Association Between Vascular Endothelial Growth Factor Gene Polymorphisms with Breast Cancer Risk in an Iranian Population

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ABSTRACT: Breast cancer (BC) is one of the most causes of death in women worldwide. It affects Iranian female population approximately a decade earlier than those in other parts of the world. Previous studies have shown that vascular endothelial growth factor (VEGF) gene variants were associated with BC risk. The current study aimed to evaluate the impact of *VEGF* rs3025039 (+936C>T), rs2010963 (+405C>G), rs833061 (−460T>C), rs699947 (−2578C>A), and rs35569394 (18-bp I/D) polymorphisms on BC risk in an Iranian population in southeast of Iran. This case–control study was done on 250 BC patients and 215 healthy women. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) or PCR was used to genotype the polymorphisms. Our findings showed that *VEGF* rs699947 variant increased the risk of BC (OR = 1.71, 95% CI = 1.15–2.54, $P = 0.009$, CA vs CC; OR = 2.12, 95% CI = 1.14–3.93, $P = 0.021$, AA vs CC; OR = 1.78, 95% CI = 1.22–2.60, $P = 0.004$, CA+AA vs CC; OR = 1.47, 95% CI = 1.12–1.92, $P = 0.005$, A vs C). The *VEGF* rs3025039, rs2010963, rs833061, and rs35569394 variants were not associated with risk/protection of BC. In conclusion, our results proposed that *VEGF* rs699947 polymorphism may increase the risk of BC development. Furthers studies with larger sample sizes and different ethnicities are necessary to confirm our findings.

KEYWORDS: vascular endothelial growth factor, VEGF, breast cancer, polymorphism

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Introduction

Breast cancer (BC) is one of the most prevalent malignancies in women worldwide, which affects more than 1 million women annually.[1,2] It comprises 21.4% of all cancers among Iranian females. Although the etiology of BC is entirely unknown, there is abundant evidence that genetic factors play key roles in the pathogenesis and progression of BC.[3–6]

The human *VEGF* gene is located on the short arm of chromosome 6 (6p12–p21) and consists of eight exons separated by seven introns that exhibit alternative splicing to form a family of proteins.[7] VEGF, also known as VEGFA, is an important regulator of physiological angiogenesis during embryogenesis, skeletal growth, and reproduction functions. It is also involved in pathological angiogenesis associated with tumors.[8,9] Angiogenesis is an important step in the development of cancer and is necessary for primary tumor growth, invasiveness, and metastases.[8] Overexpression of VEGF was found in several tumor tissues.[10–12] Breast cancer is among the well-known malignancies involving lymphangiogenesis, which is the recruitment of blood and lymphatic vessels, to a growing tumor.[13] Evidence from in vitro and in vivo studies has shown that increased expression of VEGF is associated with tumor growth and metastasis. Moreover, the inhibition of VEGF signaling results in suppression of tumor-induced angiogenesis as well as tumor growth.[14]

Single nucleotide polymorphisms (SNPs) in the promoter, intron, exon, or untranslated regions (3′- and 5′-UTR) may affect the production or function of the corresponding protein. A number of SNPs have been designated in the *VEGF* gene, which have been described to be associated with differential *VEGF* expression.[15–18] Two of these SNPs are located in the VEGF promoter region (–2578 and –1154),[15,18] one SNP (+405) in exon 1 of the *VEGF* gene[16] and a further SNP (+936) located in exon 8, corresponding to the 3′UTR region of the gene.[17] In addition, other SNPs have also been defined, although an association with *VEGF* expression has not been revealed.[16]

Several studies investigated the *VEGF* genetic polymorphisms in BC in different ethnic groups and led to different conclusions.[19–27] To the best of our knowledge, there are no reports concerning the impact of *VEGF* polymorphisms

on BC risk in Iranian women. Hence, this study aimed to evaluate the possible association between *VEGF* rs3025039 (+936C>T), rs2010963 (+405C>G), rs833061 (−460T>C), rs699947 (−2578C>A), and rs35569394 (18-bp I/D) polymorphisms with risk/protection of BC in an Iranian population.

Patients and Methods

Patients. This population-based case–control study was done on 250 histologically confirmed BC patients admitted to Ali Ebneh Abitaleb Hospital (Iran) and 215 age-matched healthy women with no history of any types of cancer and not related to the patients.

The local ethics committee of Zahedan University of Medical Sciences approved the study, and written informed consent was obtained from all the subjects before enrollment. The research complied with the principles of the Declaration of Helsinki. Blood samples were collected from participants in tubes containing EDTA, and genomic DNA was extracted by the use of salting-out method.[28]

Genotyping. Genotyping of *VEGF* variants was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) or PCR methods. The primers used for detection of polymorphisms are listed in Table 1. PCR was done using commercially available Prime Taq premix (GeNet Bio, South Korea) according to the manufacturer's recommended protocol. In each 0.20-mL PCR reaction tube, 1 µL of genomic DNA (~100 ng/mL), 1 µL of each primer (10 µM), 10 µL of 2X Prime Taq Premix, and appropriate amount of ddH_2O were added. The PCR-cycling conditions were 5 minutes at 95°C, followed by 30 cycles of 30 seconds at 95°C, 30 seconds at 62°C for rs2010936, 63°C for rs3025039 and rs833061, 61°C for rs699947, 60°C for rs35569394, and 30 seconds at 72°C with a final extension step of 72°C for 5 min. The 10-µL of PCR product was digested by appropriate restriction enzymes (Table 1). The PCR products were electrophoresed on agarose gel containing 0.5 µg/mL ethidium bromide and visualized on a UV transilluminator.

To verify genotyping quality for each polymorphism, ~20% of random samples were regenotyped and the results confirmed the preceding genotyping outcomes.

Statistical analysis. The statistical analysis was done with statistical package SPSS version 20. Cases and controls were compared by chi-square test for categorical data or independent Student's *t*-test for continuous data. The association between each variant and BC was evaluated by computing the odds ratio (OR) and 95% confidence interval (95% CI) from unconditional logistic regression analyses. For the cases, the chi-square test was used to estimate the association between each variant and the clinicopathological characteristics. Haplotype analysis was done using SNPStats software.[29] The statistical level of significance was defined as $P < 0.05$.

Results

The study group consisted of 250 BC patients with an average age of 48.7 ± 11.0 years and 215 healthy women with a mean age of 50.4 ± 13.1 years. There was no significant difference between the groups concerning age ($P = 0.144$).

The genotype and allelic frequency of *VEGF* polymorphisms are listed in Table 2. The findings indicated that *VEGF* rs699947 variant increased the risk of BC in codominant (OR = 1.71, 95% CI = 1.15–2.54, $P = 0.009$, CA vs CC; OR = 2.12, 95% CI = 1.14–3.93, $P = 0.021$, AA vs CC) and dominant (OR = 1.78, 95% CI = 1.22–2.60, $P = 0.004$, CA+AA vs CC) inheritance models tested. The A allele increased the risk of BC (OR = 1.47, 95% CI = 1.12–1.92, $P = 0.005$) when compared with C allele. We found that the genotype and allele frequencies of *VEGF* rs3025039, rs2010963, rs833061, and rs35569394 variants were not associated with risk/protection of BC in our population.

The results of haplotype analysis are given in Table 3. The findings indicated no statistically significant associations of *VEGF* haplotypes with BC risk.

The association between clinicopathological characteristics of BC patients with *VEGF* polymorphisms is

Table 1. Primers and restriction enzymes used in PCR-RFLP analysis for detection of *VEGF* polymorphisms.

VEGF POLYMORPHISMS	PRIMER SEQUENCE (5′–>3′)	RESTRICTION ENZYME	FRAGMENT (bp)
+936C>T (rs3025039)	F: ACACCATCACCATCGACAGA	Hin1II	C allele, 443;
	R: TCTCCTCCTCTTCCCTGTCA		T allele, 311 + 132
−460T>C (rs833061)	F: TGAGTGTGTGCGTGTGGGGTTGAGCG	HinpI	T allele, 162;
	R: AGAGCCGTTCCCTCTTTGCTAG		C allele, 137 + 25
−2578C>A (rs699947)	F: GGCCTTAGGACACCATACC	BstyI	C allele, 455;
	R: CACAGCTTCTCCCCTATCC		A allele, 209 + 246
+405C/G (rs2010963)	TTGCTTGCCATTCCCCACTTGA	FaqI	C allele, 469;
	CCGAAGCGAGAACAGCCCAGAA		G allele, 272 + 197
18-bp I/D (rs35569394)	AAGATCTGGGTGGATAATCAGACT	–	I allele, 188;
	AACTCTCCACATCTTCCCTAAGTG		D allele, 170

Table 2. VEGF gene polymorphisms in BC patients and control subjects.

POLYMORPHISMS	BREAST CANCER n (%)	CONTROL n (%)	OR (95% CI)	*P*-VALUE
rs3025039 (+936C>T)				
Codominant				
CC	179 (71.6)	159 (74.0)	1.00	–
CT	67 (26.8)	52 (24.2)	1.14 (0.75–1.74)	0.593
TT	4 (1.6)	4 (1.8)	0.89 (0.22–3.61)	0.897
Dominant				
CC	179 (71.6)	159 (74.0)	1.00	–
CT+TT	71 (28.4)	56 (26.0)	1.13 (0.75–1.70)	0.603
Recessive				
CC+CT	256 (98.4)	211 (98.2)	1.00	–
TT	4 (1.6)	4 (1.8)	0.82 (0.20–3.33)	0.830
Allele				
C	425 (85.0)	370 (86.0)	1.00	–
T	75 (15.0)	60 (14.0)	1.09 (0.75–1.57)	0.709
rs2010963 (+405C>G)				
Codominant				
GG	101 (40.4)	73 (34.0)	1.00	–
GC	102 (40.8)	106 (49.3)	0.69 (0.46–1.04)	0.081
CC	47 (18.8)	36 (16.7)	0.94 (0.56–1.60)	0.893
Dominant				
GG	101 (40.4)	73 (34.0)	1.00	–
GC+CC	149 (69.6)	142 (66.0)	0.76 (0.52–1.11)	0.178
Recessive				
GG+GC	203 (81.2)	179 (83.3)	1.00	–
CC	47 (18.8)	36 (16.7)	1.51 (0.71–1.86)	0.628
Allele				
G	304 (60.8)	252 (58.6)	1.00	–
C	196 (39.2)	178 (41.4)	0.91 (0.70–1.19)	0.503
rs833061 (–460T>C)				
Codominant				
TT	69 (26.6)	66 (30.7)	1.00	–
TC	148 (59.2)	131 (60.9)	1.08 (0.72–1.63)	0.753
CC	33 (13.2)	18 (8.4)	1.75 (0.90–3.41)	0.102
Dominant				
TT	69 (26.6)	66 (30.7)	1.00	–
TC+CC	181 (73.4)	149 (69.3)	1.16 (0.78–1.74)	0.479
Recessive				
TT+TC	217 (86.8)	197 (91.6)	1.00	–
CC	33 (13.2)	18 (8.4)	1.66 (0.91–3.05)	0.103
Allele				
T	286 (57.2)	263 (61.2)	1.00	–
C	214 (42.8)	167 (38.8)	1.18 (0.91–1.53)	0.229
rs699947 (–2578C>A)				
Codominant				
CC	76 (30.4)	94 (43.7)	1.00	–
CA	138 (55.2)	100 (46.5)	1.71 (1.15–2.54)	0.009
AA	36 (14.4)	21 (9.8)	2.12 (1.14–3.93)	0.021

(*Continued*)

Table 2. (*Continued*)

POLYMORPHISMS	BREAST CANCER n (%)	CONTROL n (%)	OR (95% CI)	*P*-VALUE
Dominant				
CC	76 (30.4)	94 (43.7)	1.00	–
CA+AA	174 (69.6)	121 (56.3)	1.78 (1.22–2.60)	0.004
Recessive				
CC+CA	214 (85.6)	194 (90.2)	1.00	–
AA	36 (14.4)	21 (9.8)	1.55 (0.88–2.75)	0.156
Allele				
C	290 (58.0)	288 (76.0)	1.00	–
A	210 (42.0)	142 (33.0)	1.47 (1.12–1.92)	0.005
rs35569394 (18-bp I/D)				
Codominant				
DD	78 (31.2)	52 (24.2)	1.00	–
ID	135 (54.0)	134 (62.3)	0.67 (0.44–1.03)	0.069
II	37 (14.8)	29 (13.5)	0.85 (0.47–1.55)	0.646
Dominant				
DD	78 (31.2)	52 (24.2)	1.00	–
ID+II	172 (68.8)	163 (75.8)	0.70 (0.47–1.06)	0.098
Recessive				
DD+ID	213 (85.2)	186 (86.5)	1.00	–
II	37 (14.8)	29 (13.5)	1.11 (0.66–1.88)	0.790
Allele				
D	291 (58.2)	238 (55.3)	1.00	–
I	209 (41.8)	192 (44.7)	0.89 (–.69–1.16)	0.389

Note: OR (odds ratio) and 95% CI (confidence interval) was calculated by logistic regression analysis.

Table 3. Haplotype frequencies of *VEGF* polymorphisms in breast cancer (BC) and control subjects.

rs3025039	rs2010963	rs833061	rs699947	rs35569394	BC (FREQUENCY)	CONTROL (FREQUENCY)	OR (95% CI)	*P*
C	G	T	C	D	0.2144	0.1999	1.00	–
C	C	T	C	D	0.154	0.1855	0.63 (0.32–1.25)	0.19
C	G	C	A	I	0.1269	0.1182	0.99 (0.49–1.99)	0.97
C	C	C	A	I	0.0526	0.0722	0.58 (0.24–1.39)	0.22
T	G	C	A	I	0.0637	0.0162	1.37 (0.58–3.19)	0.47
C	G	C	C	D	0.0468	0.0543	0.89 (0.38–2.11)	0.79
C	G	C	C	I	0.0295	0.0339	0.56 (0.18–1.73)	0.31
C	G	T	A	I	0.0276	0.0448	0.57 (0.20–1.67)	0.31
C	C	T	C	I	0.0292	0.0338	0.82 (0.30–2.27)	0.70
T	G	T	C	D	0.0157	0.0542	0.26 (0.06–1.10)	0.068
C	G	C	A	D	0.0392	0.0114	0.94 (0.27–3.31)	0.93
C	G	T	C	I	0.0157	0.0419	0.29 (0.06–1.38)	0.12
C	C	T	A	I	0.0313	0.0219	2.39 (0.68–8.44)	0.18
T	C	T	C	D	0.0326	0.0155	1.14 (0.19–6.83)	0.88
C	C	C	C	I	0.0173	0.0213	0.85 (0.16–4.44)	0.84
C	C	C	C	D	0.0069	0.0227	11.18 (0.64–196.77)	0.10
C	C	C	A	D	0.0269	0.0023	0.23 (0.02–2.10)	0.19
C	C	T	A	D	0.0185	0.0055	4.26 (0.11–158.25)	0.43

Note: Haplotype analysis was performed by SNPStats software that is available online.

Table 4. Correlation between *VEGF* polymorphisms and clinicopathological characteristics of breast cancer (BC) patients.

VARIABLES	rs3025039 (+936C>T)			*P*	rs2010963 (+405C>G)			*P*	rs833064 (−460T>C)			*P*	rs699947 (−2578C>A)			*P*	rs35569394 (18-bp I/D)			*P*
	CC	CT	TT		GG	GC	CC		TT	TC	CC		CC	CA	AA		DD	ID	II	
Age (years)				0.322				0.956				0.124				**0.030**				0.213
≤50	56	22	0		33	34	15		28	40	9		32	39	9		23	46	7	
>50	98	41	4		59	56	27		34	85	24		33	87	22		41	77	26	
Histological type				0.981				0.489				0.183				0.340				0.703
Ductal carcinoma	118	44	3		61	72	32		47	91	25		52	88	25		48	88	27	
Others	49	18	1		31	25	14		16	47	6		17	44	8		19	41	9	
Tumor size (cm)				0.328				0.733				0.342				**0.025**				0.420
≤2	62	23	0		37	33	15		23	50	8		32	48	6		25	50	9	
>2	91	40	3		52	57	27		39	75	23		33	76	24		38	73	23	
TNM stage				0.469				0.670				0.487				0.059				0.718
I	29	14	0		14	20	8		13	25	4		19	20	4		15	24	4	
II	64	23	2		46	33	17		30	49	14		32	48	11		25	49	15	
III	54	13	1		23	27	13		12	43	11		11	44	11		19	35	14	
IV	24	14	1		14	17	7		10	23	4		9	22	7		12	22	4	
Grade				0.391				0.290				0.740				0.499				0.831
I	36	7	1		14	21	6		13	25	7		16	20	8		16	22	6	
II	91	39	3		57	55	22		34	78	20		41	68	20		38	73	22	
III+IV	32	12	0		15	15	12		13	24	4		11	29	5		11	25	6	
ER status				**0.016**				0.220				0.257				0.599				0.151
Positive	111	29	3		54	66	25		44	78	22		46	76	21		45	73	24	
Negative	48	30	1		35	27	18		17	51	10		21	48	12		19	48	12	
PgR status				0.666				0.801				0.190				**0.005**				0.634
Positive	102	34	3		54	58	28		34	79	24		32	81	26		41	73	25	
Negative	57	24	1		35	34	14		27	49	8		35	42	7		23	47	11	
HER2 status				0.119				0.862				0.062				**0.012**				0.103
Positive	88	33	0		48	52	20		27	80	13		25	74	19		33	75	15	
Negative	81	31	4		49	45	24		36	60	20		46	60	13		37	54	22	

Note: All the analyses were performed by chi-square test.

shown in Table 4. Patients with rs3025039 CT genotype had a higher risk of developing ER negative (ER^-) BC (OR = 2.39, 95% CI = 1.30–4.41, $P = 0.007$) than those with CC genotype.

Regarding the rs699947 variant, patients with CA genotype were more prone to develop BC at age >50 years (OR = 2.16, 95% CI = 1.17–4.00, $P = 0.0176$) than those with CC genotype. Patients with rs699947 AA genotype had a higher risk of BC developing with tumor size >2 cm (OR = 3.88, 95% CI = 1.40–10.74, $P = 0.0074$) in comparison with CC genotype. Patients with rs699947 CA genotype had a lower risk of developing progesterone receptor negative (PgR^-) BC (OR = 0.47, 95% CI = 0.26–0.87, $P = 0.020$) than those with CC genotype. In addition, patients with rs699947 CA and AA genotype significantly decreased the risk of developing HER2 negative BC (OR = 0.44, 95% CI = 0.24–0.79, $P = 0.008$ and OR = 0.37, 95% CI = 0.16–0.88, $P = 0.031$) than those with CC genotype. No significant association between *VEGF* rs2010963 (+405C>G), rs833061 (−460T>C), and rs35569394 (18-bp I/D) polymorphisms and clinicopathological characteristics of BC patients was found ($P > 0.05$).

Discussion

The present study investigated the potential impact of VEGF gene polymorphisms on the susceptibility and clinicopathological features of BC in an Iranian population in southeast of Iran. Our findings proposed that *VEGF* rs699947 variant significantly increased the risk of BC. The results did not support an association between rs3025039, rs2010963, rs833061, and rs35569394 variants of *VEGF* gene and BC risk.

We performed a stratified analysis by clinicopathological characteristics of BC patients, and our findings proposed that patients with rs3025039 CT genotype had a higher risk of developing ER^- BC. Regarding the rs699947 variant, CA genotype increased the risk of developing BC in old

individuals (age > 50 years), as well as in patients with tumor size >2 cm. However, patients with CA genotype had a lower risk of developing PgR^- and HER negative BC. No other variants were associated with clinicopathological characteristics of the patients.

The –2578 C/C genotype of the *VEGF* promoter has been associated with higher levels of VEGF in blood mononuclear cells.[18] It has been shown that –2578 C>A variant correlates with disease stages in which angiogenesis plays a critical role.[30–32]

The results of meta-analysis indicated that rs2010963 (+405C>G) and rs699947 (–2578C>A) variants of *VEGF* were not associated with BC susceptibility.[33,34] The results of a meta-analysis performed by Li and Ju[35] indicated that the functionally important +936 C/T polymorphism of *VEGF* is not associated with BC risk. The results of meta-analysis indicated that *VEGF* +936CC genotype and +936C allele increased the risk of BC and tumor growth, especially in Asians. *VEGF* –634G allele has no effect on BC susceptibility in all subjects, whereas decreases BC susceptibility in Asians and inhibits tumor growth.[36]

Kapahi et al[25] investigated the impact of *VEGF* –2578C/A, –2549I/D, –460T/C, and –7C/T polymorphisms on BC in North Indian population. They found that II genotype of –2549I/D polymorphism significantly increased the risk of BC when compared with DD genotype. *VEGF* –2578AA genotype and A allele were found to be associated with increased risk of BC. The CC genotype and C allele of *VEGF* –460T/C variant significantly increased the risk of BC. The +405G>C and –1154G>A variants of *VEGF* were not associated with BC risk.[20]

No significant association between the +936 C/T polymorphism and BC risk was found in Moroccan women.[19] However, the +936 C/T variant was associated with HER2/neu expression. In a meta-analysis performed by Chen et al[21] revealed no significant association between *VEGF* –2578C/A and the risk of cancer. Subgroup analyses showed that the *VEGF* –2578C/A polymorphism is associated with colorectal and lung cancers but not with bladder and breast cancers. Furthermore, the polymorphism may decrease the risk of cancer in the Asian population. The results of a meta-analysis showed a significant association between *VEGF* +936C/T polymorphism and the risk of BC.[26] It has been reported that *VEGF* –634G/C polymorphism is associated with increased BC risk and aggressiveness.[27]

Luo et al[22] investigated the impact of *VEGF* –634 G/C, +936 C/T, and +1612 G/A polymorphisms on breast cancer in Chinese Han patients. They proposed that individual who carries +936 TT genotypes or 936 T-allele had a protective effect concerning the disease. They found that the –634CC genotype was significantly associated with high tumor aggressiveness. The +1612G/A polymorphism was not associated with BC risk. Rodrigues et al[23] observed that women carriers of +936 CT+TT *VEGF* genotypes have a protective effect concerning BC. However, rs1109324, rs1547651, and rs833052 variants of *VEGF* were not associated with BC in Spanish population. The association between *VEGF* +936C/T, –1154A/G, –2578C/A, –634G/C, and –460T/C polymorphism and risk of BC was evaluated in a meta-analysis performed by Wang et al.[24] The overall results of combined analyses revealed that five polymorphisms of *VEGF* were not associated with the risk of BC. VEGF –2578 A allele and A carrier genotypes have been shown to be associated with an increased risk of renal cell carcinoma (RCC).[37]

As the nature of breast carcinogenesis pathways is complex, there is no clear reason for the discrepancies in different studies. Ethnic, genetic, and/or environmental factors may interact in various ways to affect the risk of BC in different areas.

In conclusion, our findings revealed that *VEGF* rs699947 (–2578C>A) polymorphism was associated with the risk and clinicopathological characteristics of BC patients.

Author Contributions

Conceived and designed the experiments: MR, MH. Analyzed the data: MH, SS. Wrote the first draft of the manuscript: MR, MAM, MT. Contributed to the writing of the manuscript: MR, MH, SS, MAM, MT. Agree with manuscript results and conclusions: MR, MH, SS, MAM, MT. Jointly developed the structure and arguments for the paper: MR, SS, MAM, MT. Made critical revisions and approved final version: MH. All authors reviewed and approved of the final manuscript.

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