



## Sera Level and Polymorphism of Interleukin-33 Gene in Iraqi Females Patients with Breast Cancer

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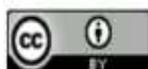
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Article history: Received 29May 2020, Accepted 31August 2020, Published in January 2021

Doi: [10.30526/34.1.2806](https://doi.org/10.30526/34.1.2806)

### Abstract

Interleukin-33 [IL-33] is a specific ligand for the ST2 receptor, and a member of the IL-1 family. It is a dual-function protein that acts both as an extracellular alarmin cytokine, and an as an intracellular nuclear factor participates in maintaining barrier function by regulating gene expression of IL-33 modulating tumor growth and anti-tumor immunity in cancer patients. The present study aimed to investigate the role of IL-33 serum level and gene polymorphism in Iraqi women with breast cancer. **Materials and methods:** Blood samples were collected from 66 Iraqi patient women diagnosed with breast cancer, which were divided into two groups: pre-treatment [PT] and under treatment with chemotherapy [UTC] patients in addition to 34 apparently healthy women who were matched with patients as a control. ELISA technique was used to determine the IL-33 serum level. The PCR-RFLP technique was performed to determine IL-33 gene polymorphisms at single nucleotide polymorphism SNP [rs1929992]. **Results:** IL-33 serum level recorded lower significant in PT [ $182.22 \pm 29.86$ pg/ml] and UTC [ $129.87 \pm 45.11$ pg/ml] patients compared with control [ $258.08 \pm 39.54$ pg/ml] under [ $p < 0.05$ ]. In a polymorphism study on IL-33 SNP [rs1929992] showed AG genotype recorded the high frequency in control than in patients [76.47, 58.46] compared to other genotypes AA, GG with no significant difference according to fisher's exact probability. **In conclusion:** IL-33 serum level decreased after performing surgery on breast cancer patients. There was no association between breast cancer development and IL-33 SNP [rs1929992]. The heterozygous genotype AG was a common genotype in the Iraqi population. Allele G had an environmental fraction, while allele A had preventive fraction, which related



to increasing the level of IL-33 with both genotypes AA, AG in the healthy control group, in contrast with GG genotype which showed the highest level in the patients' group.

**Keywords:** Breast cancer, IL-33, IL-33 gene, IL-33 Polymorphism.

## **1. Introduction**

Breast cancer is considered the most important and frequently cancer among women worldwide and the leading cause of cancer-related deaths in developing countries [1]. In Iraq Breast cancer was considered the most common cancer and there were 4529 cases in 2013 considered 4422 females and 107 males, the percentage of total constitute around 18.84% with rate 12.9 for each 100000 populations, and considered the commonest ten cancers in Iraq for the year 2013 [2]. A few recent studies indicated a potential role of IL-33 in cancer by tumor growth and modulating anti-tumor immunity in breast cancer patients [3]. IL-33 is a member of the IL-1 family, playing important roles in inflammation, immune regulation, host defense and neuronal injury [4]. The human IL-33 gene was located on chromosome 9p24.1 [5]. Including 10 introns and 11 exons [seven coding exons], which produced protein of 270 amino acids with a weight of 31 kDa. The IL-33 N-terminal domains required for nuclear localization, encode by exons 1–3, whereas the C-terminal IL-1-like cytokine domain encode by exons 4–7. Recently, a number of the IL-33 gene polymorphisms have been identified, and the relationships between some diseases and the IL-33 gene polymorphisms, such as rheumatoid arthritis and systemic sclerosis have been reported [6, 7, 8]. In 2003 IL-33 was identified as a nuclear protein, highly expressed in high endothelial venules [HEV], and initially named NF-HEV [nuclear factor from HEV] [9], and identified as a ligand for ST2 the orphan IL-1 family receptor in 2005 [5].

IL-33 was released upon cell necrosis and it drives inflammation as a DAMP [damage associated molecular pattern]; IL-33 is expressed by many cell types and precursor form is enzymatically processed and can regulate gene transcription [10]. It is released from producing cells upon cellular stress or cellular damage, and appears to function as an alarm signal rapidly. It is constitutively expressed in lymphoid organs and epithelial barrier tissues. It plays an important role in innate immunity [11]. It is a dual-function protein that acts both as an extracellular alarming cytokine that has a crucial role in the regulation of adaptive and innate immune responses, wound healing, tissue repair. As well as the initiation of the acute local inflammation, and other function as intracellular nuclear factor participates in maintaining barrier function by regulating gene expression [12]. IL-33 binding to its heterodimeric receptor ST2 and IL-33 accessory protein, induces synthesis of various cytokines such as IL-5 and IL-13. IL-33 plays various but important roles in immune-mediated diseases such as autoimmune diseases, infection and allergy [13]. IL-33 contributes to enhanced immunosuppressive function and the abnormal expansion of MDSCs [myeloid-derived suppressor cells] within the microenvironment of tumor, which can be probably targeted to reverse MDSC-mediated tumor immune evasion, MDSCs mediated immune dysfunction is an important mechanism that leads to tumor immune escape and the inefficacy of cancer immunotherapy [14].

The IL-33/ST2 axis has been implicated in numerous disease states, including cancer and asthma [15, 16], Alzheimer's disease [17] and Inflammatory bowel diseases [18]. Some evidence is supportive of a critical role for the IL-33/ST2 axis in the initiation and

maintenance of wound healing responses [19]. Initially the link between cancer and IL-33/ST2 axis was identified in breast cancer [20]. IL-33/ST2 signaling inducing the pro-tumorigenic cytokine IL-6, possibly contributes to intestinal tumorigenesis in mice [21]. IL-33/ST2 pathway facilitated expression of pro angiogenic vascular endothelial growth factor [VEGF] in tumor cells and attenuating tumor necrosis and that critically involved in the growth of breast tumor [22]. The aim of the present study is the association of IL-33 serum level and polymorphism with breast cancer in Iraqi women suffers from breast cancer.

## 2. Materials and Methods

Blood samples were collected from 66 Iraqi patient women diagnosed with breast cancer [underwent surgery] from the Oncology Teaching Hospital of the Medical City. Samples divided into two groups pre-treatment [PT] and under treatment with chemotherapy [UTC] patients [surgery was performed to remove the tumor], during the period from September 2017 to December 2017, and 34 healthy women were matched with patients in age and gender. The volume of 5 ml of whole blood samples was collected under sterilized condition by using disposable Syringe then distributed to two parts 2 ml in EDTA tube and 3 ml in Gel tube left for half an hour, then centrifuged for 15 minutes at 3000 RPM Serum was transferred into 2ml Eppendorf tubes and stored at -20 C for further analysis. The serum level of IL-33 measured by using an ELISA kit [Human IL-33 ELISA].

### 2.1. DNA Extraction and Polymorphism Genotyping

Total genomic DNA extracted from the whole blood was applied using genomic DNA extraction kits [Geneaid, Taiwan]. Then, DNA concentration and purity were measured by nanodrop. DNA bands were visualized using UV light after electrophoresis in a 1% agarose gel in 75 volts for 1 hour. Extracted DNA samples were stored at -20°C for further used. The polymerase chain reaction [PCR] performed in a 25µl reaction mixture, pre-mix 5µl [Bioneer, Korea], 2µl DNA, 2µl of each primer and 11µl of distilled water. The primer sequence of IL-33 SNP [rs1929992] was shown in **Table 1**. The program of PCR reaction was shown in **Table 2**. The length of PCR products was 217bp. The polymerase chain reaction- restriction fragment length polymorphism [PCR-RFLP] method was performed to determine IL-33 gene polymorphisms at rs1929992. The product of PCR restricted with restriction enzyme SspI [New England Biolabs, England]. The results observed, three genotypes were in the homozygote [GG] without any digest 217bp, in the heterozygote [AG] three different fragments with 217, 134 and 83bp, while in the homozygote [AA] two different fragments 134 and 83bp.

**Table 1:** sequence of the IL-33 SNP rs1929992 primers utilized in this study.

Sequences [5' →3']	Size	References
<b>Forward:</b> 5`GAAGTCATCATCAACTTGAACC-3` <b>Revers:</b> 5`-GGATTGGAATCCCATGGTC-3`	217bp	23

Table 2: PCR amplification program IL-33 gene.

Steps	Temperature [C]	Time	No. Of cycles
Initial denaturation	95	10 minutes	1
Denaturation	95	30 second	35
Annealing	57	30 second	
Extension	72	30 second	
Final extension	72	5 minutes	1

### 3. Statistical Analysis

The Statistical Analysis System- SAS [2012] program was used for outcome of different factors of parameters in this study. Least significant difference –LSD test [ANOVA] was used to significant compare between means in study parameters [24]. WINPEPI computer programs [version 11.63] was used to calculate the statistical significance of P-value that was calculated by Odd Ratio as well as Fisher’s exact test. Hardy-Weinberg equilibrium was tested by chi-squared test that was used OEGE - Online Encyclopedia for Genetic Epidemiology studies [25].

### 4. Results and Discussion

Sixty-six samples have been collected from patients with breast cancer. The median age [ $<40$ ,  $40-50$ ,  $>50$ ] and the number and percentages were 14 [21.21%], 34 [51.51%], 18 [27.27%], respectively. The results of the present study showed that most of the patients were in age between [40-50 year] which represents the high frequency group 51.51%. The result of the present study which agreed with severed studies indicated that there was a dramatic increase of breast cancer incidence in Iraqi females over the age 40 years [26, 27, 28]. Age as a breast cancer risk factor and the increasing incidence of cancer in women over than 40 years may be due to increased chromosome damage as a result of the repeated divisions, which led to the accumulation of mutation in the DNA that led to cancer development and “the age-related increase in chromosomal damage occurred faster in women than in men” because of the increase level of aberrations, and increase in the level of X chromosome damage that was the leading contributor of aging in women [29, 30]. During the period from 1991 to 2000 in Iraq in the Basrah city, the mean age of women with breast cancer was 45 years and no change in the age distribution in the 10year period [31].

#### 4.1. Interleukin-33 [IL-33] concentration in serum.

As shown in **Figure 1** which illustrated the IL-33 serum level for the studied groups as compared with control, the results of the mean  $\pm$  SE for the PT and UTC groups were  $182.22 \pm 29.86$ ,  $129.87 \pm 45.11$  pg/ml, respectively, as compared to control  $258.08 \pm 39.54$  pg/ml where there was a significant difference under  $[p<0.05]$ .

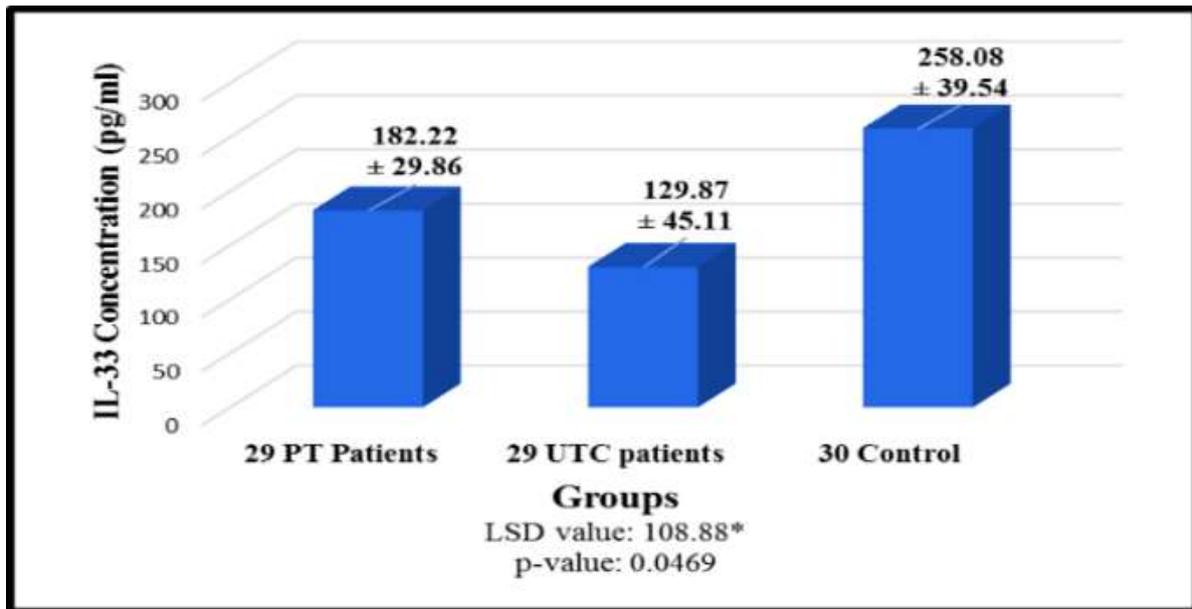
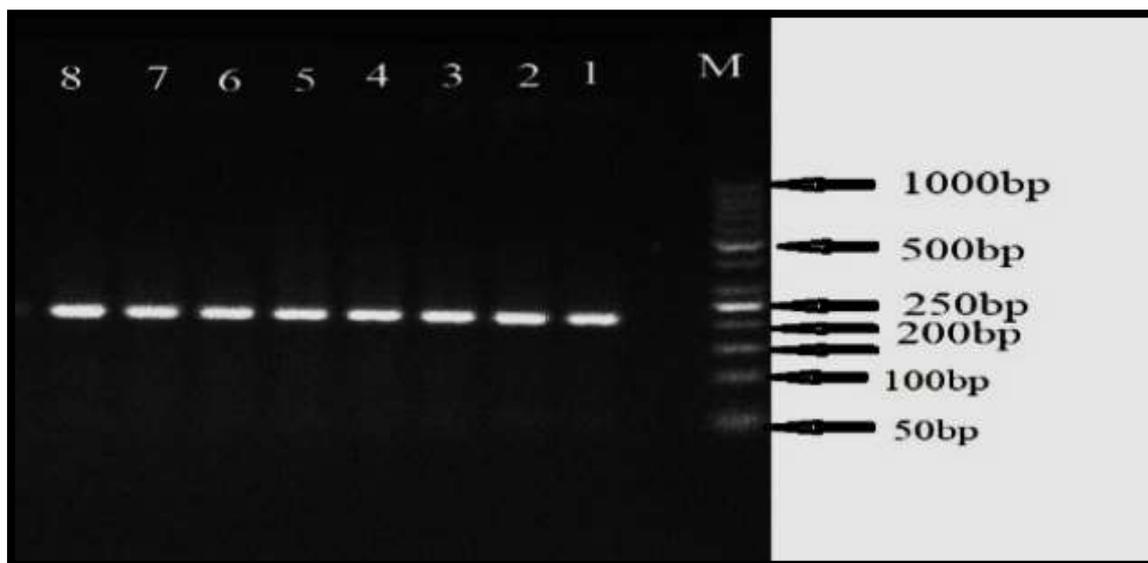


Figure 1: Comparisons between different groups in IL-33 concentration [pg/ml].

The results of the current study showed a low level of IL-33 in serum of PT, followed by UTC patients compared to the high level in the serum of healthy control. This result agreed with another study indicated that IL-33 serum levels was decreased after modified radical mastectomy in breast cancer patients [32]. While it was disagreed with other studies which found an elevating in IL-33 serum level in breast cancer patients [23, 33, 34]. Moreover Saranchova *et al.* indicated that the cancer cell evolved over time and suffered genetic changes and it through their developers lose the ability to manufacture IL-33 and reduce its expression in many metastatic carcinomas and because that immune system loses the ability to distinguish cancer cells and their start transformation and metastasis, releasing of IL-33 serum level may help the immune system to recognition malignant tumor cells by expression of MHC-1 molecules [35]. According to present result, low level of IL-33 in serum of current patients may relate to some limitation of the study which need a cohort study to follow the serum level of IL-33 in the same patients before and after mastectomy or resection surgery and then after treated with chemotherapy.

#### 4.2. IL-33 Gene Amplification

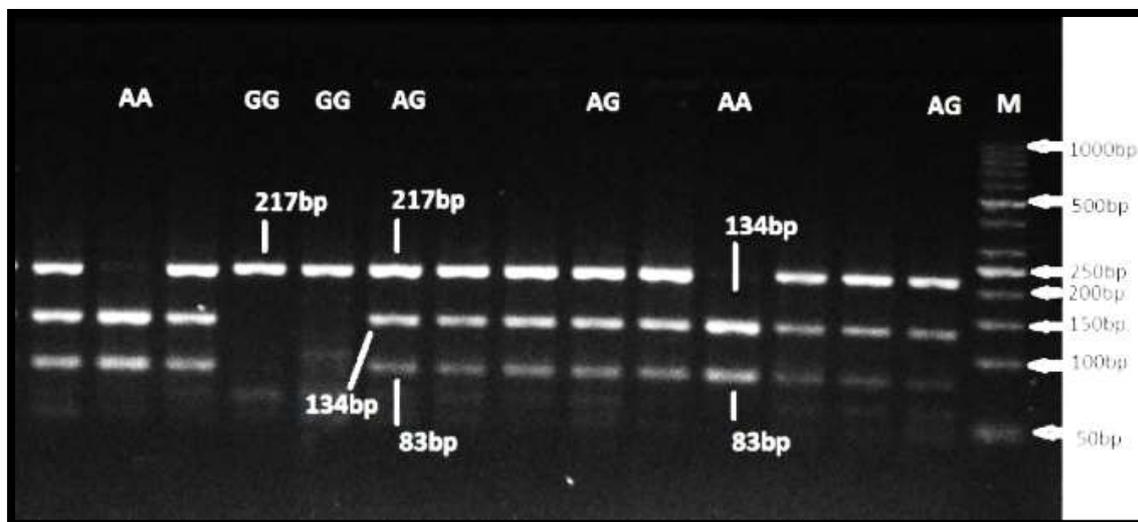
The region of single nucleotide polymorphism [SNP] [rs1929992] from *IL-33* gene was amplified from extracted DNA for each sample of patients with breast cancer and healthy control. By using specific primer, the polymerase chain reaction [PCR] was performed under optimum condition, then PCR product was electrophoresis on agarose gel [2%] in [100v, 1 hour] the results were a single clear band with molecular size [217bp] compare with DNA ladder **Figure 2**.



**Figure 2:** Gel electrophoresis for PCR product of *IL-33* gene [217bp] with DNA ladder [M] on agarose gel [2%] in [100v, 1 hour].

#### 4.2.1. Genotype of *IL-33* Gene

*IL-33* gene PCR product digested with *SspI* restriction enzyme [sequence of restrictions AAT/ATT in allele A]. The PCR-RFLP product recognized on gel electrophoresis as a homozygote [GG] 217bp without any digestion, in the homozygote [AA] two different fragments 134bp and 83bp, while three different fragments in the heterozygous form [AG] its 217bp, 134bp, 83bp were observed in **Figure3**.



**Figure 3:** Gel electrophoresis of PCR-RFLP product which illustrated genotype of *IL-33* SNP on agarose [3%] in [150v, 1hour]. The restriction process showed three types of genotype AA [134bp, 83bp], AG [217bp, 134bp, 83bp] and GG [217bp].

Genetic polymorphism of *IL-33* gene [rs1929992] that was observed with three genotypes [AA, AG, GG] as shown in **Table 3** which illustrated the distribution of genotypes of *IL-33* in patients and control groups. Heterozygous genotype AG showed a high frequency in the control [76.47%] than in patients [58.46%] with a highly significant difference between patients and the control. The Homozygote genotype AA frequency [23.08%] showed a non-significant difference in patients compared to the control [17.65%], and the Homozygote

genotype GG frequency [18.46%] had a significant difference in patients compared to control [5.88%]. Allele frequency for A allele was [52.31%] in patients compared to control [55.88%] while for the G allele [47.69%] in patients compared with control [44.12%] with no significant difference.

**Table 3:** distribution of genotype of IL-33 and allele frequency.

IL-33 Genotype	Patients NO [%]	Control NO [%]	Chi-square	P- value
AA	15 [23.08%]	6 [17.65%]	2.41 NS	0.039
AG	38 [58.46%]	26 [76.47%]	6.94**	0.0087
GG	12 [18.46%]	2 [5.88%]	5.27*	0.082
Allele frequency				
A	68 [52.31%]	38 [55.88%]	0.822NS	0.239
G	62 [47.69%]	30 [44.12%]	0.822NS	0.239

\* [P<0.05]: significant, \*\* [P<0.01]: highly significant

**Table 4:** Expected frequencies of genotypes and allele of the IL-33 using Hardy- Weinberg equilibrium.

Samples		AA	AG	GG	A	G	$\chi^2$
Patients	O.no [%].	15 23.08%	38 58.46%	12 18.46%	0.52	0.48	1.92
	E.no [%].	17.78 27.35%	32.43 49.89%	14.78 22.73%			
Control	O.no [%].	6 17.65%	26 76.47%	2 5.88%	0.56	0.44	10.32
	E.no [%].	10.62 31.23%	16.76 49.29%	6.62 19.47%			

\* $\chi^2 > 3.84$ : the expected frequencies of genotypes, results showed no significant differences in patients and significant difference in control under [p < 0.05] between observed and expected frequencies. [O; Observed. E; Expected].

Results of patients were agreed with expected Hardy- Weinberg equilibrium in patients, while in the control the result was not in agreement with expected Hardy- Weinberg equilibrium, and there was a highly significant difference between the observed and expected frequencies [ $\chi = 10.32$ ]. This deviate may due to the small sample size overlap marriages deviated from Hardy-Weinberg **Table 4**. Although the heterozygous represented the most common type in patients and control groups so, it is common in the Iraqi population.

The present study illustrated that the genotyping distribution of IL-33 [rs1929992] gene was the genotype AG, which showed the highest percentage in patients and control compared with genotypes AA, GG which recorded the lowest percentage in the patient and control, the chi- square recorded a non-significant difference for the AA genotype in patients compared to controls while genotype AG and GG recorded a highly significant difference. The results of the present study disagreed with the previous studies that showed the IL-33 [rs1929992] gene was as follows, the genotypes AG, AA showed a high percentage [46%, 39%], respectively, in patients compared with genotype GG which showed the lowest percentage [15%] in patients. While in the control the genotypes AG, AA showed a high percentage [38%, 49%], respectively, compared to other genotype GG which showed the lowest percentage [13%] in control [23].

In the statistical evaluations of *IL-33* gene between patients and control by using Fisher's test as shown in **Table 5**. The statistical analysis found that, in the patients the frequency of genotype AA recorded odd ratio [OR] [1.40] with a confidence intervals [CI] value between [0.49 - 4.31] under [95%] and showed etiological [ET] fraction of the diseases

[0.066] and it also showed a non- significant difference [0.525] according to fisher’s exact probability. The genotype AG recorded OR [0.43] with a CI between [0.16 – 1.10] under [95%] and showed a Preventive [PR] fraction [when OR less than one] of the diseases was [0.434] and it showed a non- significant difference [0.064] according to fisher’s exact probability. The frequency of genotype GG recorded OR [3.62] with a CI between [0.84- 24.94] under [95%] and showed ET fraction was [0.134] with a non- significant difference [0.079] according to fisher’s exact probability. The allele frequency of A showed OR [0.87] with CI rang between [0.48 – 1.57] under [95%] and PR fraction [0.075] with a non- significant difference [0.602], while allele G showed OR [1.15] with CI rang between [0.64 - 2.09] under [95%] and ET fraction [0.064] with a non- significant difference [0.602] according to fisher’s exact probability.

**Table 5:** The Statistical evaluations of *IL-33* gene between groups.

Genotype	OR	ET or PR	Fisher’s exact probability	CI 95 %
AA	1.40	0.066	0.525 NS	0.49 - 4.31
AG	0.43	0.434	0.064 NS	0.16 - 1.10
GG	3.62	0.134	0.079 NS	0.84- 24.94
Allele distribution				
A	0.87	0.075	0.602 NS	0.48 - 1.57
G	1.15	0.064	0.602 NS	0.64 - 2.09
NS: Non-Significant.				

The statistical evaluations of *IL-33* gene between patients and control by using Fisher’s exact probability showed that the genotype AG was a high frequency in studying groups and recorded odd ratio value [0.43] and also showed the preventive fraction in breast cancer patients, the genotypes AA, GG record as etiological fraction in breast cancer patients with more than one odd ratio [1.40, 3.62], respectively; the frequency of A allele record preventive fraction with odd ratio was less than one [0.87]. While fluency of G allele recorded an etiological fraction with odd ratios [1.15] with a non-significant difference in the frequencies of genotypes [AA, AG, GG] and alleles A and G at *IL-33* SNP [rs1929992] between breast cancer patients and healthy control. This result agreed with a previous study where there was no any association between breast cancer disease and *IL-33* SNP [rs1929992] [23]. As shown of the *IL-33*, polymorphism may have no influence of [rs1929992] on breast cancer risk. Another study on non-malignant disease [36] record *IL-33* rs1929992 polymorphism may be a potential biomarker for susceptibility to Systemic lupus erythematosus. According to the present study the conclusion was that AG genotype could be considered as a common type in the Iraqi population, while GG was a lower frequency genotype in the Iraqi population.

#### 4.3. Related Genotype with the Level of *IL-33* in Serum

The present study showed a comparison of *IL-33* level according of the *IL-33* genotype in patients and control groups. In patients the genotype AA showed a significant difference with a mean [142.27 ± 25.83] compared to control [340.07± 130.45]. As well as genotype AG recorded a significant difference in mean [136.82 ± 24.21] compared to control [242.53 ± 41.34]. While the genotype GG showed a non-significant increasing [238.27 ± 120.44] compared to control [183.24 ± 40.75] as shown in **Table 6**.

The result of the current study showed that the *IL-33* serum level was lowest in patients with genotypes AA, AG with a significant difference as compared to control than

patients with GG genotype and this result disagreed with the previous study, which demonstrated the mean serum level of IL-33 was higher in patients with the genotypes AA, AG than patients with the genotype GG and the difference not significant between patients and control [23].

**Table 6:** The comparison level of IL-33 concentration according to type of genotype in PT patients and control.

IL-33 A/G Genotype	Patients Mean $\pm$ SE	Control Mean $\pm$ SE	P- value
AA	142.27 $\pm$ 25.83	340.07 $\pm$ 130.45	0.0366 *
AG	136.82 $\pm$ 24.21	242.53 $\pm$ 41.34	0.0394 *
GG	238.27 $\pm$ 120.44	183.24 $\pm$ 40.75	0.082 NS
* [P<0.05], NS: Non-Significant.			

## 5. Conclusion

This study found that the IL-33 serum level decreased after surgery performed in patients with breast cancer. No association was found between breast cancer development and IL-33 SNP [rs1929992] in the Iraqi population, the heterozygous genotype AG was a common genotype in the Iraqi population. The G allele was an environmental effect allele, while allele A had preventive fraction, which was related with increasing level of IL-33 for both genotypes AA, AG in healthy control group, in contrast with GG genotype which showed the highly level in patients' group. This study needs a cohort study to follow the serum level of IL-33 in the same patients before and after mastectomy or resection surgery and then after treating with chemotherapy.

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