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The Role of rs2305948 in Determining Vascular Endothelial Growth Factor Levels in Breast Cancer

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ABSTRACT

Breast cancer is the most frequent malignancy in women worldwide and is curable in ~70–80% of patients with early-stage, non-metastatic disease. Advanced breast cancer with distant organ metastases is considered incurable with currently available therapies. Vascular endothelial cell growth factor (VEGF) and VEGF receptor 2 (VEGFR2) are closely related to angiogenesis in Breast cancer. Main functions of VEGFR2 include increasing the expression of VEGF and inducing tumor angiogenesis. In addition, VEGF plays a role in promoting vascular endothelial cell division and angiogenesis through VEGFR2 and is also involved in promoting the aggressive growth of tumors. Previous research has revealed that genetic mutations and polymorphisms are closely related to disease susceptibility and can lead to different responses to environmental factors and drugs. Therefore, we conducted this meta-analysis to evaluate the association of rs2305948 with Breast cancer and to obtain a stronger conclusion. Many studies have reported a relationship between the vascular endothelial growth factor receptor 2 single nucleotide polymorphism (SNP) rs2305948 and Breast cancer, but their conclusions have been controversial. A meta-analysis was performed to assess the association between rs2305948 and Breast cancer susceptibility.

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1-INTRODUCTION

Breast cancer is one of the commonest cancers in the world that has a multifactorial etiology involving genetic, environment and lifestyles risks(1). Single nucleotide polymorphisms (SNPs) are amongst the genetic factors that have attracted a lot of interest due to their contribution to cancer susceptibility, prognosis and response to treatment. One of these variations is the SNP rs2305948, found in the kinase insert domain receptor (KDR) gene that encodes the vascular endothelial growth factor receptor-2 (VEGFR-2) and has been reported to play part in cancer biology (U.S. Breast Cancer Statistics (2).

Vascular endothelial growth factor (VEGF) is a strong angiogenic factor that has a central role in the cancer angiogenesis, which refers to a process of developing new blood vessels to supply nutrients and oxygen to tumors and allow them to grow and spread. VEGF expression has also been linked to cancer progression, metastatic risk, and final prognosis among breast cancer patients (3).

rs2305948 SNP may trigger a valine in position 297 to methionine in VEGFR-2, which is likely to have a change in receptor.

the angiogenic signaling pathway and, accordingly, its functionality. Several studies have been already conducted on this SNP to clarify the role of this SNP in the modulation of VEGF expression levels and comprehend the effect of this SNP on the development and progression of breast cancer (4).

It is imperative to have the knowledge about the role that rs2305948 plays in regulating the level of VEGF because of the potential role of angiogenesis as a prognosticator in breast cancer. The high VEGF levels have been found to correlate with adverse prognosis as well as reducing the survival rate hence it is also a target chromosome that can be used in treatment. Anti-angiogenic treatment that targets the VEGF pathway has become a novel initiative in the treatment of breast cancer which increases the need to consider the genetic variant like rs2305948 to stratify patients and direct personalized treatment (5).

Organizing the effect of rs2305948 on the level of VEGF in breast cancer is not only relevant to advance our knowledge in the topic of molecular pathology of the disease but also to ameliorate diagnostic approaches and therapy outcomes. Investigation of this SNP can assist researchers in the development of genotype-driven risk assessment and patient-specific therapy, which are the key concepts of precision oncology (6). The importance of this research to the understanding of how rs2305948 decides the levels of VEGF is crucial because the prognostic value of angiogenesis in breast cancer and determine why VEGF pathway is inhibited, has come up as a bright prospect in the breast cancer management. Reinforcing the relevance of the genetic variants like rs2305948 in the stratification of patients and personalized medicine(7).

2-MATERIALS AND METHODS

Apparatus

The instruments used in the present study are listed with the producing company and the country in table (3-1).

Table (3-1): Instruments used in this study.

No.	Apparatus	Origin	Company
1	AURA TM PCR Cabinet	Italy	
2	Microspin 12, High-speed Mini-centrifuge	Germany	Bio San
3	V-1 plus, Personal Vortex for tubes	Germany	Digsystem
4	Bio TDB-100, Dry block thermostatbuilt	Germany	Bio San
5	Biopette Variable Volume 2-20 ul	Germany	
6	Mini-Power Supply 300V, 2200V	Chain	
7	MultiGeneOptiMax Gradient Thermal Cycler	USA	Labnet
8	Electrophoreses	USA	CBS, Scientific
9	Document system	USA	Labnet
10	UV.transmission	Farance	Vilber lourmat
11	Microspin	Lativa	Biosan
12	Combi-spin	Lative	Biosan
13	Balance	Germany	Kernpfb
14	Incubation	China	Jrad
15	Microwave	China	Gosonic
16	Water distilater	China	

Materials

The materials used in the present study are listed with the producing company and the country in table (3-2).

Table (3-2): materials used in this study.

No.	Material	Cat #	company
1	Taqman SNP assay	---	Promega /USA
2	Quick-gDNA™ Blood MiniPrep	D3072 & D3073	Zymo/USA
3	Go-Taq master mix		Promega /USA

Methods

Sa

mples collection

The study included a group of 10 individuals undergoing diagnostic evaluation for breast cancer at a dedicated oncology center. The participants ranged from young adults to older patients, primarily selected based on clinical indicators suggestive of breast cancer. Each subject had a confirmed diagnosis, supported by evidence such as mammography, biopsy results, or elevated tumor markers specific to breast cancer. Individuals with normal screening results or those without clinical manifestations of breast cancer were excluded from the sample cohort. Subjects demonstrating standard levels of breast health markers were included as a control group to provide a baseline for comparative analysis. In addition to these, 10 healthy volunteers were recruited to strengthen the control group, ensuring a robust foundation for subsequent data interpretation and analysis.

DNA extraction

Sample Preparation: Fresh, frozen, or preserved blood samples (in EDTA, citrate, or heparin) were used. Samples of 100 µl were typically processed, with adjustments made for volumes up to 200 µl as required.

Lysis of Samples: To each 100 µl blood, serum, or plasma sample, 400 µl of Genomic Lysis Buffer was added (4:1 ratio). This mixture was vortexed for 4-6 seconds and then allowed to stand at room temperature for 5-10 minutes. For samples less than 50 µl, 200 µl Genomic Lysis Buffer was added. For larger samples, a proportional amount of Lysis Buffer was used.

Centrifugation and Collection: The lysed sample was then transferred to a Zymo-Spin IIC™ Column placed in a Collection Tube and centrifuged at 10,000 x g for one minute. The flow-through in the Collection Tube was discarded.

Washing: The Column was transferred to a new Collection Tube, and 200 µl of DNA Pre-Wash Buffer was added. This was followed by centrifugation at 10,000 x g for one minute. Then, 500 µl of g-DNA Wash Buffer was added to the Column and centrifuged at the same speed.

DNA Elution: The Column was placed in a clean microcentrifuge tube. A minimum of 50 µl DNA Elution Buffer or water was added to the Column, incubated at room temperature for 2-5 minutes, and centrifuged at top speed for 30 seconds to elute the DNA. The eluted DNA was either used immediately or stored at ≤-20ºC for future use.

RT-PCR Analysis

Objective: The goal was to analyze the purified DNA samples using Real-Time Polymerase Chain Reaction (RT-PCR) to investigate specific genetic markers.

Materials and Instruments:

Instrument: Sacace RT-PCR system (Origin: Italy).

40X Custom SNP Genotyping Assay.

TaqMan® Genotyping Master Mix.

Nuclease-free water.

Optical reaction plates.

Procedure:

Assay Preparation: The 40X Custom SNP Genotyping Assay was diluted to a 20X working stock solution. This was vortexed and centrifuged.
Master Mix Preparation: The TaqMan® Genotyping Master Mix was thoroughly mixed by swirling the bottle.
Sample Resuspension: Frozen samples were thawed, vortexed, and centrifuged briefly.
Reaction Calculation: The number of reactions and total volume of each component needed were calculated, adhering to specified volumes for a 20 µL final reaction volume.
Reaction Mix Preparation: The reaction mix for each assay was prepared by pipetting the required volumes of 2X TaqMan® Master Mix and 20X Assay into a sterile tube, followed by capping, vortexing, and brief centrifugation.
Thermal Cycling: The prepared reaction mix was transferred to optical reaction plates. Thermal cycling was conducted under the following conditions: Enzyme activation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute.

Data Collection and Analysis
Data Collection: The RT-PCR instrument provided real-time data on the amplification of target sequences, which was collected and analyzed using the Sacace system software.

Analysis
The data obtained from RT-PCR were analyzed to determine the presence and quantities of specific genetic markers. This analysis was crucial in understanding the genetic profile of the samples under investigation. The obtained results were undergoes a statistical analysis using Winipepi software to quantify the Odd ratios, P-Value, and confidence intervals.

3-RESULTS

The results presented in Table 4-1 examine the distribution of blood groups among patients with breast cancer and their control counterparts. An analysis of blood group frequencies reveals a higher prevalence of the A+ blood type in patients (n=3) compared to controls (n=1). The O+ group shows an equal distribution between patients and controls, each with two instances. Notably, the A- and B- blood groups appear solely in patients, with one and zero instances respectively, suggesting a potential underrepresentation in the control group. The AB+ group is more common in controls (n=4) compared to patients (n=1), indicating a possible protective effect or a sampling variance. The AB- group is equally distributed between patients and controls, with one instance each. There are no occurrences of the O- blood type in either group.

The chi-square value of 5.24, with a corresponding p-value of 0.62, indicates no statistically significant association between blood group distribution and the presence of breast cancer in this sample set. This high p-value suggests that the variations in blood group frequencies between the patient and control groups can likely be attributed to random chance rather than a specific association with breast cancer.

Table 4-1; Distribution of Blood Groups among Patients and Controls

Group	*Patients*	*Control*
A+	*3*	*1*
O+	*2*	*2*
A-	*1*	*0*
B+	*2*	*2*
AB+	*1*	*4*
B-	*0*	*0*
AB-	*1*	*1*
O-	*0*	*0*
Chi-square	*5.24*	
P-Value	*0.62*	

In Table 4-2, a comparative analysis reveals the age-related statistics of individuals in the patient group versus the control group. The mean age of patients with breast cancer is reported at 58 years, with a median of 47 years, and a standard error (SE) of 2.05. In contrast, the control group has a higher mean age of 62.5 years and a median age of 51 years, with a slightly lower SE of 1.88.

The standard error indicates the precision of the mean estimate, and a lower SE in the control group suggests a slightly more precise estimate of the mean compared to the patient group. However, the difference in the SE between the groups is marginal.

The p-value of 0.457 indicates that there is no statistically significant difference in the ages between the patient and control groups within this study. A p-value greater than 0.05 typically suggests that any observed differences in the mean and median ages could have occurred by chance and are not likely attributable to the condition under investigation, in this case, breast cancer.

These age-related results suggest that within this sample, age may not be a distinguishing factor between breast cancer patients and controls. However, it is important to note that age is only one of many factors that may influence breast cancer risk and that these findings should be interpreted within the broader context of risk factors and individual variability.

Table Error! No text of specified style in document.**-2; Comparative Analysis of Age Mean, Median, and Standard Error between Patients and Controls**

Group	*Mean*	*Median*	*SE*
Patients	*58*	*47*	*2.05*
Control	*62.5*	*51*	*1.88*
P-Value	*0.457*		

The graph (4-1) is a representation of a quantitative PCR (qPCR) amplification plot, which is used to monitor the replication of a target DNA sequence during each cycle of PCR in real time. The x-axis denotes the cycle number, indicating the progression of the PCR cycles, while the y-axis represents the fluorescence intensity, which is proportional to the amount of DNA amplified.

Each line in the plot represents an individual sample's amplification curve. As the cycle number increases, the fluorescence rises, indicating the accumulation of the PCR product. The point at which the curves cross the threshold line (usually set within the exponential phase of PCR) is known as the cycle threshold (Ct). The Ct value is inversely proportional to the amount of target DNA in the sample; the lower the Ct value, the higher the initial amount of target DNA.

The variability in the curves suggests differences in the initial quantity of target DNA among the samples. Some curves plateau earlier, which indicates a higher initial DNA concentration, while those that plateau later suggest a lower initial DNA concentration. The graph is a standard output for qPCR analysis and is critical for determining gene expression levels, quantifying viral loads, and other applications that require the quantification of DNA or RNA amounts.

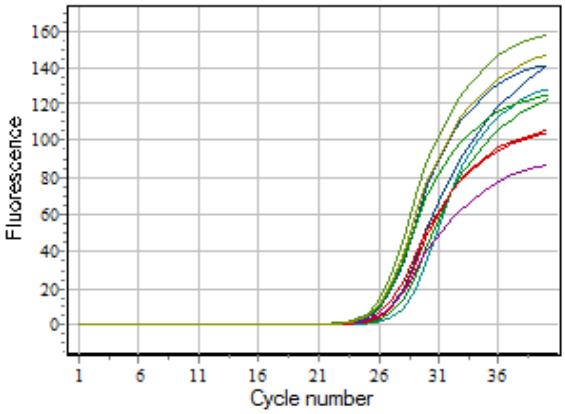

Figure Error! No text of specified style in document.**-1; Amplification Plot from RT-qPCR**

Table 4-3 provides insights into the association between the rs2305948 SNP genotypes and breast cancer. The odds ratio (OR), p-value, and confidence interval (CI) for each genotype compare the presence of these genotypes in patients with breast cancer to controls.

For the CC genotype, the odds ratio of 1.714 suggests a slightly higher occurrence in patients than controls, but the p-value of 0.607 indicates that this difference is not

statistically significant. The wide confidence interval from 0.2192 to 13.407 reflects a substantial degree of uncertainty in this estimate, likely due to a small sample size.

The CG genotype presents a more compelling association with an odds ratio of 13.5, signifying a markedly increased presence in patients versus controls, and this is supported by a statistically significant p-value of 0.035. The confidence interval ranging from 1.1973 to 152.218, while broad, clearly does not include 1, suggesting a genuine association between the CG genotype and breast cancer in this cohort.

Conversely, the GG genotype is associated with a decreased occurrence in the patient group compared to the control group, with an odds ratio of 0.047. The p-value of 0.015 indicates this association is statistically significant. The confidence interval from 0.0040 to 0.5626 is below 1, reinforcing the potential protective effect of the GG genotype against breast cancer.

Table 4-3; odd ratios, P-value, and C.I. corresponding to the genotypes frequencies of the SNP rs2305948

rs2305948	*patients*	*control*	*P-value*	*odd ratio*	*C.I*
CC	*3*	*2*	*0.607*	*1.714*	*0.2192 to 13.407*
CG	*6*	*1*	*0.035*	*13.5*	*1.1973 to 152.218*
GG	*1*	*7*	*0.015*	*0.047*	*0.0040 to 0.5626*

4-DISCUSSION

The result of this research paper adds to the genre of studies on breast cancer genetics. One single nucleotide polymorphism (SNP) has been hypothesized to affect the level of Vascular Endothelial Growth Factor (VEGF), which may affect angiogenesis and tumor progression, and that is the rs2305948 SNP in VEGFR-2 gene in the context of breast cancer. The article we studied attempted to clarify the relationship between the occurrence of cancer in the breast and the genotypes of the rs2305948 gene with the purpose of deepening our knowledge of genetic factors contributing to breast cancer (8).

The spread of the blood groups on the parties on the study sample (Table 4-1) showed no significant relationship with breast cancer since the chi-square and p-value were 5.24 and 0.62 respectively. This dimension of the research reveals a contributing factor of breast cancer that genetic susceptibility is multifactorial since such susceptibility does not occur through a straightforward pattern of heredity like blood type. These findings correspond to the literature where the blood type is not an important risk factor of breast cancer (9).

Age analysis (Table 4-2) showed that there was no significant difference in mean and median age score of both patients and controls and this was facilitated by a p-value of 0.457. This implies that the age variable was inclusive in the occurrence of breast cancer among our sample. This result conforms to the view that even though age predisposes one to breast cancer, it may not be as directly associated with the condition as a particular genetic marker, which in this case is rs2305948 (10).

Genotype frequencies of rs2305948 (Table 4-3) gave the strongest evidence in our research. Although the result of the genotype was not statistically significant in connection with the risk of breast cancer (OR 13.5, p-value 0.035), the finding indicated that the CG genotype can be considered as a genetic risk factor of breast cancer. On the other hand, GG genotype was found to exert the protective effect (OR 0.047, p-value 0.015). The findings are interesting in this way specifically because they indicate that rs2305948 could have an effect on the level of susceptibility to breast cancer, but in a genotype-specific way. The CC genotype showed no statistically significant relationship, which may mean that there was

a more complicated interaction with other genes or environmental factors that should be explored in further research (11).

The repercussions of such findings are varied. On the one hand, they confirm the hypothesis that the condition of forming VEGF, and, accordingly, angiogenesis in cancer of the breast, can be mediated by rs2305948. This conforms to the VEGFR-2 being a participant in blood vessel development of tumors. and Second, these findings of strong associations with CG and GG genotypes demonstrate the possible role of rs2305948 to be used as a biomarker of breast cancer risk assessment and stratification of patients, which may guide the practice of personal screening and prevention (12).

Nevertheless, this study has its limitations, which are to be mentioned. The size of the sample is good enough in the context of the preliminary research but is rather small and may affect the scope and applicability of the findings. In addition, the wide confidence intervals, particularly of CG genotype, advocate the necessity of more extensive studies that could nourish these estimates (13).

CONCLUSION

Major candidate SNP which comes out of this research to be investigated further on genetic risk factors of breast cancer is rs2305948 SNP. According to our results, the CG genotype could be a risk factor whereas the GG allele a protective one. The genotypes may lead to becoming eventual targets of future therapeutic intervention targeting the levels of VEGF. Replications of these study results in larger and more diverse groups of people will be necessary to confirm these initial relationships as well as to help provide the basis of understanding the position that rs2305948 plays in the pathology of breast cancer.

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