metastasis is accepted as the most powerful prognostic factor (Tavassoli, 2003). Seventy percent of Indonesian breast cancer patients presented with lymph node metastasis at diagnosis and with more than 3 nodes involvement in 38.3% patients (Aryandono et al., 2000).

Breast cancer primarily metastasizes via lymphatic system and need lymphangiogenesis as the initial process. The most important protein involved in this lymphangiogenesis is VEGF-C. The binding of this protein to its receptor, VEGFR-3, may induce lymphatic endothelial cell proliferation, maturatian and differentiation (Yavuz et al., 2005; Sundar et al., 2007). Several studies of human cancers found that

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strong expressions of VEGF-C were about of 30-83.7% (Yonemura et al., 1999; Nakamura et al., 2003; Ogawa et al., 2004; Schoppmann et al., 2006; Zhang et al., 2008). The work of VEGF-C has already started before metastasis and its expression decreased after (Teramoto et al., 2008).

Lymphovascular density (LVD) is the hallmark of lymphangiogenesis and it can be detected by D2-40, a specific marker of new lymphatic endothelial cells (Sundar et al., 2007). Previous researches of breast cancer suggested that LVD is correlated with tumor lymphovascular invasion (LVI) and could be used as an independent parameter of disease free survival, overall survival (Schoppmann et al., 2006), and also as a predictor of disease relapse (Tezuka et al., 2007). Lymphovascular invasion as the main route of lymphnode cells metastasis , is defined by the presence of cancer cells in the lymphatic vessel. The number of relapse during ten years in breast cancer patients with positive lymphnode metastasis was 70% compared to 20-30% in patients without lymphnode metastasis (Donegan, 1997).

Studies of lymphangiogenesis among human cancers and their correlation with several clinicopathological parameters showed controversial results. In colorectal, ovarium and head and neck cancer, VEGF-C expression has been proven to be a good prognostic factors (Beasley et al., 2002; Furudoi et al., 2002; Yokoyama et al., 2003). While in breast cancer, VEGF-C was negatively correlated with survival 17, and can be used as a marker of long survival (Nakamura et al., 2003).

Lymphangiogenesis in breast cancer and their correlation with several clinicopathological parameters are needed to be defined in order to determine more accurate tumor prognosis and treatment. Treatment strategies of anti lymphangiogenesis could be developed by directly inhibiting VEGF-C pathways or its ligand as well as its receptor, both in a protein and mRNA levels. Another treatment strategy is gene therapy to inhibit lymphangiogenesis by blocking antibody or molecule competitor of VEGF-C/ VEGFR-3, and molecule inhibitor tyrosine kinase as well as other molecules (Sundar, 2007).

The aim of this research is to investigate the clinicipathological significance of VEGF-C expression, LVD and LVI in Indonesian breast cancer patients.

Materials and Methods

Design of this research was a quantitative non-experimental, perfomed by cross sectional method. Research samples consisted of 48 embedded-paraffin tissue of breast cancer, which were taken from Dr. Sardjito General Hospital (Pathology Laboratory archive) Jogjakarta, Indonesia year 2009-2010. Samples taken from core biopsy or containing small focus of tumor or were diagnosed other than infiltrative duct carcinoma were excluded from this study. Hematoxyllin Eosin slides were examined to classified cancer grade into well, moderate and poorly grade, based on the Elston-Ellis criteria (Tavassoli, Devilee (2003). Lymph node metastasis was categorized as: no cancer metastasis, cancer metastasis into ≤3 lymphnodes, and cancer metastasis into >3 lymphnodes.

Immunohistochemical staining

Paraffin-embedded tissue were stained immunohistochemically with monoclonal antibody anti VEGF-C (ab 9546, Abcam, dilution 1:100), D2-40 (IP 266610, Biocare Med, dilution1:75), ER (M 7046 Dako, dilution 1:50), PR (PgR 636 Dako, dilution 1:50), Her-2 (CB 11 Dako, dilution 1:100), p-53 (ab 911 abcam, dilution 1:100) and Ki-67 (ab 16667 abcam, dilution 1:100) using DAB chromogen and counter stain Hematoxyllin Mayer. Positive control was taken from breast cancer tissue with positive IHC for those markers. Paraffin-embedded tissue of breast cancer without antibody was used as the negative control.

Immunohistochemical results were analyzed using 200 x microscopic fields by two independent observers based on following criteria. Samples showing >1% of nuclear ER/PR expression were considered as ER/PR positive. Her-2 positive was determined according to the Her-2 score. Samples with >30% membranous staining and uniform of Her-2 expression were considered as Her-2 positive. Cell proliferation rate was classified as low (samples with <10% of Ki-67 expression) and high (samples with >10% of Ki-67 expression). Over expression of p-53 was defined by the presence of nuclear expression ≥ 10%. VEGF-C expressions, which were graded according to the extent of cytoplasmic staining, were graded as 0: negative, 1: <25%, 2: 25-50%, 3: 50-75%, 4: >75%. LVD was determined by mean number of peritumoral lymphovascular densities in which endothelial cells expressed D2-40 in 5 microscopic fields. LVI was defined as the presence of tumor cells into the lymph vessels.

The correlation between LVD with several clinicopatholocigal parameters was analyzed with Linear regression and that between VEGF-C expression, LVI

Table 1. Correlation between LVD with Tumor Size, Lymphnode Status, Grade, ER/PR and Her-2 Status, p-53 Expression, Cell Proliferation Rate and LVI in Indonesian Breast Cancer Patients

Characteristic	Mean of LVD	Statistical analysis	
Tumor size		8.64±3.76	R=0.301, P=0.037
Lnn: no metastasis		7.88±3.05	R= 0.36
	≤3 Lnn positive	7.47±3.94	P= 0.036
	>3 Lnn positive	10.73±3.71	
Grade:	Well	4.20±1.64	R= 0.519
	Moderate	7.65±3.13	P= 0.000
	Poorly	10.47±3.51	
ER status:	Negative	9.41±3.82	R= -0.267
	Positive	7.87±3.61	P= 0.067
PR status:	Negative	8.54±4.05	R= 0.027
	Positive	8.72±3.35	P= 0.870
Her-2 status:	Negative	8.35±3.87	R= 0.397
	Positive	9.06±3.64	P= 0.532
p-53 expression:	Low	8.42±3.40	R=0.223
	High	8.73±3.98	P=0.119
Cell proliferation:	Low	7.53±3.02	R=0.176
	High	9.87±3.97	P=0.223
Tumor invasion:	No	6.00±4.06	R= 0.415
	Yes	9.62±3.17	P= 0.003

Table 2. Correlation between VEGF-C Expression with Tumor Size, Lymph Node Status, Grade, ER/PR and Her-2 Status, p-53 Expression, Cell Proliferation Rate and LVI in Indonesian Breast Cancer Patients

Characteristic		VEGF-C expression					Statistical
		Negatif	<25%	26-50%	51-75%	>76%	analysis
Tumor size		4±0.8	7.6±4.1	3.9±2.3	1.7±0.6	5.8±2.9	C C=0.79 Sig=0.595
Lnn:	negative	2	5	7	1	3	C C=0.458
	£ 3 Lnn +	2	2	1	2	8	Sig=0.120
	>3 Lnn +	0	5	6	0	4	
Grade:	Well	1	2	2	0	0	C C=0.323
	Moderate	1	7	7	1	4	Sig=0.006
	Poorly	2	3	5	2	10	
ER status:							
	negative	1	7	9	1	6	C C=0.258
	positive	3	5	5	2	9	Sig= 0.492
PR status:							
	negative	3	8	10	2	5	C C=0.362
	positive	1	6	3	1	9	Sign= 0.133
Her-2 status:							
	negative	3	9	9	2	8	C C=0.05
	positive	2	5	4	1	5	Sign=0.975
P-53 expression:							
	low	2	8	4	1	4	C C=0.294
	over	2	5	10	2	10	Sign=0.328
Cell proliferation:							
	low	1	7	4	1	4	C C=0.156
Rate	high	3	6	10	2	10	Sig=0.875
Tumor invasion :							
	No	2	4	5	1	1	C C=0.309
	Yes	2	8	9	2	14	Sig=0.282

Table 3. Correlation between LVI with Grade, Lymph Node Status, Grade, ER/PR and Her-2 Status, p-53 Expression and Cell Proliferation Rate in Indonesian Breast Cancer Patients

Characteristic		Tumor invasion		Statistical
		Yes	No	analysis
Grade:	Well	1	4	CC=0.448
	Moderate	13	7	Sig=0.001
	Poor	21	2	
Lnn:	Negative	12	6	CC=0.117
	<3 lnn +	11	4	Sig=0.383
	>3 lnn +	12	3	
ER status:	Negative	19	5	CC=0.082
	Positive	16	8	Sig=0.554
PR status:	Negative	10	4	CC=0.180
	Positive	18	16	Sig=0.201
Her-2 status:	Negative	9	5	CC=0.130
	Positive	21	13	Sig=0.925
p-53expression:	<10%	5	9	CC=0.314
	>10%	24	10	Sig=0.020
Cell proliferation rate:	Low	4	10	CC=0.439
	High	27	7	Sig=0.001

with several clinicopathological parameters was analyzed with Coefficient Contingency. A p-value of <0.05 was considered statistically significant.

Results

Mean of patient age at diagnosis was 53.0±10.52 year (range from 34-75 years old), mean of tumor size was 5.32±3.28 cm (range from 1-15 cm) and mean of LVD

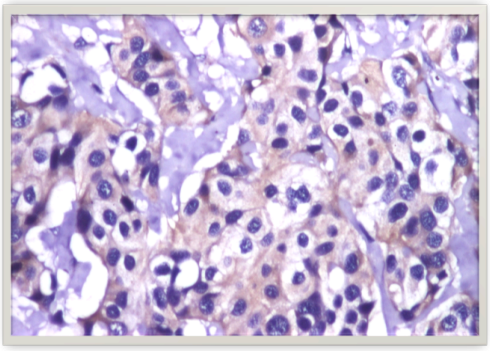

Figure 1. Positive VEGF-C Expresion in Breast Cancer

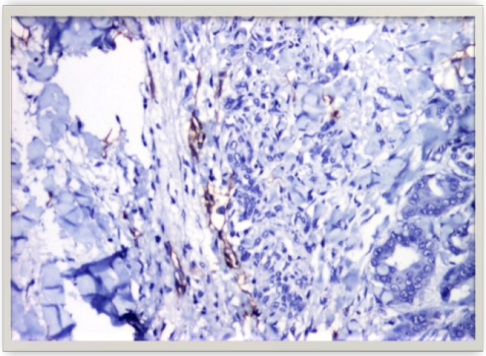

Figure 2. Lymphovascular Density in Breast Cancer (stained by D2-40)

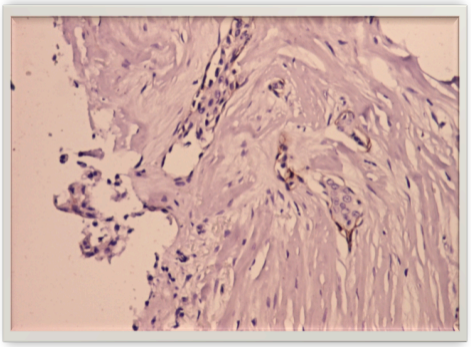

Figure 3. Tumor Lymphovascular Invasion in Breast Cancer

was 8.65±3.76 (range from 3-18). The number of patients ≤50 year old was 56.25% and patients >50 year old was 43.8%. The samples could be grouped into: 10.4% low grade, 41.7% moderate grade and 47.9% poorly grade. The percentage of patients ER positive was 50%, with PR positive was 31.25%, and with Her-2 positive was 33.3% % of total number of samples. The percentage of breast cancer samples with over expression of p-53 was 64.6% and with high cell proliferation rate was 56.3%. Lymph node metastasis was found 63.5% (more than 3 nodes in 30.2%) and LVI in 72.9%.

The highest number of LVD was big size cancer, cancer metastasis into >3 lymphnodes and high grade. Even not statistically significant, high number of LVD was found in cancer with ER negative, Her-2 positive, over expression of p-53, high cancer cell proliferation (Table 1). Data for VEGF expression are summarized in Table 2 and for correlataions wiht LVI in Table 3.

Discussion

Mean of age of Indonesian breast cancer patient studied was 53.0±10.5 year (range: 34-75 years). Pre menopausal patients was 56.3%. This result was similar with some previous studies (Schoppmann et al., 2004; Yavuz et al., 2005), but different from other (Marinho et al., 2008). High frequency of Indonesian premenopausal breast cancers patients should be taken into consideration because they are frequently associated with negative hormonal status, poorly grade, and worse prognosis. Based on the results that mean of tumor size studied was 5.32±3.28 cm, 61.4% samples was positive lymph node metastasis and 89. 6% was high grade tumor, meaning that most of Indonesian breast cancers studied were cancer with high grade and late stage.

Lymphovascular densities (LVD) were counted in the hot spot areas, which are biologically important as the main route for cancer metastasis. Lymphovascular densities are analyzed in the peri cancerous areas. Intra cancerous LVD usually collaps, has ovally and small size due to trapped by ductus, lobulus, blood vessels and tumor growth, and also can be misinterpreted as retraction artifact (Tezuka et al., 2007; El Ghohar et al., 2008; Botting et al., 2010). Cancer with high LVD significantly more often invade lymphatic vessels namely lymphangiogenesis carcinomatosa (Schoppmann et al., 2004). The precise mechanisms whereby the newly formed lymphatics promote cancer progression are not well understood. A change of lymphatic function and an increase in LVD alter the microenvironment and its prophecy for metastasis. Thus, the location of cancerous lymphatics may be an important factor in the metastatic spread of cancer cells (Botting et al., 2010).

This study found significant correlation between LVD with tumor size, grade, lymphnode status and LVI. Similar result was found by Schoppmann et al. (2004) but not by El Ghohary et al. (2008). In breast cancer, tumor size is the most powerful predictor for local recurrence, regional and systemic spread, therefore for overall survival. High grade fast growing tumor may produce more growth factors and offers a bigger clonal variety of tumor cells capable of invading lymphatic vessels compared with low grade slow growing tumor. The significant correlation between LVD and LVI in this study could be explained through a lymphangiogenesis-induced increase of the lymphatic window, providing cancer cells with opportunities to enter into lymphatic vessels (Schoppmann et al., 2004).

In this study, number of breast cancer patients with LVI were 72.9% and they were poorly grade cancer. LVI had significant correlation with tumor grade, p-53 expression and cell proliferation. Even not statistically significant, high number of patients with LVI were cancer with ER negative and Her-2 positive. Studies performed by Tezuka et al. (2007) and Marinho et al. (2008) found the percentage of breast cancer with LVI was 42% and 30.9%, respectively, and they were correlated with poorly grade, p-53 over-expression, high cell proliferation rate and negative hormone expression.

This study confirmed that D2-40 stains the endothelium of lymphatic vessels and is useful and reliable in detecting lymphovascular invasion. This antibody also stained myoepithelial cells of normal ducts and lobules of the adjacent peritumoral parenchyma, and basal epithelial cell layers of the epidermis, but they easily can be differentiated from lymph vessels due to the different cell type and location. Lymphovascular invasion of tumor cells is a prerequisite for dissemination via the lymphatic system. Thus, its presence represents a major criterion for evaluating the potential prognosis of breast cancer patients and predicts the choice of additional chemotherapy and/ or radiotherapy after surgery of the primary tumor. Therefore, anti-lymphangiogenesis therapies have been suggested as novel therapeutic concepts (Schoppmann et al., 2004).

VEGF-C was expressed not only in neoplastic cell but also in inflammatory cells especially macrophages. Some evidences suggested that inflammatory cells plays important role in pathological lymphangiogenesis. VEGF-C expression in neoplastic cell has been linked to lymphangiogenesis and lymph node metastasis (Papper et al., 2001; Ogawa et al., 2004; Schoppmann et al., 2004) and it can be used as a prognostic predictor in gastric and cervical cancer (Yonemura et al., 1999; Botting et al., 2010). In non small cell carcinoma of the lung, only VEGF-C in tumor cells were correlated with poor prognosis, but their expression in tumor cells and macrophages had correlation with lymph node status (Ogawa et al., 2004). In this study, VEGF-C was only correlated with tumor grade. The result was similar with Yavuz et al. (2005) but different from others (Yonemura et al., 1999; Kinoshita et al., 2001; Yavuz et al., 2005). The different number of samples, type of clone of antibody, positive cells interpretation and statistical analysis highlight the problem relating different results.

In conclusion, this study suggests that in Indonesian breast cancer patients, lymphangiogenesis has correlation with tumor size, grade, lymphnode status and tumor lymphovascular invasion. Meanwhile, tumor lymphovascular invasion is correlated with p-53 over expression and cell proliferation.

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