

## Prognostic Value of Lymphangiogenesis Determinants in Luminal and Non-luminal Breast Carcinomas

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### Abstract

**Background:** Breast carcinomas (BCs) are sub-classified according to the molecular characteristics into luminal and non-luminal subtypes that clinically show different biological behavior, treatment and prognosis. BCs spread primarily through lymphatic vessels using cascade processes of lymphangiogenesis in which VEGF-C plays an important role during lymph node metastasis. Prognostic value of VEGF-C in luminal and non-luminal BC is still unclear and has not been studied thoroughly to clarify and define prognosis and therapeutic monitoring. **Aim:** To define the prognostic value of lymphangiogenesis on survival rates of luminal and non-luminal subtypes BC. **Materials and Methods:** This study applied prospective cohort design, using 130 patients of invasive duct carcinoma of the breast, stage I-IIIa, from Sardjito General Hospital, Indonesia and subsequent longitudinal follow-up. Immunohistochemical staining was carried out using anti-ER, -PR, -Her-2, VEGF-C, VEGFR-3 and D2-40 antibodies. The related clinicopathologic characteristics of BC patients and lymphangiogenesis determinants, including VEGF-C expression, were statistically analyzed. **Results:** In non-luminal BC subtypes, VEGF-C expression (HR=0.04; 95% CI=0.01-0.41), lymph node metastasis (HR=0.14; 95% CI=0.04-0.55) and stage (HR=0.30; 95% CI= 0.02-0.76) were determined as independent prognostic factors on survival rates. However, the lymphangiogenesis determinants were not associated with the survival rates of luminal BC subtypes. **Conclusion:** This study suggested that lymphangiogenesis affects survival rates of non-luminal subtype rather than the luminal subtypes of BC.

**Keywords:** Breast carcinoma- luminal and non-luminal subtype- lymphangiogenesis- VEGF-C-survival

*Asian Pac J Cancer Prev*, 19 (9), 2461-2467

### Introduction

Breast carcinoma (BC) is a heterogeneous disease that varies in histopathology, therapeutic response, metastatic patterns and outcome. Intrinsic subtypes of BCs are hormonal positive (luminal subtype) cancers which arise from luminal epithelial cells of mammary gland and hormonal negative (non-luminal subtype) cancers that derive from myoepithelial and basal cells of mammary gland (Goldhirsch et al., 2005; Kao et al., 2009; Onitilo et al., 2009; Blows et al., 2010).

Around 60% of BC cases are luminal subtypes. The luminal subtype of BC is generally associated with better prognosis compared to the non-luminal subtype because the luminal subtype is hormone-receptor positive and is relatively sensitive to anti-hormonal treatment that is widely available for BC treatment (Kao et al., 2009; Onitilo et al., 2009; Widodo et al., 2017). On contrary, non-luminal BCs have been associated with poorer prognosis and more frequent and early recurrence as well as regional and distant metastasis (Sorlie et al., 2003;

Chanrion et al., 2007; Campbell et al., 2011).

Breast carcinoma primarily metastasizes through the lymphatic vessels to the regional lymph nodes in which lymphangiogenesis plays an important role in breast cancer lymphatogenous metastasis. The main protein involved in the lymphangiogenesis is VEGF-C (Vasculo Endothelial Growth Factor – C). Binding of VEGF-C to its receptor, VEGFR-3, may induce proliferation, maturation and differentiation of lymphatic endothelial cells (Nathanson et al., 2000; Yonemura et al., 2006). High number of lymphovascular density (LVD) stimulates tumor cells to invade the new vessels as well as subsequent lymph nodes (Cabioglu et al., 2005).

VEGF-C expression was found in 30% to 83.7% across different types of cancer (Nakamura et al., 2003; Ogawa et al., 2004; Schoppmann et al., 2006; Zhang et al., 2008). In BC, VEGF-C expression increases during tumor invasion to the lymphatic vessels. However, its expression decreases after cancer cells successfully metastasize to the lymph nodes (Teramoto et al., 2008). The correlation between lymphangiogenesis and tumor

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lymphovascular invasion (LVI) in cancers remains unclear and several studies reported controversial results (Yavuz et al., 2005; Tezuka et al., 2007; Zhang et al., 2008). Lymphangiogenesis also differs among several molecular subtypes of BC as well as among the pattern of metastasis (Raica et al., 2011; Jaime Jans et al., 2014).

The aim of this study was to define the prognostic value of lymphangiogenesis on survival rates of luminal and non-luminal subtypes BC to further determine precise prognosis and direct individual therapy.

## Materials and Methods

A prospective cohort study was used in this study. Follow up or reverse back intervention to patients was not performed in this study. Samples involved in this study were 130 paraffin-embedded tissues of invasive duct carcinoma of the breast (NST), stage I-IIIa, from Sardjito General Hospital, Yogyakarta, Indonesia. Data were collected from medical record including age, pathology laboratory number, methods of operation and pathological diagnosis. Data were analyzed anonymously and identity of patients will not be disclosed. Samples containing small focus of tumor were excluded from this study. Hematoxylin Eosin (HE) slides were examined to classify cancer morphology into well, moderate and poorly grade based on WHO criteria. Cancer stage was determined from the TNM system (Tavassoli and Devilee, 2003).

Adjuvant chemotherapy was determined by different types of chemical agents given to the patients after surgery, and was grouped into Anthracycline and Taxane-based chemotherapy. Survival was determined from the follow-up of patients from January 1, 2008 to June 30, 2013. The follow up was carried out to measure survival rates.

### Immunohistochemistry staining

Samples were stained using monoclonal antibody anti-ER (Biocare, 6F 11, dilution 1:50), -PR (Biocare, PGR 636, dilution 1:50), -Her-2 (Biocare, cb 11, dilution 1:100) to classify breast carcinoma into luminal and non-luminal subtypes. Immunohistochemical staining is also conducted using antibody anti-VEGF-C (AF 752, R&D system, dilution 1:100), VEGFR-3 (AE 349, R&D system, dilution 1:100) and D2-40 (Biocare IP 266 G10, dilution 1:100) to determine lymphangiogenesis and tumor lymphovascular invasion. DAB chromogen and counter stain Hematoxyllin Mayer were used in this study.

### Interpretation of results

Expression of ER, PR and Her-2, number of LVD and LVI status were determined under light microscopy by two independent and experienced pathologists. Expression of VEGF-C and VEGFR-3 were counted quantitatively using separation color of Adobe Photo Shop software. Number of VEGF-C and VEGFR-3 expression were determined from number of positive pixel divided by number of positive and negative pixel under light microscopy 200x fields, times by 100 (Lehr et al., 1999).

Samples are considered positive for ER and PR if >1% of tumor cells show clear positive nuclear staining

(Hammond et al., 2010). Samples are considered positive for Her-2 if >30 % of tumor cells show clear membrane staining (Wolff et al., 2007). High expression of VEGF-C and VEGFR-3 is determined if  $\geq 10\%$  of tumor cells show positive cytoplasmic staining (Mylona et al., 2007). Lymphovascular density was defined from the number of peritumoral lymphovascular where endothelial cell expressed D2-40, in 3 high microscopic fields (Botting et al., 2010). Tumor lymphovascular invasion is the existence of a tumor emboli in the lymph vessel where endothelial cell expressed D2-40 (Marinho et al., 2008).

### Statistical analysis

Bivariate analysis was used to identify correlation between each clinicopathological factors, lymphangiogenesis determinants and LVI and survival rates of luminal and non-luminal breast carcinoma subtypes. Survival analysis was performed using product limit of Kaplan Meier curve using log-rank test with significance limit of  $< 0.05$ . Multivariate analysis was used to determine prognostic values independent of the survival rates. To control influence of other prognostic factors, Proportional Hazards (Cox) Regression was used.

## Results

Mean of patient age participated in this study was 53.13 years-old (ranged from 31-81 years-old). Moderate to poorly differentiated tumor was found in 85.4% of total samples. Large size tumor (>2cm) and lymph node metastasis were found in 82.31% and 60%, respectively. Breast cancer patients were then divided based on IHC staining into luminal (53.85%) and non-luminal (46.15%) subtype. Lymphangiogenesis is characterized by  $\geq 10\%$  of VEGF-C and VEGFR-3 immunostaining. Patients with high lymphangiogenesis of VEGF-C and VEGFR-3 immunostaining were 83.8 % and 87.6%, respectively. High Lymphovascular Density (LVD) and Lymphovascular Invasion (LVI) were found in 43.8% and 70.8% of total samples, respectively. Number of dead patients were 29.2% (Table 1).

The luminal BC subtype in this study tends to occur in older women with relatively smaller sized, less lymph node metastasis, and also lower mean of VEGF-C, VEGFR-3 and LVD, compare with the non-luminal subtype (Table 2).

Bivariate analysis showed that VEGF-C expression, lymph node status, LVI and LVD were factors that influenced on survival rates of luminal BC subtype. However, multivariate analysis showed that they were not independent prognostic factors for survival rates (Table 3).

Meanwhile, independent prognostic factors of non-luminal BC survival were VEGF-C expression (HR=0.04; 95% CI=0.01-0.41), lymph node metastasis (HR=0.14; 95% CI=0.04-0.55) and cancer stage (HR=0.30; 95% CI= 0.02-0.76) after being adjusted with LVI (Table 4).

Table 1. Clinicopathological Features and Other Variables of Breast Carcinoma

Characteristics		n (%)
Age	< 50 years	52 (40)
	≥ 50 years	78 (60)
Grade	moderate-poor	111 (85.4)
	well	19 (14.6)
Size	≥ 2 cm	107 (82.3)
	< 2cm	23 (17.7)
Lymph node	positive	78 (60)
	negative	52 (40)
Stage	IIIA	32 (24.6)
	I-II	98 (75.4)
Sub- types	Luminal	70 (53.8)
	Non-Luminal	60 (46.2)
VEGF-C	≥ 10%	109 (83.8)
	< 10%	21 (16.2)
VEGFR-3	≥ 10%	114 (87.7)
	< 10%	16 (12.3)
LVD	High	56 (43.1)
	Low	74 (56.9)
LVI	Yes	92 (70.8)
	No	38 (29.2)
Survival	Dead	38 (29.2)
	Alive	92 (70.8)
Therapy	Anthracycline based	60 (46.5)
	Taxane based	70 (53.5)

Table 2. Differences in Lymphangiogenesis and Clinicopathological Features between Luminal and Non Luminal Breast Carcinoma

Characteristics	Luminal subtype	Non-luminal subtype
	Mean ± SD	Mean ± SD
VEGF-C	30.71 ± 21.59(5.2 -86.26)	33.32 ± 20.05(4.8-77.93)
VEGFR-3	21.75 ± 10.08(4.19-50.96)	23.54 ± 14.47(5.55-69.21)
LVD	9.51 ± 3.61 (3-19)	11.84 ± 3.62 (5-18)
Age	54.35 ± 9.5 (31-75)	51.70 ± 11.2 (32-81)
Size (cm)	4.4 ± 2.22 (1-10)	5.05 ± 2.84 (1-11)
Lymph node	1.7 ± 2.46 (0-10)	2.4 ± 2.64 ( 1-11)

Table 3. Independent Prognostic Factors for Survival of Luminal BC Subtype

Prognostic factors	Survival (n)		p	Unadjusted HR (95% CI)	Adjusted HR (95% CI)
	Dead	Alive			
VEGF-C	≥ 10%	14	45	0/084	0.04 (0.00-14.22)
	< 10%	0	11		
LVD	High	12	51	0/037	1033 (0.11-0.98)
	Low	2	5		
LVI	Yes	11	33	0/141	0.39 (0.11-1.42)
	No	3	23		
Lymph node	Positive	10	29	0/136	0.42 (0.13-1.35)
	negative	4	27		

Table 4. Independent Prognostic Factors for Survival of Non-luminal BC Subtype

Prognostic factors		Survival (n)		p	Unadjusted HR (95% CI)	Adjusted HR (95% CI)
		Dead	Alive			
VEGF-C	≥ 10%	23	27	0/06	0.18 (0.03-1.39)	0.04 (0.01-0.41)
	< 10%	1	9			
LVI	Yes	23	25	0/024	0.14 (0.02-1.03)	0.18 (0.02-2.40)
	No	1	11			
Stage	IIIA	13	8	0/029	0.56 (0.16-0.82)	0.30 (0.02-0.76)
	I-II	11	28			
Lymph	Positive	21	18	0/002	0.19 (0.55-0.63)	0.14 (0.04-0.55)
	negative	3	18			

## Discussion

Our cohort study involved 130 BC patients with mean of age was 53.13 years old. Median age of breast cancer patients in Asia was similar with our findings as shown by studies Turkey (Gurleyik et al., 2007), Thailand (Chuthapisith et al., 2012) and China (Zhao et al., 2012) as well as from Brazil (Marinho et al., 2008). In Caucasian, age at breast cancer diagnosis is generally older (> 60 years-old) as shown by studies from Miami (El-Gohary et al., 2008) and Winconsin (Onitilo et al., 2009). Meanwhile, younger than 50 years-old of breast cancer diagnosis was reported from Maroco-North Africa (El Fatemi et al., 2012), Iran (Najafi et al., 2013) and Sahara- Africa (Galukande et al., 2014). It seems that race, ethnicity and genetic as well as national policy of breast cancer management affects outcome of breast cancer patients (Parise et al., 2010).

Proportion of luminal subtype of BCs in this study was higher (53.85%), compare to non-luminal subtype (46.15%). Similar result from previous studies has been reported (Perou et al., 2000; Sorlie et al., 2003; Millikan et al., 2008; Raica et al., 2011; Chuthapisith et al., 2012; Galukande et al., 2014; Widodo et al., 2014). In African- American population, however, proportion of the non-luminal BC subtype was higher than the luminal one (Carey et al., 2006; Khokher et al., 2013). Genetic factors seem to play an important role in the incidence as well as molecular subtypes of BC in different race and ethnicities (Brewster et al., 2014).

In this study, luminal subtype cancer tends to occur in older women, low graded, small sized, lymph node metastasis negative and early staged BC (Table 2). Our results supported previous studies that luminal BC subtype was associated with good prognostic factors (Onitilo et al., 2009; Su et al., 2011; Yanagawa et al., 2012; Widodo et al., 2014; Liao et al., 2015).

Poorer prognosis was demonstrated in non-luminal subtype in which average survival rates were only 54 weeks. In addition, non-luminal subtype was commonly diagnosed in older patients with larger tumor size (7cm in diameter). Non-luminal BC subtype is considered as more aggressive cancer with poorer prognosis and low survival rates. Non luminal BC subtype is also associated with high cell proliferation, frequent p53 mutation with relatively limited targeted therapeutic

options (Onitilo et al., 2009; Blows et al., 2010; Campbell et al., 2011). Elder breast cancer patients had generally lower survival rate, with higher frequency of immunodeficiency and lower tolerance to aggressive treatments. In addition, older BC patients are often diagnosed at advanced stage in which therapeutic options are limited (Bultitude and Fentiman, 2002).

In this study, lymphangiogenesis and tumor LVI were not independent prognostic factors on survival rates in luminal BC subtype (Table 3). Higher mortality rates of luminal BC subtype are associated with several factors including tumor stage at diagnosis. In this study, 84.2% of luminal BC subtype were diagnosed at early stage and mortality was found in 14 (20%) of total luminal BC patients. Zaha et al., (2010) reported that 5-year survival rate of early stage BC was 88%. Higher survival rate in luminal BC subtype carcinoma is associated with relatively good therapeutic response to anti-hormonal therapy. During the first 4-years follow up, luminal BC subtype is able to enter a dormant state but subsequently might turn more aggressive tumor within 10 to 15 years after therapy. It is likely due to the molecular changes from positive to negative hormonal status (Kennecke et al., 2010).

Luminal BC subtype tends to metastasize to lymph nodes. Among luminal BCs, 56% were invasive ductal carcinoma, 23% were lobular carcinomas, and the 21% were mucinous and papillary carcinoma which is relatively less aggressive (Zaha et al., 2010). VEGF-C and VEGFR-3 mediated lymphangiogenesis in less aggressive breast carcinoma is limited (van Iterson et al., 2007) because VEGF-C interacts with inactive isoforms of VEGFR-3L causing inactivation of downstream lymphangiogenesis pathway (Gunningham et al., 2000; Ran et al., 2010).

Luminal BC subtype often presents with distant metastasis to the bone (Kennecke et al., 2010). However, patients with metastatic focus confined to the bone might have a better prognosis than those with distant metastatic in non-osseus sites. Elder post-menopausal BCs were more likely to have bone metastasis in comparison to pre- or peri-menopausal patients. Estrogen receptor expression is associated with higher probability to the development of bone metastasis. However, ER was not an important determinant in patients whom metastatic disease are clinically confined to the bone (Coleman and Rubens, 1987; Koenders et al., 1991).

In this study, independent prognostic factors on survival of non-luminal BC were VEGF-C expression (HR=0,04; 95% CI=0,01-0,41), lymph node metastasis (HR=0,14; 95% CI=0,04-0,55) and stage (HR=0,30; 95% CI= 0,02-0,76) after being adjusted with LVI (Table 4).

Around 75-90% of non-luminal BC subtype is invasive ductal carcinoma, meanwhile the other 10-25% are medullary, metaplastic, and inflammatory carcinomas (Yang et al., 2007). Non-luminal BC subtype is hormonal negative carcinoma with relatively higher proliferation and development rates which requires significant supply of nutrients (Carey et al., 2006; Campbell et al., 2011). In contrast to normal healthy cells, energy balance in tumor cells is obtained from aerobic glycolysis, and oxydative phosphorylation. Although the energy production from aerobic glycolysis is less effective than from oxydative phosphorylation, this state still allows cells to remain alive but resistant to therapeutic intervention under hypoxic conditions (Demetrius et al., 2010).

Hypoxic conditions during carcinogenesis induce expression of Hypoxia-Inducible Factor-1 (HIF-1) that plays as a master regulator of cellular homeostasis. HIF-1 has a vital role in tumor progression, by inducing gene expression involved in anaerobic metabolism, angiogenesis, cell survival, and drug resistance. HIF-1 is a heterodimer protein composed by HIF-1 $\alpha$  and HIF-1 $\beta$  subunits. HIF-1 $\alpha$  stabilize the function of HIF-1. One of the target genes is a VEGF-C gene that encodes important protein factors for lymphangiogenesis. Hypoxia induces not only VEGF-C production, but also its ligand, VEGFR-3, (Ke and Costa, 2006; Schoppmann et al., 2006).

Hypoxia attracts inflammatory cells and aggravates hypoxia-related responses. Hypoxia induces tumor cells migration as well as alter several inflammatory mediators including IL-8, in order to stimulate migration of tumor cells. IL-8 also plays an important role in Epithelial Mesenchymal Transition (EMT) that mediate cellular resistance into cancer therapy (Waugh and Wilson, 2008; Voss et al., 2011). Inflammatory cells stimulate lymphangiogenesis and VEGF-C expression through the Nuclear factor  $\kappa$ B (NF- $\kappa$ B) pathway. NF- $\kappa$ B encoded proteins such as TNF- $\alpha$ , IL-6, IL-8 and COX-2, to stimulate lymphangiogenesis indirectly. NF- $\kappa$ B regulate metalloproteinase enzymes and urokinase-type plasminogen activator (uPA), to degradate the matrix and activate VEGF-C, in order to bind to VEGFR-3. NF- $\kappa$ B also activates VEGFR-2 transcription factor receptor that binds to VEGF-C to further induce lymphangiogenesis (Timoshenko et al., 2007; Ran et al., 2010). In addition, NF- $\kappa$ B expression is associated with poor response to chemotherapy in locally advance breast cancer (Prajoko and Aryandono, 2014).

Although lymphangiogenesis in non-luminal subtype carcinomas is relatively higher, the propensity of lymph node metastasis is low. Non-luminal carcinoma tends to metastasize to the visceral organs (Raica et al., 2011; Jaime Jans et al., 2014). Visceral metastasis occurs through the lymphatic vessels, blood vessels or afferent lymphatic vessels and finally forms colonies on the distant organ sites. Distant metastasis is an organo-specific

event, tumor cells in the lymph vessels can only grow in a specific organ. Lymph node infiltrations also collectively mediates the initial process of distant metastasis. A study using animal model shows that lymphangiogenesis is not initiated in condition with low VEGF-C expression. Higher levels of VEGF-C expression is able to induce lymphangiogenesis and pulmonal metastasis (Hirakawa et al., 2007; Ran et al., 2010).

In conclusion, this study revealed that lymphangiogenesis determinants are associated with survival rates of non-luminal BC subtype in comparison to luminal BC subtype.

## Acknowledgements

This study was supported by Dana Masyarakat 2009, 2010 and 2011, Faculty of Medicine Universitas Gadjah Mada, Yogyakarta Indonesia.

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