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Associated of Variable Number Tandem Repeat (VNTR) Polymorphism in IL 4 with Susceptibility to Breast Cancer in Iraqi Women

Noorhan Ihsan Oun[1] and Maan Hasan Salih[2]
[1]Department of Biology, College of Science, Tikrit University, IRAQ.
[2]Department of Biology, College of Science, Tikrit University, IRAQ.

[1]Corresponding Author: nwrhanahsanwnly@gmail.com

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ABSTRACT

Background: Breast cancer (BC) is common disease in the worldwide. Furthermore genetic mutations are known to enhance BC risk. The aim of this research is to examine correlation between the frequency of BC among Iraqi women and a variable number tandem repeat (VNTR) polymorphism in the IL-4 gene.

Methods: The study included 74 patients and 54 healthy women. To determine the genotypes of the variable number of tandem repeats (VNTR) in the IL-4 gene, a Polymerase Chain Reaction (PCR) technique was employed. Additionally, a complete blood count (CBC) test was conducted. Logistic regression analyses were performed to evaluate the association between genotypes and the occurrence of BC. Measures of association were computed in the form of odds ratios (OR) and 95% confidence intervals (95% CI). The threshold for statistical significance in both CBC analysis and genotyping analysis was established as a p-value below 0.05.

Results: Among the CBC analysis, only the results of RBC (red blood cell count) and WBC (white blood cell count) showed statistical significance at the p = 0.05 level when comparing the study population. However, our investigation identified no association between IL-4 gene variant genotypes and breast cancer in Iraqi women. Other than tumor differentiation and body mass index($p < 0.05$), no clinical or pathological features of BC patients were linked to variant genotypes.

Conclusion: The study concluded a substantial RBC and WBC relationship between patients and control. However, the IL-4 genetic variation does not appear to affect breast cancer development or progression. However, the IL-4 genetic variation may affect disease prognosis. Thus, more research is needed to determine how IL-4 genetic variation affects breast cancer prognosis.

Keywords- breast cancer, IL-4, genetic variation, VNTR.

سرطان الثدي هو مرض شائع في جميع أنحاء العالم .كما أن الطفرات الجينية تزيد من خطر الإصابة بالسرطان الثدي .الهدف :هدفت هذه الدراسة الى العلاقة بين نسبة حدوث سرطان الثدي في النساء العراقيات وتعدد الأشكال .الطريقة :شملت الدراسة 74 مريضا و 54 امرأة سليمة .IL-4 في جين (VNTR) لتحديد الأنماط الجينية لـ IL-4، في جين (VNTR) تم استخدام تقنية تفاعل البوليميراز المتسلسل .(PCR) بالإضافة إلى ذلك، تم إجراء فحص تعداد الدم .(CBC) الكامل تم إجراء تحليلات الانحدار اللوجستي لتقييم العلاقة بين الأنماط الجينية وحدوث سرطان الثدي .تم حساب مقاييس الارتباط في شكل نسب .(OR) الأرجحية كما واظهرت الدراسة .$p = 0.05$)عدد خلايا الدم البيضاء (فرق معنوي عند مقارنة مجاميع الدراسة عند مستوى احتمالية WBCخلايا الدم الحمراء (و لم ،(P <0.05) وسرطان الثدي لدى النساء العراقيات .بخلاف تمايز الورم ومؤشر كتلة الجسم IL-4 عدم وجود ارتباط بين الأنماط الجينية المتغيرة لجين يتم ربط أي سمات سريرية أو مرضية لمرضى سرطان الثدي بالأنماط الجينية المتغيرة .الاستنتاج :خلصت الدراسة إلى وجود علاقة كبيرة بين كرات الدم يؤثر على تطور سرطان الثدي أو تقدمه .ومع ذلك، فإن IL-4 الحمراء وخلايا الدم البيضاء بين المرضى والسيطرة .ومع ذلك، لا يبدو أن الاختلاف الجيني على IL-4 قد يؤثر على تشخيص المرض .وبالتالي، هناك حاجة إلى مزيد من الأبحاث لتحديد كيفية تأثير الاختلاف الجيني لـ IL-4 الاختلاف الجيني VNTR، تشخيص سرطان الثدي .الكلمات المفتاحية :سرطان الثدي، الإنترلوكين 4، التباين الوراثي.

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I. INTRODUCTION

Breast cancer is the leading cause of cancer-related deaths among women and accounts for a large percentage of oncological cases. Its incidence continues to rise across all regions (1). BC has emerged as the most often diagnosed form of cancer in recent times, it represents a substantial burden, with 1 in 8 cancer diagnoses and approximately 2.3 million new cases annually (2,3).

Modifiable risk factors (e.g., alcohol use, physical activity , obesity, smoking) and non-modifiable (e.g., family history, BRCA1 or BRCA2 mutations, and familial syndromes) causes (4). Interestingly, the median Arab BC diagnostic age is younger, around 48-52 years, compared to industrialized countries like Europe and the USA, where it is around 63 years, with a significant proportion of diagnoses occurring in individuals under 50 (5).

Inflammatory blood markers (IBM) may predict histological response to neo-adjuvant treatment in breast cancer and other cancers (6,7). IBM has been inconsistently associated with histological full response in early breast cancer patients undergoing neo-adjuvant chemotherapy (8,9).

Several studies have been conducted to investigate genetic variations in key genes that could potentially play a role in the development of breast cancer (10,11). The variable number tandem repeat (VNTR) polymorphism, which denotes differences in the length of adjacent repeated nucleotides, has received significant attention among these genetic variants (12). The variable number tandem repeat (VNTR) located in the IL-4 gene has been subject to substantial investigation about its potential correlation with the advancement of different types of malignancies, such as breast cancer (13). Interleukin-4 (IL-4), a type of cytokine secreted by T lymphocytes, has been extensively studied for its notable anticancer characteristics. These include its ability to trigger apoptosis in BC cells and its role in modulating the manufacture of estrogen(14,15,16).

Given the aforementioned roles of IL-4 and the extant body of literature on this topic, it is evident that the IL-4 gene holds significant potential as a candidate for association analysis (17,18,19). The gene encoding interleukin-4 (IL-4) is situated on chromosome 5 and encompasses an intron 3 region that harbors a variable number tandem repeat (VNTR) polymorphism spanning 70 base pairs. This phenomenon of polymorphism encompasses two prevalent alleles, namely allele 1 (consisting of a single repeat) and allele 2 (comprising two repeats), whilst allele 3 (comprising three repeats) is regarded as an infrequent variety (20,21).

II. MATERIALS AND METHODS

2.1 Selection of Study Participants

This study included 74 women with BC patients and 54 control The control group comprised individuals who volunteered for the study and did not have cancer or any systemic condition. These individuals were carefully chosen to match the experimental group's age. Patients having mastectomy, chemotherapy, hormone therapy, radiation therapy, recurrent samples, or non-breast neoplasms were excluded. Informed consent was obtained from all study participants to guarantee ethical compliance. Using conventional intravenous sampling, 5 mL blood samples were taken into anticoagulant tubes.

2.2 Hematological Analysis

The study evaluated various hematological indices, such as mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), the total white blood cell count (WBC), hemoglobin count (Hb), total red blood cell count (RBC), hematocrit (HCT) and platelet count (PLT). These indices were measured using an automated hematology analyzer (Sysmex KX-21 N, Germany).

2.3 Genotyping of IL-4

The Qiagen DNA isolation kit (QIAamp DNA Blood Mini Kit, Qiagen, Hilden, Germany) was used to isolate genomic DNA from WBC according to protocols. Using appropriate primers, allele-specific PCR genotyped the IL-4 gene polymorphism. Salimi et al. (22) utilized 5′AGGCTGAAAGGGGGAAAGC3′ for the forward primer and 5′CTGTTCACCTCAACTGCTCC3′ for the reverse primer. The PCR process began with a 5-minute 94°C denaturation. A series of 30 cycles of denaturation at 94°C for 50 seconds, annealing at 61°C for 30 seconds, and extension at 72°C for 45 seconds followed. The PCR ended with a 5-minute 72°C extension phase. The PCR product was separated and visualized using a 2% agarose gel and compared to a 100 bp ladder to determine fragment sizes.

2.4 Statistical analyses

A t-test was used to compare CBC parameter distributions in healthy and BC patients. P-values equal or below 0.05 were considered significant. This study used logistic regression to analyze allelic and genotypic frequencies and breast cancer risk. This odds ratio was estimated using a 95% confidence interval. The most common allele and genotype were homozygous wild type alleles and genotypes. Based on this premise, association analyses compared the wild type genotype to the heterozygous and homozygous genotypes (which contain the most frequent allele). Long and short allele cumulative counts were used to calculate additional genotypes. Thus, logistic regression investigations were used to confirm genotype correlation utilizing codominant, dominant, recessive, and over-dominant genetic inheritance models.

III. RESULTS

Patient samples were separated by age into two groups: 49 (66.2%) patients under 60 and 60 (33.8%) patients over 60.

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Table 1 presents the RBC, HBG, and PLT count findings, indicating that the minimum rate reported in patients with BC was (0.6, 8.200,and 81.00) while the maximum rate was (5.26, 15.00, 744.0) . In comparison, the control group exhibited a minimum rate of (3.01, 7.100, 129.0) and a maximum rate of (5.52, 14.00, 461.0). The mean ± SD of the RBC count in the BC group (3.945± 0.697), (11.27 ±1.461 versus 11.43 ±1.407), (255.3 ±102.0versus 269.2 ±79.18). There was a significant difference observed for RBC p-value <0.05 and no-significant difference observed for HBG, and PLT p-value > 0.543.

Table 1: The summary statistics compared between patients and control for RBC count; HBG count and PLT count.

Parameters	Minimum	Maximum	Mean ± SD	Coefficient of variation	P value
			RBC		
Cases	0.6	5.26	3.945± 0.697	17.68%	0.0007
Control	3.01	5.52	4.34 ± 0.534	12.31%	
			HBG		
Cases	8.200	15.00	11.27 ±1.461	12.96%	0.543
Control	7.100	14.00	11.43 ±1.407	12.32%	
			PLT		
Cases	81.00	744.0	255.3 ±102.0	39.96%	0.385
Control	129.0	461.0	269.2 ±79.18	29.41%	

As shown in table (2), Illustrate WBC, Neutrophil , Lymphocyte, Monocyte, Eosinophil, Basophil count reported Minimum and Maximum rate were(1.000, 40.00), (16.00, 87.00), (7.000,61.00), (0.000, 38.00), (0.000, 68.00), (0.000,19.00) respectively in patients with BC, as compared to a control group that were (2.000, 20.00), (27.00, 88.00), (3.000,58.00), (3.000,18.00), (0.000, 66.00), (19.00,13.00) respectively, the mean ± SD of the WBC count in BC comparing with the control group (6.338±5.065 versus 7.833±3.155), (60.36±10.87 versus 61.54±10.64), (29.26 ±10.87 versus 28.65 ±10.64), (8.054 ±5.810 versus 6.759 ±2.691), (10.72 ± 12.73versus 12.6 ± 14.52), (4.595 ±4.118 versus 4.852 ± 2.987). The result was significant difference for WBC, Monocyte (p-value <0.05), no significant difference for Neutrophil, Lymphocyte, Eosinophil, Basophil p-value >0.05.

Table2: the summary statistics compared between patients breast cancer and control for WBC count; Neut. count; LYMPH. count; Mono. count; EO**. count and Baso. count**

Parameters	Minimum	Maximum	Mean ± SD	Coefficient of variation	P value
WBC					
Cases	1.000	40.00	6.338±5.065	79.91%	0.04
Control	2.000	20.00	7.833±3.155	40.27%	
Neut.					
Cases	16.00	87.00	60.36±10.87	21.54%	0.6
Control	27.00	88.00	61.54±10.64	19.15%	
LYMPH.					
Cases	7.000	61.00	29.26 ±10.87	37.14%	0.75
Control	3.000	58.00	28.65 ±10.64	37.13%	
Mono.					
Cases	0.000	38.00	8.054 ±5.810	72.13%	0.09
Control	3.000	18.00	6.759 ±2.691	39.82%	
EO.					
Cases	0.000	68.00	10.72 ± 12.73	118.8%	0.6
Control	0.000	66.00	12.6 ± 14.52	120.4%	
Baso.					
Cases	0.000	19.00	4.595 ±4.118	89.62%	0.763
Control	0.000	13.00	4.852 ± 2.987	61.56%	

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In the study, a total of 128 individuals underwent genotyping using standard PCR followed by electrophoretical separation. Genotyping was successful in 100% of all attempts. Two different VNTRs of IL-4 alleles were detected (IL-4 VNTR-253 and VNTR-183 ; Figure 1) as well as four genotypes (253/253 (L\L), 253/183 (L\S) and 183\183 (S\S)) were identified with respect to IL-4 tandem repeats in *IL-4* gene in the study participants. Based on the DNA bands, the genotypic frequencies were determined along with the allelic frequencies.

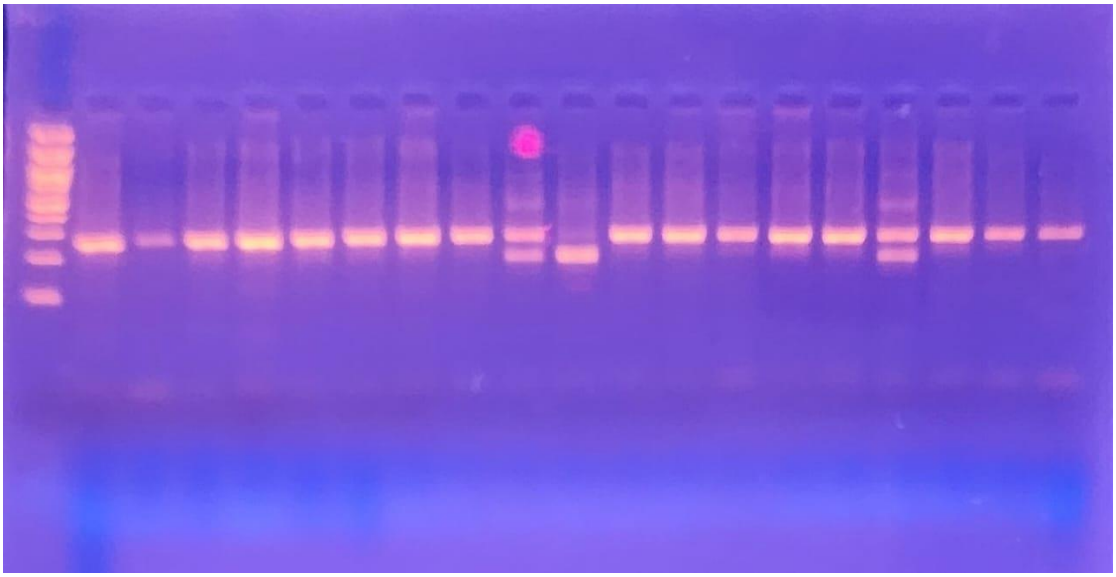

Figure 1: The PCR products of the IL-4 gene are electrophoresis on an agarose gel (2%). M = Marker 100 bp. Wells 9 and 16 represent the LS genotype. Well, 10 represents the ss genotype. Wells 1,2,3,4,5,6,7,8,11,12,13,14,15,17,18 and 19 represent the LL genotype.

Only one patient and two control possessed the short allele carrying SS 183\183, which had an allele frequency of 0.26. The VNTR-253 allele was observed to have a higher frequency in both the patient group (0.891) and the control group (0.769), indicating its prevalence in both populations. The allele frequency of the S 183 allele was found to be the second most prevalent, with a frequency of 0.109 in the patient group and 0.231 in the control group. However, it was shown that there was no significant association between allele frequency and the risk of breast cancer as shown in Table3

Table 3: Allelic distribution of IL-4 in breast cancer patients and control along with statistical analyses.

Alleles	Study participants									
	Breast cancer NO.	Allele frequency in Breast Cancer	control no.	Allele frequency in control	Allele frequency in total study participants	OR	95% CI		$\chi 2$	p-value
							Lower	Upper		
RP1 253 (reference)	132	0.891	83	0.769	0.84	1				
PRP2 183	16	0.109	25	0.231	0.16	0.40	0.2	0.79	7.06	P = 0.009
Total	148		108							

OR= odd ratio; Cl; confidence interval; $\chi 2$: Chi square

In the present study, three genotypes were identified. When considering the genotype 253\253 as the reference group, the genotype 253\183 (L\S), which contains heterozygous repeats (both short and long alleles), was found to be highly prevalent in both the breast cancer group (14 individuals) and the healthy group (21 individuals).

However, based on the analysis of genotypic distribution and various models of inheritances, no genotypes were found to be associated with the risk of breast cancer. This indicates that the different genotypes identified in the study do not appear to have a significant influence on the risk of developing breast cancer.

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Table 4: Genotypic distribution of IL-4 in breast cancer patients and in Control along with statistical analyses.

genotype	Breast cancer No.	control No.	OR	95% CI		χ2	p-value
				Lower	Upper		
253/253 (RP1\ RP1)(reference)	59	31	1				
253/183 (RP1\ RP2)	14	21	0.35	0.15	0.78	6.77	P = 0.01
183/183 (RP2\ RP2)	1	2	0.26	0.02	3.01	1.31	P = 0.28
Group of genotypes and models of inheritances							
Co-dominant model							
(RP2/RP2) vs. (RP1/RP1)	1 vs 59	2 vs 31	3.8	0.33	43.65	1.31	P = 0.28
Dominant model							
(RP1\ RP1) vs (RP1\ RP2) + (RP2\ RP2)	59 vs 15	31 vs 23	0.34	0.15	0.74	7.45	P = 0.007
Recessive model							
(RP1\ RP1) + (RP1\ RP2) vs (RP2\ RP2)	73 vs 1	52 vs 2	0.35	0.03	4.03	0.75	P = 0.4
Over-dominant model							
(RP1\ RP1) + (RP2\ RP2) vs (RP1\ RP2)	60 vs 14	33 vs 21	0.36	0.16	0.81	6.26	P = 0.01

IV. DISCUSSION

Although the age of breast cancer initiation in the Arab world is uncertain (22,23), Arab physicians believe it appears earlier and at a more advanced state than in Western countries. This view is supported by scant statistical data. Young Arab women (30–59 years old) have similar breast cancer rates to their global counterparts. Older Arab women (60+) had far lower rates (5). The immunological response of the body is generally strongly correlated with the clinical stage of breast cancer(23, 24, 25).

CBC, a vital diagnostic test, must be performed on cancer patients before treatment. Pre-treatment CBC and liver function test are recommended by the National Comprehensive Cancer Network for BC staging to discover concealed metastatic illness (24,25).

In the present case-control investigation, we observed a differential association between WBC count and BC burden based on the minimum and maximum rates. A growing amount of studies suggests that prolonged low-grade inflammation contributes to cancer growth. WBCs like neutrophils, monocytes, and eosinophils produce ROS and NOS. ROS and NOS can damage proteins, lipids, and DNA if the antioxidant defense system fails to neutralize them. This damage can cause genomic instability, affecting SNPs (26).

Park et al. (2021) found that BC patients had higher WBC counts than controls. However, it is worth noting that the study did not account for the menopausal status of the participants. The study conducted by Okuturlar et al. (22) demonstrated a significant correlation between neutrophil numbers and the susceptibility to breast cancer. A recent meta-analysis was conducted to evaluate the correlation between the neutrophil-to-lymphocyte ratio, a biomarker utilizing white blood cell subsets, and the prognosis of breast cancer (23).

This study agree with the research conducted by Chen et al. (2020), in which they assessed peripheral blood parameters such as WBC, monocyte count, and RBC count. These parameters are commonly suggested as prognostic variables for various malignancies, as they serve as indications of systemic inflammatory response (23). In contrast to the findings of Divsalar et al. (2020), who observed a statistically significant distinction in Hb, HCT, MCV, RDW parameters, and MPV/PLT ratio ($p < 0.05$). Furthermore, a statistically important difference distinction was seen among both groups in MPV and MCH (24).

In this study, a range of hematological parameters was assessed. Modifications to any of these factors may provide deeper exploration of the fundamental potential reasons and subsequent tailored treatment interventions. Therefore, it contributes to the enhancement of the patient's overall well-being, immune response, and capacity to endure medical interventions (26,27). Similarly, it is imperative to do a thorough examination for those diagnosed with BC before commencing any form of treatment, such as chemotherapy, radiotherapy, or surgery (28,29). This study has not been able to establish the diagnostic use of the CBC test in the detection of breast cancer. However, it suggests that the CBC test may serve as a potential marker for overall health in individuals diagnosed with breast cancer. A significant correlation has been observed between the immunological response of the human body and the clinical stage of breast cancer (25). Multiple studies have demonstrated a statistically significant distinction in RBC, HBG, and PLT characteristics

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between individuals diagnosed with breast cancer and those comprising the control group (30).

According to other research, it shows no significant difference in Hb, WBCs, and PLTs based on the kind of breast surgery, namely mastectomy. On the contrary, an elevated lymphocyte count may serve as a positive prognostic indicator, as it has been documented to promote the survival of breast cancer patients (31). Consequently, the lymphocyte count can either be raised or remain within the normal range, contingent upon the expressions of cancer cell antigens and the immune response. Therefore, the quantification of WBCs using absolute counts is a valuable tool for illness management (32). BC is a significant issue that impacts individuals of all genders, although it is notably prevalent among females (26, 27). Although the exact etiology of breast cancer (BC) remains uncertain, it is widely hypothesized that a multitude of risk factors, encompassing environmental influences, physiological factors, and individual genetic predispositions, contribute to its development (33, 34, 37). Numerous studies have endeavored to explore the association between various genetic polymorphisms and the heightened susceptibility to BC across diverse populations. The given data point is represented as (35.36, 38). In this particular investigation, a total of 54 individuals who were in good health were subjected to screening. The screening specifically focused on the sequence found within intron 3 (39,40) of the IL-4 gene, and BC. This study presents a contrasting perspective to the findings of Al-Eitan et al. (2019), imply that the variable genotypes in the IL-4 gene are not associated with breast cancer in women in Jordan. Statistical research also found a strong correlation between tumor differentiation and body mass index ($p < 0.05$) (40).

Two distinct alleles were identified inside the VNTR region of the IL-4 gene, specifically consisting of two repeats and three repeats. IL-4 is a multifunctional cytokine that is produced by various types of epithelial cells, including BC cells. This particular cytokine has been associated with the advancement of breast cancer and the development of resistance to conventional treatment protocols due to the presence of tumor-initiating cells (TICs) (41).

As an illustration, it is projected that the prevalence of (PR1, PR2) is (62.81%, 37.19%), (76.9%, 23.1%), and (21.7%, 78.8%) in Malaysia, China, North India, and respectively. (42, 43, 44). In the present investigation, it was shown that the prevalence of PR1 among healthy individuals and breast cancer patients in Iraq was 0.84%, but the frequency of PR2 was 0.16%. Notably, in Taiwan(45), the presence of the 1R allele of IL-4 VNTR has been found to be associated with the occurrence of bladder and oral cancers in China(46). Furthermore, the presence of the PR1/PR2 genotype of IL-4VNTR was associated with an elevated susceptibility to cervical cancer in women of North Indian (47). In study conducted by (48), it was proposed that individuals with RP2\RP2 genotypes may have a strong protective effect against BC. Furthermore, Jia et al. (49) show statistically significant relationship between IL-4 VNTR polymorphism and breast cancer risk.

The present study agreement with the study Zhao *et al.,* (2020) who reported significant difference IL-4 in breast cancer patients (50). Also agreement with Myers (2010) shows that Cytokines, stimulated by cancer and causing a local immune response and enhancing oxidative stress (51). In addition to nitric oxide and reactive oxygen species, microglia can also produce pro-inflammatory cytokines (52).

V. CONCLUSION

The CBC test showed their potential as a screening tool and a significant prognostic role in BC patients. Our study showed that the IL-4 gene variant no is associated with the risk of breast cancer development in Iraqi populations.

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