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RESEARCH PAPER

Association of three single nucleotide polymorphisms of interleukin -35 for detection and prediction of women with breast cancer in Erbil city

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A B S T R A C T:

Background: Interleukins have a substantial role in the improvement of many types of cancer. Interleukin genes have many single nucleotide polymorphisms (SNPs) that may alter protein synthesis or function, and many of them have been related to cancer risk. The objective of this study is to determine whether particular SNPs in interleukin 35 contributed to breast cancer susceptibility and severity.

Methods: For IL-35 interleukin SNP detection, seventy benign breast disease, BC patients, 70 malignant BC and control healthy donors were obtained from patient's sera. We utilized an allelic discrimination approach that could genotype a large number of samples quickly. The long-term consequences and clinico-pathological aspects were also investigated.

Results: study is focused on three distinct IL-35 SNPs (IL12 G/A, IL12 ACT, and EBI3) and revealed a substantial upsurge in the chance of developing malignant breast cancer exclusively when compared to the control group. Both benign and malignant patients had significantly lower blood levels of IL-35 than controls ($p< 0.05$). Regardless of your age or BMI, there are a few things to consider (BMI) on the level of serum IL-35.

Conclusion: Polymorphisms in the interior of key interleukin genes (IL12 ACT, IL12 G/A & EBI3) seem to play a substantial complete role in breast cancer vulnerability or strictness.

KEY WORDS: Breast cancer, Interleukin 35, Single Nucleotide Gene polymorphism, Age and BMI.
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1. INTRODUCTION:

According to the American Cancer Society (ACS), 2 million new instances of breast cancer will be detected in 2018 (representing 23% of all cancers) (10.9 percent of all cancers). It has now surpassed lung cancer in terms of global prevalence, and this is true for both developed and developing nations. As well, East Africa has incidence rates of 19.3 per 100,000 women, whereas Western Europe has rates of 89.7 per 100,000 women. The occurrence proportions are high (over 80 per 100,000) in industrialized districts of the world (except Japan) and truncated (less than 40 per 100,000) in the bulk of emergent nations (Zaidi and Dib, 2019).

Furthermore, based on the latest cancer statistics worldwide (Bray et al., 2018), BC resembles the most communal overall diagnosed cancer in females (8.6 million, 24.2% of total) and the prominent reason of cancer associated demises in women (15 % of total cancer related deaths in women) (Sung et al., 2021). In 2021, about 281,500 estimated BC new cases, 43,220 estimated deaths are recorded among women in the United States (US) of America representing the leading cause of death among US women correspondingly guesstimate the entire number of cancer deaths averted because of the continuous reduction in the rate of cancer death during the

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early 1990s mainly due to mammography screening programs (Siegel et al., 2021).

Notwithstanding, in terms of the most recent Iraqi Cancer Registry data, BC is the most common form of female cancer in Iraq, composing approximately one-third of all female cancer cases recorded. Thus, breast cancer has surpassed bronchogenic cancer as the most recurrent cancer type in Iraqi women as a whole. People in Karbala city were discovered to have a wide range of malignancies during (2008- 2015). Iraqi women are most commonly diagnosed with breast cancer (BC) (ALSHashimi and Wang, 2014). Further, according to the data, the frequency and age-specific rate of several types of cancer differed between men and women. Women were more likely than males to develop breast cancer (Al-Janabi et al., 2017). BC's recurrence and metastasis rates are still high despite advances in surgical and pharmacological therapy, and immune suppression is a key component in tumor growth even now (Hao et al., 2018).

Epstein-Barr virus–prompted gene 3 (EBI-3) β-chains and the IL 12 p35 α-chain combine to generate Interleukin 35 (IL35), a cytokine mostly created by controlling T cells (Treg cells). In mice, IL35 increases carcinogenesis, shields cancer cells from apoptosis, and hastens disease development (Yazdani et al., 2020).

Correspondingly, IL-35 has recently been shown to have several functions in the tumor microenvironment as an EBI-3 and IL-12p35 heterodimeric cytokine. Many cytokines, such as IFN-γ, are inhibited by IL-35 and have a pro-tumor effect, according to research. This includes the growth of benign and malignant tumors, such as advanced breast cancer (BRCA1) receptor and IL-35 (Liu et al., 2021).

This study suggests a role of the IL-12 signaling and BC risk. SNPs in this passageway may change IL-12 encourage antitumor repayment and modify BC predisposition in a population-specific state. Functional studies will be needful to emphasize these findings, which conclusively may gain IL-12 related immunotherapeutic application towards BC.

Materials and Methods

Subjects

One hundred and forty female patients who referred for operation with breast disease were enrolled in the current study. None of the infected women had been remedied with chemical therapeutics or radiotherapy before sample collection. Breast cancer was diagnosed and confirmed by oncologists according to the surgical and pathological reports on the basis of a clinical review and laboratory test results.

On the other hand, this study was conducted on patients affiliated simultaneously to several general and private hospitals in Erbil city. 70 patients were having Malignant BC and another 70 patients were having benign breast disease. The mean age for BC patients were 33.5 and 52.5 respectively. Seventy healthy female subjects (seemingly), were considered as a control group and their age range was 21 - 66 years (mean 43.5 years).

Sample collection

Each group had five milliliters of peripheral blood drawn and split approximately equally into two tubes. DNA (2 ml) was quarantined from entire blood or mononuclear cells and stored at -70°C until genomic extraction was complete in heparinized tubes. The remaining 3 mL of blood were put into a gel tube to get the serum. The tubes were spun at 10,000 rpm for 10 minutes before the serum was relocated to a 1.5 ml Eppendorf tube and deposited at -20°C until the cytokine level was measured.

Determination of serum IL-35

Serum IL-35 pg/mL levels were measured using an ELISA kit from Cloud Clone Corp. succeeding the manufacturer's procedure.

Genotyping of Cytokine Polymorphisms

There are several approaches for assessing genotypes of polymorphic genetic loci in individual patients. For their determination, most loci have only a limited number of identified methods. The polymorphic loci investigated in this analysis were four particular nucleotide polymorphisms (SNPs) in the loci IL-35 (IL-12A ACT, IL-12 A G/A, EBI3 G/C and EEBI3 T/C), although EBI3 T/C did not give any response and its results were omitted.

Moreover, the intensification-recalcitrant modification structure (ARMS), also referred as an allele-precise polymerase restraint response (ASPCR) or PCR intensification of definite alleles, is an easy, quick and dependable system for identifying some single-base mutation or small deletions. For the SNPs of IL-35 genotype, the (ARMS-PCR) was applied. The investigations were achieved in a 20 µL rejoinder capacity comprising 40 ng genomic DNA. The

manufacturer's instruction was performed using 2X Prime Taq Premix (GeneAll® ExgeneTM Blood SV mini (105-101/ 105-152), Korea), as well, peripheral blood mononuclear cells were used to collect genomic DNA from patients and control group. Correspondingly, each reaction contained two forward primers, one for each of the two alleles, as well as a general antisense primer. Each 0.20 mL reaction comprised 1 l of each primer, 1 l of genomic DNA (100 ng/mL), 10 l of 2X Prime Taq Premix (GeneAll® ExgeneTM Blood SV small (105-101/ 105-152), Korea) and 7 l ddH2O. Electrophoresis was employed on a 2% agarose gel to separate the amplified products from the originals. Here is a list of the primers that were utilized in this study: (as shown in table 1):

Table 1. List of Primers applied in this research

Gene	Primer	Primer Sequence 5′ - 3′	Length (bp)	PCR Product size (bp)
IL-12 A/C/T FC	FA	TGG GTG TCC CAA CTC TAG GAA	21	106
	FC	TGG GTG TCC CAT CTC TAG GAA	21	
	FT	TGG GTG TCC CAC CTC TAG CAA	21	
	R	CCC AAA TGA CCA GGA GGC AT	20	
IL-12A G/A	FG	ATG CCA TTC AAA AAC CAA ACA TTT	24	739
	FA	ATG CCA TTC AAA AAC CAA ACG TTT	24	
	R	CAG ACA GAG GTA ACA GCA TGA G	22	
EBI3 G/C	FG	AAA ACC AAA AAG AAA CCA AAG GAA TG	26	440
	FC	AAA ACC AAA AAG AAA CGA AAG GAA TG	26	
	R	CCT CTC AAG TCT CCC ACG A	19	

For the PCR, the cycling settings indicated in table 2 were used in a thermal cycler (PX2).

Table 2. Studied gene polymorphisms ARMS-PCR protocol and product size.

Cytokines	PCR protocol &Conditions
IL-35= IL-12A+EBI3 A/C/T	Preliminary denaturation for 5 minutes at 95ºC followed by first step (Denaturation) by 40 cycles of 35 seconds at 94˚C, the 2nd step (Annealing) for 35 seconds at 57˚C, the 3rd step (Extension) for 35 seconds at 72˚C, subsequenced by 10 minutes at 72˚C as the final extension.
IL-12A G/A	Preliminary denaturation for 5 minutes at 95ºC followed by first step (Denaturation) by 40 cycles of 35 seconds at 94˚C, the 2nd step (Annealing) for 35 seconds at 59˚C, the 3rd step (Extension) for 35 seconds at 72˚C, subsequenced by 10 minutes at 72˚C as the final extension.
EBI3 G/C	Preliminary denaturation for 5 minutes at 95ºC followed by first step (Denaturation) by 40 cycles of 35 seconds at 94˚C, the 2nd step (Annealing) for 35 seconds at 61˚C, the 3rd step (Extension) for 35 seconds at 72˚C, subsequenced by 10 minutes at 72˚C as the final extension.

Statistical Analysis

In this current case control study, regularly distributed variables were represented by the mean and SD. ANOVA was applied to make comparison among categorical variables and the absorptions of serum cytokines across groups. Direct allele counting was used to all alleles for the IL-35 gene polymorphisms (IL-12A ACT, IL-12 A G/A, and EBI3 G/C). Accordingly, genotype-BC susceptibility risk was assessed using multivariate logistic regression, which computed the risk factor or adjusted odd ratio (OR) as well as 95% confidence intervals for each (CI). Hardy–Weinberg equilibrium was detected using the chi square (X2) test. We looked at the genotype and allele frequency differences between the two groups using 2 x 2 contingency tables and z statistics. Data were deliberated statistically important if their P-values have been less than 0.05.

Results and Discussion

The intensities of IL-35 in BC disease and control groups

Results of IL-35 cytokine demonstrated a significant variation in benign and malignant BC patients. The serum level of IL-35 in benign and malignant infected women were ominously ($p<0.05$) decreased when compared with controls (83.51±13.55 vs. 301.20 ± 55.20 pg/ml, and 114.20 ± 18.67 vs. 301.20 ± 55.20 pg/ml) respectively (Table 3).

Table 3. Serum level of IL-12 and IL-35 in total Benign and Malignant BC patients and controls.

Cytokine Serum Mean Level ± S.D. (pg/ml)			
Cytokine (N=70)	**Breast disease Patients**		**Control**
	Benign (N=70)	**Malignant BC (N=70)**	
IL-35	83.51 ± 13.55b	114.20 ± 18.67c	301.20 ± 55.20a

The proportion of the variance in the dependent variable (benign & malignant) that is explained by the independent variables (control group) as showed in table 4, the coefficient of determination was non-significant regarding the age group and BMI, and there were negative correlation (r) in control and malignant but, there was a positive correlation in benign breast disease regarding age group, while there were positive correlation between control, benign breast disease and malignant BC regarding BMI. This means there was no substantial correlation between BC neither with age, nor with BMI in the current study.

Table 4. Coefficient of determination & correlation of breast disease groups and control group regarding age & body mass index under the impact of IL-35.

Characters			Control	Benign	Malignant
IL-35	**Age**	**R^2**	0.01990	0.002270	0.01990
		r	-0.2713	0.06146	-0.02171
		P- value	ns	ns	ns
	Body Mass Index	**R^2**	0.004443	0.004374	0.005040
		r	0.06529	0.1233	0.03089
		P- value	ns	ns	ns

Table 3 shows a substantial difference in levels of the studied cytokine (IL-35) between the patient and control groups. Most studies so far have revealed low levels of the interleukin 35 (IL-35) in cancer patients. A genetic connection between the BC patients' genotypes and genes may be at fault here, and the function of IL-35 in tumor growth is now becoming more evident as a result of the drug's capacity to constrain effector resistant responses. Consequently, these findings imply that IL-35, whether generated by insusceptible cells or cancer cells, may have a particular role in cancer (Mirlekar and Pylayeva-Gupta, 2021). Research has shown that the expression of IL-35 in peripheral blood mononuclear cells of patients infected by invasive ductal carcinoma (IDC) is firmly organized as compared with the same cells in healthy individuals with the same age (Hamidinia et al., 2015). Additionally, TIL (tumor infiltrating lymphocytes) IL-35 expression is linked to TNM (tumor nodes metastasis) stage and metastasis in patients with IDC, although cytokine expression and stage metastasis have not been linked (Zhao et al., 2017).

High plasma levels of IL-35 and incremented expression of IL- 35 in the tumor microenvironment (TME) denotes destitute prediction in sundry malignancies, such as pancreatic ductal adenocarcinoma, acute myeloid leukemia. IL-35 is ordinarily generated in malignant tissues by some cancer cells and Treg cells. Tumor-infiltrating dendritic cells have been testified to express EBI3. Thus, dendritic cells are also considered as sources of IL-35 in the TME. A number of studies have shown that IL-35 inhibits the antitumor activity of lymphocytes, which increases the cumulation of myeloid cells and ameliorates angiogenesis and lastly procures to the expansion of tumor cells and cancer development (Long et al., 2016).

Genetic Polymorphism of Cytokine Genes
IL-35 (IL12 A + EBI3)
IL-12 A rs582054 ACT

Gene polymorphism of the studied cytokine (IL35) were done by ARMS-PCR and identified in benign, malignant BC patients and controls with six genotypes (AA, AC, AT, CC, CT & TT) derived from Erbil city women. Tables 5 & 6, demonstrate clearly the consequences of allele precise PCR for the SNP of IL-12 ACT in BC infected women and monitors, correspondingly.

Moreover, non-significant departures from H-W equilibrium were recorded in benign, malignant BC patients and controls. While, the results demonstrated significant difference for both benign, malignant BC patients versus the controls for the heterozygous mutant genotype AT (95% CI = 1.02 to 4.40 & 0.90 to 3.92) at $P \leq 0.05$). Heterozygous AT genotype of 12 A rs582054 ACT SNP can be of risk nearly two-fold (RR was 2.12 for benign and 1.88 for malignant BC) as compared to control groups, and makes it as a risk genotype for BC, which means a higher susceptibility of this genotype carriers to get the disease. As well, according to the genotypes, high producers (CT & TT) were found higher in the control group than in patient groups, this means the carriers of genotypes CT & TT are strongly protective (preventive) factor for BC progression, while high producers AA were found in the benign breast disease as preventive factor. Accordingly, the etiologic fraction of allele A as a risk factor for benign & malignant BC are proposing that this allele deliberated enlarged danger for breast infection improvement in Erbil women, as shown in table (6). Nevertheless, variant alleles C & T were more frequent in control than in breast patient groups, and may be considered as a preventive fraction and the gel electrophoresis of PCR products for patients genotyping is shown in fig. 1.

Table 5. Observed numbers and proportion occurrences and H-W stability of *IL-12 ACT* genotypes and alleles in benign, malignant BC patients and controls.

Groups			***IL12 ACT Genotype* or Allele**									**H-W**
			AA	**AC**	**AT**	**CC**	**CT**	**TT**	***A***	***C***	***T***	**P ≤ 0.05**
	Observed	**No.**	10	11	27	5	12	5	58	33	49	
		%	14.28	15.71	38.57	7.14	17.14	7.14	41.43	23.57	35	

BENIGN (N=70)	**Expected**	**No.**	7.82	12	25	9.38	13	2.81	Not Estimated			Not Significant
		%	11.17	17.14	35.71	13.4	18.57	4.01				
Malignant (N=70)	**Observed**	**No.**	5	11	25	6	18	5	46	41	53	Not Significant
		%	7.14	15.71	35.71	8.57	25.71	7.14	32.86	29.29	37.85	
	Expected	**No.**	4	10.23	26.54	8	17.23	4	Not Estimated			
		%	5.7	14.61	37.91	11.43	24.61	5.74				
CONTROLS (N=70)	**Observed**	**No.**	6	10	16	7	23	8	38	47	55	Not Significant
		%	8.57	14.28	22.86	10	32.86	11.43	27.14	33.57	39.29	
	Expected	**No.**	4.3	6.61	22.77	10.4	19.62	6.3	Not Estimated			
		%	6.14	9.44	32.53	14.86	28.03	9				

Table 6. Statistical appraisals of associations between IL-12 ACT genotypes or alleles and benign, malignant BC patients.

IL12 ACT Genotype or Allele	Statistical Evaluations/ Benign				Statistical Evaluations/ Malignant			
	Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals	Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
AA	1.78	0.06	NON-SIGNIFICANT	0.61 to 5.15	0.82	0.02	NON-SIGNIFICANT	0.24 to 2.80
AC	1.12	0.02	NON-SIGNIFICANT	0.44 to 2.81	1.12	0.02	NON-SIGNIFICANT	0.44 to 2.81
AT	2.12	0.20	SIGNIFICANT	1.02 to 4.40	1.88	0.17	SIGNIFICANT	0.90 to 3.92
CC	0.69	0.03	NON-SIGNIFICANT	0.21 to 2.28	0.84	0.02	NON-SIGNIFICANT	0.27 to 2.63
CT	0.42	0.19	SIGNIFICANT	0.19 to 0.93	0.71	0.1	NON-SIGNIFICANT	0.34 to 1.46
TT	0.6	0.05	NON-SIGNIFICANT	0.19 to 1.91	0.6	0.05	NON-SIGNIFICANT	0.19 to 1.91
A	1.9	0.20	SIGNIFICANT	1.15 to 3.13	1.31	0.08	NON-SIGNIFICANT	0.79 to 2.19
C	0.61	0.13	SIGNIFICANT	0.36 to 1.03	0.82	0.06	NON-SIGNIFICANT	0.50 to 1.36
T	0.83	0.06	NON-SIGNIFICANT	0.51 to 1.35	0.82	0.02	NON-SIGNIFICANT	0.24 to 2.80

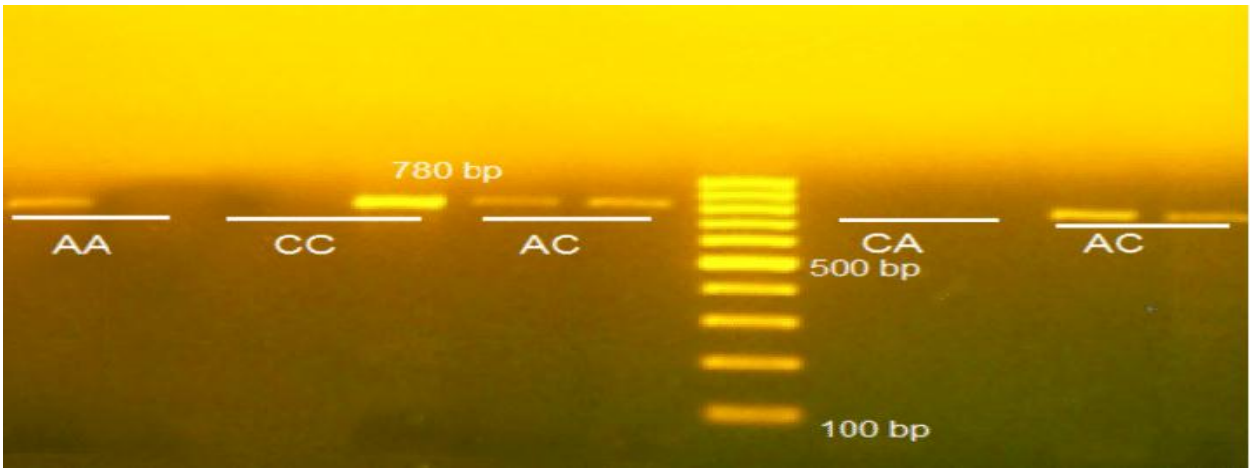

Figure1. Gel electrophoresis visualized under UV light of IL-12 A rs582054 ACT using ARMS-PCR.

To date, researchers have determined which subgroups of benign and malignant breasts express the most or least of IL12 A ACT. There were variations in the way people responded to the stimuli, which affected the amount of cytokine produced. Different polymorphisms in the promoter regions of the gene influence this shift, which can increase a person's susceptibility to cytokine-induced chronic inflammation. Pro-inflammatory cytokines in the tumor microenvironment have been related to a poor prognosis in breast cancer patients, according to research (Ali et al., 2020). In addition, SNPs, for example, may disrupt normal gene expression patterns and lead to inefficient immune responses, which in turn elevate cancer risks.

Although certain polymorphisms have been associated with cancer risk in the past, the relationship has now been lost as a result of interactions with other genetic and environmental factors.

IL12 A rs583911 G/A

In position IL-12 G/A which was associated with three genotypes (GG, GA and AA) in benign, malignant BC patients and controls. Hardy-Weinberg (H-W) equilibrium testing showed that benign breast disease patients and controls presented a substantial (P<0.05) variation in the distribution of IL12A G/A genotypes.

However, according to the results of the current investigation, the wild type (reference) IL12-A rs583911 genotype was GA, whereas the variant (mutant) genotype was AA, which was shown to be the least common. There was no significant difference between the observed and anticipated distribution according to Hardy Weinberg equation (P = 0.05) when considering the control group's IL12-A rs583911 genotypes of GG, GA, and AA, which were 10, 59, and 1, respectively (7).

Nonetheless, the prevalence of allele G varied substantially across the study groups (Benign breast disease and BC) and control groups (P=0.05). Allele G is a risk factor for malignant BC, with a relative risk of 2.96 and a 95 percent confidence interval of 1.75 to 5.00. The etiologic fraction of allele G as a risk factor for malignant BC was therefore 0.52, as shown in the table 8. Despite this, variation allele A was more predominant in the control cluster than in the breast cancer patient cluster, indicating that it might be a preventive factor.

Table 7 shows the number and percentage frequencies in benign and malignant BC patients, as well as controls.

Groups			***IL12 G/A* Genotype or Allele**					**H-W**
			GG	**GA**	**AA**	***G***	***A***	**P ≤ 0.05**
BENIGN	**Observed**	**No.**	16	53	1	85	55	Significant
		%	22.86	75.71	1.43	60.71	39.29	
		No.	25.8	33.4	10.8			

(N=70)	**Expected**	**%**	36.86	47.71	15.43	Not Estimated		
Malignant (N=70)	**Observed**	**No.**	41	29	0	85	55	Not Significant
		%	58.57	41.43	0	60.71	39.29	
	Expected	**No.**	44	23	3	Not Estimated		
		%	62.86	32.86	4.29			
CONTROLS (N=70)	**Observed**	**No.**	10	59	1	79	61	Significant
		%	14.29	84.29	1.43	56.43	43.5	
	Expected	**No.**	22.3	34.4	13.3	Not Estimated		
		%	31.86	49.14	19			

Table 8. Statistical evaluations of associations between IL12 G/A genotypes and alleles in benign, malignant BC patients and controls.

IL12 G/A Genotype or Allele	Statistical Evaluations/ Benign				Statistical Evaluations/ Malignant			
	Relative Risk	**Etiological or Preventive Fraction**	**Fisher's Exact Probability**	**95% Confidence Intervals**	**Relative Risk**	**Etiological or Preventive Fraction**	**Fisher's Exact Probability**	**95% Confidence Intervals**
GG	0.21	0.46	Significance	0.10 to 0.43	8.48	0.51	Significance	3.75 to 19.17
GA	1.72	0.35	Not significance	0.74 to 3.98	0.13	0.73	Significance	0.06 to 0.29
AA	1	0	Not significance	0.06 to 15.99	0.33	0.01	Not significance	0.01 to 8.02
G	0.84	0.09	Not significance	0.52 to 1.35	2.96	0.52	Significance	1.75 to 5.00
A	1.17	0.07	Not significance	0.74 to 1.92	0.34	0.28	Significance	0.20 to 0.57

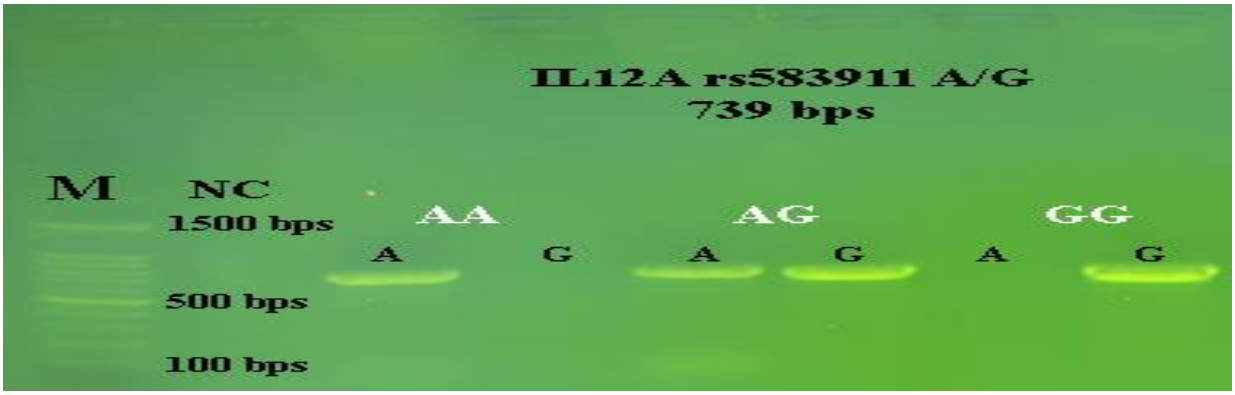

Figure 2. IL-12 G/A product of ARMS-PCR on agarose gel (2%) amplicon size (739 bp): M: 100bp size DNA ladder.

However, there are a few studies that have examined the relationship between IL-35 SNPs and the risk of breast cancer. As a result, there is no debate on how to account for substantial variations in genotype and allele frequency among patients with BC (heterozygous or homozygous). The 3'UTR of the IL-12 coding gene contains the G/A SNP. However, even in the absence of protein-coding material, it is still possible to influence the amount of translated protein via altering mRNA stability and transcriptional and posttranscriptional activity. (Matoulkova et al., 2012)The SNP may affect gene silencing and IL-12 mRNA expression (Kårvatn et al., 2012).

Conversely, in a research by Chang and his team in 2010 (Chang et al., 2010), only IL12A rs583911 remained significant after accounting for multiple testing (odds ratio for each copy of the variation G allele = 1.52, 95 percent confidence interval: 1.25–1.85, p = 2.95 $x10^{-5}$). A higher risk of BC in women was shown to be associated with the allele G variation. According to these findings, there was allelic diversity in the IL-12 a gene among people with BC.

Additionally, a study by (Pistiner et al., 2008), showed the immunological sensitivity to cockroach antigen may be caused by a mutation in intron 2 situated 739 base pairs distant from rs583911. Fine mapping and functional investigations are needed to establish causative variations, and it appears that there is some significance to IL12A function in the area surrounding rs583911.

As well, further study has demonstrated that the TME promotes the activation of Treg cells while suppressing conventional T cells, normal T cells were converted to Treg cells when exposed to IL-35 and Similar data have been published by another researcher, which corroborate the existence of IL-35 in TME while besides highlighting its exceptional role in carcinogenesis and correlated angiogenesis (Xue et al., 2019).

Interleukin-EBI3 rs428253 G/C

The EBI3 (Epstein-Barr virus induced 3) gene located on chromosome 19p13.3, its gene synonyms are IL27B, IL35B (Gene Cards, 2021) and was investigated at position rs428253 which was presented with three genotypes (GG, GC and CC) in benign, malignant BC patients and controls. The genotype distribution for each polymorphism was all in agreement with HWE (P>0.05).

In addition to that, while GC is the most common IL12-A rs583911 genotype, CC is the least frequent, thus CC will be regarded as the variant (mutant) genotype. As indicated in table (3.5) and Fig. 2, the frequency distribution of IL12-A genotypes GG, GC, and CC in the control group was 20, 34, and 16, respectively, and this observed distribution did not differ substantially from that anticipated by the Hardy-Weinberg equation (P0.05).

Correspondingly, the malignant BC group (P 0.10) varied substantially from the control group for allele G. The 95 percent confidence interval for allele G is between 1.75 and 5.00, and its etiologic fraction as a risk factor for breast cancer is 0.52. Colon cancer risk is increased by 296% when this allele is present, compared to a control group that has an odds ratio of 2.96. as shown in the table 10. While this is true, variation allele C was more prevalent in the healthy control group than in the malignant BC group (47.14% vs. 41.43%), and may be considered a preventive factor (Tables 9 and 10).

Table 9. Observations of EBI3 genotypes and alleles in benign and malignant BC patients as well as controls.

Groups			***EBI3* Genotype or Allele**					**H-W**
			GG	**GC**	**CC**	**G**	**C**	**P ≤ 0.05**
BENIGN (N=70)	**Observed**	**No.**	14	46	10	74	66	Significant
		%	20	65.71	14.29	52.86	47.14	
	Expected	**No.**	19.55	34.88	15.55	Not Estimated		
		%	27.93	49.83	22.21			
Malignant (N=70)	**Observed**	**No.**	29	24	17	82	58	Significant
		%	**41.43**	**34.29**	**24.29**	**58.57**	**41.43**	
	Expected	**No.**	24	34	12	Not Estimated		
		%	34.29	48.57	17.14			
CONTROLS	**Observed**	**No.**	20	34	16	74	66	Not Significant
		%	28.57	48.57	22.86	52.86	47.14	
	Expected	**No.**	19.55	34.88	15.55			

(N=70)		**%**	27.93	49.83	22.21	Not Estimated	

Table 10. Statistical evaluations in individuals with benign, malignant, or BC conditions, genomic and allele analyses revealed a link to the EBI3 gene.

EBI3 **Genotype or Allele**	**Statistical Evaluations/ Benign**				**Statistical Evaluations/ Malignant**			
	Relative Risk	**Etiological or Preventive Fraction**	**Fisher's Exact Probability**	**95% Confidence Intervals**	**Relative Risk**	**Etiological or Preventive Fraction**	**Fisher's Exact Probability**	**95% Confidence Intervals**
GG	**8.48**	**0.51**	**Significance**	**3.75 to 19.17**	0.63	0.1	**Not significance**	0.29 to 1.36
GC	**0.13**	**0.73**	**Significance**	**0.06 to 0.29**	**2.03**	**0.33**	**Significance**	**1.03 to 3.99**
CC	0.33	0.01	Not significance	0.01 to 8.02	0.56	0.1	**Not significance**	0.24 to 1.34
G	**2.96**	**0.52**	**Significance**	**1.75 to 5.00**	1	ref	**Not significance**	0.63 to 1.60
C	**0.34**	**0.28**	**Significance**	**0.20 to 0.57**	1	ref	**Not significance**	0.63 to 1.60

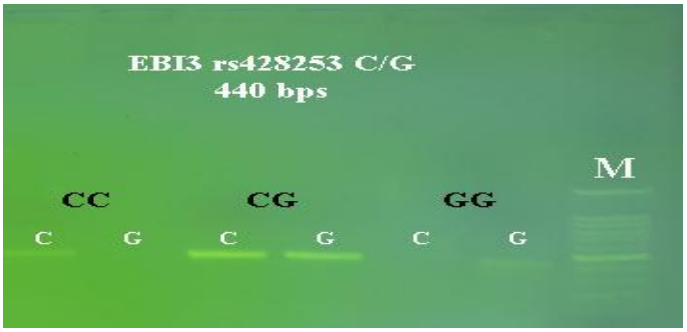

Figure 3. EBI3 rs428253 C/G product of ARMS-PCR on agarose gel (2%) amplicon size (440 bp): M: 100bp size DNA ladder

The outcomes of the existing research regarding the relative risk of EBI3 with breast cancer are designated that the manifestation of EBI3 was meaningfully lower in the BC infected women than in the corresponding control groups ($P < 0.05$), and not compatible with those reviews that done till now.

This study also looked at the medical effectiveness of EBI3 in predicting prognosis in patients with BC-breast cancer. The survival research found that cancer patients with higher EBI3 expression had a worse probability of surviving. Results also indicated that the level of EBI3 expression alone was a predictor for BC patients. In contrast, no other experimental variables were linked with an increased overall survival in breast cancer patients. The current study looked at the prognostic value of EBI3 in women with breast cancer. Additionally, researchers found that EBI3 expression patterns and functional participation in the development of BC were both revealed in this study.

Furthermore, as a prognostic biomarker for breast cancer, EBI3 has been proven to be useful; but it is yet unknown what particular molecular mechanisms underlie it, necessitating more study. In addition, inflammatory factors have been found to be important in the development of inflammatory breast cancer (Jiang and Liu, 2018), and IL-35 is a widely investigated inflammatory aspect associated to innumerable inflammatory syndromes (Choi et al., 2015). As a consequence, EBI3 might have a role in the progression of inflammatory BC, although this has to be shown in future research (Wolfe et al., 2016).

All the same, EBI3 expression was shown to be decreased in breast cancer patients compared to healthy women, and this was linked to tumor growth. This low level of EBI3 expression has high predictive value and might be utilized in the treatment of breast cancer (Jiang and Liu, 2018).

Conclusions

This project has focused on the relationship of three single nucleotide polymorphisms of interleukin -35 for recognition and expectation of women with BC in Erbil city. Overall, it has been concluded that malignant BC risk was found to be associated with the IL-35 gene. IL12 G/A, IL12 ACT, and EBI3 are all SNPs linked with this gene. Even if you are young or slim, there are a few factors to keep in mind regarding your serum IL-35 level (BMI). Furthermore, polymorphisms in major interleukin genes (IL12 ACT, IL12 G/A, and EBI3) were found to have a substantial impact on breast cancer susceptibility or severity. According to this study's findings, the IL12B rs3212227 SNP major allele A contributes to an elevated risk of human breast cancer development, but the minor C allele may have a protective role in BC.

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