Prognostic Significance of Microvessel Density in Breast Cancer of Thai Women

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Objective: *Angiogenesis is important in the process of tumor growth and progression of breast cancer. Microvessel density (MVD) is the most commonly used technique to quantify intratumoral angiogenesis in breast cancer. In the present study, the authors investigated the prognostic indicator of intratumoral MVD in predicting overall survival, disease recurrence, and distance metastasis of breast cancer.*
Material and Method: *Two hundred patients who were diagnosed as invasive breast cancer from January 2000 to December 2004 were included in the present study, but only 64 patients had complete pathological specimens and tumor receptor studies. Representative paraffin sections of the primary tumor including the tumor border were immunostained with a monoclonal anti-CD34 antibody. The area of highest vascular density ("hot spot") was identified and the average count of three hot spots in each tumor was used for analyses. The distribution of MVD was categorized into high and low MVD as more than 76 and less than 76 respectively. Overall survival probability was estimated by the Kaplan-Meier method. A multivariate Cox regression was employed to examine the relationship between MVD and disease outcomes while adjusting for other concomitant variables.*
Result: *The tumor size and more advanced disease have correlated with poor outcomes of invasive breast cancer. Tumor size is a poor predictor for local recurrence [Harzard ratio1.02 (95%CI 0.99-1.04)] and more disease staging have correlated with distance metastases [Harzard ratio1.48 (95%CI 0.98-2.24)]. The cancer staging only predicted poor outcome in invasive breast cancer in overall recurrence [Harzard ratio1.51 (95%CI 1.05-2.16)]. MVD is not correlated with both tumor recurrence and distance metastases [Hazard ratio for local recurrence1.01 (95%CI 0.99-1.04) and Harzard ratio 1.00 (95%CI 0.97-1.02)].*
Conclusion: *The microvessel density (MVD) has not predicted poor outcomes of invasive breast cancer in Thai woman.*

Keywords: *Angiogenesis, Anti-CD34, Survival*

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Breast cancer is one of the most common site-specific cancers in women and is the leading cause of death from cancer for women aged 35 to 55 years. There is a tenfold variation in breast cancer incidence among different countries worldwide. Women living in less industrialized nations tend to have a lower incidence of breast cancer than women living in industrialized nations, although Japan is an exception[1].

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Disease outcome, both disease-free survival (DFS) and overall survival (OS) of the disease, depends on several prognostic indicators, including tumor size, axillary lymph node status, histological grade, tumor type, vascular invasion and estrogen receptor status[2,3]. Among all of these prognostic factors, lymph node status remains the most important indicator. In the USA, the 5 years overall survival was 92% in node-negative patients, 81% in patients with one to three axillary lymph nodes, and 57% in those with more than four involved nodes[4].

Angiogenesis, the process of new blood vessel formation, play a central role in both local tumor growth and distant metastasis in breast cancer[5]. When a new tumor reaches the size of 1-2 mm, its growth requires the induction of new blood vessels, which may consequently lead to the development of metastases, via the penetration of malignant cells into the circulation. Extensive laboratory data suggest that angiogenesis plays an essential role in breast cancer development, invasion and metastasis. Hyperplastic murine breast papillomas and histologically normal lobules adjacent to cancerous breast tissue support angiogenesis in preclinical models, suggesting that angiogenesis precedes transformation of mammary hyperplasia to malignancy. Transformation of tumor cells with angiogenic stimulatory peptides has been shown to increase tumor growth, and metastasis. Conversely, transfection of tumor cells with inhibitors of angiogenesis decrease growth, and metastasis[6].

Concerning the relationship between angiogenesis and clinical outcome, breast cancer has been the most studied tumor. For more than 10 years, microvessel density (MVD), a surrogate marker of tumoral angiogenesis, has been proposed to identify patients at high risk of recurrence more precisely than classical indicator, particularly in node negative patients. The identification of such patients especially at an early stage of their disease may allow for a more appropriate and effective treatment (by adjuvant chemotherapy or in the near future by specific antiangiogenic drugs) of patients at high risk.

Microvessel density assessment is the most commonly used technique to quantify intratumoral angiogenesis in breast cancer. It was first developed by Weidner et al[7] in 1991 and uses panendothelial immunohistochemical staining of blood microvessels [mainly with Factor VIII antigen (Factor VIII Ag or von Willebrand's factor), CD31, PECAM-1, or CD34; rarely with integrin $\alpha_v \beta_3$, CD105, or type IV collagen]. The first step in Weidner's approach is the identification by light microscopy of the area of highest microvessel density (the "hot spot"). The individual microvessel are counted at a high power (x 200 field) in an adequate area after scanning the whole tumoral section (include the tumor border) at low power. Any stained endothelial cell or clusters separate from adjacent vessels are counted as a single microvessel, even in the absence of vessel lumen[8]. Each single count is expressed as the highest number of microvessels identified at the hot spot.

Chalkley count or computerized image analysis system are used by some authors to minimize the subjectivity in the qualification of MVD. The Chalkley count consists of applying a 25-point eyepiece graticle on several hot spots (usually 3). The graticle is oriented to allow the maximum number of points to hit on or within the area of stained microvessel profiles[8]. MVD is a prognostic factor in invasive breast cancer concluded by many observational studied, but the opposite conclusion was reached by the others.

Although recent meta-analysis by Uzzan et al[8] revealed that high MVD significantly predicted poor survival in terms of both disease free survival (DFS) and overall survival (OS), many studies remained varied as a result of patient selection criteria, techniques to stain and count microvessels and cutoff selection. Finally, standardization of MVD assessment is needed.

The present article aims to investigate the prognostic indicator of intratumoral MVD in predicting disease free survival and overall survival of breast cancer patients.

Material and Method

This is a study of sixty-four patients who had complete pathological specimen of primary tumor and receptor, which were selected from two hundred patients diagnosed with invasive breast cancer at Ramathibodi Hospital, Bangkok, Thailand from January 2000 to December 2004.

Table 1 shows the distribution of patient characteristics. The patients had a mean age of 50 years (range, 26-75 years) and most had been previously treated by mastectomy with axillary lymph node dissection. Surgery followed by adjuvant hormonal therapy (44 patients, tamoxifen or aromatase inhibitor), radiotherapy (24 patients, chest wall and axillary radiation) and combination chemotherapy (49 patients, either 5-fluorouracil/doxorubicim/cyclophosphamide [FAC] or intravenous cyclophosphamide/methotrexate/5-fluorouracil [CMF] as conventional schedule) were administered depending on pathologic stage.

Staging analysis was done according to the 1992 TNM classification by American Joint Committee on Cancer. Tumors were graded histopathologically according to Bloom-Richardson histological grading system. Tumor estrogen, progesterone receptor and HER 2/neu status had been determined by the immunohistochemical method.

Follow up

The median follow-up duration of patients was 31 months (range, 4-68 months). Patients were observed

every 3-4 months for 2 years, every 6 months for the next 2 years and yearly thereafter. Complete blood cell count (CBC) and biochemical profile had been investigated every 6 months. Radiological studies had been performed every 12 months or earlier if necessary.

Immunohistochemical Procedures

Microvessel Density Quantization

Representative paraffin block for each tumor (proper slides that included tumor border) were selected for immunohistochemical examination. Full cross sections of each tumor for evaluation were used. These sections were stained for CD34 (Monoclonal Mouse Anti-Human CD34 Class II Clone QBEnd-10) (Dako code No. M 7165). Endothelial cells of tumor vessels were highlighted by this method. After deparaffinization in xylene and washing in ethanol, antigen retrieval was performed by incubation with Ethylene diamine tetraacetic acid (EDTA) at 95°C for 40 minutes. Sections were incubated for 10 minutes in 3% hydrogen peroxide in distilled water for blocking of endogenous peroxidase. Thereafter, the slides were processed according to the standard method with the Envision (Dako code No. K4001). The monoclonal antibody CD34 (QBEnd/10) was diluted with Tris buffered saline solution 1:50 and reacted with tissue specimen for 60 minutes at room temperature. Diaminobenzedine (DAB) was used as chromogen, followed by hematoxylin counterstaining. Normal mouse IgG was substituted for primary antibody as a negative control.

Microvessel density was determined as previously described by Weidener et al microvessel quantization was performed with light microscopy by a pathologist (one examiner who did not know about patient data). First, the area of the highest microvessel density of the tumor (hot spot) was identified by systematically scanning in the whole section at x40 magnification (Fig. 1), and then the single field with the highest number of microvessels at x200 magnification (0.2 mm^2 per field) was identified (Fig. 2). Any brown-stained endothelial cell that had clearly separated from adjacent microvessels was considered a single countable microvessel. Undefined endothelial cells that appeared to be fragments were not counted as microvessels. Branching structures were counted as single vessel unless there was a break in continuity of the structure. The presence of a vessel lumen was not required to classify a structure as a vessel[9]. All blocks were counted three times at different periods; the average of these counts was taken as the MVD of the tumor. The median MVD (value 76) of all patients was used to classify patients in high and low MVD groups.

Table 1. Tumor characteristics of 64 patients

Characteristics	Number of patient (%)
Age (years)	
≤ 39	15 (23.44)
40-50	17 (26.56)
> 50	32 (50)
Plan age (range)	50 (26-75)
Menstrual status	
Premenopause	28 (43.75)
Postmenopause	36 (56.25)
Stage	
I	10 (15.63)
IIA	16 (25)
IIB	15 (23.44)
IIIA	12 (18.75)
IIIB	6 (9.37)
IIIC	3 (4.69)
IV	2 (3.12)
Tumor size (cm)	
≤ 2	22 (34.38)
> 2	42 (65.62)
Nuclear grading	
I	10 (15.63)
II	39 (60.93)
III	15 (23.44)
Angiolymphatic invasion	
Absence	34 (53.13)
Presence	30 (46.87)
Mean vessel density	
High (> 76)	30 (46.85)
Low (< 76)	34 (53.12)

Statistical analysis

Results were expressed as means and standard deviations (SD) for continuous variables and as percentage for discrete variables. Survival analysis was displayed by the Kaplan-Meier method, and the effect of covariates on lifetime and recurrence was tested by Cox regression analysis. Result was considered significant at the 5% critical level ($p < 0.05$).

Results

The patient characteristics and clinicopathological, factors are shown in Table 1, more than half of the patients were post menopause (56.25%) with a mean age of 50 years (range, 26-75 years). Most of the patients were operable breast cancer (82.82% including stage I 15.63%, IIA 25%, IIB 23.44%, and IIIA 18.75%), they underwent mastectomy with axillary

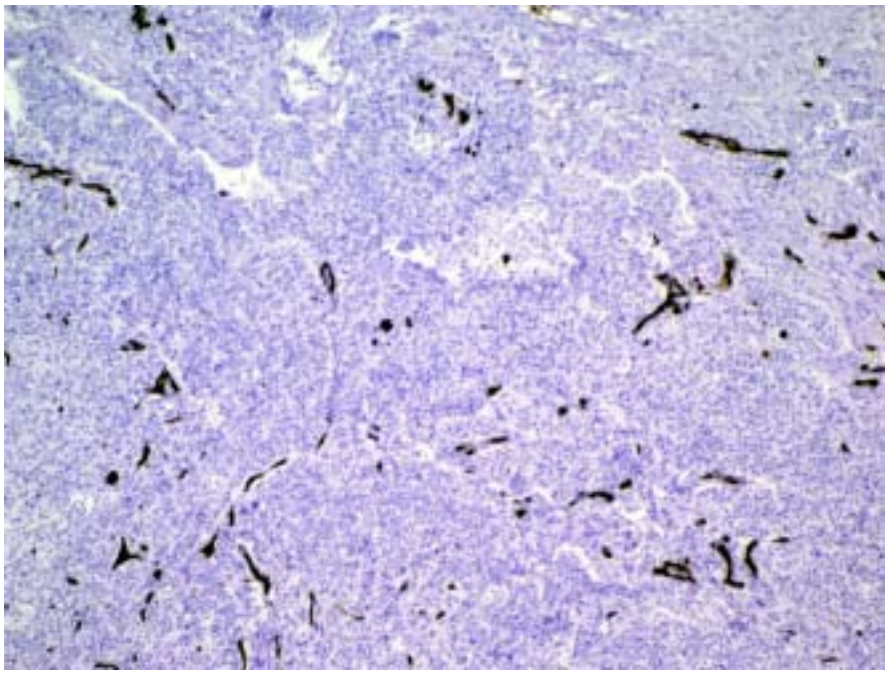

Fig. 1 Photomicrograph of angiogenic pattern. Formalin fixed, paraffin-embeded breast cancer tissue was stained by anti-CD34 antibody. Example of hot spot with numerous discrete stromal vessels (x40). In this sample the mean MVD was 47.66 (low)

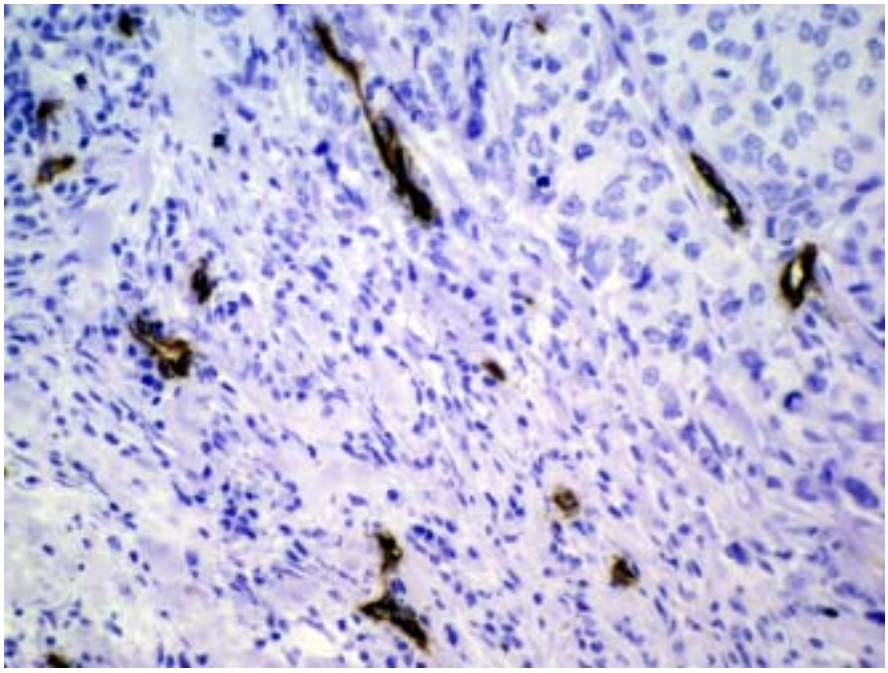

Fig. 2 Example of hot spot area, cleary discrete stromal microvessel in high magnification (x200). In this sample the mean MVD was 47.66 (low)

lymph node dissection and some had received neo-adjuvant chemotherapy before surgery. Most of the primary tumors were larger than 2 cm (65.62%). Forty-seven percent of tumors represented angiolymphatic invasion. According to tumor receptor study, they tended to be hormonal responsive tumors (ER positive 65.62% and PR positive 65.63%) without HER-2 over expression (HER-2/neu negative 71.88%) (Table 2). In node positive patients (56.25%), more than half of them were positive 1-3 nodes. Kaplan-Meier survival estimation (Fig. 3-5) showed good prognosis in the studying population with median follow up time 29.9 and 31.1 months for local recurrence and metastasis respectively (Table 3, 4). MVD, tumor receptors and other clinicopathological factors including age, menopausal status, tumor size, axillary lymph node status, stage, histological and nuclear grade were studied. Multivariate analysis showed that staging of cancer was the strongest independent prognostic factor for disease outcome in both distance metastases probability [Hazard ratio 1.92 (95%CI 1.14-3.24) p 0.015] and overall recurrence probability [Hazard ratio 1.511 (95%CI 1.05-2.16) p 0.024] (Table 5). The tumor size was the second independent prognostic factor for overall recurrence probability [Hazard ratio1.022 (95%CI 1.00-1.04) p 0.017]. Microvessel density (MVD) was not a prognostic factor influencing disease outcome in both local recurrence probability [Hazard ratio 1.02 (95%CI 0.99-1.04) p 0.076] and distance metastases probability [Hazard ratio 1.00 (95% CI 0.98-1.03) p 0.86].

Table 2. Receptor and nodal status of 64 patients

Characteristics	Number of patient (%)
Receptor status	
ER negative	22 (34.38)
1+	17 (26.56)
2+	11 (17.19)
3+	14 (21.87)
Pg R negative	22 (34.38)
1+	15 (23.44)
2+	12 (18.75)
3+	15 (23.44)
Her-2/neu negative*	46 (71.88)
1+	2 (3.12)
2+	8 (12.50)
3+	8 (12.50)
Nodal status	
negative	28 (43.75)
1-3	20 (31.25)
≥ 4	16 (25)

* Her-2 neu negative and 1+ considered as negative receptor status

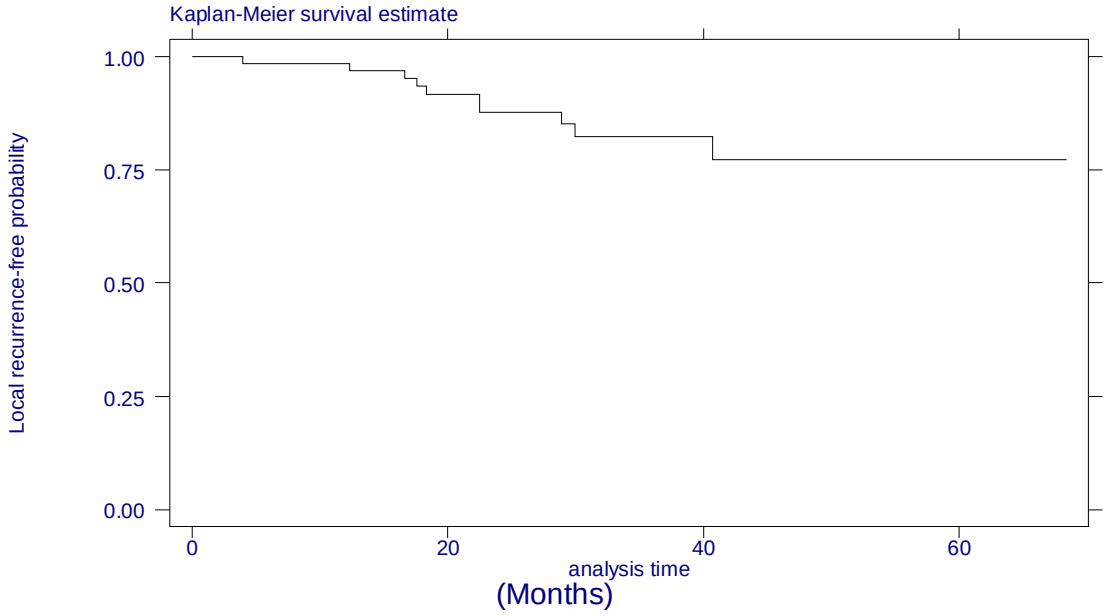

Fig. 3 Kaplan-Meier survival estimation of local recurrence free probability

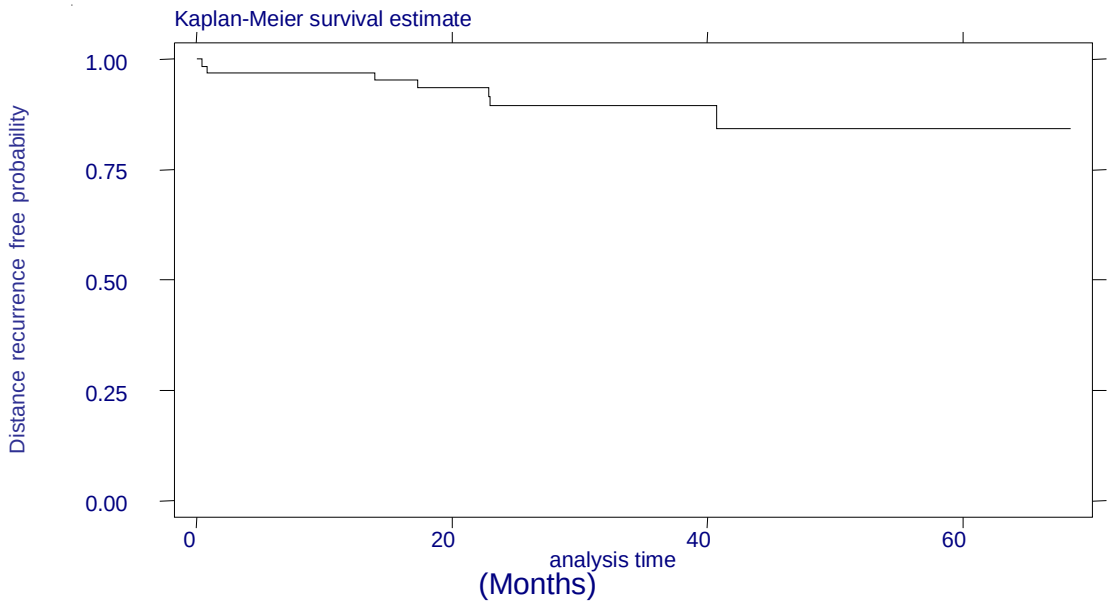

Fig. 4 Kaplan-Meier survival estimation of distance recurrence free probability

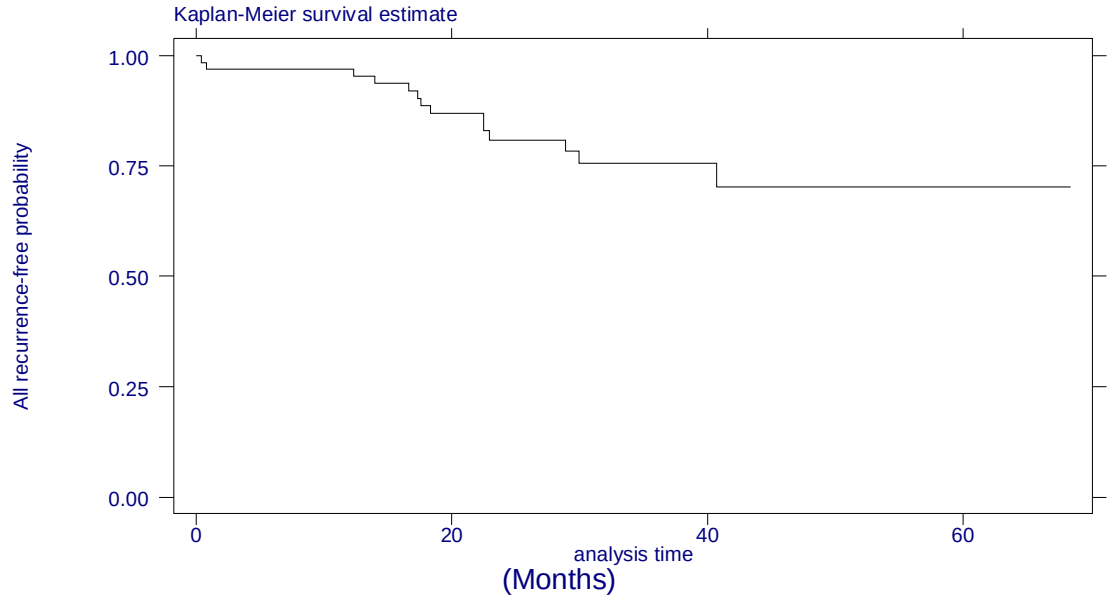

Fig. 5 Kaplan-Meier survival estimation of all recurrence free probability

Discussion

Tumor progression is critically dependent on the ability of malignant cells to induce a vascular stroma. In breast cancer, angiogenesis begins at the earliest stages of in situ disease. After invasion occurs, neovascularization is required for tumor growth and metastases. Weidner et al were the first to demonstrate that quantifying tumor neovascularization yields prognostically useful information in patients with invasive breast cancer[10].

Subsequently, a large number of studies have examined the prognostic utility of MVD counts in a variety of human tumors, including breast cancer. In general, an inverse relationship has been observed between MVD counts in primary breast cancer or axillary lymph node metastases, disease free survival, and overall survival. However, not all studies have demonstrated this association. These previous studies have differed in many respects, including the number and characteristics of patients studied, the length of follow-up, the treatment modalities used, the method of microvessels quantization, and statistical methods used in data analysis. These variations have made it difficult to reach firm conclusions. The prognostic utility of MVD counts may be useful in specific subgroups of breast cancer patients.

Table 3. Multivariate analysis of local recurrence probability in 64 patients

Local recurrence	Hazard ratio (95%CI)	p-value
Size (mm)	1.02 (0.99-1.05)	0.082
Stage	1.49 (0.98-2.24)	0.057
MVD	1.02 (0.99-1.04)	0.076

Table 4. Multivariate analysis of distance recurrence probability in 64 patients

Metastases	Hazard ratio (95%CI)	p-value
Age (yr)	1.073 (0.99-1.15)	0.062
Size (mm)	1.014 (0.99-1.04)	0.318
Stage	1.917 (1.14-3.24)	0.015
MVD	1.000 (0.98-1.03)	0.86

Table 5. Multivariate analysis of all recurrence probability in 64 patients

All recurrence	Hazard ratio (95%CI)	p-value
Age (yr)	1.045 (0.99-1.09)	0.08
Size (mm)	1.022 (1.00-1.04)	0.017
Stage	1.511 (1.05-2.16)	0.024
MVD	1.007 (0.99-1.02)	0.39

In this current study, the authors have examined angiogenesis using MVD in patients who had invasive breast cancer. MVD was not associated with prognostic of this group of patients (Thai population). MVD may not be a good test to select a patient who is a good candidate for subsequent adjuvant treatment of anti-angiogenesis.

Recent systematic review by Uzzan et al[8] showed that MVD is a weak prognostic factor of invasive breast cancer. This present study compared both positive and negative literatures[11-16] in the systematic review as shown in Table 6. They used statistical test to evaluate factors thought to be influencing the results of studies, e.g. the year of studies, study design, number of patients, median follow-up time, MVD assessment, and type of immunohistochemical stained. These factors did not influence nor predicted the result of the studies. The systematic review concluded that high MVD significantly predicted poor survival for invasive breast cancer and Final cutoff selection of MVD should be standardized. The present study showed a negative result of MVD on prognosis of breast cancer. This is the first study in a Thai population. The authors analyzed MVD from this group of patients and used MVD of 76 as a cutoff point. In the future, MVD still needs to be evaluated in its clinical use of reliable and standardized predictive markers of angiogenesis in breast cancer.

Table 6. Comparison of recent study and previous study in Meta-analysis literature reviews

Characteristic	Positive study	Negative study	Recent study
Age	Young	Old	Old
	< 50 yrs	> 50 yrs	>50 yrs
Menstrual	Premenopausal	Postmenopausal	Postmenopausal
Tumor size	> 2 cm	< 2 cm	2.8 cm
Nodal status	Positive	Negative	Positive
ER	Positive	Positive	Positive
	< 50%	75%	65%
MVD	10.5	110	76.6
Nation	Oriental	Western	Oriental

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ความสำคัญในการพยากรณ์โรคมะเร็งเต้านมโดยใช้ค่าความหนาแน่นของหลอดเลือดขนาดเล็ก

สิโรจน์ กาญจนปัญจพล, ศันสนีย์ วงศ์ไวศยวรรณ, สามารถ ภูวไพรศิริศาล, จุมพล วิลาศรัศมี

ภูมิหลัง: *การสร้างหลอดเลือดใหม่เป็นกระบวนการสำคัญในการเจริญเติบโตและลุกลามของมะเร็ง การวัดความหนาแน่นของหลอดเลือดขนาดเล็กเป็นเทคนิคที่สำคัญในการบอกจำนวนของการสร้างหลอดเลือดใหม่ในมะเร็งเต้านม*

วัตถุประสงค์: *เพื่อที่จะหาความสามารถในการทำนายโรคของค่าการวัดความหนาแน่นของหลอดเลือดเล็กในมะเร็งเต้านม โดยพิจารณาความสัมพันธ์ของค่าความหนาแน่นของหลอดเลือดขนาดเล็กกับอัตราการปราศจากโรค และอัตราการอยู่รอดของผู้ป่วยมะเร็งเต้านม*

วัสดุและวิธีการ: *ในการศึกษานี้ทำในผู้ป่วยมะเร็งเต้านม 64 ราย จากทั้งหมด 200 ราย ที่มีข้อมูลโรค สถานะของฮอร์โมน และชิ้นเนื้อทางพยาธิวิทยาครบ การวัดความหนาแน่นของหลอดเลือดขนาดเล็กทำโดย ย้อมชิ้นเนื้อด้วย anti-CD34 และตรวจนับบริเวณที่มีความหนาแน่นของหลอดเลือดมากที่สุด 3 แห่ง ผู้ป่วยถูกจัดออกเป็นกลุ่มที่มีความหนาแน่นของหลอดเลือดสูง (มากกว่า 76) และกลุ่มที่มีความหนาแน่นของหลอดเลือดต่ำ (น้อยกว่า 76) อัตราการอยู่รอดของผู้ป่วย ความสัมพันธ์ของค่าความหนาแน่นของหลอดเลือด และผลลัพธ์ของการรักษามะเร็งเต้านม ได้ถูกนำมาวิเคราะห์หาค่าความสัมพันธ์โดยใช้วิธี Kaplan-Meier และ multivariate Cox regression วิเคราะห์โอกาสที่จะ มีชีวิตอยู่*

ผลการศึกษา: *พบว่าขนาดของก้อนเนื้องอกและระยะของโรคมีความสัมพันธ์กับผลลัพธ์การรักษาที่ไม่ดีในผู้ป่วยมะเร็งเต้านม ขนาดของก้อนเนื้องอกเป็นตัวทำนายการกลับเป็นใหม่ของโรค ค่าความหนาแน่นของหลอดเลือดเล็กไม่มีความสัมพันธ์กับการกลับเป็นใหม่และการแพร่กระจายของมะเร็งเต้านม*

สรุป: *การศึกษานี้พบว่าความหนาแน่นของหลอดเลือดเล็กไม่ได้เป็นตัวทำนายผลลัพธ์ที่ไม่ดีของโรคมะเร็งเต้านม*