Journal of Al-Farabi for Medical Sciences volume (3), No (3) September (2025)

ISSN: 2957-7675 (Print)
Journal of Al-Farabi for Medical Sciences
https://iasj.rdd.edu.iq/journals/journal/view/96
Issued by Al-Farabi University

IL-6 rs1800797 Polymorphism in Iraqi Breast Cancer Patients: A Genotypic Study with Supporting Histopathology

١*Naba Qahtan Abdul Hamza Alhilali

٢Prof. Dr.Mohammed Abdulwahab Alaskeri

١College of Biotechnology Department of Medical Biotechnology

***Email:N.Q.Abdulhamza@qu.edu.iq**

Email:mohammed.ati@qu.edu.iq

Abstract

BC in Iraq is a worrying health issue for Iraqi women after the increase in cases of infection in recent years. Although the disease is associated with many causes and infection factors, current studies are directed at molecules and genetic components that have a significant role in disease development. The most important of these factors are inflammatory gene such as the IL-6 gene, which has importance in the inflammatory response. When a mutation occurs in this gene, IL-6, at the rs1800797 variant, it may affect the gene expression of interleukin-6, thus creating a chronic inflammatory environment by raising the expression levels of IL-6, which is considered an ideal environment for the tumor. From this standpoint, the research aimed at finding the relationship between IL-6 rs1800797 and the development of BC in Iraqi women. The study included two types of samples: 100 blood samples for genetic analysis, divided into two parts: 50 patients with BC and 50 healthy individuals as a control group. The second type, tissue samples, supported the genetic study, including 4 tissue samples from breast cancer patients after surgical resection, and the demographic data represented by age and family history. Analysis for genotyping using the T-ARMS-PCR. The results of the studies showed that there is a significant relationship between IL-6 rs1800797 and BC, women carrying the AC genotype (OR=3.66 Ci95%=1.45-9.19, P=0.005) are and CC genotype (OR=2.98 CI95%=0.95-9.3 P=0.048)more susceptible to progression of breast cancer), and the C allele (OR=2.04 CI95%=1.14-3.62, P=0.014)in addition to the fact that the genotype AA had a protective effect(OR=0.24 CI95%=0.12-0.68, P=0.003).

Keywords: *(breast cancer, IL-6, rs1800797, polymorphsims, Histopathology)*

1.Introduction

Breast cancer is a leading cause of morbidity and mortality among women worldwide.(1), with a marked increase in incidence, particularly among Iraqi women, where it accounts for the largest proportion of all cancers(2). Despite traditional predisposing factors, including age, family history, lifestyle, and several environmental influences(3), Interest in recent years has focused on studying molecular and genetic compounds that play a significant role in understanding the mechanism of disease development or even early detection. IL-6 has a pivotal role in the chronic inflammatory response that causes carcinogenesis. It is one of the main cytokines involved in many biological processes, including cell proliferation, survival, and metastasis.(4). Numerous studies in different populations have shown a significant link between genetic changes in IL-6 and the risk of breast cancer. Genetic mutations, such as rs1800797, can affect the level of IL-6 expression(5). On the other hand, histological examination is considered the essential basis for diagnosing breast cancer, as it allows classification by grade (DCIS (*Ductal Carcinoma in Situ*), IDC (Infiltration and Ductal Carcinoma)), revealing microscopic features that indicate tumor severity and degree of differentiation, in addition to the state of lymphatic invasion and the presence of borders. Surgical sections. Since the inclusion of selected histological images as a supporting example for this study enables us to enhance the visual understanding of tumor mechanisms and supports genetic interpretation, from this standpoint, our study focuses on the genetic analysis of the rs1800797 variant and its association with the BC risk in BC patients. This study was supported by selected illustrative histological images from diagnosed cases in support of clinical interpretation.

2.Methods& Materials

2.1 Study population: This study included 50 blood samples from patients diagnosed with breast cancer and 50 healthy blood samples as a control group, along with tissue samples from breast cancer patients. All samples were collected from Al-Diwaniyah Teaching Hospital after obtaining ethical approval from all participants. Demographic data, such as age and family history, were gathered from the patients' medical records. The control group included healthy individuals who had not experienced any previous cancerous tumors, and individuals under the age of 18 years were excluded.

2.2 Molecular study

2.2.1-Sample Preparation

(5 ml) Blood was collected from patients with BC after confirming their infection through clinical diagnosis and was placed in an EDTA tube containing an anticoagulant to prevent clotting. The blood was then stored at -20°C.

Using the FAVORGEN extraction kit and following the manufacturer's protocol, DNA was extracted, followed by ethidium bromide staining and analysis with 1% agarose gel electrophoresis. This was then viewed under UV illumination to assess the quality and concentration of the DNA.

2.2.2 Genotype

TARMS-PCS was used for the genotypic(analysis of the rs1800797(A/C) variant as stated (6). 4 primers designed by (Primer 3) were used as shown in Table 2-1. Then, the reaction mixture was prepared by adding 1µL of each primer, 25µLof master mix(2× EasyTaq® PCR SuperMix kit), and 16µL of nuclease-free water with 2µL of the DNA sample until the full reaction volume of 50 µL for each sample was completed in the PCR tube. Then, the mixture was mixed well using an Exispin vortex centrifuge for 1 minute at 3000 rpm to ensure complete homogeneity. Then, the reaction tube was transferred to a PCR thermocycler (USA: Labnet Technology), which includes a thermal protocol consisting of 35 cycles divided into three parts. The first part: the denaturation at 95°C for 30 seconds, the second part: the annealing at 54°C for(30 sec), finally part: the extension at 72°C for (45 sec), then by using 2%agarose of electrophoresis with the addition of 1µL of ethidium bromide, was analyzed product sizes for rs1800797 were 162 bp (A allele), 136 bp (C allele), and 242 bp for the control band***Table(2- 1): Sequences of primers and product sizes for IL-6 SNPs genotyping***

Primer rs1800797	Sequence	Product Size (bp)
FIP	GCCTTGAAGTAACTGCACGAAATTTGAAGA	162 (A ALLELE)
RIP	CGGCTGTTGTAGAACTGCCTGGCTAG	136 (C ALLELE)
FOP	CTCTAAGTGGCTGAAGCAGGTGATGA	242 (OUTER)
ROP	AGTTTCCTCTGACTCATCGCAGGCC	242

2.3 Histopathology Study

Histological data were collected from four breast cancer patients. The tissue sections were sectioned after receiving the samples following surgical resection. They were processed and embedded in paraffin wax. Afterwards, they were transferred to a laboratory protocol where the samples were stained with hematoxylin(H&E). These standard stains were used for their efficiency in highlighting histological details and cellular changes under the microscope. The results were then confirmed by microscopic examination, which determined the tumor type and histological grade, as well as evaluated the axillary lymph node status to detect the presence or absence of cancerous metastasis. The surgical resection margins were also examined to ensure they were free of tumor, and all information was collected in a medical report accompanying the histological image.

2.4 Statistical analysis

using SPSS version 26(Statistical Package for the Social Sciences) of was used in the statistical analysis to the data. The variables were represented using numbers and percentages. The mean and standard deviation (SD) for the normally distributed variables were calculated. also used to ascertain the differences between the qualitative variables and the independent The test is known as chi-square. (ANOVA) was used to evaluate the difference between the 2 tests, less than 0.05 of the P-value to assess the level of statistical significance.

2.5 Ethical Approval

The Declaration of Helsinki has been adopted as the ethics guide for this research. Also, obtaining ethical agreement from the Ethics Committee of the College of Biotechnology, all procedures involving human participants adhered to moral principles at the institutional and national levelsAll study participants offered written informed consent after being informed of the potential advantages and risks of the study, as well as the research methods and objectives. Furthermore, all participant data was treated with complete confidentiality.

The results of this study and its publication do not involve any personal or even financial conflict. This was confirmed by the study's researchers.

3.Results & discussion

3-1 Demographic features of patients with Breast Cancer and the healthy control groups

In the middle of breast cancer patients, the mean age was 50.94±11.7 years, with a range was 25–76 years, whereas control group, the mean age was 47.70 ± 10.50 years, with a range was 28–72 years. No significant difference between patients and healthy controls in mean age (P= 0.150). Table 3-1 displays the age group of both groups (the patients with BC and the control group). Similarly, in patients as well as control subjects, according to age group, and no significant difference in the frequency distribution (P = 0.331)**Table (3-1): patients with Breast Cancer and healthy controls demographic details**

characteristic	patients with BC n=50	*Healthy control* n=27	*P*
age (years)			
Mean ±SD	50.94±11.7	47.70 ± 10.50	0.150 † NS
Range	25 –76 years	28– 72 years	
< 40, *n* (%)	9 (18.0%)	10 (20.0%)	0.331 ¥ NS
40-49, *n* (%)	12 (24.0%)	18 (36.0%)	
≥ 50, *n* (%)	29 (58.0%)	22 (44.0%)	

n: number of cases; SD: standard deviation; †: independent samples t-test; ¥: Chi-square test; NS: not significant at P > 0.05.

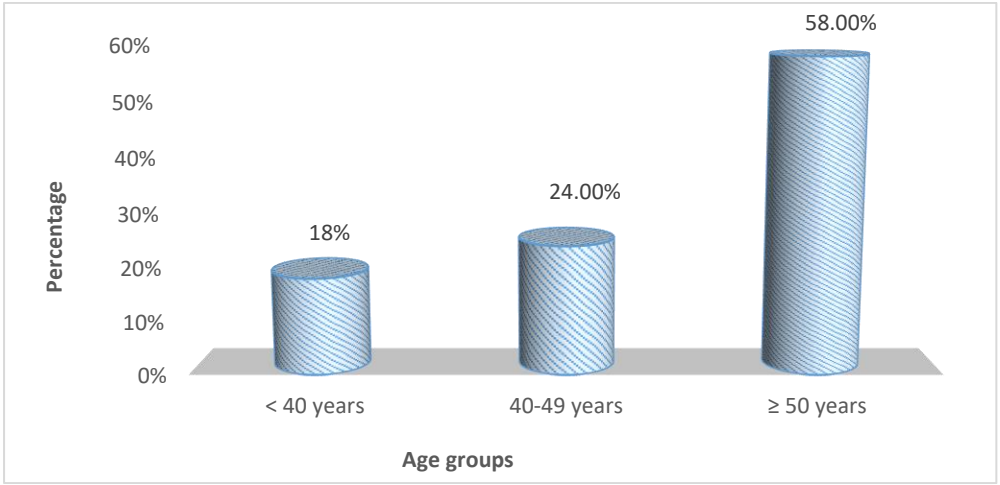


Figure (3-1): Distribution of patients according to age groups of patients.

With many local and international studies that have presented a correlation between breast cancer and age. showing that the risk of developing malignant breast tumors increases with age. In Iraq, studies by ((7–9))

reported the highest incidence rates among those aged 50-59 and 60-69. These results confirm the findings of((10)) that the highest frequency was in the age groups 35-40, 60-64 years. Additionally,(11) Showed that women over the 50 age are more prone to breast cancer. Several Arab studies also supported our finding, for example, the Saudi Cancer Registry report for 2017 indicated that the most frequent diagnosis of cancer, and the diagnosis was 50 years, is the average age (12) . In another study conducted in Lebanon(13) , reported that the age groups 45-49 and 50 - 59 years have the highest incidence rates Internationally, many studies have also concurred with our findings. According to a study by(14). showed that incidence rates rise with age, especially in women who have delayed menopause. These age trends in studies are attributed to hormonal changes associated with menopause, in addition to environmental factors, lifestyle, dietary habits, and genetic predisposition, which play a significant role in developing breast cancer(13).

3.2 Family History of breast cancer.

The important contributory factor in breast cancer is family history. This study showed 23 (46.0%) of BC patients have positive family history, and 27 (54.0%) of BC patients have negative family history, Figure (3-2).

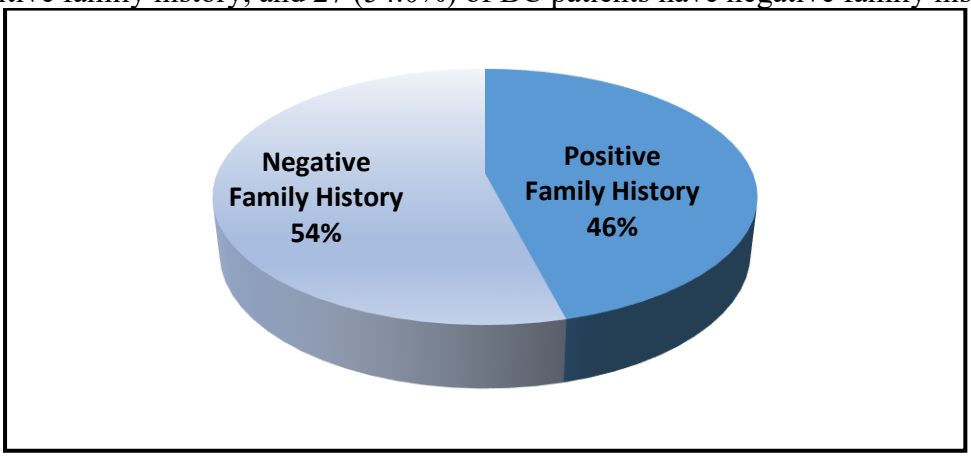


Figure (3-2): Distribution of patients with BC according to family history.

This indicates that genetic factors influence the development and progression of the disease. This relatively high percentage of family history among patients is evidence of the potential importance of genetic compounds in determining the risk of developing the disease. This is consistent with numerous studies and scientific literature that have shown that the presence of relatives among patients increases the likelihood of developing the disease among other women in the family. This includes a study, which showed that 24% of patients had a first- or second-degree family history. Also, a meta-analysis study conducted by(15) on European women showed that family history is significantly linked with the risk of developing BC. Also, this study suggested that genetic factors have an important role in explaining the risk of developing the disease. In addition, numerous studies have demonstrated the same trend, including his study (16), which clarified that family history is an important risk factor. Conversely, many studies have shown no association between a positive family history and BC. This study, conducted by (17), revealed that the percentage of 46% is relatively high. This is a very important indicator, not only from an epidemiological perspective, but also from a genetic and hereditary perspective, which includes the presence of a hereditary factor within affected families, such as a genetic mutation in the BRCA or BRCA2 genes. This mutation may consist of other genetic variants, such as the IL-6 gene. The recurrence of infection within a single family is not a coincidence, but rather the result of an inherited genetic pattern that contributes to a defect in immune responses and cell proliferation. These genetic factors may be intertwined with other factors, such as lifestyle and nutrition within the family, which may accelerate the onset of the disease. This result suggests that family history should be considered as a risk indicator and an early warning sign, requiring screening for all close relatives, not just those affected.

3.3 Detection of Gene Polymorphisms

3.3.1 IL-6 rs1800797A/C The distribution of IL-6 (rs1800797) A/C Polymorphism was detected by a technique known as T-ARMS-PCR. At this locus, there are three genotypes (AA, AC, and CC). The homozygote genotype(wild-type) showed amplification product size of (162 bp) only the A allele. The homozygote genotype (mutant type) showed amplification product size at(136bp) only C allele. Whereas, the A and C allele heterozygote genotype showed amplification of a product size of 162bp and 136bp, respectively, figure (3-3). The genotype frequencies were consistent with the Hardy-Weinberg equilibrium no deviation. Additionally, a common control band at 242bp was present in all samples, which is used as an element to confirm the success of the interaction

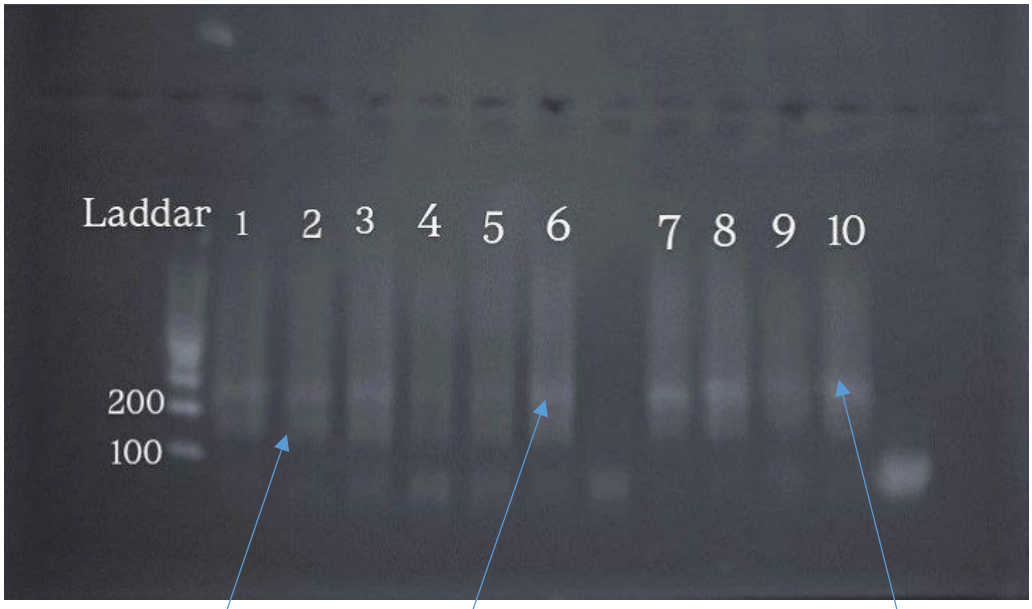


Figure (3-3): depicts the T-ARMS-PCR product analysis of the IL-6(rs1800797) A/C gene polymorphism using an agarose gel electrophoresis picture. M: the marker (100- 1500bp). the 162 bp product of T-ARMS -PCR result for the lane (AA) homozygote (wild-type) only showed A allele. at bp 162 and 136 bp (T-ARMS-PCR) product the (AC) heterozygote displayed both the G and C allele, the lane (CC) mutant type homozygote only displayed the C allele at 136 bp. The 242 bp (T-ARMS-PCR) product was where the external internal control was seen

The Hardy-Weinberg equation was applied to IL-6 (rs1800797) A/C genotypes across three loci: AA, AC, and CC, within the control group, and the results are shown in Table 3-1. The homozygous AA genotype was observed in 26 out of 50 control subjects; the homozygous CC genotype was observed in 8 out of 50 control subjects; and the heterozygous AC genotype was observed in 16 out of 50 control subjects, as shown in Table 3-6. The distribution of control subjects according to IL-6 (rs1800797) A/C genotype was not significantly different from the expected distribution (P=0.173).

Table (3-1): Hardy-Weinberg equation

Genotypes	Observed	Expected	χ^2	*P*
Homozygote reference AA	26	23.1	3.503	0.173 ¥ NS
Heterozygote AC	16	21.8		
Homozygote variant CC	8	5.1		

¥: Chi-square test; NS: Non-significant at P < 0.05

3.3.2 Genotypic and Allele Analysis for the studied gene in patients and healthy controls.

The comparison between patients and healthy controls is shown in Table 3-2, which details genotypes and allele frequencies concerning IL-6 (rs1800797) A/C. In the co-dominant model, there was a significant difference in genotype distribution between the patient and control groups. Risk analysis indicated that the homozygous CC genotype (OR= 2.98) was a significant risk factor, and the heterozygous A/C genotype (OR of 3.66) was also a significant risk factor. This means that patients with the homozygous CC genotype are approximately 3 times more likely to develop the disease compared to those with other genotypes. In the dominant model, a significant difference was observed between patients and controls (p > 0.05). Additionally, allele analysis showed a significant difference between patients and controls (P= 0.014). However, in the recessive model, there was no significant difference between patients and controls (p < 0.05).

Table (3-2): IL-6 (rs1800797) A/C genotype frequency in patients and healthy controls.

mode	*IL-6 (rs1800797)*	patients n=50	control *n* = 50	*P*	OR	95% CI
Co-dominant	CC	11 (22.0%)	8 (16.0 %)	0.048	2.98	0.95-9.3
	A/C	27 (54.0%)	16 (32.0 %)	0.005	3.66	1.45-9.19
	AA	12 (24.0 %)	26 (52.0%)	Reference		
Dominant	CC+ A/C	38 (76.0 %)	24 (48.0 %)	0.003 ¥ S	Reference	
	AA	12 (24.0 %)	26 (52.0%)		0.291	0.12-0.68
Recessive	CC	11 (22.0%)	8 (16.0 %)	0.444 ¥ NS	1.48	0.53-4.06
	A/C +AA	39 (78.0%)	42 (84.0%)		Reference	
Alleles	C	49 (49.0%)	32 (32.0%)	0.014 ¥ S	2.04	1.14-3.62
	A	51 (51.0%)	68 (68.0%)		Reference	

¥: Chi-square test; S: significant at P > 0.05.

Our findings suggest that the rs1800797 variant in the IL-6 gene may contribute to the high likelihood of progression of breast cancer. It showed that women carrying the genotype (CC/AC) in addition to the C allele are more vulnerable to breast cancer compared to women carrying the genotype AA, which has a protective role against breast cancer.

These results agree with several previous studies that investigated the role of the rs1800797 variant and its relationship to an increased risk of BC, including a study on Bangladeshi women by[18], which showed a relationship between rs1800797 and breast cancer. An Iranian study conducted by[19] also demonstrated a strong acorrelation between this variant and breast cancer risk. A study that was highly consistent with our findings demonstrated the strength of the relationship between IL-6 rs1800797 and a higher risk of BC. Despite these studies that agree with our findings, some studies have not found an association between rs1800797 and breast cancer risk. These studies include the[20], in addition to the study[21], which also showed that there is no relationship between the variant and breast cancer. The biological explanation behind the association was that the IL-6 rs1800797 is a genetic polymorphism located within the promoter region of the IL-6 gene. It is well known that this gene plays an important role in regulating the immune and inflammatory response. The presence of the C allele may lead to altered pleural efficiencies and increased IL-6 levels, which leads to an increased inflammatory response, thus enhancing the chronic inflammatory environment that stimulates tumor growth and cancer cell proliferation. This, in turn, increases the risk of BC for women who carry one or more copies of this allele. Despite the strength of these results, this association requires further research in a larger sample population and across more geographical areas, as well as focusing on directly studying IL-6 gene expression to verify the actual effect

3.4 Histopathological descriptions were included from four breast cancer patients to support the genetic findings.Table(3-3): Histopathological descriptions for cases

n. case	Histological type	grade	Lymph Node Involvemeny	Desmoplastic stroma	Axillary vascular invasion	Nipple involvement	Free Resection margins	TNM staging
1	Ductal Carcinoma in Situ (DCIS)	N/A	Not applicable	No	No	No	Yes	N/A
2	Infiltration and Ductal Carcinoma (IDC)	Grade II	1/14 positive	Yes	Yes	No	Yes	T2N1 mx
3	Infiltration and Ductal Carcinoma (IDC)	Grade II	16/20 positive	Yes	Yes	Yes	Yes	T2N3 mx

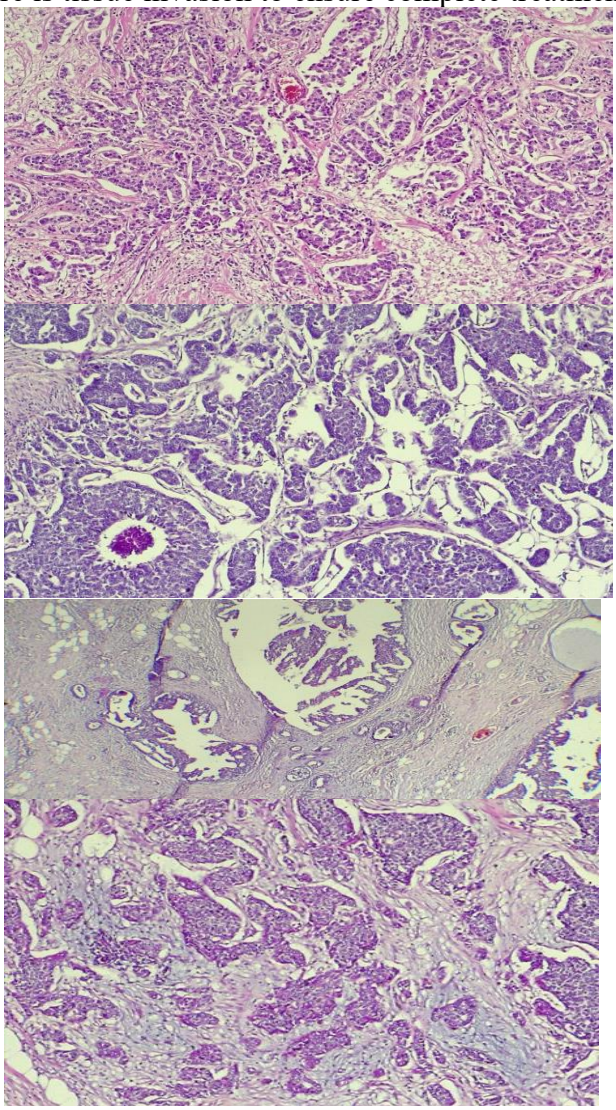
4	Infiltration and Ductal Carcinoma (IDC)	Grade II	none	Yes	No	No	Yes	N/A

A B

After analyzing the four cancer cases of the study participants as an illustrative example of the histological characteristics among the participating samples, they were divided according to tumor condition, grade, and spread. The first case in Figure C was diagnosed as ductal carcinoma in situ (DCIS), showing no lymph node involvement or even Axillary vascular invasion. The surgical incision margins were completely free of any tumor, indicating an early stage of the disease. This is considered a favorable diagnosis and prognosis until the disease is largely controlled. This finding is consistent with one of the studies, such as the study by (22)

In contrast, the second case in Figure A and the third case in Figure B were diagnosed with grade II invasive ductal carcinoma (IDC). These two cases demonstrated significant lymph node involvement of (1/14 and 16 /20), respectively. Additionally, a Desmoplastic

stroma reaction was observed. There was also axillary vascular invasion extending to the nipple. This diagnosis indicates an aggressive, invasive form of the disease, suggesting that the patients are in advanced stages (T2N1mx, T2N3mx) and have a rapidly spreading aggression. Systemic involvement of patients was observed. This finding aligns with a study suggesting increased disease aggressiveness and heightened risk of relapse for the patient (23). The fourth case in Figure D was also classified as grade II invasive ductal carcinoma (IDC), but exhibited no lymph node involvement. There was no axillary vascular invasion or extension to the nipple. Despite this, a Desmoplastic stroma reaction was present, which was observed as a positive indicator. However, there is tissue invasion even though the lymph nodes are not affected. Therefore, the patient must be closely monitored when there is tissue invasion to ensure complete treatment.



C D

Figure(3-4): A Infiltrative ductal carcinoma, Grade II Microscopic section demonstrates moderately differentiated infiltrative ductal carcinoma (Grade II, Nottingham system), with desmoplastic stroma, irregular tumor borders, and vascular invasion. Tumor involves the nipple and 16 axillary lymph nodes (H&E stain, 10X)

B: shown Invasive ductal carcinoma, Grade II.

Histological section reveals features consistent with Grade II ductal carcinoma according to the Nottingham modification of the Bloom-Richardson grading system. The tumor appears as irregular infiltrative nests of malignant epithelial cells within fibrous stroma. (H&E stain, 10X)

C: shown Ductal carcinoma in situ (DCIS).

Microscopic section shows fenestrated neoplastic proliferation with punched-out lumens and polarized tumor cells. Histological features are consistent with DCIS. Surgical margins are free (~1 cm) (H&E stain, 10X)

D: Infiltrative ductal carcinoma, Grade II. Microscopic section shows moderately differentiated infiltrative ductal carcinoma (Grade II, Nottingham grading), with desmoplastic stroma, irregular tumor borders, and vascular invasion. The nipple and resection margins are free of tumor (H&E stain, 10X)

After studying these cases, it was found that three cases had an aggressive histological behavior associated with an increased likelihood of relapse. This is consistent with many studies showing that a Desmoplastic stroma reaction is responsible for enhancing tumor invasion into neighboring tissues, including the study (24). In conclusion, this study summarized the relationship between BC and the variant in Iraqi women. This study was supported by demographic and histological analyses that showed a significant link between the variant and BC. Despite the difference in histological cases, it supported known histological patterns in aggressive tumors that are associated with increased expression of the gene responsible for the chronic inflammatory response, which is the interleukin-6 gene, as mentioned in previous studies that demonstrated a significant association between this variant (rs1800797) and increased expression of IL-6. These results combined indicate the importance of this variant in disease progression and the urgent need for further studies on larger samples and a broader study.

4. **Conclusion**The importance of this study centers on the genetic analysis of IL-6 rs1800797 and its correlation with BC. The results revealed a significant relationship between women carrying the A/C genotype and the C allele and the development of BC, whereas women with the AA genotype appeared to have a protective effect against malignant breast tumors. This study was further supported by an analysis of the patients' demographic data, which highlighted potential risk factors for developing breast cancer, alongside histological evaluations that assessed the aggressiveness of the tumors and identified their types and clinical patterns, emphasizing the significance of early disease detection. Given that the results suggest IL-6 rs1800797 may serve as a promising molecular indicator for early patient detection and appropriate treatment methods, future studies involving larger samples and diverse populations are recommended to validate the diagnostic value of this research.

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