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Association of Single-Nucleotide Polymorphism (rs833061) of Vascular Endothelial Growth Factor with Breast Cancer Risk

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ABSTRACT

Background: Breast cancer is the most common cancer among women and one of the most important causes of death among them. Angiogenesis is an important step for primary tumor growth, invasiveness, and metastases. Vascular endothelial growth factor (VEGF) is involved in angiogenesis associated with tumors.

Objective: To investigate the association of single nucleotide polymorphisms (SNPs) of VEGF gene located in the promoter region at position 460T/C (rs833061) with the risk of breast cancer.

Patients and Methods: This study was conducted on (40) Egyptian female patients who were diagnosed as breast cancer according to histopathological examination of breast biopsy (Group 1, Patients), in addition to (40) age-matched apparently healthy females serving as controls (Group II, Controls). Detection of SNP of VEGF gene located in the promoter region 460T/C (rs833061) was done by real-time polymerase chain reaction technique.

Results: There was no significant difference in frequency of the (C/C, T/C and T/T) genotypes of VEGF 460T/C (rs833061) in group I compared with group II ($p>0.05$). None of the studied VEGF genotypes had shown significant difference in distribution related to clinico-pathological parameters as tumor size, histological grade, hormone receptors, HER2/new status, regional lymph node and distant metastasis ($p>0.05$).

Conclusion: This study revealed that there was no significant association of the (C/C, T/C and T/T) genotypes of VEGF 460T/C (rs833061) and breast cancer risk. Moreover, VEGF polymorphism 460T/C (rs833061) was not associated with breast cancer patients' clinico-pathological characteristics and tumor markers levels.

Keywords: Breast cancer, SNP, VEGF polymorphism 460T/C

INTRODUCTION

Breast cancer is the most common cancer in women and accounts for 29% of all cancers diagnosed worldwide [(1)]. It is the fifth leading cause of cancer death in women. The incidence of breast cancer has increased steadily over the past few decades, but breast cancer mortality appears to be declining [(2)]. This suggests a benefit from early detection and more effective treatment. In Egypt, breast cancer is the most common type of cancer in females as it accounts for approximately 32 % of total reported malignancies[(3)].

In general, carcinoma of the breast is a heterogeneous disease with a variety of pathological entities, clinical behaviour and molecular alterations involved in tumor growth, survival of individuals and response to treatment [(2)].

Angiogenesis is an important step in the development of cancer and is necessary for primary tumor growth, invasiveness, and metastases. Breast cancer is among the well-known malignancies involving lymphangiogenesis, which is the recruitment of blood and lymphatic vessels, to a growing tumor [(4)].

Vascular endothelial growth factor (VEGF), also known as VEGFA, specifically stimulates endothelial cell proliferation, promotes endothelial cell migration, and participates in the formation of new blood vessels. VEGF promotes mitotic activity mainly through its interactions with 2 receptors: VEGF receptor-1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2). In particular, VEGFR-1 plays a role in vascular permeability, while VEGFR-2 plays a role in angiogenesis [(5)].

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. This gene is highly polymorphic with multiple polymorphisms in the promoter 5′ untranslated region (5′-UTR) and 3′ UTR [(6)]. The single-nucleotide polymorphisms (SNPs) in the promoter and 5′ UTR have been reported to regulate VEGF expression via alternative initiation of transcription and internal initiation of translation [(7)].

Several SNPs in the promoter and 5' UTR of VEGFA have been reported to be associated with variation in VEGF protein production. The common sites of SNPs are at position 2578, 46o, 1154, and 634. The detection of VEGF SNPs and the validation of their associated risk in different studies are crucial to understand the effect of these polymorphisms in breast cancer susceptibility [(7)], with subsequent developing of anti-angiogenetic drugs targeting the VEGF pathway that could be used in tumour therapy [(8)].

A number of molecular epidemiological studies have been conducted to examine the association between VEGF 460T/C polymorphism (rs833061) and cancer susceptibility as lung, ovarian, cervical and endometrial, colon, prostate, pancreatic, renal cell carcinoma and breast cancer with disparate results [(9)].

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The objective of the current case–control study was to investigate the association of functional single-nucleotide polymorphisms (SNPs) of vascular endothelial growth factor (VEGF) gene 460T/C (rs833061) with the risk of breast cancer.

SUBJECT AND METHODS

This study was conducted on 40 Egyptian female patients (Group I, mean age: 53 ± 8 years) who were referred to the Oncology and Radiology Departments and Outpatient Clinics of Ain Shams University Hospitals. They were diagnosed as breast cancer patients according to histopathological examination of breast tissue biopsy. This study was also conducted on 40 age-matched apparently healthy, mammogram-free, females served as a control group (Group II**,** mean age of 52 ± 10 years).

Exclusion Criteria: Patients suffering from any malignancy other than breast cancer were excluded from the study.

Ethical Approval:

The research followed the tents of the Declaration of Helsinki. The Ethics Committee of Ain Shams University approved this study. The Institutional Ethical Committee at Ain Shams University approved all study protocols. Accordingly, written informed consent was taken from all participants before any intervention.

All individuals enrolled in this study were subjected to full history including detailed family history, reproductive history and predisposing factors to breast cancer as well as thorough clinical examination with special emphasis on breast examination and mammogram. For patients only, further radiological investigations as CT scan or MRI, in addition to breast tissue biopsy were done. Laboratory investigations included assessment of estrogens, progesterone and HER2/neu (Human epidermal growth factor receptor2/neu) receptor status by immunohistochemistry. Assay of serum CA15.3 and CEA was done on Cobas e411 autoanalyzer (Roche Diagnostics, Indianapolis, USA.) by electrochemiluminescence immunoassay technique. In addition, detection of SNP of VEGF gene located in the promoter region 460T/C (rs833061) by real-time polymerase chain reaction technique was done for all individuals.

Detection of SNP 460T/C (rs833061) of VEGF gene by real-time polymerase chain reaction was carried out through two steps including DNA extraction then amplification and detection. First, DNA extraction was performed on the EDTA-K3 plasma samples. The plasma was aliquoted and stored at -70^{0}C till assay which was done by PureLink® Genomic DNA Mini Kit Thermo Fisher Scientific (Life Technology, Carlsbad, USA), according to manufacturer instructions.

DNA in the sample was extracted using proteinase K solution and lysis solution. Any residual RNA was removed by digestion with RNase prior to binding samples to the silica membrane. The lysate was mixed with ethanol and PureLink® Genomic Binding. Buffer allowed high DNA binding to the silica-based membrane in the column and impurities were removed by thorough washing with wash buffers. The genomic DNA was then eluted in low salt elution buffer. Finally, measuring of DNA concentration and purity using Nano drop spectrophotometer (NanoDrop products Wilmington, UeSA) was performed and the eluted purified DNA was stored at -80°C until used.

The extracted DNA was amplified using TaqMan Universal Master Mix II and ready-made TaqMan SNP genotyping assay supplied by Applied Biosystems (Waltham, MA, USA) that consists of sequence specific forward and reverse primers to amplify the polymorphic sequence of interest (rs833061) :

Forward: 5'TTTCTCGTAATTTTCCCGTGA-3'

Reverse: 5'AAACGAAAGACAGCGATAAAAA3'

According to the manufacturer instructions, each TaqMan SNP Genotyping Assay includes two allele-specific TaqMan Minor Groove Binder (MGB) probes and a PCR primer pair to detect specific SNP targets.

The wild-type TaqMan MGB probes were FAM labelled that detects the T Allele sequence, and the mutant probes were VIC labelled that detects the T Allele sequence. PCR amplification reactions were performed in 20 µL containing 2 µL of genomic DNA, 10 µL of TaqMan Universal PCR master mix (Applied Biosystems, **USA**), 1 µL of Assay Mix and 7 µL DNAse free water. Thermal cycling and detection were performed on the StepOnePlus™ Real-Time PCR System (Applied Biosystems). The PCR amplification cycles were 60 ºC for 30 s and 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. Allelic discrimination was automatically determined by StepOne™ software (Applied Biosystems).

Statistical Methodology:

The data were coded, entered and analyzed using Statistical Package for the Social Sciences (SPSS) software computer program version (V. 22.0, IBM Corp., USA, 2013). Data were statistically described in terms of mean ± SD (standard deviation) for quantitative parametric data, median and interquartile range for quantitative nonparametric data and number and percentage for qualitative data. For comparing categorical data, Chi square test or Fisher's exact test was performed. A probability value (*P* value) more than 0.05 was considered non-statistically significant.

RESULTS

Characteristics of breast cancer patients as regards the clinical, histopathological data and tumor marker levels are shown in table 1. In our study, the intra ductal carcinoma was the most common histological type in the studied breast cancer patients. In addition, T2 tumor size and histological grade of Scarff-Bloom-Richardson grading system 2 (SBR2) were the most frequently found, followed by T3 and SBR3. Moreover, 75% of cases had a regional lymph node metastasis. Regarding hormone receptor status, the majority of the studied breast cancer patients were positive for estrogen receptor (90%) whereas only 50 % of them were progesterone receptor positive.

Table (1): Characteristics of breast cancer patients' group regarding the clinical, histopathological data and tumor markers levels

Parameters		**Patients' Group (n=40)**
Tumor size: No. (%)	T1	6 (15.0%)
	T2	22 (55.0%)
	T3	12 (30.0%)
LNs metastasis: No. (%)	Negative	10 (25.0%)
	Positive	30 (75.0%)
Distant metastasis: No. (%)	Negative	36 (90.0%)
	Positive	4 (10.0%)
Histological type: No. (%)	IDC	36 (90.0%)
	Lobular invasive	4 (10.0%)
Histological grade: No. (%)	SBR1	4 (10.0%)
	SBR2	24 (60.0%)
	SBR3	12 (30.0%)
ER: No. (%)	Negative	4 (10.0%)
	Positive	36 (90.0%)
PR: No. (%)	Negative	20 (50.0%)
	Positive	20 (50.0%)
HER2/neu: No. (%)	Negative	22 (55.0%)
	Positive	18 (45.0%)
CEA (ng\mL) **Median (IQR)**		1.85 (1.1 – 3.45)
CA15.3 (U\mL) **Median (IQR)**		18.55 (15.3 – 25)

T1 < 20 mm, T2 < 50 mm, T3 < 50 mm, IDC: intra ductal carcinoma, SBR: Scarff-Bloom-Richardson grading
ER: Estrogen receptor, PR: Progesterone receptor, HER2: Human epidermal growth factor receptor 2.

There was no significant difference in frequency of the (C/C, T/C and T/T genotypes) in the breast cancer patients' group compared with control group [Table 2]. None of the studied VEGF genotypes had shown significant difference in distribution related to clinico-pathological parameters as (tumor size, histological grade, hormone receptors and HER2/neu status, regional lymph node and distant metastasis [Table 3], as well as tumor marker levels**.**

Table (2): Frequency association of (VEGF) gene polymorphism in the breast cancer patients and control groups

Parameters		**Patients' Group (n=40)**		**Control group (n=40)**		**P-value**
		No.	**%**	**No.**	**%**	
Genotyping	CC	12	30.0%	14	35.0%	> 0.05
	TC	16	40.0%	14	35.0%	
	TT	12	30.0%	12	30.0%	

CC: Homozygous mutant type, TC: Heterozygous mutant type, TT: Wild type

Table (3): Distribution of (VEGF) genotypes in the breast cancer patients in relation to clinical and histopathological characteristics

Parameters		**CC** (No. = 12)	**CT** (No. = 16)	**TT** (No. = 12)	**P-value**
Tumor size	T1 (n=6)	0 (0.0%)	4 (25.0%)	2 (16.7%)	> 0.05
	T2 (n=22)	6 (50.0%)	8 (50.0%)	8 (66.7%)	
	T3 (n=12)	6 (50.0%)	4 (25.0%)	2 (16.7%)	
LNs metastasis	Negative (n=10)	0 (0.0%)	6 (37.5%)	4 (33.3%)	> 0.05
	Positive (n=30)	12 (100.0%)	10 (62.5%)	8 (66.7%)	
Distant metastasis	Negative (n=36)	10 (83.3%)	16 (100.0%)	10 (83.3%)	> 0.05
	Positive (n=4)	2 (16.7%)	0 (0.0%)	2 (16.7%)	
Histological type	IDC (n=36)	10 (83.3%)	16 (100.0%)	10 (83.3%)	> 0.05
	Lobular invasive (n=4)	2 (16.7%)	0 (0.0%)	2 (16.7%)	
Histological grade	SBR1 (n=4)	0 (0.0%)	6 (37.5%)	2 (16.7%)	> 0.05
	SBR2 (n=24)	12 (100%)	4 (25.0%)	6 (50.0%)	
	SBR3 (n=12)	0 (0.0%)	6 (37.5.0%)	4 (33.3%)	
ER	Negative (n=4)	0 (0.0%)	4 (25.0%)	0 (0.0%)	> 0.05
	Positive (n=36)	12 (100%)	12 (75.0%)	12 (100.0%)	
PR	Negative (n=20)	6 (50.0%)	8 (50.0%)	6 (50.0%)	> 0.05
	Positive (n=20)	6 (50.0%)	8 (50.0%)	6 (50.0%)	
HER2/neu	Negative (n=22)	6 (50.0%)	10 (62.5%)	6 (50.0%)	> 0.05
	Positive (n=18)	6 (50.0%)	6 (37.5%)	6 (50.0%)	

T1 < 20 mm, T2 < 50 mm, T3 < 50 mm, IDC: intra ductal carcinoma, SBR: Scarff-Bloom-Richardson.
ER: Estrogen receptor, PR: Progesterone receptor, HER2: Human epidermal growth factor receptor 2: Results of Immunohistochemistry test (0 or 1 +: negative, 3 +: positive)

DISCUSSION

Breast cancer is the most common cancer in women and it is the leading cause of cancer related mortality, representing 15% of deaths per year worldwide [10, 11]. Excessive abnormal angiogenesis plays a pivotal role in tumor progression and is a hallmark of solid tumors as breast cancer. This process is driven by an imbalance between pro- and anti-angiogenic factors dominated by the tissue hypoxia-triggered overproduction of vascular endothelial growth factor (VEGF) [12].

Vascular endothelial growth factor (VEGF) is a potent angiogenic cytokine. It is also a survival factor for endothelial cells during physiological angiogenesis and tumour angiogenesis with vasodilatation, vascular permeability and antiapoptosis functions [7]. VEGF-superfamily members can be expressed and secreted by several cell types including tumor cells, tumor-infiltrating inflammatory cells and cancer-associated fibroblasts [13].

In the current study, no significant difference was detected in frequency of the (C/C, T/C and T/T genotypes) of VEGF polymorphism 460T/C in the breast cancer patients' group compared with control group. Our results agreed with those of **Wang *et al.*** [14], who performed a meta-analysis of 10 case-controlled studies which included 8175 cases and 8528 controls. The overall results did not show any association of VEGF polymorphism 460T/C (rs833061) with breast cancer susceptibility especially for Caucasian. In addition, **Sa-Nguanraksa and O-Charoenrat** [15] found that determination of VEGF levels in breast cancer tissue, or serum did not prove to show any association with 460T/C polymorphisms.

Moreover, studies on Chinese and white Caucasian performed by **Rezaei *et al.*** [9], which included 250 cases and 215 controls did not detect any association of VEGF polymorphism 460T/C with breast cancer.

However, **Kapahi *et al.*** [5] investigated the impact of VEGF 460T/C polymorphism on breast cancer in North Indian population which included 204 cases and 2o4 controls. They found that the CC genotype variant [odd ratio (OR)=2.23] significantly increased the risk of breast cancer. On the contrary to **Kapahi *et al.*** [5] findings, study the relationship between genetic variation of VEGF polymorphism 460T/C and breast cancer risk among participants of Shanghai Breast Cancer Genetics Study (SBCGS) which included 1,093 cases and 1,184 controls revealed increased risk of breast cancer with TT genotype (wild type) (OR = 1.26, 95% CI: 1.05 – 1.52, p = 0.016) [16]. Moreover, **Rahoui *et al.*** [4] case–control study on Moroccan women which included 7o cases and 7o controls demonstrated that carriers of 46o C alleles (mutant type) seem to have a protective effect against breast cancer.

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 breast cancer in different population [9].

Interestingly, the increased risk or protective role of VEGF polymorphism 460T/C in breast cancer in other studies might be explained as a false positive association due to linkage disequilibrium**.** The non-random association of alleles of linkage disequilibrium at different loci in a given population may have a role in increasing or decreasing the frequency of association of VEGF 460T/C polymorphism than what would be expected if the mutation was independent and associated randomly between 460T/C and other positions. Therefore, this polymorphism may be in linkage disequilibrium with another unknown polymorphism elsewhere leading to a change of mRNA structure.

As regards the association of VEGF 460T/C polymorphisms in relation to clinico-pathological characteristics which affected breast cancer aggressiveness as (tumor size, lymph nodes metastasis, distant metastasis, histological type, histological grade, estrogen receptor, progesterone receptor and HER2neu) our study showed no association.

Our results agreed with those of case–control study performed by **Rahoui *et al.*** [4] in Moroccan population in which no association between VEGF SNP 460T/C and breast cancer aggressiveness was found. They reported that these different results of the VEGF polymorphism 460T/C might influence the delivery of chemotherapy to the cancer cells and might consequently hold predictive information in relation to response.

As regard other cancers, previous studies on the association of VEGF 460T/C polymorphism with cancer susceptibility as lung [17], ovarian, cervical and endometrial [18], Colon [19], prostate, pancreatic [20], and renal cell carcinoma [21], produced contradicting results; some were showing an association with 46oC/T SNPs in the VEGF gene and others were showing no association.

VEGF-A itself does not represent a single mature molecular species; it is a group of molecules appearing in several alternatively spliced isoforms. Among the VEGF-A isoforms with relevance to both physiological and pathological angiogenesis, splice form VEGF-A165 appears to be the most critical factor in pathological neovascularization. VEGF-A isoforms number reaches 16 isoforms that differ in their length, which reflects the length of the particular molecule. VEGF-A isoforms are obtained by alternative splicing mechanism and they differ significantly in their heparin-binding affinity, and in turn has a crucial influence over their bioavailability and interactions with defined co-receptors [22]. Thus, post-translational proteolytic processing, in addition to the molecular diversity generated by alternative RNA splicing, might have a further impact on receptor-binding affinity and interactions and this in turn modifies the spreading and biological availability of certain members of VEGF family that might explain the discrepancy of results regarding VEGF gene polymorphism [23].

Moreover, although VEGF-A (especially VEGF-A165)-triggered VEGFR-2 signalling is clearly central to the pathogenesis of neovascularization, it is not the sole pathway involved in tumor neoangiogenesis as several lines of evidence suggest that fibroblast growth factor

and angiopoietin has been linked to pathological angiogenesis in different organs [(12)].

CONCLUSION

This study revealed that there was no significant association of the (C/C, T/C and T/T) genotypes of VEGF 460T/C (rs833061) and breast cancer risk. Moreover, VEGF polymorphism 460T/C (rs833061) was not associated with breast cancer patients' clinico-pathological characteristics and tumor markers levels. Our results agree well and go in accordance with previous results in various ethnic and geographical distribution studies. Limitations of our study are the limited sample size and lack of study of different VEGV isoforms. Further largescale studies are needed to clarify the role of VEGF isoforms in different SNP and their association with breast cancer susceptibility.

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