Role of Inhibitory Check Point (CTLA-4) in Iraqi Women with Breast Cancer

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Abstract

Breast cancer is the most important and frequently diagnosed cancer among women worldwide. The CTLA-4 is a member of Immunoglobulin super family and binds to the CD80 and CD86 co-stimulatory molecules. (binds to CD80/86 on APCs with a higher affinity than CD28, thereby inhibiting co-stimulatory signals. *CTLA-4* (Gene ID:1493, MIM number: 123890) is a new member of the immunoglobulin super family known as insulin-dependent diabetes mellitus 12 (IDDM 12) ,and cluster of differentiation 152 (CD152). The CTLA-4 protein consists of a 37 amino acid leader peptide, an extracellular immunoglobulin (Ig) V like domain or the ligand-binding domain (116 amino acid), a hydrophobic trans membrane region (37 amino acid), and a cytoplasmic domain. The aim of the present study of the association of CTLA-4 serum level and polymorphism with breast cancer in Iraqi women suffers from breast cancer. **Materials and Methods**: peripheral Blood samples were collected from 45 Iraqi patients women diagnosed with breast cancer patient, and 45 healthy women were matched with patients as a control. ELISA technique has been used to determine the serum level of sCTLA-4.The polymerase chain reaction (PCR) performed on 45 patients and 45 control, to determine the genetic variation in the +49 exon 1 region of *CTLA-4* gene. **Results**: the serum level of understudying groups recorded an non-significant difference increasing in the serum level of CTLA-4 under ($p>0.05$). in mean ± SE (3.17 ± 0.82 ng/ml) patients as compared to control (2.72 ± 0.29 ng/ml) under ($p>0.05$). Genetic polymorphism of CTLA.4 gene (rs231775) which illustrated the distribution of genotypes of CTLA.4 in patients and control. The heterozygous genotype AG recorded high frequency in patients (35.56%) than control (0.00%) with a highly significant difference under ($P<0.05$). Homozygote genotype AA recorded high frequency in control (95.56%) than patients (64.44) and has a highly significant difference, and the homozygous genotype GG frequency (0.00%) was non- significant in patients compared to control (4.44%). The allele frequency for allele A was (0.82%) in patients compared with control (0.96%) while for the frequency of allele G was (0.18%) in patients compared with control (0.04%) with a significant difference. **Conclusion**: elevation of serum concentration of sCTLA-4 could consider as a clinical biomarker for prognosis breast cancer. There are some difference noticed in CTLA-4 gene with SNP (rs231775A/G) which showed a significant differences between patients and control, A allele of SNP (rs231775A/G) may have role to prevent the risk of breast cancer in Iraqi females, while allele G has an etiological fraction.

Keywords: *CTLA-4, Iraq, Breast cancer, Women*

Introduction

Breast cancer is the most important and frequently diagnosed cancer among women worldwide in developing countries, it is considered the leading cause of cancer-related deaths . It is affecting women worldwide with an increasing incidence from (26.6/100 000) in the year of 2000 to (31.5/100 000) in 2009. Although it was reported that the disease incidence is higher in the developed countries than in the developing ones, however, in Iraq breast cancer incidence increased significantly from (30/100 000) to (40 /100 000) in the period between 2006 to 2012 [2].

Cluster of differentiation 152 (CD152), also known as cytotoxic T lymphocyte-associated protein 4 (CTLA-4), is a homologue of CD28, and functions as an inhibitor receptor for B7)CD80/CD86) which is a co-stimulatory molecule on mature antigen-presenting cells [3,4]. CD152 acts as a negative regulator of T cells involved in antitumor immune responses [5]., and its blockade can promote immune responses [6], and reject tumors [7].

The hypothesis has been put forward that CD152 may attenuate the antitumor responses and increase cancer susceptibility via elevating the activation threshold of T cells in early stage of tumorigenesis [8]. Cytotoxic T-lymphocyte antigen-4 expressed by Treg cells impairs maturation of APCs, such as DCs, by binding to CD80/86 [9]. The CTLA-4is amember of Immunoglobulin superfamily and binds to the CD80 and CD86 co-stimulatory molecules.(binds to CD80/86 on APCs with a higher affinity than CD28, thereby inhibiting co-stimulatory signals.However, CTLA4 can interact with B7 ligands with a 20-to 50-fold higher affinity than CD28 [10]. The balance between CD28 and CTLA4 signaling is important for the regulation of immune response. B7–CD28 interactions induce T-cell proliferation, differentiation and survival; in contrast, CTLA4 negatively affects proliferation and activation of T-cells [11]. For CTLA4 inhibitory effects on T-cell functions, it has to compete with the co-stimulatory CD28 molecule in tems of interacting with their commonB7 ligands [12]. It is believed that because regulatory T (Treg) lymphocytes, particularly bearing CD4+CD25+ forkhead box P3, and natural killer cells may cause cancer immune evasion through the suppresion of antitumor immune responses, blocking the activity of Tregs may improve the efficacy of tumor vaccines or immunotherapy of cancer [13].

In addition, CD80/86bound to CTLA-4 can be physically transferred from APCs to the surface or the cytoplasm of Treg cells by trogocytosis (14).*CTLA-4* (Gene ID:1493, MIM number:123890) is a new member of the immunoglobulin superfamily known as insulin-dependent diabetes mellitus 12 (IDDM 12) [15], and cluster ofdifferentiation 152 (CD152) [16]. It is mapped to chromosome 2q33 with a nucleotide size of about 6.2 kb and consists of four exons and 3 introns [17]. The CTLA-4 protein consists of a 37 amino acid leader peptide, an extracellular immunoglobulin (Ig) V like domain or the ligand-binding domain (116 amino acid), a hydrophobic transmembrane region (37 amino acid), and a cytoplasmic domain [118,19]. In this regard, significantly increased expression ofCTLA-4 protein and mRNA have been shown in individualscarrying thymine at position −318 of theCTLA4 promoter (T-318) and those homozygous foradenine at position 49 in exon 1 (20**).**Apart from polymorphism studies, a native soluble form of CTLA4 (sCTLA4), an alternate transcript of *CTLA4* gene encoding a protein without a transmembrane region, was described in human serum [(21, 22)]. It has important roles in immunoregulatory functioning [22,23]. The serum level of sCTLA4 increases in various autoimmune diseases [(21)], as well as in some cancer types, including breast cancer [24]. According to the all above the present study designed to evaluate the association of CTLA-4 serum level and its polymorphism with breast cancer in Iraqi patient women.

Materials and Methods

Blood samples were collected from 45 Iraqi patient women diagnosed with breast cancer from the Oncology Teaching Hospital of the Medical City, there were 45 patients with an age average (30-70), As well as 45 match ages of the apparently healthy women from different area of Baghdad, during the period from September 2019 to February 2020. The volume of 5 ml of peripheral blood samples was collected by disposable syringe and divided into 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 -20ºC for further analysis. The main data collected from patients in this study were: age, weight, tumor primary site, tumor type, stage and histological grade.Serum level of CTLA-4 was measured by using an ELISA technique (Human CTLA-4 ELISA Kit, , Elabscience, China).

DNA extraction and Polymorphism Genotyping

Total genomic DNA extracted from the whole blood was applied using G-spin™DNA Extraction Kit (Intron biotechnology, Korea). Then, DNA concentration and purity were measured by Nanodrop. DNA bands were visualized using UV light after electrophoresis in a 2% agarose gel in 100 volts for 1:30 hour. Extracted DNA samples were stored at - 20C^{o} for furthered used. The polymerase chain reaction (PCR) performed in a 25µl

reaction mixture, pre-mix 5µl (Intron, Korea), 2µl DNA, 2µl of each primer and 16µl of distilled water. The primer sequence of the CTLA-4 shown in (Table 1). The program of PCR reaction as shown in (Table 2). The length of PCR product was 162bp.Enzyme *BbvI* were used for detection of genotype using RFLP. Visualization of digestion products was under ultraviolet light after agarose gel electrophoresis. PCR conditions for *CTLA4* +49 A>G were initially a melting step of 45 s at 95˚C, then 35 cycles of 45 s at 94˚C, 45 s at 55˚C and 45 s at 72˚C, and finally an elongation step of 5 min at 72˚C. The PCR products (162 bp) were digested with *Bbv*I restriction enzyme (65˚C for 3 hours). The digested G allele yielded 88 bp and 74 bp fragments and undigested A allele yielded 162 bp fragment.

Table 1: sequence of the primers utilized in this study.

Primers	Sequences (5`→3`)	Product size	References
CTLA-4	Forward: 5` GCTCTACTTCCTGAAGACCT- 3`- Revers:5` AGTCTCACTCACCTTTGCAG- 3`-	162bp	25

Table 2: PCR amplification program CTLA-4 gene (rs 231775).

Steps	Temperature (ºC)	Time	No. of cycles
Initial denaturation	95 ºC	3 minutes	1 cycle
Denaturation	95 ºC	45 second	35 cycle
Annealing	55ºC	45 second	
Extension	72 ºC	45 second	
Final extension	72 ºC	7 minutes	1 cycle

Statistical Analysis

The data were examined for normality, homogeneity and normal distribution, mean ± SE of mean by using the IBM SPSS version 26.0 (26). The probability also examined by using student T-test and ANOVA table. For non-parametric data, Pearson's chi-square test used to calculate the probability. A Pearson's correlation used to determine the relationship between the studied parameters. For genotyping and alleles frequencies, the odd ratio, 95% confidence interval and Fisher's exact probability calculated by WinPepi version 11.65 (27). Such. Online Hardy-Weinberg calculator (28) used for genotyping and alleles frequencies calculations

Results and Discussion

Samples collected from patients with median age (<40, 40-50, >50) the number and percentages were 14 (21.21%), 34 (51.51%), 18 (27.27%), respectively. The results of the present study showed that, the most patients were in age between (40-50 year) represents the high frequency was group 51.51%. This study has been agreement with several studies which indicated that breast cancer was developing an increase in Iraqi females after the age 40 years (29, 30). During the period from 1991 to 2000 in Iraq the mean age was 45 years and no change in the age distribution in the 10year period (31). But this time breast cancer developed at an early age about 25 (personal communication).

1- sCTLA-4 concentration in serum.

As shown in (Figure1) which demonstrate the levels of CTLA4 in the serum for patient and control groups (Figure1) . The mean ± SE for patient group (3.17 ± 0.82 ng/ml), as compared to control (2.72 ± 0.29 ng/ml) there was a non-significant difference increasing in the serum level of CTLA-4 under (p>0.05).

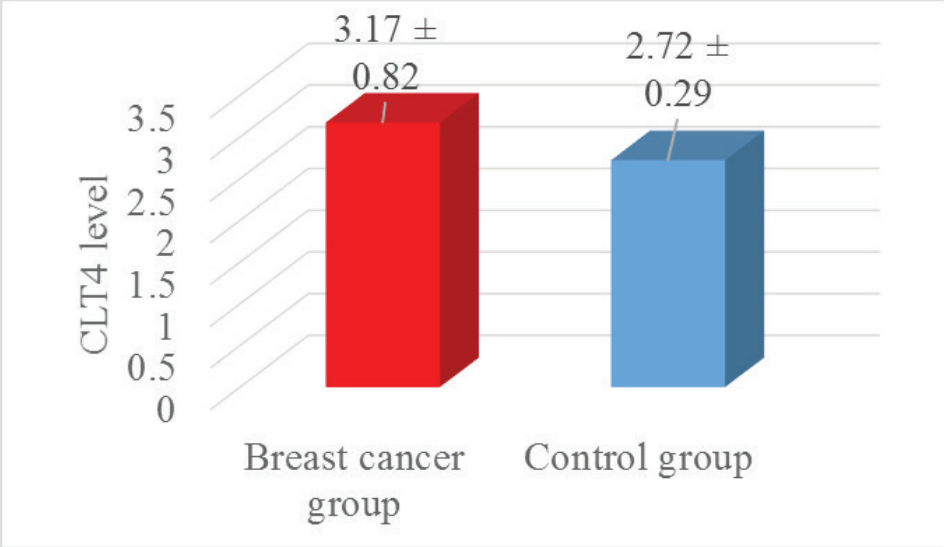

Figure: Serum level of sCTLA-4 in patients as compared with control..

These results were in agreement with several studies indicated that the elevation of serum concentration of sCTLA-4 may considered as a valuable indicator of prognosis in breast cancer (32,25).Another researcher pointed that sCTLA4 has been reported to play an imperative role in immune regulation by binding with the B7 molecules and inducing indoleamine2,3-dioxygenase enzyme activity in dendritic cells(33).indicated that parts of sCTLA4 function are also mediated through the ability of sCTLA4 to compete for B7, thus interfering withB7–CD28 ligation. Huurman*et al*. have recently indicated thatsCTLA4 is able to bind to APCs and inhibit the expression of CD80/CD86 as well (34).

2- *CTLA.4* Gene SNP(rs231775)Amplification

The region that contain single nucleotide polymorphism (SNP) (rs231775) from *CTLA.4* gene was amplified by PCR, using the extracted DNA of each sample of patients with breast cancer and control with a specific primer under optimum condition, then PCR product was electrophoresed on (2%) agarose gel, the result showed a single band for each sample with molecular size (162bp) as compared with DNA ladder bands, figure 2.

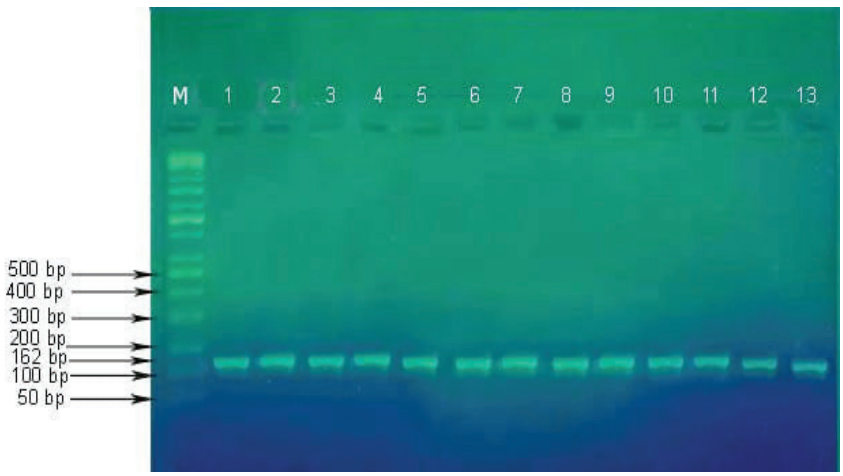

Figure (2): Gel electrophoresis for PCR product of *CTLA.4* gene (162bp) with (M) DNA molecular weight marker, samples (1-13) , 2%agarose gel (100v, 1:30 hour)

3- **Genotype of *CTLA.4* Gene SNP(rs231775)**

CTLA.4 gene PCR product digested with *BbvI* restriction enzyme, sequence of the restriction (5`-GCT/CCT-3`) in A allele (restriction fragment blunt ended). The polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) product recognized on gel electrophoresis as a homozygote (AA) 162bp without any digestion, in the homozygote (GG) two different fragments 88bp and 74bp, while threedifferent fragments in the heterozygous form (AG) its 162bp,88bp, 74bp observed in figure (3).

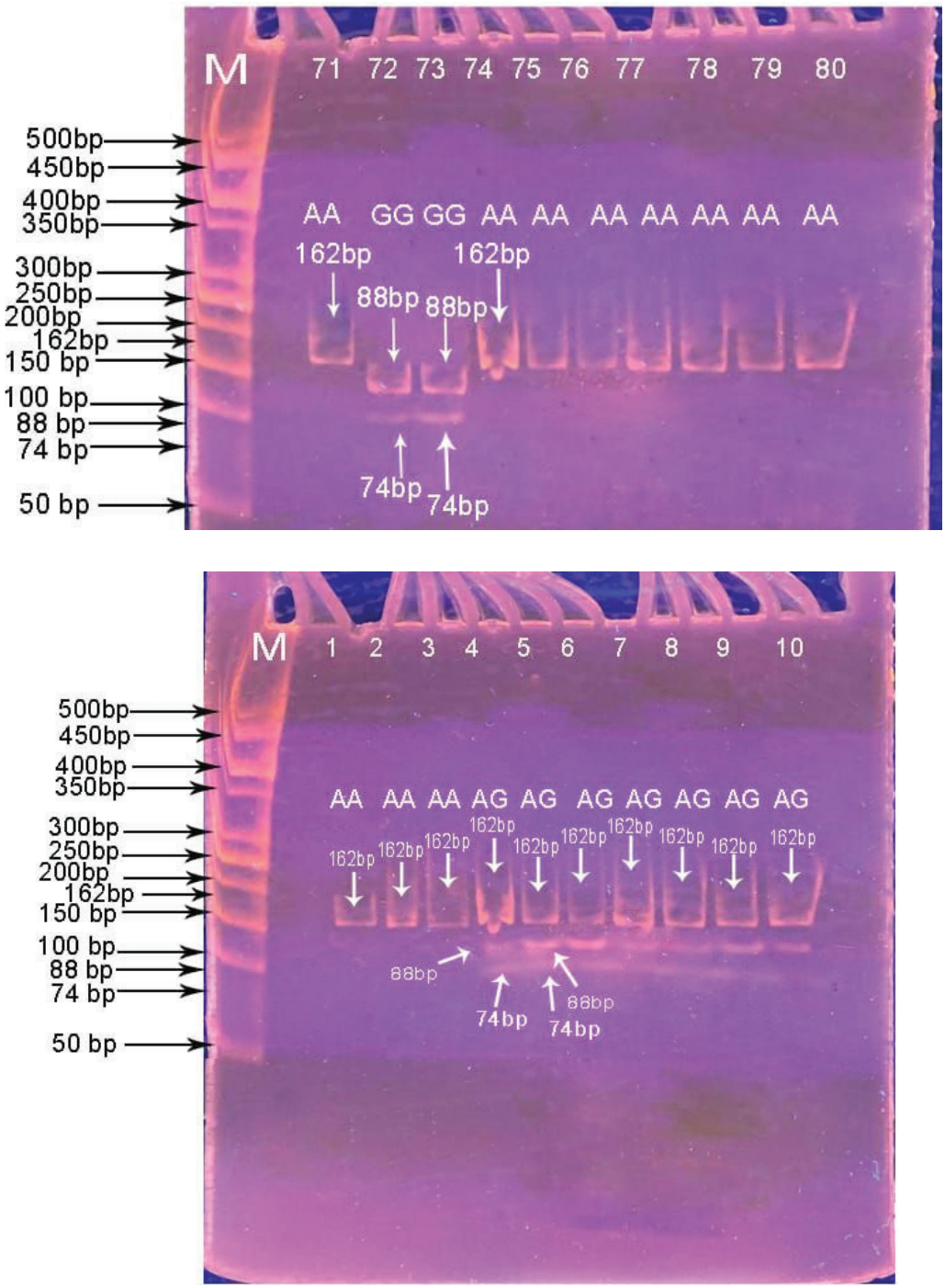

Figure (3): Gel electrophoresis of PCR-RFLP product which illustrated genotype of CTLA.4 SNP(rs231775) on agarose (3%) in (150v, 1hour). The restriction process showed three types of genotype GG (88bp, 74bp), AG (162bp, 88bp, 74bp) and AA (162bp).

Table(3): Distribution of genotype of CTLA.4 SNP(rs231775) and allele frequency.

CTLA.4 Genotype	Patients NO (%)	Control NO (%)	Pearson's chi-square	P- value
AA	29 (64.44%)	43 (95.56%)	13.611**	2.2 x 10-4
AG	16 (35.56%)	0 (0.00%)	19.459**	1.0 x 10-5
GG	0 (0.00%)	2 (4.44%)	2.045 NS	0.153
Total	45	45	---	---
Allele frequency				
A	74 (0.82%)	86 (0.96%)	8.10*	4.4 x 10-3
G	16 (0.18%)	4 (0.04%)	8.10*	4.4 x 10-3
* (P<0.05): significant, ** (P<0.01): highly significant, NS: non significant				

Genetic polymorphism of CTLA.4 gene (rs231775) which was observed with three genotypes (AA, AG, GG) as shown in (Table 3) which illustrated the distribution of genotypes of CTLA.4 in patients and control. The heterozygous genotype AG recorded high frequency in patients (35.56%) than control (0.00%) with a highly significant difference under (P<0.05). Homozygote genotype AA recorded high frequency in control (95.56%) than patients (64.44) which was a highly significant difference, and the homozygous genotype GG frequency (0.00%) which was a non- significant in patients compared to control (4.44%). The allele frequency for allele A was (0.82%) in patients compared with control (0.96%) while for the allele G was (0.18%) in patients compared with control (0.04%) with a significant difference. These results disagreed with(25) which founded that the frequency of homozygous (GG + AA) variants of CTLA4 +49 A>G was higher in patients with breast cancer than in controls, and no significant differences were found in the distribution of the genotype between patients and controls (p>0.05).These results agreed with(35).study on Iranian women patients with breast cancer demonstrated that the frequency of GG genotype was considerably decreased compared with controls.

Results of patients were agreed with expected Hardy- Weinberg equilibrium results in patients, while in control result was disagreed with expected Hardy-Weinberg equilibrium results, and there was a highly significant difference between observed and expected frequencies($2x10^{-12}$)This deviation may due to the small sample size overlap marriagesdeviated from Hardy-Weinberg (Table 4). Since the AA homozygousrepresent the common type in patients and control groups so it common in the Iraqi population.

Table (4): Expected frequencies of genotypes and allele of the CTLA.4 SNP(rs231775) using Hardy-Weinberg equilibrium

Genotypes		AA	AG	GG	A	G	P-HWE
Patients Genotypes	Observed no (%).	29 64.44%	16 35.56%	0 0.00%	74 0.82	16 0.18	0.1469
	Expected no (%).	30.42 67.60%	13.16 29.23%	1.42 3.16%			
Control Genotypes	Observed no (%).	4395.56%	0 0.00%	2 4.44%	86 0.96	4 0.04	2x10-12
	Expected no (%).	41.09 91.31%	3.82 8.49%	0.09 0.20%			

The statistical analysis as showed in (Table 5) it has been observed that, in patients, the frequency of genotype AA recorded odd ratio (OR) (0.08)with a confidence intervals (CI) value between (0.02-0.39) under (95%)and showed Preventive fraction (PR)(when the OR less than one) of the diseases. It showed a high significant difference (3.6×10^{-4}) according to Fisher's exact probability. The genotype AG recorded OR (50.90)with CI between (3.03-854.10) under (95%)and show etiological fraction(ET)(when OR more than one) of the diseases .It showed a high significant difference (6.0×10^{-6}) according to Fisher's exact probability. The frequency of genotype GG recorded OR (0.19) with CI between(0.01-3.96) under (95%)and showed Preventive fraction (PR)of the diseases. It showed a non- significant difference (0.494) according to Fisher's exact probability. The allele frequency of A showed OR (0.22)with CI rang between (0.07-0.67) under (95%) and showed Preventive fraction (PR)of the diseases. it showed a high significant difference (7.7×10^{-3}), while allele G showed OR (4.65)with CI rang between (1.50-14.43) under (95%)and show etiological fraction(ET)of the diseases. it showed a high significant difference (7.7×10^{-3}) according to Fisher's exact probability.

Table (5): The statistical evaluations of *CTLA.4* SNP(rs 231775) between groups.

CTLA.4 A/G Genotypes	OR	Fisher's exact probability	CI 95 %
AA	0.08	3.6 x 10-4	0.02-0.39
AG	50.90	6.0 x 10-6	3.03-854.10
GG	0.19	0.494 NS	0.01-3.96
A	0.22	7.7 x 10-3	0.07-0.67
G	4.65	7.7 x 10-3	1.50-14.43

The present study illustrates that the higher genotyping distribution for CTLA.4(rs231775) gene was the genotype AA, which showed the highest percentage in patients and control compared with genotypes AG, GG which recorded lowest percentage, the chi- square recorded a non-significant difference for the GG genotype in patients compared with controls while genotype AA and AG recorded a highly significant difference.

In regard to the statistical evaluations of *CTLA.4*(rs231775) gene for patients and control, and by using Fisher's exact probability it has been found that the genotype AA has, The higher frequency in study groups and recorded odd ratio value both the genotypes AA, GG(0.08,0.19), respectively, and showed preventive fraction in breast cancer patients, the genotype AG recorded as etiological fraction in breast cancer patients with more than an odd ratio (50.90)the frequency of A allele recorded as preventive fraction with odd ratio less than one (0.22). While frequency of G allele recorded as etiological fraction with odd ratio (4.65)and has a significant frequencies of genotypes (AA, AG,) for alleles A and G at CTLA.4 SNP (rs231775) between patients and control. While a non-significant difference in the frequencies of genotype (GG).These results disagreed with the previous study which recorded a non-significant association between CTLA.4 SNP (rs231775) and disease (36).CTLA-4 +49 G/A polymorphism was not found to be associated with breast cancer risk in North Indian population, and disagreed with(25).Which founded that homozygous AA genotype at CTLA4 +49 A>G is associated with increased susceptibility to breast cancer, also results disagreed with(37).Which found that the A allele was associated with increased risk of many types of cancer, including breast cancer in Chinese women. These results were agreed with several studies(35,38,37).CTLA-4 +49 G/A polymorphism was found to be associated with breast cancer risk. Another study on a non-breast cancer women CTLA-4(rs231775) polymorphism is known to be associated with the risk of colorectal cancer in Chinese but no such association was seen in a study on Turkish patients (39,40).According to a recent meta-analysis report CTLA4+49A/G polymorphism is associated with anincreased risk of Hashimoto's thyroiditis in Asian but not Caucasian populations (41).Present result suggest that AA genotype could consider as a common type in the Iraqi population.

4- Associated of CTLA.4 in serum level with the Genotype

The present study showed a comparison of CTLA.4 level according of the CTLA.4 genotype(rs231775) in patients and control groups. In patients IDC, the genotype AA showed a non- significant difference with a mean of (3.13 ± 1.0), and in patients ILC, the genotype AA showed a significant difference with a mean of (10.36 ± 8.95) compared to control (2.76 ± 0.31). As well as the genotype AG recorded only in patient group IDC with a mean of (1.89 ± 0.25). While the genotype GG recorded only in control group with a mean of (1.94 ± 0.39) as shown in (Table 6). The result of the current study showed that the CTLA-4 serum level in patients IDC with genotype AA was higher and a non-significant difference shown when compared with control, and in patients ILC, with genotype AA was higher and a significant difference when compared with control, this result agreed with the previous study, which demonstrated that the mean of CTLA-4 serum level was higher in patients with the genotypes AA(25).no significant association was found between study groups, CTLA4 +49AA genotypic frequency, and sCTLA4 where sCD28 levels were higher in patients.

Table (6): The comparison of CTLA.4level concentration according to genotype(rs231775) in patients and controls.

CTLA.4 A/G Genotype	AA	AG	GG
Patients IDC Mean ±SE	3.13 ± 1.0Aa*	1.89 ± 0.25A	....
Patients ILC Mean ±SE	10.36 ± 8.95a	....	....

Cont... **Table (6): The comparison of CTLA.4level concentration according to genotype(rs231775) in patients and**

Control Mean ±SE	2.76 ± 0.31Aa	....	1.94 ± 0.39B
* The superscript capital letters for the horizontal comparison, while the subscript small letters for the vertical comparison. The similar letters stated to non- significant variances, the different letters stated to significant variances			

Conclusions

In concluding this study foundnon-significant difference increasing in serum level of sCTLA-4 in patients compared with control this elevation of serum concentration of sCTLA-4 could consider as a clinical biomarker in breast cancer female patients. According to the results SNP (rs231775) showedassociation between breast cancer development and CTLA-4 SNP (rs231775) and significant differences between patients and control, A allele from (rs231775) SNP may have role to prevent the risk of breast cancer in Iraqi females. while AG represnt the risk genotype. Further studies about the *CTLA-4* gene should conducted in order to shed a light on the relationship between polymorphism and susceptibility for breast cancer.

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Conflict of Interest: None to declare.

Ethical Clearance: All experimental protocols were approved under the University of Baghdad and all experiments were carried out in accordance with approved guidelines.

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