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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 remains one of the most prevalent malignancies affecting women worldwide, with a multifactorial etiology encompassing genetic, environmental, and lifestyle factors. Among the genetic influencers, single nucleotide polymorphisms (SNPs) have garnered significant attention for their potential role in cancer susceptibility, prognosis, and response to treatment. The SNP rs2305948, located within the kinase insert domain receptor (KDR) gene, which encodes for the vascular endothelial growth factor receptor-2 (VEGFR-2), is one such variant that has been implicated in cancer biology (U.S. Breast Cancer Statistics | Breast cancer.org n.d.).

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that plays a pivotal role in tumor angiogenesis, the process by which new blood vessels form to supply nutrients and oxygen to tumors, facilitating their growth and metastasis. The expression levels of VEGF have been correlated with tumor progression, metastatic potential, and overall prognosis in breast cancer patients (Karlsson *et al*. 2021).

The rs2305948 SNP results in a valine to methionine substitution at position 297 in the VEGFR-2, potentially altering receptor

function and, consequently, the angiogenic signaling pathway. This SNP has been the subject of numerous studies aiming to elucidate its impact on VEGF expression levels and to understand how it may influence breast cancer pathogenesis and progression (Denton 2017).

Understanding the role of rs2305948 in determining VEGF levels is critical due to the prognostic significance of angiogenesis in breast cancer. High VEGF levels have been associated with poor prognosis and lower survival rates, making it a potential target for therapeutic intervention. Anti-angiogenic therapies, which aim to inhibit the VEGF pathway, have emerged as a promising approach in breast cancer treatment, underscoring the importance of genetic variants such as rs2305948 in patient stratification and personalized medicine (KL *et al.* 2021).

The exploration of rs2305948's influence on VEGF levels in breast cancer is not only pertinent for enhancing our understanding of the disease's molecular underpinnings but also for improving diagnostic strategies and therapeutic outcomes. By investigating this SNP, researchers can contribute to the development of genotype-based risk assessment tools and targeted therapies, which are the cornerstones of precision oncology (KL *et al*. 2021). Aim of this study to understanding the role of rs2305948 in determining VEGF levels is critical due to the prognostic significance of angiogenesis in breast cancer and explain the inhibit of VEGF pathway, have emerged as a promising approach in breast cancer treatment. Underscoring the importance of genetic variants such as rs2305948 in patient stratification and personalized medicine.

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.

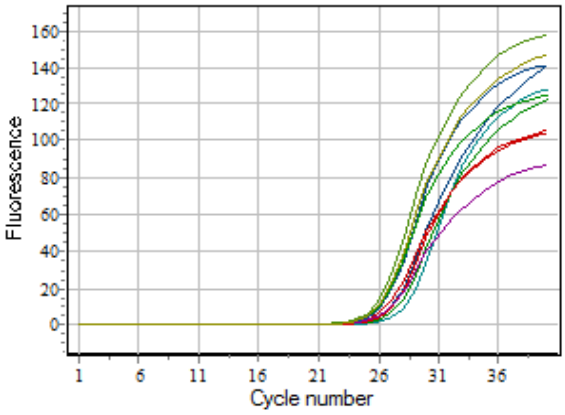
Table Error! No text of specified style in document.**-2; Comparative Analysis of Age Mean, Median, and Standard Error between Patients and Controls**

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 findings of this study contribute to the evolving landscape of genetic research in breast cancer. The rs2305948 SNP within the VEGFR-2 gene has been postulated to influence Vascular Endothelial Growth Factor (VEGF) levels, with potential implications for angiogenesis and tumor progression in breast cancer. Our study sought to elucidate the association between rs2305948 genotypes and breast cancer incidence, with the aim of enhancing our understanding of the genetic determinants of this malignancy (Tomimatsu *et al.*, 2019).

The distribution of blood groups among the study participants (Table 4-1) did not reveal a significant association with breast cancer, as indicated by the chi-square value of 5.24 and a p-value of 0.62. This aspect of the research underscores the multifactorial nature of breast cancer, where genetic susceptibility does not manifest through simple hereditary patterns such as blood type. These results align with the existing literature that suggests blood type is not a significant risk factor for breast cancer (Tavares *et al*., 2021).

Age analysis (Table 4-2) indicated no significant difference between the mean and median ages of patients and controls, which was supported by a p-value of 0.457. This suggests that within our sample, age did not play a discriminatory role in breast cancer incidence. This finding is consistent with the notion that while age is a risk factor for breast cancer, the presence of specific genetic markers such as rs2305948 may have a more direct relationship with the disease (Rebbeck *et al*. 2009).

The genotype frequencies of rs2305948 (Table 4-3) presented the most compelling evidence within our study. Individuals with the CG genotype were found to have a significantly higher risk of breast cancer (OR 13.5, p-value 0.035), suggesting that this genotype may be a genetic risk factor for the disease. Conversely, the GG genotype appeared to confer a protective effect (OR 0.047, p-value 0.015). These findings are particularly intriguing as they suggest that rs2305948 may influence breast cancer susceptibility in a genotype-specific manner. The CC genotype did not exhibit a statistically significant association, which could indicate a more complex interaction with other genetic or environmental factors

that warrants further investigation (McDonnell *et al.,* 2019).

The implications of these findings are multifaceted. Firstly, they support the hypothesis that rs2305948 may modulate VEGF levels and, by extension, angiogenesis in breast cancer. This is in line with the role of VEGFR-2 in tumor blood vessel formation. Secondly, the significant associations identified for CG and GG genotypes highlight the potential of rs2305948 as a biomarker for breast cancer risk assessment and patient stratification, which could guide personalized approaches to screening and prevention (Mohseni *et al*., 2019).

However, there are limitations to this study that must be acknowledged. The sample size, though adequate for a preliminary investigation, is relatively small, which could influence the breadth and generalizability of the conclusions. Additionally, the broad confidence intervals observed, especially for the CG genotype, point to the need for larger studies to refine these estimates (Mudduwa *et al*., 2018).

CONCLUSION

The rs2305948 SNP emerges from this study as a candidate of interest for future research into genetic risk factors for breast cancer. Our findings suggest that the CG genotype may increase risk while the GG genotype may decrease it. These genotypes could serve as potential targets for future therapeutic interventions aimed at modulating VEGF levels. Expanding upon this research with larger, more diverse populations will be essential to validate these preliminary associations and to clarify the role of rs2305948 in breast cancer pathophysiology.

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