RESEARCH ARTICLE

Chalkley Microvessel but not Lymphatic Vessel Density Correlates with Axillary Lymph Node Metastasis in Primary Breast Cancers

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Abstract

This study aimed to investigate tumor microvessel density (MVD) and lymphatic vessel density (LVD) using the Chalkley method as predictive markers for the risk of axillary lymph node metastasis and their relationship to other clinicopathological parameters in primary breast cancer cases. Forty two node-positive and eighty node-negative breast cancers were immunostained for CD34 and D2-40. MVD and LVD were counted by the Chalkley method at x400 magnification. There was a positive significant correlation of the MVD with the tumor size, coexisting ductal carcinoma in situ (DCIS) and lymph node metastases (P<0.05). In multivariate analysis, the MVD (2.86-4: OR 5.87 95%CI 1.05-32; >4: OR 20.03 95%CI 3.47-115.55), lymphovascular invasion (OR 3.46, 95% CI 1.13-10.58), and associated DCIS (OR 3.1, 95%CI 1.04-9.23) independently predicted axillary lymph node metastasis. There was no significant relationship between LVD and axillary lymph node metastasis. However, D2-40 was a good lymphatic vessel marker to enhance the detection of lymphatic invasion compared to H and E staining. In conclusion, MVD by the Chalkley method, lymphovascular invasion and associated DCIS can be additional predictive factors for axillary lymph node metastases in breast cancer. No relationship was identified between LVD and clinicopathological variables, including axillary lymph node metastasis.

Keywords: Breast cancer - angiogenesis - lymphangiogenesis - microvessel density - lymphatic vessel density - Chalkley

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Introduction

Breast cancer is the most recent common malignancy in Thai women and the incidence is substantially increasing in the forthcoming years (Attasara et al., 2009). Histopathological factors including tumor size, type, grade, vascular invasion, hormone receptor and axillary lymph node (ALN) status provide the important prognostic information for the management of patients (Uzzan et al., 2004). Amongst all of these, metastasis to axillary lymph node has a major influence on survival and prediction of ALN metastasis is crucial for therapeutic strategies in breast cancer patients (Bast et al., 2001).

Angiogenesis plays an essential role in the development and progression of a variety of malignancies, determining survival of the malignant cells, local growth and invasion, as well as in dissemination of the disease (Folkman, 2002). In breast cancer, most previous studies have demonstrated that highly neovascularised tumors have a higher likelihood of metastasis, a higher risk of tumor recurrence and decreased disease-free survival and overall survival compared with patients who have less vascularised tumors (Weidner et al., 1992; Vermeulen et al., 1996; Guidi et al., 2000; Offersen et al., 2003). However, the concept of lymphangiogenesis regarding as a predictor of lymph node metastasis is still controversial. Some investigators suggested that the lymphatic vessels have a minor role with tumor cells infiltrating pre-existing peritumoral lymphatic and lymphangiogenesis is absent during breast carcinogenesis (Vleugel et al., 2004). Conversely, other investigators have suggested that formation of new tumor-associated lymphatics plays an active role in the lymph node metastases (Skobe et al., 2001; Timar et al., 2002) and therefore, number of newly-formed lymph vessels could be a good prognostic significance to predict nodal metastasis. Previous studies have been limited by the lack of specific lymphatic endothelial markers that could be used to discriminate between lymphatics and blood vessels. The monoclonal antibodies selectively for lymphatic endothelium have been developed for clinical studies on lymphangiogenesis in various types of human cancers such as head and neck cancer (Beasley et al., 2002) and papillary thyroid carcinoma (Lee et al., 2012). They have shown a high correlation between lymphatic vessel density (LVD) and neck node metastasis.

To assess tumor vascularity, there are several methods including counting the number of immunohistochemically stained microvessels in vascular hot spots, grading of

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vascular, using image analysis systems (Sullivan et al., 2009) and applying the Chalkley grid. The Chalkley count technique was recommended in an international consensus report because it is considered to be a simple and acceptable procedure for daily clinical use and produced lower interobserver variability compared to the more frequently used conventional microvessel density method (Hansen et al., 2004; Dhakal et al., 2009).

The aim of this study was therefore to evaluate the MVD and LVD by the Chalkley method as predictive markers for the risk of axillary lymph node metastasis and their relationship to other clinicopathological factors in primary breast cancer patients. Additional evaluation of the tumoral expression of vascular endothelial growth factor (VEGF), the most potent and specific angiogenic activator was also performed.

Materials and Methods

This retrospective study represented a subset of breast cancer patients who underwent primary surgical treatment at Songklanagarind Hospital or provincial hospitals in the Southern Thailand. The sample size was calculated to provide 90% certainty of hypothetical difference of vascular count between node-positive and node-negative primary invasive breast carcinomas in a ratio of 1:2. The samples in each group were randomly selected using computer-generated random number. For each tumor, all H&E stained slides were initially reviewed by a pathologist then selected a tumor block with an invasive carcinoma, including the tumor border for immunohistochemical staining.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue was cut in 4 μm thick and mounted on coated slides. The sections were immunohistochemically stained with antibodies against D2-40 (Dakocytomation, Glostrup, Denmark; dilution 1:200), CD34 (Dakocytomation, Glostrup, Denmark; dilution 1:100), and VEGF (Dakocytomation, Glostrup, Denmark; dilution 1:100). The epitope retrieval was performed manually by using pressure cooker. Envision+HRP (Dakocytomation, Glostrup, Denmark) was subsequently used as secondary detection. The vascular and lymphatic endothelium in the adjacent non-neoplastic breast tissue served as internal control for CD34 and D2-40, respectively. Sections of resected breast cancer known to express VEGF were used as positive control for VEGF. All of the immunostained sections were evaluated by principle investigator (S.K.) without knowledge of the patient's cancer status.

Evaluation

Quantitative assessments of microvessel density (MVD) and lymphatic vessel density (LVD) were examined in the same manner under an Olympus BX41 microscope by initial low-power x40 (x10 ocular, x4 objective) screening for the 3 most vascularized areas ("hot spot" areas) before being counted at the higher power. An eyepiece Chalkley grid graticule (Pyser-SGI Limited, United Kingdom) containing 25 randomly dots was applied to each hotspot area and oriented to permit the maximum number of points to hit on highlighted vessels at x400 magnification. The mean of 3 graticule counts was recorded. Reproducibility of the method was evaluated by re-assessing 20 randomly tumor samples.

Statistical analysis

Statistical analysis was performed using the R program version 2.7.0. The correlation between the Chalkley count and patients' characteristics was analysed by Pearson's correlation coefficient, Wilcoxon rank sum test or Kruskal Wallis test when appropriated. The chi-square test was used to explore the relation between clinicopathologic parameters and the Chalkley count. Non-parametric tests were also used for data not being normally distributed. For prediction of axillary lymph node metastasis, all variables with level at alpha less than 0.2 in univariate analyses were entered into a multivariate logistic regression. The odds ratios for independent significant parameters were calculated for lymph node metastasis. Data was considered statistically significant when $p<0.05$.

Ethical approval

The study was approved by the Clinical Research Ethics Committee of the Faculty of Medicine, Prince of Songkla University.

Results

Clinical parameters

Table 1 describes the distribution of clinicopathological characteristics of the 122 patients. A high MVD Chalkley count was significantly correlated with a large tumor size ($P<0.05$), axillary lymph node metastasis ($P<0.0001$) and interestingly, existing DCIS ($P<0.00001$).

To consider of biological diversity among ethnic, the actual tertiles of the Chalkley counts which were 2.86 and 4 were used instead of the preselected cutoff points. The association between the MVD Chalkley count and patients' tumor characteristics is summarized in Table 2. The median MVD Chalkley count was 3.3 (range 2-7; mean 3.6, SD 1.21). Of the 122 patients, 37 (30%) had a MVD Chalkley count of ≤2.86, 48 (40%) a count between 2.86 and 4, and 37 (30%) a count of >4. There was a significant association between the MVD Chalkley count and grade of tumor and lymph node status.

Positive D2-40 staining highlighted lymphatic vessels whereas adjacent blood vessels were D2-40 negative.

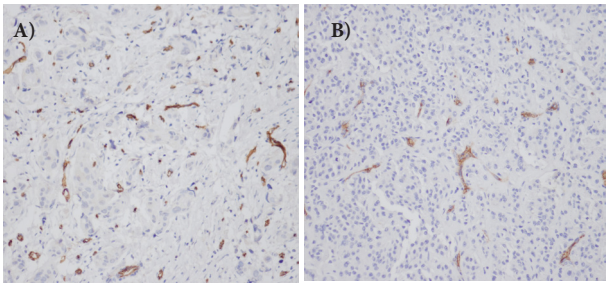

Figure 1. Immunoreactivity for CD34 is Recognized in the Endothelium of Microvessels in Cases. A) high and B) low microvessel density (x200)

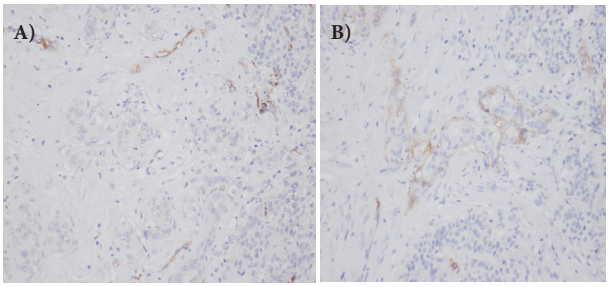

Figure 2. Example of D2-40 Facilitates Lymphatic Tumor Invasion Detection. A) CD34 immunohistochemical stain highlights new vessel formation in contrast to B) whereas D2-40 encircles lymph vessels containing tumor cells (x200)

Table 1. Clinicopathological Details of Patients Studied

Characteristics		n	%
All patients		122	100
Age (years)	< 40	11	10
	40-49	37	30
	50-59	37	30
	≥ 60	37	30
Menopausal status	Pre/perimenopausal	71	58
	Postmenopausal	51	42
Self-palpable mass	Yes	112	92
	No	6	5
Side†	Right	55	45
	Left	66	54
Surgical procedure	MRM	112	92
	Lumpectomy/partial mastectomy		
	with LN dissection	1	1
	Others	8	7
Tumor size (mm)	≤ 20	56	46
	20-50	51	42
	> 50	6	5
Histological grade	Grade I	19	16
	Grade II	37	30
	Grade III	47	39
	Non-ductal	12	10
Histological type	Ductal	104	85
	Lobular	6	5
	Special	12	10
Associated DCIS/LCIS	Present	35	29
	Absent	78	64
Microscopic vascular invasion			
	Present	29	24
	Absent	89	73
Lymph node metastasis	None	80	66
	1-3	21	17
	≥ 4	21	17
Estrogen receptor	Positive	67	55
	Negative	48	39
Progesterone receptor	Positive	60	49
	Negative	55	45

*Median (range): age 52 years (29-86 years), tumor size 21 mm (6-150 mm), metastatic lymph nodes 3.5 (1-19), Some data are missing in some of the categories

Table 2. The Relationship between the Chalkley MVD and Clinicopathological Factors in Breast Cancer

Characteristics	n	%	MVD			P
			≤ 2.86	2.86-4	>4	
Menopausal status						
Pre/perimenopausal	71	58	23 (32)	32 (45)	16 (23)	0.08
Postmenopausal	51	42	14 (28)	16 (31)	21 (41)	
Tumor size (mm)						
≤ 20	56	46	17 (30)	22 (40)	17 (30)	0.85
> 20	57	47	17 (30)	20 (35)	20 (35)	
Histological grade						
Grade I	19	16	5 (26)	12 (63)	2 (11)	0.014
Grade II	37	30	9 (24)	9 (24)	19 (52)	
Grade III	47	39	16 (34)	17 (36)	14 (30)	
Histological type						
Ductal	104	85	32 (31)	39 (37)	33 (32)	0.804
Lobular	6	5	1 (17)	4 (66)	1 (17)	
Special	12	10	4 (33)	5 (42)	3 (25)	
Associated DCIS						
Present	35	29	8 (23)	14 (40)	13 (37)	0.423
Absent	78	64	26 (33)	31 (40)	21 (27)	
Lymph node stage						
N0	80	66	34 (42.5)	34 (42.5)	12 (15)	3.15E-07
N1-2	42	34	3 (7)	14 (33)	25 (60)	

Table 3. Clinicopathological Parameters and Predictors of Axillary Lymph Node (ALN) Metastasis

Parameter		ALN-	ALN+	P value
Tumor size (mm)	≤ 20	37	19	0.609
	> 20	34	23	
Histological grade	Grade I	14	5	0.457
	Grade II	21	16	
	Grade III	30	17	
Associated DCIS	Present	18	17	0.019
	Absent	59	19	
Vascular invasion	Present	11	18	<0.001
	Absent	68	21	
Estrogen receptor	Positive	34	16	0.81
	Negative	41	23	
Progesterone receptor	Positive	42	23	0.856
	Negative	34	16	
Chalkley count	≤ 2.86	34	3	3.15E-07
	2.86-4	34	14	
	> 4	12	25	

Table 4. Predictive Factors of Axillary Lymph Node Involvement in Multivariate Analysis

Parameters	Estimate	Odds ratio (95% CI)	P value
Presence of microscopic vascular invasion	1.2404	3.46 (1.13-10.58)	0.03
Presence of existing DCIS	1.1159	3.1 (1.04-9.23)	0.037
The MVD Chalkley count			<0.001
2.86-4	1.715	5.87 (1.05-32.9)	
> 4	2.9504	20.03 (3.47-115.55)	

*CI, confidence interval

In the majority of breast cancers, the lymphatic vessels were located in the peritumoral area rather than within the tumor itself. The median LVD Chalkley count was significant lower than the median MVD. Additionally, no significantly differences of the median LVD count between tumors with and without axillary lymph node metastasis (data not shown). However, D2-40 highlighted lymphatic tumor invasion which did not recognize on H&E slides in some cases.

High VEGF expression of the neoplastic cells was identified in only 21 cases (17%). No statistically significant correlation was found between VEGF and MVD, LVD, and axillary lymph node metastases.

Histological parameters to predict axillary lymph node metastasis

The distribution of the histopathologic parameters between tumors with and without axillary lymph node

metastasis is summarized in Table 3. There were 3 significant parameters correlating with the metastatic status of axillary lymph node in the univariate model which were histologic vascular invasion (p=0.0003), the MVD Chalkley count (p<0.0001) and associated DCIS (p=0.0195). In Multivariate logistic regression models (Table 4), the three significant factors determined in univariate analysis still being significant independent factors differentiating patients with and without axillary lymph node metastasis. The other two histologic predictive factors, tumor size and histological grade, were not significantly predicted the axillary lymph node status. The relative risks of lymph node metastasis were calculated independently for each of these factors.

Discussion

The presence of axillary lymph node metastasis is important for diagnosis, treatment and prognosis in breast carcinoma. Tumor angiogenesis has been reported to have an important role in the metastasis of breast cancer and tumor blood vessel density has been reported to be associated with axillary lymph node metastasis. The first report was from Weidner et al in 1992 (Weidner et al., 1991). In the present study, we demonstrated significant difference of MVD assessed by the Chalkley counting on CD34 between breast cancers that did and did not axillary metastasized, indicating that tumor MVD can serve as a predictive factor for ALN metastasis.

The significant of associated DCIS in primary tumor to predicted ALN metastasis; these results might be explained by a synergistic angiogenic effect in DCIS and invasive cancer. As Bluff et al. (2009) showed angiogenic switch from mammary hyperplasia through carcinoma in situ and invasive carcinomas which associated with increases in HIF-1alpha expression (Bluff et al., 2009).

The recently developed monoclonal antibody D2-40 has enabled the relatively easy detection of lymphatic vessels in tissue, and lymphangiogenesis has been reported to potentially increase lymph node metastasis in head and neck (Munoz-Guerra et al., 2004) and colorectal cancer (Saad et al., 2006). Nevertheless, there are a few reports on the relationship between lymphatic vessel density (LVD) and metastasis in breast carcinoma (Bono et al., 2004). In the present study, the lymphatic vessel density was count on D2-40 immunohistochemically stained slide using the Chalkley method, to evaluate its relationship with ALN metastasis. However, no significant relationships were found between tumor LVD and ALN metastasis. This result suggests that lymphangiogenesis may not have a major role in ALN metastasis, although it may suggest the possibility that lymphangiogenesis has a certain degree of involvement in the growth, invasion and progression of the tumor. This finding is consistent with the previous studies (Faoro et al., 2008) indicating although the generation of intratumoral lymphatic vessels may enhance tumor cell metastasis, this may not be an essential requirement as lymphatic spread can occur using pre-existing vessels (Mandriota et al., 2001). Also, there are previous studies showing lymphangiogenesis is not increased in tumor tissues but is even less activated than in normal breast tissue (Boneberg et al., 2009). This phenomenon was explained by that solid tumors do not have lymphatic vessels, due to the increased interstitial pressure created by the proliferating cancer cells (Pepper, 2001). On the other hand, when the relationship between tumor lymphatic invasion and ALN metastasis was examined, the rate of ALN metastasis was substantially higher in patients with demonstrable lymphatic invasion compared to those without lymphatic invasion, suggesting that lymphatic invasion but not LVD may be a predictive factor of ALN metastasis. Moreover, different tumor types have particular behavioral characteristics in terms of preferential signaling pathways to metastasis. The finding that LVD are sparse in breast cancer and did not differ between node-positive and node-negative subgroups may point out this and LVD may not be a useful prognostic marker in breast tumors. The lower of LVD measurement than MVD reflects the necessity for angiogenesis over lymphangiogenesis as a requirement for tumor growth and lymph node metastasis. However, we are cautious regarding the Chalkley counting which we applied to evaluate LVD could be another factor affecting the negative correlation between LVD and lymph node status since the method for LVD counting is not well established. Thus, the optimal method for the quantification of LVD should be further investigated (Duff et al., 2007).

Our study indicates that it is possible histologically to estimate the risk of lymph node involvement for breast cancer patients. For those with the MVD Chalkley count less than 2.86, the probability of ALN metastasis was less than 10%. On the other hand, the percentage of lymph node metastasis is substantially increased according to increasing number of the MVD. The probability of ALN metastasis was 30% and 68% for the MVD ranged 2.86-4 and greater than 4, respectively.

The increasing breast cancer screening program facilitates detecting smaller tumors with less probability of metastatic lymph nodes questioning the need for routine axillary lymph node dissection. Predictive factors for lymph node metastasis may provide a way to avoid lymph node surgery in subgroups of patients. This study may add up additional information for pathologists to consider an evaluation of microvessel density as well as other well-documented clinicopathological predictors in low-risk breast cancer patients who may beneficial to get a limited surgical procedure.

In conclusion, we showed that MVD Chalkley but not the LVD Chalkley count can be a predictive factor for ALN metastasis in breast carcinoma. These conclusions may provide an important evidence for cancer therapy through antiangiogenesis and selective limited axillary lymph node dissection in the selected group of patients. In addition, D2-40 enhances the detection of lymphatic invasion relative to routine H&E staining.

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