

# High vascular endothelial growth factor gene expression predicts poor outcome in patients with non-luminal A breast cancer

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**Abstract.** Breast cancer is the most frequent type of cancer among women worldwide. Vascular endothelial growth factor (VEGF), the key modulator of angiogenesis, has been implicated in breast cancer susceptibility and aggressiveness. *VEGF* expression was determined in 99 breast cancer tissue samples using reverse transcription-polymerase chain reaction and the human epidermal growth factor receptor 2 (HER2) status was determined by immunohistochemistry. Subsequently, the associations of *VEGF*, HER2 and hormone receptor status with clinicopathological data were evaluated. High *VEGF* expression was found to be significantly correlated with the presence of lymphovascular invasion. In hormone receptor-positive/HER2-positive, HER2-positive and triple-negative breast cancer, high *VEGF* expression was correlated with the presence of axillary nodal metastasis and lower overall survival rates. Therefore, the assessment of the *VEGF* status along with the hormone receptor and HER2 status may help identify high-risk patients who may benefit from anti-VEGF treatment.

## Introduction

Breast cancer is the most frequent type of cancer among women worldwide, with increasing incidence rates in the majority of countries (1). In Thailand, breast cancer is also the most common type of cancer among women (2). Genetic alteration is one of the key factors involved in breast cancer initiation and progression. Human epidermal growth factor receptor 2 (*HER2*), an onco-

gene that is amplified and overexpressed in breast cancer, has been correlated with more aggressive characteristics, including negative estrogen receptor (ER) and progesterone receptor (PR) status, higher histological grading, lymph node involvement and resistance to chemotherapy.

In addition to oncogene alterations, angiogenesis, the formation of new blood vessels, is of particular significance in the process of cancer growth, invasion and metastasis (3,4). The most important key modulator in this complex process is vascular endothelial growth factor (VEGF). The expression of VEGF has been correlated with the presence of higher microvessel density (MVD), lymphovascular invasion (LVI) and shorter disease-free survival (DFS) and overall survival (OS).

The analysis of plasma VEGF levels in metastatic breast cancer patients receiving bevacizumab demonstrated that VEGF levels >32.6 pg/ml were associated with shorter time-to-progression (5). The evaluation of VEGF in a randomized control trial on HER2-negative metastatic breast cancer revealed that the pretreatment plasma concentration of VEGF was correlated with a greater treatment effect. In addition, patients with higher VEGF concentrations exhibited lower hazard ratio (bevacizumab + docetaxel vs. placebo + docetaxel) (6). A study of *VEGF* polymorphisms in advanced breast cancer patients who were treated with paclitaxel alone or paclitaxel+bevacizumab (Eastern Cooperative Oncology Group 2100) revealed that *VEGF*-2578 AA and -1154 AA were associated with better OS in the combination arm (7).

HER2 activation is one of several mechanisms that upregulate VEGF expression. The evaluation of *VEGF* along with HER2, ER and PR status may provide useful information regarding the aggressiveness of breast cancer and may help identify patients who are suitable for anti-VEGF treatment.

## Patients and methods

**Study population.** The patients were recruited from the Division of Head-Neck and Breast Surgery, Department of Surgery, Faculty of Medicine, Siriraj Hospital (Bangkok, Thailand), between 2002 and 2004. All the patients with histopathologically confirmed breast carcinoma fulfilling the selection criteria were asked to be participated in this study. Patients who were diagnosed with breast cancer, aged ≥18 years and able to provide written informed consent, were included in the

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study. Patients with history of other cancers were excluded. At recruitment, informed consent was obtained from all the subjects and each participant was interviewed to collect detailed information on demographic characteristics and family history of cancer.

This study's protocol was approved by the Institutional Review Board of the Siriraj Hospital.

**Immunohistochemistry.** The expression levels of HER2 and MVD in breast cancer tissue were assessed by immunohistochemical staining with specific antibodies. Paraffin-embedded sections from each specimen were stained with monoclonal rabbit antihuman HER2 antibody, clone 4B5 (ready to use, incubation time 8 h; catalog no. 790-289921; Roche Diagnostics GmbH, Mannheim, Germany) and monoclonal mouse anti-human antibody to transmembrane glycoprotein CD31, clone JC70A (dilution 1:300, incubation time 16 h; catalog no. M082301; Dako Denmark A/S, Glostrup, Denmark). The 3- $\mu$ m sections were incubated at 56°C overnight, deparaffinized and rehydrated. To block endogenous peroxidase activity, the sections were incubated in 3% H<sub>2</sub>O<sub>2</sub> in deionized water for 10 min and then washed with running distilled water for 5 min. Antigen retrieval was performed by boiling the sections in 10 mmol/l citrate buffer (pH 6.0). The sections were placed in phosphate-buffered saline (PBS) for 10 min and then in 2% bovine serum albumin (BSA) for 30 min. The excess BSA was removed. The sections were stained with the primary antibody at room temperature, washed twice with PBS for 5 min, incubated with secondary rabbit anti-mouse antibody (catalog no. K500711; EnVision; Dako Denmark A/S) for 30 min. Following incubation, the sections were washed twice with PBS for 5 min, incubated in 3,3'-diaminobenzidine for 5 min and washed in tap water for 5 min. The sections were counterstained with haematoxylin, dehydrated, fixed and mounted. All the immunohistochemical data were evaluated by two pathologists who were blinded to the patients' characteristics and clinical outcome.

**Assessment of VEGF mRNA expression levels.** The levels of VEGF mRNA expression were assayed by semiquantitative reverse transcription-polymerase chain reaction, as previously described (8). Each RNA sample was assayed in duplicate and in two separate settings.

**Statistical analysis.** Patient data on cancer recurrence and death were retrieved through medical record review. The dates of recurrence and death were recorded. The date of last contact was defined as the date of the patient's last visit to the department where they had received breast cancer therapy (Division of Head-Neck and Breast Surgery, Department of Surgery; Division of Oncology, Department of Medicine; and Division of Therapeutic Radiology, Department of Radiology, Siriraj Hospital). The DFS analysis endpoint was cancer recurrence/metastasis or breast cancer-related death. DFS was defined as the time from diagnosis to the endpoint (recurrence, metastasis, or breast cancer-related death), censoring at the date of last contact or non-cancer death. The OS analysis endpoint was breast cancer-related death. OS was defined as the time from diagnosis to the endpoint of the study, censoring at the date of last contact or non-cancer death. Survival curves were

constructed using the Kaplan-Meier product-limit method and statistical significance was assessed using the log-rank test. A multivariate analysis was performed to evaluate the effect of prognostic factors on OS, using the Cox proportional hazards model. The statistical analyses were conducted using SPSS software version 15.0 (IBM Corp., Armonk, NY, USA).  $P < 0.05$  was considered to indicate statistically significant differences.

## Results

**VEGF mRNA expression in breast cancer tissue.** A total of 99 breast cancer patients were recruited. The patient characteristics are summarized in Table I. The mean age at diagnosis was 51.42 years (range, 39.49-63.35 years), with a median age of 50 years. A total of 91 patients had invasive ductal carcinoma; 62 patients had tumor size >20-50 mm; 55 patients had axillary nodal metastasis and 6 patients had distant metastasis at the time of diagnosis. The assessment of VEGF mRNA expression revealed that the mRNA ratio ranged from 0 to 3.27, with a median mRNA ratio of 1.16. At this cut-off value, 49 patients exhibited low and 50 patients high VEGF mRNA expression.

**Correlation between VEGF expression and clinicopathological characteristics.** On univariate analysis, high VEGF expression was correlated with the presence of LVI [odds ratio (OR)=2.96, 95% confidence interval (CI): 1.28-6.83;  $P=0.011$ ]. High VEGF expression tended to be correlated with locally advanced breast cancer [stage III (except T3N1M0) and IV] (OR=2.30, 95% CI: 0.96-5.54;  $P=0.062$ ). However, the multivariate analysis failed to demonstrate the statistical significance of this correlation. The distribution of VEGF expression status by different clinicopathological characteristics is summarized in Table II. Breast cancer patients were classified into 4 groups according to the ER, PR and HER2 status. The numbers of patients with hormone receptor-positive/HER2-negative, hormone receptor-positive/HER2-positive, HER2-positive and triple-negative breast cancer were 47 (47.47%), 10 (10.10%), 17 (17.17%) and 25 (25.25%), respectively.

In patients with hormone receptor-positive/HER2-positive, HER2-positive and triple-negative breast cancer, high VEGF expression was correlated with axillary nodal metastasis (OR=3.56, 95% CI: 1.13-11.15;  $P=0.030$ ). High VEGF expression was also correlated with the presence of LVI in patients with hormone receptor-positive/HER2-negative (OR=3.75, 95% CI: 1.08-13.07;  $P=0.038$ ).

**Survival analysis.** The median follow-up was 58.73 months (range, 1.23-93.03 months). The univariate analysis of survival by the Kaplan-Meier method revealed that the presence of perineural invasion (PNI), PR negativity and the presence of axillary nodal metastasis were correlated with lower DFS rates ( $P < 0.001$ ,  $P=0.017$  and 0.043, respectively). The presence of PNI, PR negativity, the presence of distant metastasis at the time of diagnosis and advanced-stage breast cancer were correlated with lower OS ( $P=0.011$ , 0.035, 0.003 and 0.009, respectively). The DFS and OS rates by clinicopathological characteristics and levels of VEGF expression are summarized in Table III. In the hormone receptor-positive/HER2-positive, HER2-positive and triple-negative groups, the presence of PNI was associated

Table I. Clinicopathological and demographic characteristics of breast cancer patients.

Characteristics	Patients, no. (%) (n=99)
Age at diagnosis, years	
<50	49 (49.50)
≥50	50 (50.50)
Tumor type	
Invasive ductal carcinoma	91 (91.92)
Invasive lobular carcinoma	3 (3.03)
Others	5 (5.05)
Tumor size, mm	
≤20	20 (20.20)
20-50	62 (62.63)
>50	17 (17.17)
Axillary nodal metastasis	
No	44 (44.44)
Yes	55 (55.56)
Distant metastasis	
No	93 (93.94)
Yes	6 (6.06)
Stage at diagnosis	
I	12 (12.12)
II	52 (52.53)
III	29 (29.29)
IV	6 (6.06)
Histological differentiation	
High	3 (3.03)
Moderate	58 (58.59)
Poor	35 (35.35)
Unknown	3 (3.03)
Lymphovascular invasion	
Absent	49 (49.49)
Present	46 (46.46)
Perineural invasion	
Absent	73 (73.74)
Present	15 (15.15)
Estrogen receptor	
Negative	42 (42.42)
Positive	57 (57.58)
Progesterone receptor	
Negative	57 (57.58)
Positive	42 (42.42)
HER2	
Negative	72 (72.73)
Positive	27 (27.27)

Data for lymphovascular and perineural invasion could not be pathologically confirmed for all the patients. HER2, human epidermal growth factor receptor 2.

Table II. Proportion of *VEGF* expression among different clinicopathological characteristics.

Characteristics	mRNA expression		P-value
	Low (n=49)	High (n=50)	
Age, years			0.482
<50	26	23	
≥50	23	27	
Tumor size, mm			0.121
≤20	13	7	
>20	36	43	
Axillary nodal metastasis			0.088
No	26	18	
Yes	23	32	
Distant metastasis			0.097
No	48	45	
Yes	1	5	
Early-stage cancer			0.060
Yes	38	30	
No	11	20	
Histological differentiation			0.806
High	2	1	
Moderate	28	30	
Poor	18	17	
Lymphovascular invasion			0.010
Absent	30	19	
Present	16	30	
Perineural invasion			0.451
Absent	37	36	
Present	6	9	
Estrogen receptor			0.257
Positive	31	26	
Negative	18	24	
Progesterone receptor			0.191
Positive	24	18	
Negative	25	32	
Hormone receptor			0.257
Positive	31	26	
Negative	18	24	
HER2			0.101
Negative	32	40	
Positive	17	10	

HER2, human epidermal growth factor receptor 2.

with lower DFS rates ( $P<0.001$ ). High *VEGF* expression, the presence of distant metastasis at the time of diagnosis and

advanced-stage breast cancer were found to be correlated with lower OS rates ( $P=0.041$ ,  $<0.001$  and  $0.008$ , respectively; data not shown). In the hormone receptor-positive/HER2-negative group, the presence of PNI and distant metastasis at the time of diagnosis were correlated with lower OS rates ( $P=0.019$  and  $0.013$ , respectively; data not shown). However, the Cox

Table III. Disease-free survival (DFS) and overall survival (OS) by clinicopathological characteristics and *VEGF* expression level.

Characteristics	DFS				OS			
	Cases (n=90)	Events (n=20)	5-year survival (%)	P-value	Cases (n=96)	Events (n=9)	5-year survival (%)	P-value
Age, years				0.679				0.719
<50	45	9	80.0		47	4	91.5	
≥50	45	11	75.6		49	5	89.8	
Tumor size, mm				0.059				0.117
≤20	28	1	94.4		19	0	100.0	
>20	72	19	73.6		77	9	88.3	
Histological differentiation				0.449				0.862
High/moderate	55	13	76.4		60	5	91.7	
Poor	33	6	81.8		33	3	90.9	
LVI				0.354				0.314
Absent	46	8	82.6		49	3	93.9	
Present	40	10	75.0		43	5	88.4	
PNI				<0.001				0.011
Absent	67	9	86.6		72	3	95.8	
Present	13	7	46.2		14	3	78.6	
ER				0.822				0.141
Positive	52	11	78.8		56	3	94.6	
Negative	38	9	76.3		40	6	85.0	
PR				0.017				0.035
Positive	38	4	89.5		41	1	97.6	
Negative	52	16	69.2		55	8	85.5	
Hormone receptor				0.822				0.141
Positive	52	11	78.8		56	3	94.6	
Negative	38	9	76.3		40	6	85.0	
HER2				0.457				0.245
Negative	64	13	79.7		70	5	92.9	
Positive	26	7	73.1		26	4	84.6	
Subtype				0.813				0.110
HR+HER2-	42	9	78.6		46	2	95.7	
Others	48	11	77.1		50	7	86.0	
VEGF				0.745				0.076
Low	47	10	78.7		48	2	95.8	
High	43	10	76.7		48	7	85.4	
Axillary nodal metastasis				0.043				0.089
No	43	6	86.0		44	2	95.5	
Yes	47	14	70.2		52	7	86.5	
Distant metastasis								0.003
No					90	7	92.2	
Yes					6	2	66.7	
Early-stage cancer				0.090				0.009
Yes	67	12	82.1		67	3	95.5	
No	23	8	65.2		29	6	79.3	

Data for histological differentiation, lymphovascular and perineural invasion could not be pathologically confirmed for all the patients. *VEGF*, vascular endothelial growth factor; LVI, lymphovascular invasion; PNI, perineural invasion; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; HR+HER2-, hormone receptor-positive human epidermal growth factor receptor 2-negative breast cancer.

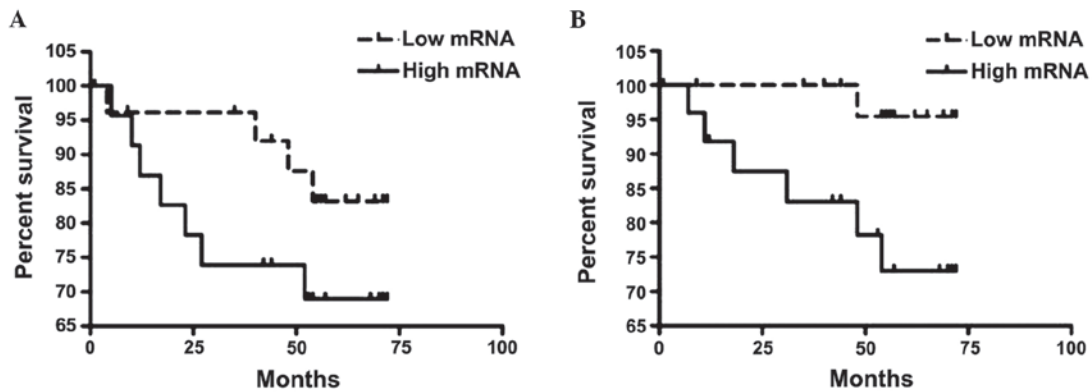


Figure 1. (A) Disease-free survival (DFS) and (B) overall survival (OS) by vascular endothelial growth factor (*VEGF*) expression among patients with hormone receptor-positive/human epidermal growth factor receptor 2 (HER2)-positive, HER2-positive and triple-negative breast cancer. High *VEGF* expression was significantly associated with lower OS ( $P=0.03$ ). However, the correlation between *VEGF* expression and DFS was not significant ( $P=0.20$ ).

regression analysis did not identify a significant correlation of clinicopathological characteristics with DFS and OS. The DFS and OS rates by *VEGF* expression in the hormone receptor-positive/HER2-positive, HER2-positive and triple-negative groups are shown in Fig. 1.

## Discussion

The results of this study demonstrated an association between high *VEGF* expression and the presence of LVI. This finding was in concordance with those of several previous studies, as reviewed elsewhere (9). We also demonstrated a significant association of *VEGF* expression with axillary nodal metastasis and lower OS in hormone receptor-positive/HER2-positive, HER2-positive and triple-negative breast cancer. However, due to the limited number of patients, the multivariate analysis failed to demonstrate a statistically significant difference.

Luminal B, HER2 and triple-negative subtypes were found to be more aggressive compared with luminal A subtype by tumor stage, lymph node status, or pathological type and also exhibited worse DFS and OS (10,11). The identification of high-risk patients and selection of an intensive regimen may improve treatment outcome. The expression of VEGF was found to be associated with reduced response to adjuvant endocrine treatment. In a retrospective study of 699 breast cancer patients conducted by Linderholm *et al* (12), the patients who received adjuvant endocrine therapy and exhibited higher VEGF expression had significantly shorter relapse-free survival and OS. In a study of 160 ER-positive advanced breast cancer patients who received tamoxifen, an above median VEGF level was correlated with shorter progression-free survival and post-relapse OS (13). In a randomized control trial of 224 breast cancer patients comparing 2 years of tamoxifen treatment with no tamoxifen treatment, regardless of hormone receptor and HER2 status, the patients with ER-positive and VEGF-negative tumors significant benefited from tamoxifen after a 10-year follow-up, whereas the patients with ER- and VEGF-positive tumors did not benefit from tamoxifen treatment (14).

In a large study on 1,788 breast cancer patients, higher frequency of VEGF expression was correlated with luminal B,

HER2 and basal-like subtypes. VEGF expression was associated with increased risks of breast cancer-specific mortality and distant recurrence among luminal A patients (15). In the present study, however, we did not identify a significant difference in *VEGF* expression frequency among breast cancer subtypes. In that study, conducted by Liu *et al* (15), VEGF immunohistochemistry was performed using VG1 antibody. VEGF positivity was defined as any positive staining in the cytoplasm of the tumor cells. By this definition, 72.5% of the patients were positive for VEGF. In our study, the median of the VEGF ratio was used as cut-off point. Using this definition, the patients were evenly distributed into low and high *VEGF* expression groups. The characteristics of the patients were also different, with higher stage and lower age at diagnosis compared with those reported earlier.

In conclusion, we demonstrated the role of *VEGF* in non-luminal A (hormone receptor-positive/HER2-positive, HER2-positive and triple-negative) breast cancer. The assessment of the *VEGF* status in this group of patients may help identify high-risk patients and may be used to guide appropriate treatment selection.

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