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The potential impact of vascular endothelial growth factor *rs699947* polymorphisms on breast tumors susceptibility in a sample of Iraqi females

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ABSTRACT

The angiogenic factor vascular endothelial growth factor (VEGF) plays a pivotal role in tumor initiation, progression, and metastasis. Polymorphisms in VEGF gene can alter the activity of the gene's promoter, leading to increased or decreased VEGF secretion. This study aimed to investigate the association of VEGF *rs699947* polymorphisms with breast cancer risk in female from Baghdad-Iraq in a case control study which was done on 60 female patients with breast cancer and 60 female patients with benign breast tumor which compared with 75 age, BMI and sex-matched healthy subjects. Blood samples were collected from fasting patients and controls and put into EDTA tube for DNA extraction for subsequent screening of VEGF rs699947 gene polymorphisms by PCR-RFLP method. Results revealed that the incidence of malignant tumor were associated with the genotypic distribution and allelic frequency of VEGF rs699947 gene polymorphisms in that the AA genotype and A allele showed a strong correlation with the risk of malignant breast tumor in comparison with controls. The findings of this study proposed that VEGF rs699947 variant (AA) significantly increased the risk of breast cancer. It was also concluded that A allele is associated with the increased risk of breast cancer.

Keywords: breast cancer, rs699947, VEGF

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INTRODUCTION

The angiogenic factor vascular endothelial growth factor (VEGF) plays a pivotal role in tumor initiation, progression, and metastasis. In addition to aiding tumor migration and invasion, VEGF signaling helps cancer cells avoid death by apoptosis[1]. Many types of solid cancer, including breast cancer, have VEGF as an upregulated biomarker. Higher levels of VEGF were found in malignant breast lesions than in benign breast tissues. Multiple studies have found that micro vessel density and poor prognosis are strongly correlated with VEGF expression in tumor tissue[2].

The VEGF gene, which encodes a family of proteins, is found on the short arm of chromosome 6 (6p12-p21) and is made up of eight exons and seven introns that undergo alternative splicing. During embryogenesis, skeletal growth, and reproductive processes, VEGF (also known as VEGFA) plays a crucial role as a regulator of physiological angiogenesis. It also plays a role in tumor-related pathological angiogenesis. Cancer cannot develop without angiogenesis, which is required for primary tumor growth, invasiveness, and metastasis. 8 Multiple tumor tissues had an increased level of VEGF expression[3]. Lymphangiogenesis, the process by which new blood and lymphatic vessels are formed to supply a growing tumor, is a hallmark of several types of cancer, including the most common type of female cancer, breast cancer. Increased expression of VEGF is linked to tumor growth and metastasis, as shown by *in vitro* and *in vivo* studies. Tumor-induced angiogenesis and tumor growth are both restrained by blocking VEGF signaling[4,5].

There is evidence that SNPs in the promoter, intron, exon, or untranslated regions (3′- and 5′-UTR) can have an effect on protein expression and function. Differential VEGF expression has been linked to several single-nucleotide polymorphisms (SNPs) in the VEGF gene. Two of these SNPs (2578 and 1154) are found in the VEGF promoter, one is in exon 1 of the VEGF gene16, and the other is in exon 8, which corresponds to the 3′UTR region of the gene[6]. Furthermore, other SNPs have been defined, but no correlation between them and VEGF expression has been found. VEGF genetic polymorphisms in BC have been the subject of a number of studies, with conflicting results[7].

This study aimed to investigate the association of VEGF *rs699947* polymorphisms with breast cancer risk in female population in Baghdad.

METHODOLOGY

Study protocol

Sixty female patients with breast cancer and sixty female patients with benign breast tumors were enrolled in a case control study between May 2022 and December 2022 at Al-Imamain Al-Kadhemain Medical City in Baghdad, Iraq. The age range of the malignant group was 29–48 years old, while that of the benign group was 29–46 years old; both groups were compared to a group of 75 age-, BMI-, and sex-matched healthy females. The research was carried out in Al-Nahrain University's Department of Chemistry and Biochemistry in the College of Medicine in Baghdad, Iraq. Women who had a suspicious breast lesion confirmed by histopathology following a clinical breast examination and/or imaging study were considered for inclusion in the current study.

Exclusion criteria

1. Subjects that had a history of any acute or chronic diseases (eg. Acute and chronic hepatitis).

2. Subjects that had a history of any type of cancer (eg. Colorectal and endometrium cancer).

3. Patients received hormonal treatment or chemotherapy.

4. Pregnant women.

5. Age above 65 years' old

The study was approved by the Institutional Review Board (IRB) of the College of Medicine, University of Al-Nahrain, Baghdad, Iraq. In addition, an informed written consent for participation in the study was signed by investigated subjects according to the Helsinki principles.

All eligible control subjects and studied patients were subjected to the following:

- Thorough clinical examinations in addition to full medical history
- Genetic screening for VEGF rs699947 gene polymorphisms in malignant breast tumor, benign breast tumor and control groups using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

Sample collection and preparation

Blood samples were collected from fasting for 6 hours patients and controls. These samples were put into EDTA tube for DNA extraction for subsequent screening of VEGF rs699947 gene polymorphisms.

DNA extraction[8]

Blood Genomic DNA Extraction Kit (Geneaid, Taiwan) is designed for rapid extraction and purification of pure genomic DNA from whole blood using the glass fiber matrix of the spin columns.

Genotyping

Single nucleotide polymorphisms (SNPs) of VEGF/rs699947 were detected by PCR-RFLP method. Amplification was performed in programmable thermal cycler gradient PCR system The primers used for detection of polymorphisms are listed in Table 1. PCR primers were obtained from previous studies with melting temperature of 68°C, primer length is 19 nucleotides, and PCR amplicon length 455 base pair[7].

Table 1. The primers used in the study

Primer Name	Seq.	Annealing Temp. (°C)	Product size	SNPs
VEGF-F	5`-GGCCTTAGGACACCATACC -3`	61	455 bp	rs699947
VEGF-R	5`-CACAGCTTCTCCCCTATCC -3`			

PCR was done using GoTaq® Green Master Mix from Promega, containing Taq DNA polymerase (5 U/µl), dNTP (400µM), MgCl2 (3mM) and reaction buffer (2X,PH=8.5) at optimal concentration for efficient amplification of DNA templates by PCR. The PCR reaction mixtures were brought together according to the manufacturer procedure of the master mix (Promega). When necessary, the appending was done on frozen cooling blocks and ice inside a laminar flow cabinet. Twenty microliter volumes were used for the PCR amplifications, with ten microliters of GoTaq Green Master Mix (2X), one microliter for each primer (10 pmol), six microliters of nuclease-free water, and two microliters of template DNA. The following temperature program was used for PCR cycling on a PCR Express (Thermal Cycler, BioRad, USA): denatured at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 61°C for 30 seconds, and extension at 72°C for 30 seconds. The reactions were halted after a 10-minute incubation at 10 degrees Celsius and a 5-minute extension incubation at 72 °C.

The PCR product for amplified gene was digested with the restriction enzyme BstYI. The PCR products were electrophoresed on agarose gel containing 0.5 μg/mL ethidium bromide and visualized on a UV transilluminator.

After the complete digestion with the restriction enzyme BstYI at 37°C for 30 min, the resulting DNA fragments were analyzed by electrophoresis on a 2% agarose gels. DNA step ladders (100 b.p) were used to determine the length of the DNA fragments. The result of the digestion showed that the DNA fragment with a homozygote C allele of VEGF / rs699947 was not digested with the restriction enzyme and appear as a single band of 455bp whereas samples with a homozygote A allele should produce two fragments of 209 and 246 bp but actually only one fragment appeared at a region of about 240bp as it digested by the enzyme while a heterozygote A and C allele samples showed two bands of 455 and 240 bp

Statistical analysis

The data of the study were analyzed using the SPSS software 20. Categorical variables were expressed as numbers and analyzed by cross tabulation to assess the frequency and percentage of each variable among studied groups. The correlation was done between all parameters using Chi square to test the relationships between categorical variables with the measurement of Phi that is considered as a chi-square-based measure of association to indicate the strength of the association (given that values ranged from 0-0.5 considered as weak association while values above 0.5 considered as strong association) in addition to *p* values which is considered as significant at ≤0.05. Logistic regression was used to calculate odd ratio (ORs) and 95% confidence interval (CI) for breast cancer risk associated with the genetic polymorphisms of VEGF gene[9].

RESULTS and DISCUSSION

Results postulated in Table 2 and Figure 1 showed that the genotypic distribution of the rs699947 polymorphism showed a nearly similar distribution between controls and benign patients in that CC, CA and AA genotypes represent 46.7, 34.7, 18.7%; respectively of controls and 45, 28.3, 26.7%; respectively in benign group. Patients with malignant tumor showed a different pattern of genotypic distribution in comparison with control and benign groups in which the CC, CA and AA genotypes represent 20, 45, 35%. These apparent differences in the genotypic distribution confirmed by Chi^2 results which showed non-significant differences in the genotypic distribution between controls and benign groups (p=0.479, Phi=0.102) while significant differences were obtained between controls and cancerous patients (p=0.004; Phi=0.287), benign and malignant groups (p=0.013; Phi=0.270), and among all studied groups (p=0.011; Phi=0.26).

Table 2. Genotypic distribution of rs699947 polymorphism among all studied groups

			rs699947 polymorphism			Total
			CC	CA	AA	
GROUP	CONTROL	Count	35	26	14	75
		% within GROUP	46.7%	34.7%	18.7%	100.0%
	BENIGN	Count	27	17	16	60
		% within GROUP	45.0%	28.3%	26.7%	100.0%
	CANCER	Count	12	27	21	60
		% within GROUP	20.0%	45.0%	35.0%	100.0%
Chi^2	Control vs benign	p-value	0.479			
		Phi	0.102			
	Control vs cancer	p-value	0.004			
		Phi	0.287			
	Benign vs cancer	p-value	0.013			
		Phi	0.27			
	Among all groups	p-value	0.011			
		Phi	0.26			

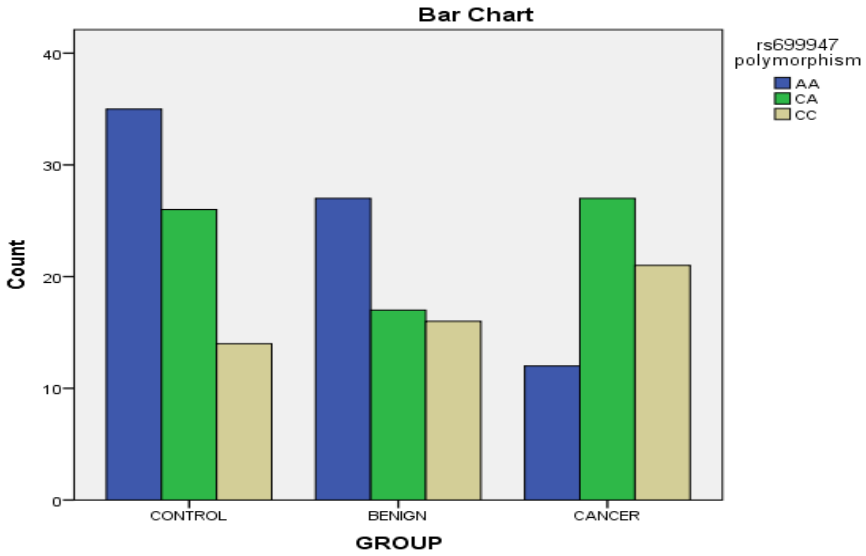

Figure 1. Distribution of VEGF rs699947 genotypes in all studied groups

Results illustrated in Table 3 revealed that the incidence of malignant tumor were associated with the allelic frequency in that the A allele showed a strong correlation with the malignant tumor in comparison with controls as indicated by the Odds ratio results which showed a significant association between A allele frequency and the prevalence of cancer (p= 0.005) and the results also showed that the risk of malignancy showed to be higher by about 2.41 times in patients with A allele than those with C allele. Additionally, A allele also showed to be associated with the incidence of malignant tumor in comparison with benign tumor as it represented by the Odds ratio results which revealed a significant association between the allelic frequency and the incidence of malignancy (p= 0.01) and the results also demonstrated that patients with A allele have a higher risk to get malignant tumor by about twice with that have C allele in benign. On the other hand, allelic frequency showed non-significant effect on the incidence of benign tumor in comparison with controls (p= 0.42, OR=1.123 and 95% CI=0.75-2.01).

Table 3. Allelic distribution of rs699947 polymorphism among all studied groups

			rs699947 polymorphism		Total
			C Allele	A Allele	
GROUP	CONTROL	Count	96	54	150
		% within GROUP	64%	36%	100.0%
	BENIGN	Count	71	49	120
		% within GROUP	59.2%	40.8%	100.0%
	CANCER	Count	51	69	120
		% within GROUP	42.5%	57.5%	100.0%
Odds ratio	Control vs benign	P-value	0.42		
		OR (95% CI)	1.23 (0.75-2.01)		
	Control vs cancer	P-value	0.005		
		OR (95% CI)	2.41 (1.47-3.93)		
	Benign vs cancer	P-value	0.01		
		OR (95% CI)	1.96 (1.17-3.28)		

Several studies reported that the polymorphism of VEGF rs699947 C/A gene showed to be correlated with an overall increased risk of breast cancer as it links to the rate of the expression of VEGF that influenced by the polymorphism of this SNP that located in the promoter region of the VEGF gene[7,10] studies also stated that this SNP responsible for differentiated VEGF gene expression as it showed a reduction in the serum concentration of VEGF in patients homozy-

gous for −2578 A alleles. In the present study, the prevalence of rs699947 in CC, CA and AA genotypes represent 20, 45, 35%; respectively in patients with malignant tumor which showed to be identified as significantly differ from that in the healthy individuals (46.7, 34.7 and 18.7%; respectively) as indicated by the significant Chi^2 results (p=0.004; Phi=0.287). The genotypic distribution of cancerous patients also showed to be significantly (p=0.013; Phi=0.270) differ from that of benign tumor which in turn showed a pattern of distribution similar to that of controls as illustrated in Table 2. Results demonstrated in the current study were consistent with the study conducted by al Balawi et al. who demonstrated that the prevalence of CC, CA, AA in the patient samples were 37%, 45% and 18% that differ significantly from that of healthy controls which provide a prevalence of 54%,37%, and 09% respectively[10] and also showed an agreement with the study conducted by Rezaei et al. who demonstrated that the prevalence of CC, CA, AA in the samples of cancerous were 30.4%, 55.2% and 14.4% which is showed to be significantly differ from the prevalence in the controls in which CC, CA, AA genotypes represented 43.7%, 46.5% and 9.8%; respectively[7].

The A allele of rs699947 was shown to be more frequent in malignant group than that of healthy subjects (57.5 vs 36 respectively) and the Odds ratio results revealed that the risk of breast cancer in patients with A allele were 2.4 time more than the patients with C allele which is comparable to the results conducted in Saudi Arabia which demonstrated that A allele increased the risk of breast cancer when compared with C allele by about 1.8 times[10]. Multiple mechanisms implicate VEGF as a critical factor in angiogenesis, including increased endothelial cell proliferation and survival, increased endothelial cell migration and invasion, increased permeability of existing vessels forming a lattice network for endothelial cell migration, and increased chemotaxis and homing of bone marrow-derived vascular precursor cells[11,12]. Independent of vascular processes, VEGF has several critical roles, including proangiogenic effects, autocrine effects on tumor cell function (survival, migration, invasion), immune suppression, and homing of bone marrow progenitors to “prepare” an organ for subsequent metastasis[13,14]. Several functional polymorphisms of the VEGF gene, including the VEGF 2578C/A polymorphism, have been identified in some studies and may influence serum VEGF expression level. Several prior studies reported that functional genetic polymorphisms could alter mRNA or protein expression, thus generating significant influence on disease development across a wide spectrum of diseases, including cancer[10,15]. Numerous studies have been conducted recently on the links between VEGF gene variations and the risk of breast cancer, but the reported results have been contradictory. Our results suggested that a variant of VEGF called rs699947 (AA)

significantly raised the risk of breast cancer which is consistent with previous literatures[7,10,16].

The findings of the current study proposed that VEGF rs699947 variant (AA) significantly increased the risk of breast cancer. It was also concluded that A allele is associated with the increased risk of breast cancer.

STATEMENT OF ETHICS

The study was conducted in compliance with the ethical guidelines derived from the Declaration of Helsinki. The procedure was conducted after obtaining verbal and analytical consent from the patients before to collecting the sample. The Institutional Review Board (IRB) of the College of Medicine, University of Al-Nahrain, Baghdad, Iraq, examined and approved the study protocol, subject information, and permission form. This approval was granted by a local ethics commission, as indicated by document number 154 dated 07/12/2021.

CONFLICT OF INTEREST STATEMENT

The authors declared no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept – E.A.; Design – H.A., E.A.; Supervision – E.A., N.F., A.N.; Resources – N.F., H.A.; Materials – H.A.; Data Collection and/or Processing – H.A.; Analysis and/or Interpretation – H.A.; Literature Search – H.A.; Writing – H.A.; Critical Reviews – H.A., E.A., N.K., A.N.

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