

Vascular endothelial growth factor polymorphisms affect gene expression and tumor aggressiveness in patients with breast cancer

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Abstract. Vascular endothelial growth factor (VEGF) is one of the key modulators of angiogenesis. The highly polymorphic promoter and 5' untranslated region of *VEGF* have been associated with susceptibility to and aggressiveness of several types of cancer. To examine the functional role of *VEGF* polymorphisms at -634 and -1498 positions, *VEGF* mRNA and protein in breast cancer tissues were analyzed by quantitative polymerase chain reaction and immunohistochemistry. A dual-luciferase assay was performed to determine promoter activity. The *VEGF*-634CC genotype demonstrated the highest *VEGF* mRNA expression. High *VEGF* mRNA expression was correlated with a tumor size of >2 cm, the presence of lymphovascular invasion and the presence of axillary nodal metastasis. The promoter containing the -1498T/-634C haplotype exhibited the highest basal promoter activity. These findings suggest that the interaction between -1498T and -634C polymorphisms increases *VEGF* expression and is involved in breast cancer aggressiveness.

Introduction

Vascular endothelial growth factor (VEGF) is one of the key modulators of angiogenesis. The *VEGF* gene is located on chromosome 6p21.3 and is organized as eight exons separated by seven introns (1,2). Alternative exon splicing was initially shown to result in the generation of four major isoforms that were 121, 165, 189 and 206 amino acids in length (2). The *VEGF* promoter, which spans 2.36 kb, contains several transcription factor binding sites, including Sp1/Sp3, activating

protein (AP)-2, Egr-1, signal transducer and activator of transcription-3 and hypoxia-inducing factor-1. These transcription factor binding sites are highly conserved in mice, rats and humans. These studies indicated that the *VEGF* promoter is critical in the regulation of VEGF expression. Several studies showed an association between *VEGF* polymorphisms and breast cancer susceptibility/aggressiveness, as well as levels of VEGF expression [reviewed in (3)].

Haplotype analysis showed that -1498C was linked with -634G (4). These two polymorphisms were associated with breast cancer susceptibility and aggressiveness (5-9). However, these polymorphisms were not located in the established transcription factor binding sites. *In vitro* models suggested a haplotypic effect of the polymorphic *VEGF* promoter on basal and stimulated promoter activity (10). In the present study, *VEGF* mRNA and VEGF protein expression in breast cancer tissue were determined and were correlated with various clinicopathological parameters. To verify the functional role of *VEGF* polymorphisms at the -634 and -1498 positions, site-directed mutagenesis was performed to generate different *VEGF* genotypes and to exclude other functional polymorphisms that may be in linkage disequilibrium with the polymorphisms of interest. The transcriptional activities of these polymorphisms were determined by a dual-luciferase assay.

Materials and methods

Study population. The study population was recruited from the Division of Head-Neck and Breast Surgery, Department of Surgery, Faculty of Medicine, Siriraj Hospital (Bangkok, Thailand) between 2000 and 2003. Patients with newly diagnosed breast cancer, aged ≥18 years with ability to provide informed consent were included. Patients with a history of other cancers were excluded. At recruitment, informed consent was obtained, and each participant was interviewed to collect detailed information with regard to demographic characteristics. This study was approved by the Siriraj Ethics Committee on Research (Bangkok, Thailand).

Genotyping of *VEGF* polymorphisms. Genomic DNA was obtained from peripheral blood according to a standard method. Briefly, venous blood samples were drawn into

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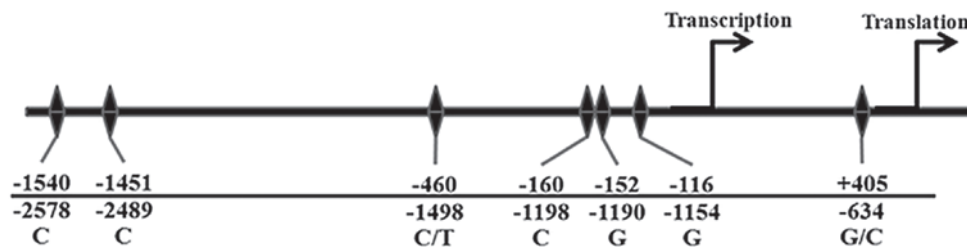


Figure 1. Haplotype of the VEGFA promoter. Transcription and translation start sites were used as references for the nomenclature of the polymorphisms in the upper line and lower lines, respectively. VEGFA, vascular endothelial growth factor A.

EDTA-containing tubes. The leukocyte cell pellet obtained from the buffy coat was resuspended with TE 20-5 solution, then digested with proteinase K (Promega Corporation, Madison, WI, USA) at 37°C overnight. DNA was isolated by the addition of phenol and chloroform-isoamyl alcohol (24:1) and centrifugation. The *VEGF* -634G/C polymorphisms were genotyped on allele refractory mutation system-polymerase chain reaction (PCR) using primers as follows: Forward, 5'-CATTGATCCGGGTTTATCCC-3'; reverse -634G, 5'-CACTCACTTTGTCCCTGTAG-3'; reverse -634C, 5'-CAC TCACTTTGTCCCTGTAC-3'; forward control, 5'-AGA TGGTCCCTCACCTTCCT-3'; and reverse control, 5'-GTC TACCCTCCTGAGCTTGC-3'. *VEGF*-1498C/T polymorphisms were genotyped by PCR-restriction fragment length polymorphisms. The forward primer was 5'-TGTGCGTGT GGGGTTGAGCG-3' and the reverse primer was 5'-TACGTG CGGACAGGGCCTGA-3'. The products were digested with the *Bst*UI restriction enzyme (New England Biolabs, Beverly, MA, USA). -1498C products were digested and resulted in 155 and 20 bp fragments. Representative PCR products were sequenced to validate the assay.

Evaluation of VEGF expression and microvessel density (MVD) in breast cancer tissue. The levels of *VEGF* mRNA expression in breast cancer tissue were determined by qPCR as described previously (11). *VEGF* protein expression was determined by immunohistochemistry as follows: Paraffin-embedded sections were stained with a monoclonal mouse antibody to human VEGF clone VG1 (dilution, 1:100; incubation time, 1 h; Diagnostic BioSystems, Pleasanton CA, USA) and a monoclonal antibody to CD31 (dilution, 1:300; incubation time, 16 h; Dako, Glostrup, Denmark). The immunohistochemical data was evaluated by two pathologists who had no knowledge of the patients' characteristics and/or clinical outcome. Expression of VEGF was assessed semiquantitatively using an immunohistochemical score (H score). The score was calculated by multiplying the percentage of positive carcinoma cells by the staining intensity (0, negative; 1, weak; 2, moderate; and 3, strong; as determined subjectively by the two pathologists). The average of the H scores from two pathologists was used. The median of the scores was used as the cutoff level to categorize tumors into low- (below the median H score) and high-expressing tumors (above the median H score), with regard to VEGF. MVD was expressed as the average number of microvessels per x200 field. The three most intense areas of angiogenesis were identified and microvessels were counted. A single microvessel was defined as any brown immunostained endothelial cell that was separated from adjacent microvessels

and other connective tissue elements. Large vessels with thick muscular walls were not counted, and the presence of a lumen was not required for scoring as a microvessel. The median of the MVD was used as the cutoff level to categorize tumors into low- and high-MVD tumors.

Construction of plasmids. pCR2.1 plasmids were purchased from Invitrogen Life Technologies (Grand Island, NY, USA). pGL3-Basic and pRL-SV40 plasmids were purchased from Promega Corporation (Madison, WI, USA). The *VEGF* promoter was amplified from human genomic DNA using the following primers: Forward primer, 5'-CAGGACTAGTGC ACGAATGA-3' and reverse primer, 5'-CTGTCTGTCTGT CCGTCAG-3'. The PCR reaction was conducted in a 100- μ l reaction containing 5 units of ProofStart DNA polymerase (Qiagen, Hilden, Germany), 1X PCR buffer (containing 1.5 mM MgCl₂), 0.2 mM dNTPs, 0.4 μ M of each primer, 1X Q-solution and 800 ng genomic DNA. The 3' A-overhang was added to the purified PCR product by means of a Qiagen A-addition kit (Qiagen). This product was immediately ligated into pCR2.1 plasmid using T4 DNA ligase (Life Technologies Corporations, Grand Island, NY, USA). The ligated plasmid was transformed into *Escherichia coli* (*E. coli*) strain DH5 α and propagated. The constructed plasmids were fully sequenced to exclude PCR errors. The promoter was excised using *Hind*III and *Xho*I (both from New England Biolabs) prior to ligation into the pGL3-Basic vector. The promoter-reporter plasmid was resequenced to confirm correct orientation of the promoter. This plasmid was used as a template to generate other plasmids containing different polymorphisms.

Site-directed mutagenesis. Plasmids containing different polymorphisms were amplified by ProofStart DNA polymerase (Qiagen). The DNA primers were as follows: -1498Mut-C forward, 5'-GTGGGGTTGAGGGCGTTGGAGCGGGG-3' and reverse, 5'-CCCCGCTCCAACGCCCTCAACCCAC-3'; -634Mut-C forward, 5'-GAGCAGCGAAAGCGACAGGGGC AAAGTG-3' and reverse, 5'-CACTTTGCCCTGTCTGCT TTCGCTGCTC-3'. The underlined base indicated the site of mutagenesis. The amplified products were digested by the addition of 10 units of *Dpn*I (Stratagene, Santa Clara, CA, USA) directly to the reaction. The *Dpn*I-treated DNA was transformed into *E. coli*. All plasmids were fully sequenced to exclude PCR errors and to confirm the presence of the polymorphisms. The polymorphisms in other positions are illustrated in Fig. 1.

Cell culture and DNA transfection. MCF7 breast carcinoma cells (derived from the American Type Culture Collection,

Table I. Determination of the distribution of *VEGF* genotypes among breast cancer patients.

A, qPCR (total=124)	
<i>VEGF</i> genotype	No. of patients (%)
-1498	
TT	65 (52.42)
CT	53 (42.74)
CC	6 (4.84)
-634	
GG	57 (45.97)
GC	55 (44.35)
CC	12 (9.68)

B, Immunohistochemistry (total=108)	
<i>VEGF</i> genotype	No. of patients (%)
-1498	
TT	58 (53.70)
CT	44 (40.74)
CC	6 (5.56)
-634	
GG	47 (43.52)
GC	50 (46.30)
CC	11 (10.18)

VEGF, vascular endothelial growth factor; qPCR, quantitative polymerase chain reaction.

Manassas, VA, USA), were cultured in Dulbecco's modified Eagle's medium with Ham's Nutrient Mixture F12 containing 10% fetal calf serum (Life Technologies, Inc., Middlesex, UK) at 37°C and in 5% CO₂. For transient transfection, 5x10⁴ cells were placed in 24-well plates and grown to 60-70% confluence. Transfection was conducted using Lipofectamine reagent Life Technologies, Inc. (Grand Island, NY, USA) according to the manufacturer's instructions. Cells were co-transfected with *VEGF* promoter-luciferase plasmid and *Renilla* luciferase plasmid (pRL-SV40). After 5 h of incubation, fetal calf serum was added to a final concentration of 10% with or without 10⁻⁷ M phorbol myristate acetate, and incubated for 16 h prior to evaluation of luciferase activity.

Dual-luciferase reporter assay. The cells were washed with phosphate-buffered saline and passive lysis buffer (Promega Corporation) was added to each well. The dual-luciferase reporter assay was performed according to the manufacturer's instructions. The experiments were performed in sextuplicate and repeated on three independent occasions.

Statistical analysis. The level of mRNA was calculated as the ratio of tissue sample to corresponding β -actin and then corrected as a ratio to the MDA-MB231 on the same scan. All luciferase results were expressed as a ratio to the luciferase activity of the empty vector and were normalized by *Renilla*

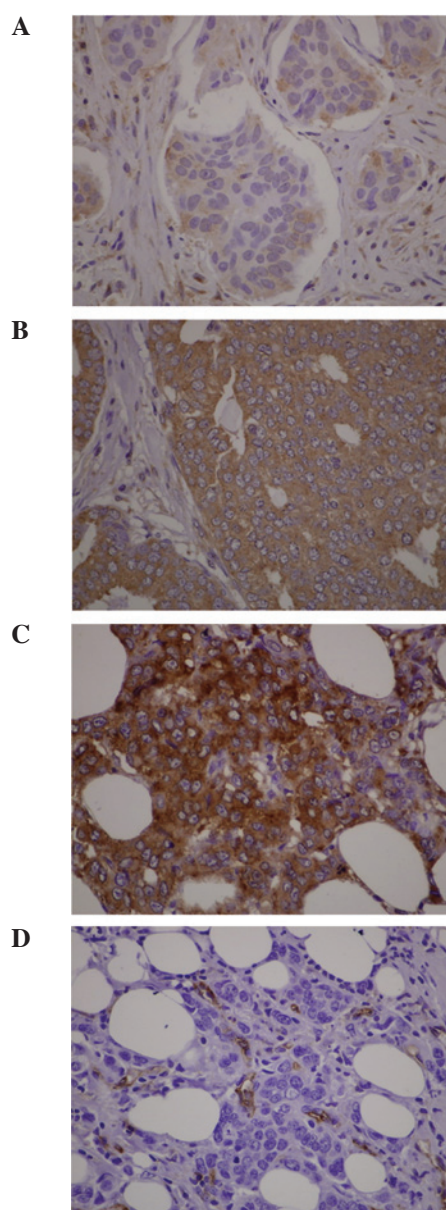


Figure 2. Representative immunohistochemical staining of VEGF and CD31. (A) 1+, Weak granular staining in the cytoplasm. (B) 2+, Moderate cytoplasmic staining. (C) 3+, Strong cytoplasmic staining. (D) Immunoperoxidase of CD31 demonstrates positive staining in endothelial cells. VEGF, vascular endothelial growth factor. Magnification, x200.

luciferase activity. Analysis of variance was used to evaluate the difference in mRNA expression among different genotypes and the difference in luciferase activity among haplotypes. Scheffe's post hoc test was performed to compare the difference between each pair of haplotypes. P<0.05 was considered to indicate a statistically significant difference.

Results

Genotyping of *VEGF* polymorphisms. The distribution of the *VEGF* genotype among breast cancer patients is summarized in Table I. The characteristics of the patients were summarized in our published data (12). Due to inadequate specimens and poor tissue quality, certain breast cancer specimens were not included in the analysis.

Table II. Normalized luciferase activity from three independent experiments.

	Normalized luciferase activity, mean (standard deviation)			
	-460T+405G	-460C+405G	-460T+405C	-460C+405C
I	15.12 (1.55)	18.56 (3.43)	26.05 (3.83)	18.04 (1.60)
II	25.98 (4.05)	29.21 (7.18)	39.41 (8.85)	22.91 (2.79)
III	37.32 (9.69)	45.03 (9.78)	53.14 (5.98)	37.79 (6.18)
Average	25.48 (10.65)	30.10 (12.79)	38.73 (12.78)	25.57 (9.13)
P-value ^a	0.003			

^aP-values were obtained from one-way analysis of variance.

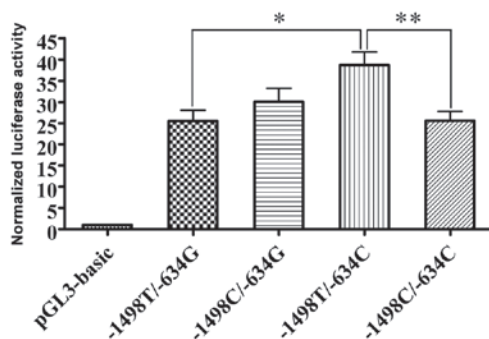


Figure 3. Luciferase activity of different *VEGF* A promoter haplotypes in MCF-7 cells. Results are expressed as corrected relative light units \pm standard deviation. * $P=0.014$, ** $P=0.015$. *VEGF* A, vascular endothelial growth factor A.

***VEGF* expression in breast cancer tissue.** Patients with the -634CC genotype had significantly higher *VEGF* mRNA in breast cancer tissue than those with -634GG or -634GC genotypes ($P<0.001$). High *VEGF* mRNA was previously shown to be associated with a tumor size >2 cm [odds ratio (OR), 2.476; 95% confidence interval (CI), 1.047-5.858, $P=0.039$], the presence of lymphovascular invasion (OR, 2.406; 95% CI, 1.142-5.070; $P=0.021$), and the presence of axillary nodal metastasis (OR, 2.288; 95% CI, 1.110-4.713; $P=0.025$) (12). Fig. 2 shows representative *VEGF* immunohistochemistry and staining of endothelial cells. All specimens were *VEGF* positive. The percentage of positive carcinoma cells ranged from 10 to 100%. The intensity was expressed as weak, moderate or strong staining. The H score ranged from 10 to 300. The median of the H score was 141.25. At this cutoff level, 54 breast carcinoma specimens were classified as exhibiting high *VEGF* expression. No association between *VEGF* genotype and H score was observed. High *VEGF* H-scores were associated with higher MVD counts ($P=0.021$) and the correlation between the H score and MVD count was statistically significant (Pearson correlation, 0.203; $P=0.035$). No significant difference was observed between MVD and any of the four polymorphisms.

Comparison of transcriptional activity of different *VEGF* promoter genotypes. Comparison of the *VEGF* promoter and empty vector activity revealed that the *VEGF* promoter increased basal luciferase activity of the pGL3-plasmid (Table II). The *VEGF* promoter bearing -1498T/-634C

had significantly higher promoter activity when compared with -1498T/-634G and -1498C/-634C ($P=0.014$ and 0.015, respectively; Fig. 3). To examine mechanisms that *VEGF* polymorphisms use to alter transcription, phorbol ester, which is known to stimulate the AP-1 site was added into the culture medium. Notably, phorbol ester increased transcriptional activity of the internal control, *Renilla* luciferase, more than the *VEGF* promoter-pGL3 Luc. This resulted in a decrease in normalized luciferase activity when compared with the basal luciferase activity of each *VEGF* promoter genotype.

Discussion

The significant correlation between the -634CC genotype and high levels of *VEGF* mRNA expression in breast cancer tissues was demonstrated and was concordant with *in vitro* promoter activity. No correlation was identified between *VEGF* protein expression and *VEGF* genotype or mRNA expression. The findings of the present study and previous studies (13-15) showed no correlation between *VEGF* protein expression which was determined by immunohistochemistry and *VEGF* genotype or mRNA expression. Failure to identify the association may be due to differences in scoring systems and the lack of reproducibility of subjective scoring in the determination of *VEGF* by immunohistochemistry (16). Although the levels of *VEGF* protein expression were not evaluated in the patients that were enrolled, several clinical trials concerning bevacizumab treatment showed satisfactory results in terms of objective response rate and progression-free survival (17-20). The majority of the patients in the present study had a *VEGF* intensity of 2+ and the median proportion of positive cells was 80%. This evidence suggests that almost all of the patients had relatively high *VEGF* levels and the expression of *VEGF* occurred in a dynamic manner as it varied with time.

In the current study, promoters bearing different haplotypes were generated by site-directed mutagenesis, which allowed desired loci to change and be compared, without altering the functional polymorphisms that may be in linkage disequilibrium with the loci of interest. Alteration of -634G to C resulted in increased luciferase activity; thus, -634G/C polymorphisms may have a direct effect on transcriptional activity. However, no transcription factor binding motif was identified at this position (21). The transcription factor binding motif predicted by the MatInspector Online Tool found no potential transcription factor bound to this position (22). Identification of the

transcription factor binding site using TFSEARCH version 1.3 (<http://mbs.cbrc.jp/research/db/TFSEARCH.html>) revealed that -634G was the potential binding site for myeloid zinc finger protein 1 (MZF1), which is expressed in hematopoietic progenitor cells that are committed to myeloid lineage differentiation (23). Watson *et al* (4) reported that alteration from G to C diminished the potential binding capacity. However, MZF1 may not have any role in the breast cancer cells used in the current study. Transcriptional activity assessed in GI-1 human glioma cell lines and Jurkat human lymphoblastic T-lymphocyte cell lines revealed that constructions bearing -1154G/-634C haplotypes exhibited higher luciferase activity than those bearing -1154G/-634G haplotypes (24). This indicated a direct effect of the alteration from G to C at -634 position on promoter activity, and polymorphisms at this position may regulate promoter activity at the post-transcriptional level. G to C alterations may affect the internal ribosome entry site and enhance transcription of the large VEGF isoform (395 amino acids) (25).

Stevens *et al* (10) constructed different haplotypes by direct amplification of genetic DNA bearing different haplotypes. This method could not ensure that the polymorphisms other than those at a specific position were identical. However, due to the high linkage disequilibrium of the VEGF promoter, this method had the advantage that the functional polymorphisms that linked with the position of interest remained linked as a block. It was reported that haplotypes containing -1198T/-1190G/-634G had higher basal VEGF promoter activity than haplotypes containing -1198C/-1190A/-634G. In the current study, alteration from T to C at position -1498 significantly decreased the VEGF promoter activity. The interactions between these two positions contributed to the difference in promoter activity and susceptibility to/aggressiveness of breast cancer.

In conclusion, the present study demonstrated the association between mRNA expression and breast cancer aggressiveness. VEGF polymorphisms altered the expression by modification of VEGF promoter activity. These findings suggested that VEGF polymorphisms influence growth and invasion of breast cancer cells through increased transcriptional activity and lead to increased angiogenesis. Genotyping of VEGF as a potential marker for identification of the high-risk patients may therefore improve the outcome of breast cancer treatment.

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