Vascular endothelial growth factor -634G/C polymorphism is associated with increased breast cancer risk and aggressiveness

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Abstract. Polymorphisms in the promoter and 5' untranslated region of vascular endothelial growth factor (*VEGF*) have been associated with *VEGF* levels. To investigate the role of *VEGF* polymorphisms in breast cancer, the *VEGF* -2578C/A, -1498C/T, -1154G/A and -634G/C polymorphisms were genotyped in 483 breast cancer patients and 524 healthy controls. *VEGF* mRNA levels in breast cancer tissue were determined using semi-quantitative RT-PCR. The genotypes, -634G/C and -634C/C, were associated with an increased risk for breast cancer when compared with the -634G/G genotype. The *VEGF* -634G/C genotype was associated with tumor size >20 mm, perineural invasion and stage II-IV. Individuals with -634C/C had lower disease-free survival. Patients with the *VEGF* -634C/C genotype exhibited the highest *VEGF* mRNA levels. High *VEGF* mRNA expression correlated with tumor size >20 mm, presence of lymphovascular invasion and axillary nodal metastasis. These observations suggested that *VEGF* -634G/C polymorphisms have a significant role in breast cancer susceptibility and aggressiveness.

Introduction

Breast cancer is the most prevalent cancer and the leading cause of cancer mortality in females worldwide, as well as in Thailand. Angiogenesis is the formation of new blood vessels and has been involved in the initiation and aggressiveness of breast cancer (1-3). The most important key modulator in this complex process is vascular endothelial growth factor (*VEGF*). *VEGF* plays a role in breast cancer (4) and the *VEGF* pathway is targeted in the treatment of breast cancer (5).

Human *VEGF* is localized on chromosome 6p21.3 and organized as eight exons, separated by seven introns (6,7). Several polymorphisms in the promoter and 5' untranslated region of (UTR) of *VEGF* have been identified (8,9). Awata *et al* previously identified seven polymorphisms in the promoter region as well as 5' and 3'UTR of *VEGF* in a Japanese population. Serum *VEGF* levels have also been found to be significantly higher in healthy subjects with the -634C/C genotype (10). However, *in vitro* experiments using lipopolysaccharide-stimulated peripheral blood mononuclear cells demonstrated that -634 G/G correlates with elevated *VEGF* production (9). In non-small cell lung cancer, a low *VEGF* expression in cancer tissue was significantly associated with the presence of the -2578C/C, -634G/G and -1154A/A and GA alleles in the *VEGF* promoter (11). The association between *VEGF* polymorphisms and breast cancer has been previously investigated (12). Based on these observations, we hypothesized that polymorphisms in the *VEGF* promoter and 5'UTR contribute to varied levels of *VEGF* expression, subsequently leading to susceptibility to aggressive breast cancer. To investigate this hypothesis, the association between *VEGF* polymorphisms and breast cancer susceptibility and aggressiveness was investigated, as well as mRNA expression in breast cancer tissue.

Materials and methods

Study population. The study population was recruited from the Division of Head, Neck and Breast Surgery (Siriraj Hospital, Bangkok, Thailand) between 2000 and 2003. Patients with histopathologically confirmed breast carcinoma were included in this prospective study and newly diagnosed breast cancer patients were included in the case group. Patients with histories

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Table I. PCR primer pairs and conditions for *VEGF* genotyping.

Polymorphisms	Primers	Product size, bp	PCR conditions (T, $MgCl_2$)[a]	Alleles	Restriction enzyme	DNA fragment sizes, bp
-634G/C	F: CATTGATCCGGGTTTTATCCC	282	65-60[b], 1			
	G: CACTCACTTTGTCCCTGTAG			G	-	-
	C: CACTCACTTTGTCCCTGTAC			C		-
	Inc F: AGATGGTCCCTCACCTTCCT	352				
	Inc R: GTCTACCCTCCTGAGCTTGC					
-1154G/A	F: GTCCGCACGTAACCTCACTT	220	62, 1.5			
	G: GACAGGCGAGCTTCAGCACC			G	-	-
	A: GACAGGCGAGCTTCAGCACT			A	-	
	Inc F: AGATGGTCCCTCACCTTCCT	352				
	Inc R: GTCTACCCTCCTGAGCTTGC					
-1498C/T	F: TGTGCGTGTGGGGTTGAGCG	175	60, 1.5	T	*Bst*UI	175
	R: TACGTGCGGACAGGGCCTGA			C		155, 20
-2578C/A	F: ATTGCTGCATTCCCATTCTC	251	60, 2.5	C	*Bst*YI	251
	R: CCCTTTTCCTCCAACTCTC	268		A		180, 88

[a]T, annealing temperature (˚C) and $MgCl_2$ concentrations (mM). [b]With decrement of temperature of 0.5˚C for each cycle until annealing temperature reached 60˚C and reaction contained 2% DMSO. *VEGF*, vascular endothelial growth factor. F, forward; R, reverse; Inc, internal control.

Table II. PCR primer pairs and conditions for semi-quantitative RT-PCR of *VEGF* and *β-actin* mRNA.

Gene	Primers	Product size, bp	PCR conditions (T, $MgCl_2$)[a]	PCR cycles
VEGF	F: CTCACCAAGGCCAGCACATAGG	159	55, 2.5	32
	R: ATCTGGTTCCCGAAACCCTGAG	291		
		363		
		414		
β-actin	F: TCGACAACGGCTCCGGCAT	239	50, 2.5	26
	R: AAGGTGTGGTGCCAGATTTTC			

[a]T, annealing temperature (˚C) and $MgCl_2$ concentrations (mM), *VEGF*, vascular endothelial growth factor. F, forward; R, reverse.

of other types of cancer were excluded. Healthy individuals and patients who attended the hospital due to benign conditions with no previous diagnosis of cancer were included in the control group and frequency matched to the breast carcinoma cases with regard to age (±5 years). Informed consent was obtained from all subjects. This study was approved by the ethics committee of the Siriraj Hospital, Mahidol University, Bangkok, Thailand.

Genotyping of VEGF polymorphisms. VEGF -634G/C and -1154G/A were genotyped by the allele refractory mutation system-PCR. PCR was performed as summarized in Table I. *VEGF* -1498C/T and -2578C/A were genotyped by the PCR-restriction fragment length polymorphisms. Representative PCR products were sequenced to validate the assay.

Assessment of VEGF mRNA expression levels. The correlation between *VEGF* polymorphisms and expression levels was determined in breast tissue. *VEGF* mRNA expression was assayed by semi-quantitative RT-PCR as described previously (13). A primer pair that amplified *β-actin* was employed to check RNA integrity and used as an internal control. Primer sequences, PCR conditions and the number of PCR cycles are presented in Table II. To account for variability between gels, an RT-PCR product from the MDA-MB-231 cell line was electrophoresed as a control on each gel. PCR product intensity was analyzed using GeneTools® software (Syngene, Cambridge, UK). mRNA levels were calculated as the ratio of tissue sample to corresponding *β-actin* and then corrected as a ratio to the MDA-MB-231 sample on the same scan. Each RNA sample was assayed in duplicate and in two separate settings.

Table III. Correlation between *VEGF* genotype and breast cancer susceptibility.

VEGF genotype polymorphisms	Controls, n (%)	Cases, n (%)	OR (95% CI), P-value
-634			
GG	234 (65.91)	223 (46.17)	1 (ref.)
GC	81 (22.82)	199 (41.20)	2.544 (1.852-3.496), 0.001
CC	40 (11.27)	61 (12.63)	1.600 (1.030-2.485), 0.036
-1154			
GG	279 (69.23)	318 (65.84)	1 (ref.)
GA	118 (29.28)	149 (30.85)	1.096 (0.819-1.467), 0.538
AA	6 (1.49)	16 (3.31)	2.320 (0.819-6.014), 0.084
-1498			
TT	215 (52.31)	243 (50.31)	1 (ref.)
CT	172 (41.85)	214 (44.31)	1.097 (0.835-1.441), 0.507
CC	24 (5.84)	26 (5.38)	1.002 (0.557-1.802), 0.995
-2578			
CC	214 (51.69)	240 (49.69)	1 (ref.)
AC	173 (41.79)	213 (44.10)	1.091 (0.830-1.435), 0.530
AA	27 (6.52)	30 (6.21)	1.016 (0.584-1.767), 0.957

ORs and 95% CIs calculated by logistic regression, age-adjusted. *VEGF*, vascular endothelial growth factor; OR, odds ratio; CI, confidence interval.

Statistical analysis. Distribution of *VEGF* allele frequencies and genotypes among the case and control groups was analyzed using the χ^2 test. Odds ratios (ORs) and 95% confidence intervals (CIs), obtained from unconditional logistic regression, were used to measure the strength of the association between *VEGF* polymorphisms and susceptibility and aggressiveness of breast cancer. Individual haplotypes were determined using the PHASE program available at http://www.stat.washington.edu/stephens/phase.html (14). The end-point of overall survival (OS) analysis was breast cancer-associated mortality. The disease-free survival (DFS) analysis end-point was cancer recurrent/metastasis or breast cancer-associated mortality. DFS and OS time was calculated as the time from diagnosis to the end point of the study, censoring at the date of last contact or non-cancer mortality. The survival curves were determined using a Kaplan-Meier product-limit method. Statistical significance between the survival curves was assessed using the log-rank test. Multivariate analysis was performed to evaluate the effect of prognostic factors on OS, using the Cox proportional hazards model. P<0.05 was considered to indicate a statistically significant difference. mRNA levels were calculated as the ratio of tissue sample to corresponding *β-actin* and corrected as a ratio to the MDA-MB-231 cell line on the same scan. Each RNA sample was assayed in duplicate and in two separate settings.

Results

Correlation between VEGF genotypes and breast cancer susceptibility. Genotyping was performed on 483 breast cancer patients and 524 controls. -634C allele distribution was significantly higher in breast cancer patients compared with control subjects (33.23 vs. 23.71%; P<0.001). *VEGF* -634G/C and -634C/C genotype distributions were significantly higher in breast cancer patients (GC, 41.20 and CC, 12.63 vs. GC, 25.06 and CC, 11.18%; P<0.001). Allele and genotype frequency distributions of other loci were not found to be different. The mean age of the control group was 48.56±14.45 years (SEM). The mean age of the breast cancer patients was 50.8±11.326 years (SEM). The mean age of the breast cancer and control groups were statistically different [OR, -2.238; 95% CI, (-5.20)-(-3.957); P=0.011], thus, ORs and 95% CIs calculated by logistic regression were adjusted for age. Individuals with the -634G/C genotype had an increased risk of breast cancer when compared with the -634G/G genotype (OR, 2.544; 95% CI, 1.852-3.496; P<0.001). Homozygous CC had an increased risk when compared with -634G/G (OR, 1.600; 95% CI, 1.030-2.485; P=0.036; Table III). *VEGF* polymorphisms in other loci did not demonstrate any increased risk for breast cancer.

Correlation between VEGF genotypes and clinicopathological parameters. Table IV shows known clinicopathological parameters and demographic factors of the breast cancer patients. Numerous patients received surgery (mastectomy in 385 patients and wide excision in 91 patients). The patients with invasive carcinoma who underwent wide excision received radiotherapy. *VEGF* -634G/C genotype was associated with tumor size >20 mm (OR, 1.638; 95% CI, 1.103-2.434; P=0.015), perineural invasion (OR, 2.261; 95% CI, 1.217-4.202; P=0.010) and stage II-IV at diagnosis (OR, 1.915; 95% CI, 1.255-2.944; P=0.003). Separate analysis of invasive ductal carcinoma revealed a marked association with tumor size >20 mm (OR, 1.722; 95% CI, 1.097-2.703; P=0.018), perineural invasion (OR, 2.36; 95% CI, 1.227-4.539; P=0.010) and stage II-IV at diagnosis (OR, 2.078; 95% CI, 1.237-3.490; P=0.006). The *VEGF* -1498C/C genotype correlated with decreased

Table IV. Clinicopathological parameters and demographic factors of breast cancer.

Characteristics	Breast cancer patients, n (%)
Age at diagnosis, years	
≤40	68 (14.08)
40-49	178 (36.85)
50-59	137 (28.36)
>60	100 (20.70)
Tumor type	
Ductal carcinoma *in situ*	36 (7.45)
Invasive ductal carcinoma	396 (81.99)
Invasive lobular carcinoma	12 (2.48)
Others	39 (8.07)
Tumor size, mm	
In situ	30 (6.21)
≤20	159 (32.92)
>20-50	250 (51.76)
>50	44 (9.11)
Axillary nodal metastasis	
No	271 (56.11)
Yes	205 (42.44)
Unknown	7 (1.45)
Distant metastasis	
No	466 (96.48)
Yes	17 (3.52)
Stage at diagnosis	
0	28 (5.80)
I	115 (23.81)
II	214 (44.31)
III	109 (22.57)
IV	17 (3.52)
Histological grading	
Well-differentiated	36 (7.45)
Moderately differentiated	230 (47.62)
Poorly differentiated	146 (30.23)
Unknown	71 (14.70)
ER	
Negative	191 (39.54)
Positive	261 (54.04)
Unknown	31 (6.42)
PR	
Negative	236 (48.86)
Positive	210 (43.48)
Unknown	37 (7.66)
Surgery	
Yes	476 (98.55)
No	3 (0.62)
Unknown	4 (0.83)
Chemotherapy	
Yes	308 (63.77)
No	166 (34.37)
Unknown	9 (1.86)
Radiotherapy	
Yes	198 (40.99)
No	272 (56.31)
Unknown	13 (2.69)

ER, estrogen receptors; PR, progesterone receptor.

risk of lymphovascular invasion (LVI; OR, 0.308; 95% CI, 0.102-0.927; P=0.036).

Haplotype analysis. The -2578C/-1498T/-1154G/-634G haplotype was the most common haplotype in the two groups (frequency, 0.3778 and 0.4739, respectively). Permutation testing revealed a significant difference between haplotype frequencies in the breast cancer and control groups (P=0.01). CTGG and CTGC haplotype copy number distributions were significantly different between the groups. Bearing 1 or 2 copies of the CTGG haplotype had a protective effect against breast cancer (OR, 0.55; 95% CI, 0.42-0.73; P<0.001). By contrast, bearing 1 or 2 copies of the CTGC haplotype was found to have an increased risk for breast cancer (OR, 1.81; 95% CI, 1.39-2.35; P<0.001). Patients with 1 or 2 copies of the CTGC haplotype significantly correlated with a tumor size >20 mm, (OR, 1.60; 95% CI, 1.09-2.35; P=0.0126), presence of perinural invasion (PNI; OR, 1.84; 95% CI, 0.97-3.52; P=0.046) and stage II-IV (OR, 1.73; 95% CI, 1.14-2.61; P=0.0062). Patients with 1 or 2 copies of ACAG haplotype exhibited a reduced risk for LVI and poorly differentiated cell types (OR, 0.74; 95% CI, 0.48-1.15; P=0.1656 and OR, 0.74; 95% CI, 0.46-1.17; P=0.1768, respectively).

Survival analysis. The median follow-up time was 65 months (range, 1.25-136.7 months). Among 446 patients, there were 37 mortalities during the study period, 30 of which were breast cancer-related. Locoregional recurrence was observed in 29 patients. Distant metastasis occurred in 86 patients. The Kaplan-Meier survival curves of various *VEGF* genotypes are shown in Fig. 1. Patients with the -634CC genotype (5 mortalities of 57 patients) succumbed to breast cancer in the first 21 months following breast cancer diagnosis. Univariate analysis between clinicopathological parameters, *VEGF* genotypes and corresponding 5-year survival are provided in Table V. Age >50 years correlated with lower 5-year OS. Larger tumor size, LVI, PNI, estrogen receptor- and progesterone receptor-negative, regional lymph-node positive, distant metastasis and advanced staging were the major prognostic factors for OS and DFS. No statistically significant correlation was found between *VEGF* genotypes and 5-year survival in the univariate analysis. Factors with a P-value ≤0.2 were included in the Cox regression analysis and poor differentiation, presence of PNI, PR-negative, axillary nodal metastasis and having the -634C/C and -1154G/A genotypes were found to significantly correlate with increased hazard ratio (HR) for DFS. HRs were 3.050 (95% CI, 1.354-6.871; P=0.007) and 2.452 (95% CI, 1.384-4.343; P=0.002) for the -634C/C and -1154G/A genotypes, respectively.

Table V. DFS and OS by clinicopathological parameters and *VEGF* polymorphisms.

	DFS				OS			
Parameters	Cases, n	Event	5-year survival, %	P-value	Cases, n	Event	5-year survival, %	P-value
Age at diagnosis, years								
≤50	250	50	79.70	0.863	253	12	95.02	0.047
>50	197	40	78.46		205	19	90.89	
Tumor size, mm								
In situ and ≤20	177	12	93.13	<0.001	180	4	97.66	0.001
>20	274	78	70.03		285	27	90.18	
Histological grading								
Well-/moderately differentiated	245	44	81.48	0.014	253	15	94.93	0.158
Poorly differentiated	134	38	70.23		137	13	88.45	
LVI								
Absent	277	39	84.80	<0.001	284	15	95.22	0.047
Present	147	45	69.31		154	15	89.15	
PNI								
Absent	332	51	83.79	<0.001	343	15	95.80	0.003
Present	49	20	55.11		52	7	85.92	
ER								
Positive	244	42	82.24	0.032	254	10	96.36	0.003
Negative	179	44	74.48		183	20	88.19	
PR								
Positive	196	25	87.31	<0.001	204	7	97.39	0.007
Negative	222	59	72.06		228	22	89.38	
Regional nodal metastasis								
No	258	25	89.24	<0.001	262	7	97.41	<0.001
Yes	187	64	65.05		197	23	87.48	
Distant metastasis								
No	N/A	N/A	N/A	N/A	449	28	93.72	0.007
Yes	N/A	N/A	N/A	N/A	16	3	74.29	
Staging								
0-I	134	8	94.06	<0.001	133	1	99.25	0.001
II-IV	312	82	72.73		324	30	90.55	
634G/C								
GG	209	35	82.87	0.088	215	12	94.18	0.490
GC	187	40	77.54		193	14	92.98	
CC	55	15	71.14		57	5	90.29	
-1154G/A								
GG	297	52	81.23	0.166	304	18	93.99	0.627
GA	138	35	74.45		145	12	91.83	
AA	16	3	81.25		16	1	92.31	
-1498C/T								
TT	225	38	82.33	0.224	231	13	93.72	0.542
CT	201	48	74.66		208	17	92.52	
CC	25	4	87.50		26	1	94.74	
-2578C/A								
CC	223	39	81.82	0.207	229	13	93.67	0.162
AC	199	47	74.82		206	18	91.73	
AA	29	4	88.82		30	0	100.00	

Proportion of survival obtained from Kaplan-Meier analysis. *VEGF*, vascular endothelial growth factor; DFS, disease-free survival; OS, overall survival; ER, estrogen receptors; PR, progesterone receptors; LVI, lymphovascular invasion; PNI, perinural invasion.

Table VI. Characteristics of 124 breast cancer patients included in *VEGF* mRNA evaluation.

Characteristics	Patients, n (%)
Age at diagnosis, years	
≤50	66 (53.23)
>50	58 (47.77)
Tumor type	
Ductal carcinoma *in situ*	2 (1.61)
Invasive ductal carcinoma	108 (87.10)
Invasive lobular carcinoma	4 (3.23)
Others	9 (7.26)
Tumor size, mm	
In situ	2 (1.61)
≤20	28 (22.58)
>20-50	68 (54.84)
>50	26 (20.97)
Axillary nodal metastasis	
No	59 (47.58)
Yes	65 (52.42)
Distant metastasis	
No	117 (94.35)
Yes	7 (5.65)
Stage at diagnosis	
0	2 (1.61)
I	20 (16.13)
II	61 (49.19)
III	34 (27.42)
IV	7 (5.65)
Histological grading	
Well differentiated	3 (2.61)
Moderately differentiated	69 (60.00)
Poorly differentiated	43 (37.39)
LVI	
Absent	68 (57.14)
Present	51 (42.86)
PNI	
Absent	93 (83.78)
Present	18 (16.22)
ER	
Negative	60 (48.78)
Positive	63 (51.22)
PR	
Negative	70 (56.91)
Positive	53 (43.09)

VEGF, vascular endothelial growth factor; ER, estrogen receptor; PR, progesterone receptor; LVI, lymphovascular invasion; PNI, perinural invasion.

VEGF mRNA expression in breast cancer tissue. VEGF mRNA expression was evaluated in 124 breast cancer tissues. Characteristics of breast cancer patients are provided in Table VI. Expression ranged between 0 and 3.27 with a median of 1.10. Patients with the *VEGF* -634C/C genotype had significantly higher *VEGF* mRNA in breast cancer tissue compared with those with the -634G/G or -634G/C genotype (Fig. 2). Patients with heterozygous -1154G/A, -1498C/T and -2578A/C exhibited lower *VEGF* mRNA when compared with homozygous -1154G/G, -1498T/T and -2578C/C. Due to the presence of outliers and a small number of patients with the homozygous -1154A/A, -1498C/C and -2578A/A genotypes,

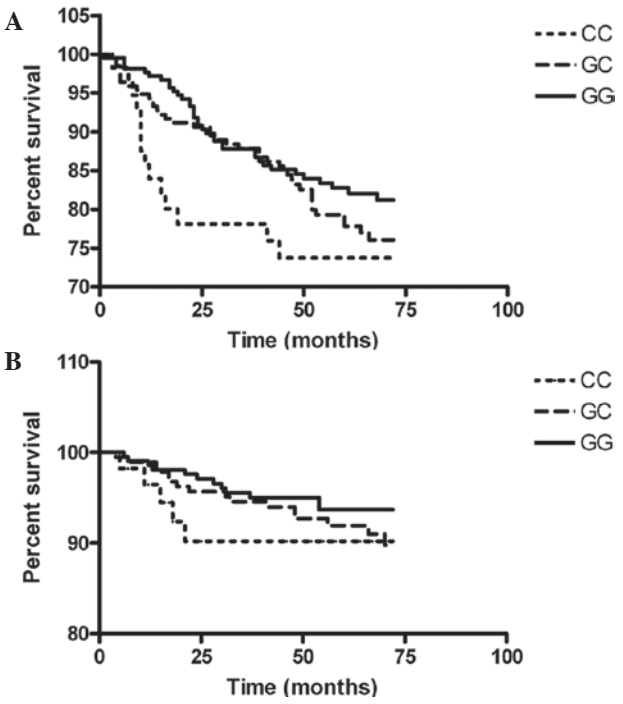

Figure 1. Patient survival following diagnosis by *VEGF* genotypes. (A) Patients with the -634C/C genotype correlated with lower DFS compared with those with the -634G/G genotype. (B) Lower OS rate correlated with -634G/C and CC compared with -634G/G genotype. *VEGF*, vascular endothelial growth factor; DFS, disease-free survival; OS, overall survival.

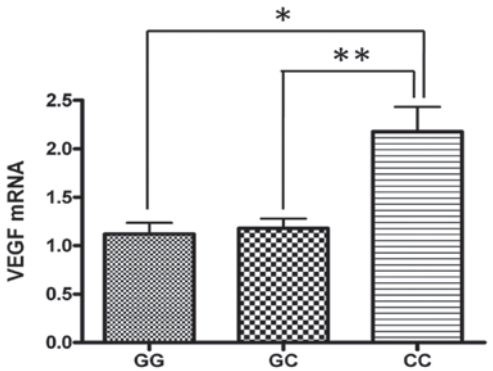

Figure 2. *VEGF* mRNA expression in patients with -634G/C polymorphisms, presented as a ratio to *β-actin* mRNA levels. One-way analysis of variance revealed that the three genotypes exhibited significantly different *VEGF* mRNA expression levels (P<0.001). Patients with the -634C/C genotype exhibited a significantly higher *VEGF* mRNA expression when compared with -634G/G and -634G/C genotype (Scheffe post test; P<0.001 and 0.001, respectively). The number of patients was 57, 55 and 12 for the -634GG, GC and CC genotypes, respectively. *P<0.001, vs. -634G/G; **P=0.001, vs. -634G/C. *VEGF*, vascular endothelial growth factor.

Table VII. Correlation between *VEGF* mRNA expression and clinicopathological parameters.

Characteristics	*VEGF* mRNA expression		OR (95% CI)	P-value
	Low	High		
Age, years				
≤50	36 (58.06)	30 (48.39)		
>50	26 (41.94)	32 (51.61)	1.477 (0.727-3.001)	0.281
Tumor size, mm				
≤20	20 (32.26)	10 (16.13)		
>20	42 (67.74)	52 (83.87)	2.476 (1.047-5.858)	0.039
Axillary nodal metastasis				
No	36 (58.06)	23 (37.70)		
Yes	26 (41.94)	38 (62.30)	2.288 (1.110-4.713)	0.025
Distant metastasis				
No	60 (96.77)	56 (91.80)		
Yes	2 (3.23)	5 (8.20)	2.679 (0.499-14.369)	0.250
Staging				
0-II	46 (74.19)	37 (59.68)		
III-IV	16 (25.81)	25 (40.32)	1.943 (0.906-4.163)	0.088
Histological grading				
Well-/moderately differentiated	38 (65.52)	34 (59.65)		
Poorly differentiated	20 (34.48)	23 (40.35)	1.285 (0.603-2.740)	0.516
LVI				
Absent	40 (67.80)	28 (46.67)		
Present	19 (32.20)	32 (53.33)	2.406 (1.142-5.070)	0.021
PNI				
Absent	48 (87.27)	45 (80.36)		
Present	7 (12.73)	11 (19.64)	1.676 (0.598-4.701)	0.326
ER				
Positive	36 (58.06)	27 (44.26)		
Negative	26 (41.94)	34 (55.74)	0.574 (0.281-1.171)	0.127
PR				
Positive	31 (50.00)	22 (36.07)		
Negative	31 (50.00)	39 (63.93)	0.564 (0.274-1.161)	0.120
Hormone receptor				
Negative	39 (62.90)	30 (49.18)		
Positive	23 (37.10)	31 (50.82)	0.571 (0.278-1.172)	0.126

VEGF, vascular endothelial growth factor; OR, odds ratio; ER, estrogen receptor; PR, progesterone receptor; LVI, lymphovascular invasion; PNI, perinural invasion; CI, confidence interval.

VEGF mRNA expression in these groups appeared to be high. Following exclusion of the outlier, *VEGF* mRNA expression was decreased in patients with homozygous -1154A/A, -1498C/C and -2578A/A (data not shown).

Correlation between VEGF mRNA levels and clinicopathological parameters. Breast cancer patients were classified into low and high expression groups using a median value of 1.10. Patient distribution in each group and clinicopathological parameters are provided in Table VII. Elevated *VEGF* expression correlated with a tumor size >20 mm (OR, 2.476; 95% CI, 1.047-5.858; P=0.039), axillary nodal metastasis (OR, 2.288; 95% CI, 1.110-4.713; P=0.025) and presence of LVI (OR, 2.406; 95% CI, 1.142-5.070; P=0.021).

Discussion

In the current study, it was observed that alteration of nucleotides from G to C at -634, resulted in an increased risk of breast cancer. However, previous studies have not reported this correlation in breast cancer (15-20). In the present study, the -634G/C genotype was significantly associated with more aggressive features. Due to a limited number of patients with the -634C/C genotype, the difference was not observed to be statistically significant. This was consistent with a previous study by Balasubramanian *et al* and Jin *et al* which reported that an alteration of G to C at this position was associated with a larger tumor size and high grade breast cancer (16,18). By contrast, Langsenlehner *et al* observed a significant correlation between the -634G/C and -634C/C genotypes and smaller tumor size (19). Survival analysis revealed a significant correlation between the -634C/C genotype and lower DFS. However, OS of the patients with different -634G/C genotypes was similar, which may be due to the relatively short term follow up of this study. By contrast, the survival analysis of 1,455 Chinese breast cancer patients revealed that patients with the -634G/G genotype had a lower OS compared with those with the -634C/C genotype, however, this polymorphism was not found to correlate with DFS (21). The variance in the demographic results of the cancer population may contribute to discrepancies observed. In the present study, 26.09% of the patients were diagnosed as stage III and IV and 63.77% of the patients received chemotherapy, while in a study by Lu *et al,* only 11.34% of the patients were diagnosed as stage III and IV and a large number of patients received chemotherapy (93.95%) (21).

In the present study, no correlation was found between -1154G/A polymorphisms and breast cancer risk, consistent with previous studies in Caucasian populations (18,20,22). No correlation between breast cancer aggressiveness and -1154G/A polymorphisms was observed, consistent with a study by Jin *et al* (18). Breast cancer susceptibility or aggressiveness was not associated with -1498C/T polymorphisms, consistent with previous large case-control studies in Asian and Caucasian populations (15,16). There was no association between -2578A/C polymorphisms and breast cancer susceptibility/aggressiveness as previously observed in Caucasian populations by Jin *et al* and Langsenlehner *et al* (18,19). By contrast, two additional studies in Caucasian populations revealed conflicting results. Schneider *et al* reported an association between the -2578A/A genotype and breast cancer risk while Jacobs *et al* reported that -2578C was associated with an increased risk of invasive breast cancer (17,20). However, the latter study stated the importance of LD of -2578A/C and -1154G/A. Thus, the association of the two polymorphisms and breast cancer risk may not be individually demonstrated.

Haplotype analysis revealed that the -2578C/-1498T/-1154G/-634G haplotypes had a protective effect against breast cancer. Patients with the -2578C/-1498T/-1154G/-634C haplotype had an increased risk of breast cancer and were associated with a tumor size >20 mm, stage II-IV and PNI. In a Swedish population, haplotypes -2578C/-634C were significantly associated with a large tumor size and higher grade. Having 2 copy numbers of haplotypes -2578A/-634G was associated with lower tumor grade (18). Jacobs *et al* reported that the -2578A/-1154A/-634G haplotypes correlated with a reduced risk of breast cancer in an American population (17). These observations and the observations of the present study indicate that haplotypes bearing the-634C allele contribute to increased breast cancer risk and aggressiveness. Non-replication of genetic association results is common in genetic epidemiology. In addition, the polymorphisms on the promoter region and 5'UTR were in LD. Interpretation of haplotype analysis revealed that the alleles included in the haplotypes may be associated with other functional polymorphisms that were not assessed. Comparison of haplotype analysis may be complicated due to variation in alleles in the haplotype, different software used to generate the haplotype and determining haplotype frequency.

The *VEGF* -634CC genotype correlated with elevated levels of mRNA expression. Elevated *VEGF* mRNA expression correlated with tumor size >20 mm, lymph node involvement and presence of LVI. These observations are consistent with a previous study employing the RT-PCR technique. Gomez-Esquer *et al* demonstrated a correlation between *VEGF* mRNA expression higher than the 25th percentile and more aggressive features in 103 breast cancer patients (23).

Incorporation of bevacizumab, a humanized monoclonal antibody that targets *VEGF* in chemotherapy, is a rapidly evolving area in the treatment of breast cancer. An association study of *VEGF* polymorphisms in 180 advanced breast cancer patients treated with paclitaxel alone or with bevacizumab and 183 untreated patients, revealed that the *VEGF* -2578A/A genotype was associated with an improved median OS in the combination arm when compared with AC combined with the CC genotype. The *VEGF* -1154A/A genotype also demonstrated an improved median OS when compared with GG combined with the GA genotype in the combination arm (24). These observations indicate that selection methods to identify the patients suitable for anti-*VEGF* therapy must be established. -634G/C polymorphisms may identify populations at risk and predict the outcome of breast cancer. It is possible that genotyping of *VEGF* -634 polymorphisms in breast cancer patients may be used to select appropriate patients for anti-angiogenesis treatment.

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