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Immuno-genetic Study of the clinical Role of CTLA4(+49 A/G rs231775) Polymorphisms and Soluble CTLA4 in Celiac Disease patients

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ABSTRACT

Background

Celiac disease (CD) is a rare condition where a genetic susceptibility and the intake of gluten-containing foods like Wheat, Barley, and Rye are the primary causes of autoimmune reactions that impact the Gut and other organs. For decades, Celiac disease's pathogenesis has been studied and linked to other human autoimmune diseases . Numerous variables, such as genetic, environmental, and local immunological problems, are contributing to the disease's genesis.

OBJECTIVES

This study investigates the correlation between polymorphisms in CTLA4(+49A/G rs231775) with the progression and intensity of CD in patients. also Explore the correlation between CTLA4 SNPs and soluble CTLA-4 (sCLLA-4) serum levels on the active progression of CD patients.

MATERIALS AND METHODS

The current investigation identified celiac disease among of sixty Iraqi participants aged above 18 years were included in a case-control study, were divided into two groups: 30 individuals who participated as the control group(they had negative endoscopy and serological test for CD) and a patient group included 30 individuals, and they were categorized into 3 groups: the Newly Diagnosed group, the Gluten-Free Diet group, and Non-Gluten Free Diet group. Each one of these groups included (10) patients. Samples were obtained from two Baghdadi hospitals.: Imameinkadhimein Medical City, Digestive and Liver Diseases Teaching Hospital /Medical City during the period (February to July 2024).Using a serum sample to measure soluble CTLA-4 by enzyme linked immune sorbent (ELISA) assay, For CTLA-4 genotyping, Human DNA extracted out of samples of whole blood of CD patients using DNA extraction kit, then conventional PCR was employed for (CTLA-4)gene amplification with specific primers, the products of PCR were undergone gel electrophoresis and the results were carried out for DNA sequencing and examined for the presence of the polymorphism CTLA4 (+49A/G rs231775.

RESULTS
The results obtained revealed the predominance of females (35) than males(25) among the CD patients and statistical significance of the difference between them at (P=0.02). High serum sCTLA-4 concentrations were observed in the majority of the Newly Diagnosed and Non-Gluten Free Diet groups , compared to low concentrations in Gluten Free Diet group and normal controls with the difference between them being statistically significant at $p < 0.001$**. the results of gene amplification and genotyping, the results of the current investigation indicated that the polymorphism of CTLA4 (+49G/A rs231775) had three genotypes in CD patients, they were (GA, AA, and GG), the prevalence of the genotype (AA) percentage in patients with CD is lower than that of healthy people . In contrast, the prevalence of the genotype (AG) is higher in CD patients compared to control people and the statistical significance of the difference between them at (P=0.015). On the other hand, the data about the serum levels of sCTLA-4 to CTLA-4 (rs231775) genotypes and alleles demonstrated that A significant difference was found at P-value = (0.039) between sCTLA-4 and CTLA-4 rs231775 alleles.

Conclusion: According to the results of this study strongly suggest that sCTLA-4 production is regulated by Gluten's presence or absence in the diet, which may have an immunomodulatory effect on the functions of cytotoxic T lymphocytes. Thus, we demonstrated that CTLA4 (rs231775) Polymorphisms changing the protein's actual function (sCTLA-4) & The CTLA4 (+49A/G rs231775) SNP's (AG) genotype is one of the risk factors for the development of celiac disease .

Keywords: Celiac disease (CD), CTLA4 Polymorphism , CTLA4 (+49A/G rs231775) , sCTLA4, soluble cytotoxic T lymphocytes, Gluten Free Diet group.

INTRODUCTION

Celiac illness is a gluten-sensitive enteropathy that is typified by the small intestine's mucosal atrophy. The disease may be mostly caused by a T- Lymphocytes(T-cell) mediated immunological response to certain Wheat's immunodominant gliadin peptides. Consuming grains that contain gluten such as (wheat, barley and rye) likely triggers immunologic mediated intestinal destruction in people who are genetically predisposed who possess the (DQ2) & (DQ8) haplotypes (1). Throughout the previous few decades, the prevalence of CD has grown, with a global prevalence of around 1.5%. This is partly because diagnostic technologies have become more sensitive and the altered environmental reaction to dietary gluten (2). CDs can occur at different stages of patients' lives from weaning off to adulthood for both sexes, but it often remains undiagnosed or misdiagnosed because of the vast range of symptoms(3,4). CD's pathophysiology centers on a dysregulated immune response to gluten, which damages and inflames the small intestine, particularly the villi, which are in charge of absorbing nutrition, the villi destruction makes it more difficult to absorb vital nutrients like vitamins and minerals(5). This damage may lead to several gastrointestinal symptoms such as (bloating, diarrhea, weight loss, bloating and diarrhea) . It's proposed that the etiology of CD results from a combination of a vulnerable genetic (HLA- DQ2 and HLA -DQ8 haplotypes) background and various environmental elements (most notably, consuming gluten, viral and bacterial infection)(4). Although CD has been most often linked to Human Leukocyte- Antigen (HLA) Class II molecules, family studies indicate that this relationship only explains one-third of hereditary vulnerability, and an unknown number of Non-HLA genes contribute to genetic factors that are involved in the risk of CD susceptibility including; X-linked Fork head Box (P3) FOXP3, and Cytotoxic T - Lymphocyte Antigen -4 CTLA-4 **(5, 6)**. CTLA-4 located on chromosome region 2q33, is one non-HLA candidate gene involved in CD due to its essential role in the co-stimulatory , suppression response of T lymphocyte cells through negative signal and peripheral tolerance (7), and connection to the genetic diversity that determines the development of different autoimmune disorders and increases vulnerability. Therefore, a CTLA-4 deficit may result in an overreactive T cell population, which might cause excessive T cell proliferation and organ invasion. **(8)**.

According to various papers' recent findings an SNP in the CTLA4 gene was discovered in the untranslated 3' region (UTR) of exon (1) resulting in the exchange of an alanine at position 49 (49A/G; rs231775) for a threonine amino acid, has been associated to autoimmune disease vulnerability, such as Type -1 Diabetes, Rheumatoid arthritis and Celiac disease (9). This research strives to detect the clinical function of CTLA4 (rs231775) polymorphism, evaluate the sCTLA4 level in individuals with CD and explanation the correlation between this marker with immune response, T-cell activation and gluten tolerance in CD.

MATERIALS & METHODS

Study setting and population

This case control study which included 60 Iraqi subjects, sorted into two primary categories; a Control group and a CD patients' group, each group consisted of 30 participants. Patients group was categorized into three subgroups including newly diagnosed group, gluten-free diet group, and non-gluten-free diet group. All participants' samples were collected from two hospital in Iraq/ Baghdad included Imamein kadhimein Medical City, and Teaching Hospital /Medical City, during the period of (January 2024 to May 2024). Celiac Patients were diagnosed depending on a combination of Clinical manifestations typically proposed for CD, serological markers, and histological international guidelines from the European Society for Pediatric Gastroenterology and Hepatology and Nutrition **(10)**. While the control group had negative endoscopy OGD and Histological tests for celiac disease. Furthermore, the participants needed to meet two requirements; the clinical history of celiac disease presentation comes first, followed by serological evidence of autoantibodies (Anti-tTG, Anti-DGP IgA, and IgG) for celiac disease diagnostic testing.

Inclusion criteria:

People with celiac disease aged 18 years and older, who meet the clinical signs of the disease and have positive results from serological and histological laboratory tests and examination related to the disease. Control people aged 18 years and older who negative endoscopy OGD and Histological test for CD.

Exclusion criteria:

Individuals aged below 18years. Patient was diagnosed with histological criteria (Marsh I, II, III type) of CD in duodenal mucosa with serological markers (negative). suspected but not proven by biopsy or serological testing to have CD. Existence of Diabetic Mellitus, Chronic Inflammatory Bowel Diseases such as Crohn's disease or Liver diseases, Autoimmune Thyroiditis, patient with active *H. Pylori* gastritis and duodenitis infection.

Institutional ethical approval:

The study received ethical approval from Iraq Ministry of Health's and Institutional Review Board's (IRB) standards, as well as the University of Al-Nahrain's College of Medicine's research ethics committee, approved this project with ethical permission (No:2023.11.25).

Collection and processing of blood samples:

Each participant (patient and control)in this research had a venipuncture to draw 5 ml of venous blood, and placed 3 ml in a particular gel tube, then allowed for fifteen minutes at room temperature, and separated for 10 minutes in a 4000xg centrifuge before being stored at -20 C^o until testing sCTLA-4 level utilizing a Human ELISA kit that is sold commercially (E0277Hu) .For the CTLA4 (+49A/G rs231775) SNP molecular detection, direct dispensing of (2 milliliters) of blood into tubes of EDTA used for DNA extraction by whole genomic kit according to company instruction manual using DNA kit from samples using the protocol Relia Prep™ Blood gDNA Miniprep System kit (Promega/ USA) . Total DNAs are separated following extraction on a 0.8% agarose gel and investigated with ultraviolet (UV) light, with Nanodrop being used to verify the concentration and purity of genomic DNA and kept at (-20 C^o) until molecular assay.

Quantification of serum level soluble CTLA-4:

The technique of Enzyme Linked Immunosorbent Assay (ELISA) is employed to detect sCTLA-4 in sampled serum; the microtiter wells are coated with the specific monoclonal ab towards a unique antigenic site on the sCTLA-4 molecule. The optical density (OD) is measured at 450nm, and the color intensity is appropriate for sCTLA-4 concentration. The OD was plotted to create the standard curv for each standard on the vertically (**Y**) axis in relation to the horizontally (**X**) concentration, then calculated for each sample. The normal reference of fasting insulin is (<245mlU/ml) as show in figure (1) .

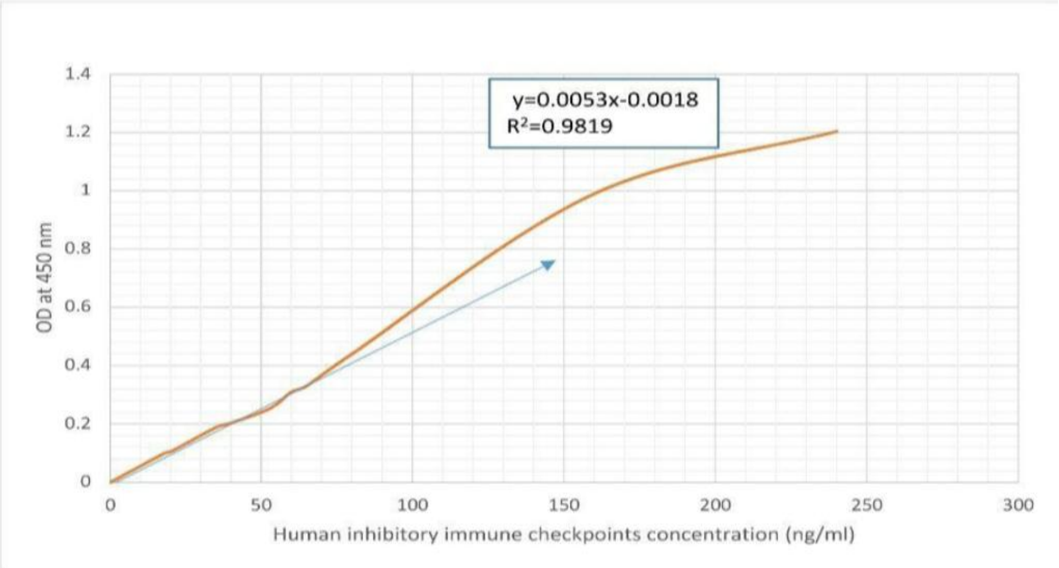

Figure 1: Standard curve demonstrates optical density at 450 nm and concentration of human inhibitory immune checkpoints(ICP).

Detection of CTLA4 gene SNP

The high-purity genomic DNA and online design primers using to investigate SNP based on NCBI-SNP accessible data. The primers sequence for CTLA4(+49A/Grs231775):
(F-5'-GTAAAACGACGGCCAGTGGATGGTGCTTCACAGATAG-'3&
R-5'- AGGAAACAGCTATGACGGCTATCCTTTCCCTCTTAAAT-**'3**) supplied by (The Macrogen Company of Korea in lyophilized form. The CTLA4(+49A/G rs231775) SNP-containing region was amplified. by the reaction was completed in a 25 µl reaction that contained (1µl) from each forward and reversed primer mixed with 12.5X master mix, then added 3 µl from (7.5 µl) of nuclease-free water and DNA. The PCR reaction was carried out using a 25 µL total reaction volume as shown in table (1) , and this gene region's PCR amplified programs were shown in Table 2. After electrophoresing the PCR results on a (**2%**) agarose gel, they were examined after ethidium bromide staining under ultraviolet light the target bind at (1012bp).

Table .1: Components of PCR reaction for amplification of CTLA4 SNP (rs231775).

Components	**Quantity (µL)**
Master Mix	**12.5X**
Forward primer	**1µM**
Reverse primer	**1µM**
Nuclease free water	**7.5**
DNA	**3 ng/µl**
Total volume	
Aliquot per single rxn	**22µl of Master mix per tube and add 3µl of Template**

Table .2: CTLA4 gene SNP (rs231775) software for PCR amplification.

Steps	**°C**	**Time / second**	**Cycle**
Initial Denaturation	**95°**	**05:00**	**1**
Denaturation	**95°**	**00:30**	**30**
Annealing	**55°**	**00:30**	
Extension	**72°**	**00:45**	
Final extension	**72°**	**07:00**	
Hold	**10°**	**10:00**	**1**

For every sample, The Macrogen Company received 25 µL of PCR products for one-way (forward) analysis and sequencing. The bioinformatics investigation used the Chromas tool and the National Center for Biotechnology Information(**NCBI**) database.

Analysis of statistics:

SPSS version 25, a statistical software for social sciences, was used to analyze the data. The normality test was detected by the Shapiro-Wilk test, which revealed normal distribution data. The data obtained were presented as mean ± SD and t-test or variance analysis **ANOVA**. Chi-square analysis was used to examine categorical variables, which were represented as numbers and percentages. Three variables were compared using the ANOVA test; Statistical significance was established when the P-value was less than 0.05.

THE RESULTS

Demographic characterization of all the groups in the study:

The result of current study reveals the majority of patients in the study population are female, even though the random sample approach was used during patient recruitment. From the whole study populations (35) were females and (25) were males, the ratio of female to male 1.4:1as shown below in the Table .3 .The results showed that females in newly diagnosed group 7(3.4%) compare to 6 (2.7%) in the gluten free diet group and non-gluten free diet 5 (2.4%) while 17 (8.5%) in the control group on other hand , male in newly diagnosed group was 5(2.4%) higher than in non-gluten free 4 (1.8%) , gluten free diet group 3 (1.6%) while with control group 13 (6.5%).

Table (3) the sex demographic characteristic among study populations

Sex		Status of patient				Total	P-vale
		newly diagnosed (gluten diet) (10)	gluten free diet (10)	non-gluten free diet (10)	Control (30)		
Male	**Count**	5	4	3	13	25	0.022
	% within Sex	2.4%	1.8%	1.6%	6.5%	100.0%	
Female	**Count**	7	6	5	17	***35***	
	% within Sex	3.4%	2.7%	2.4%	8.5%	100.0%	
Total	**Count**	12	9	8	30	60	

The chi-squared test was used. P =< 0.05 indicates statistical significance.

Quantification of serum sCTLA4 among study groups:

The mean ±SD serum levels of sCTLA4 values in the Gluten Free Diet group and the control group were (21.90 ±5.62, 23.36± 6.52 ng /mL) respectively. These results were much lower than that of the new diagnosed group and the non-gluten free diet group (57.72±32.253 and 56.64 ± 42.71 ng /ml), respectively. The two groups showed statistically high significance at level P = ≤0.0001 as shown in figure .2 and table -4.

Table (4): Level of sCTLA4 in serum among study Populations

Groups of the study of sCTLA4	number	Mean	Std. Deviation	P-value
newly diagnosed (gluten diet)	10	57.72	32.25	<0.0001**
gluten-free diet	10	21.90	5.62	
non-gluten free diet	10	56.64	42.71	

control	30	23.36	6.525	
Total	60	34.95	27.569	

ANOVA: $p \leq 0.01$ significant, ** significant at 0.001 level.

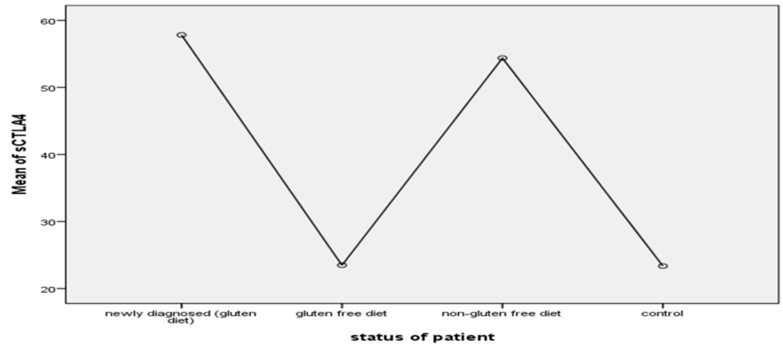

Figure .2 :Quantification of serum sCTLA4 among study groups.

Detection CTLA4 (+49A/G rs231775) SNP:

In the present investigation, as shown in figure (3), the area of the CTLA4 gene, which has the (+49A/G rs231775) SNP, produces one band with a molecular size of 1012 bp for every sample..

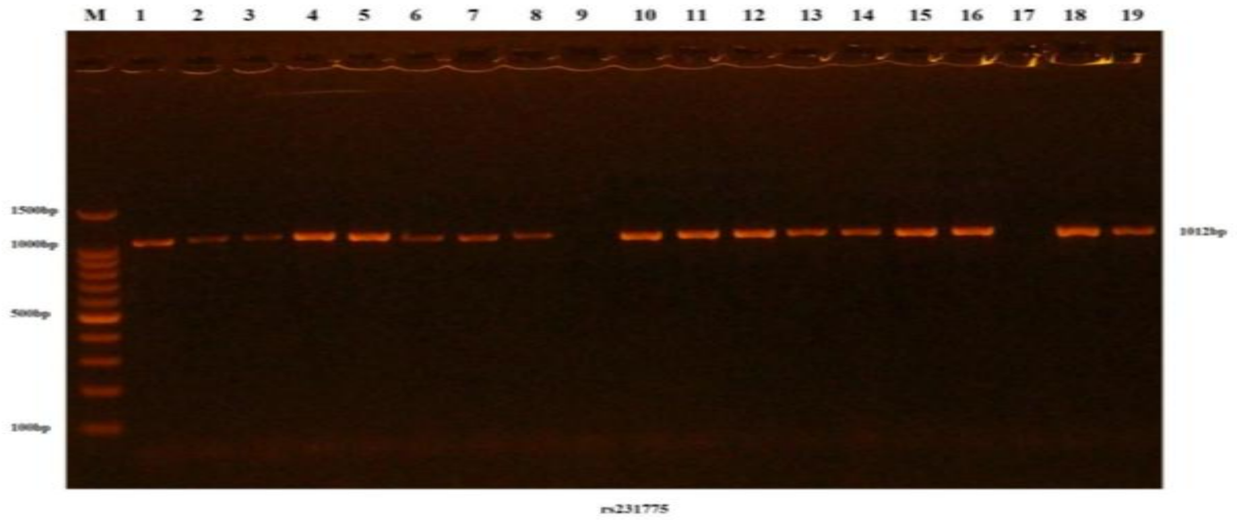

Figure .3: Gel electrophoresis CTLA4 (+49A/G rs231775) polymorphism amplify with a specific pair of primer by using conventional PCR. Using ethidium bromide as fluorescent dye. M: (100 bp) ladder marker. Lanes (1-19) resemble 1012 bp PCR products.

Among the sequencing techniques, Direct sequencing (Sanger's sequencing) was performed on CTLA4 exon region amplified PCR-product samples. The sequences were compared to an (NCBI Gene Bank) reference sequence for the CTLA4 gene & analyzed using gene ware. (Figure 4) revealed that direct sequencing was used for genotyping the forward strand of PCR products. The SNP rs231775 appeared in three genotypes: AA(wild type),GA and GG(mutant type)

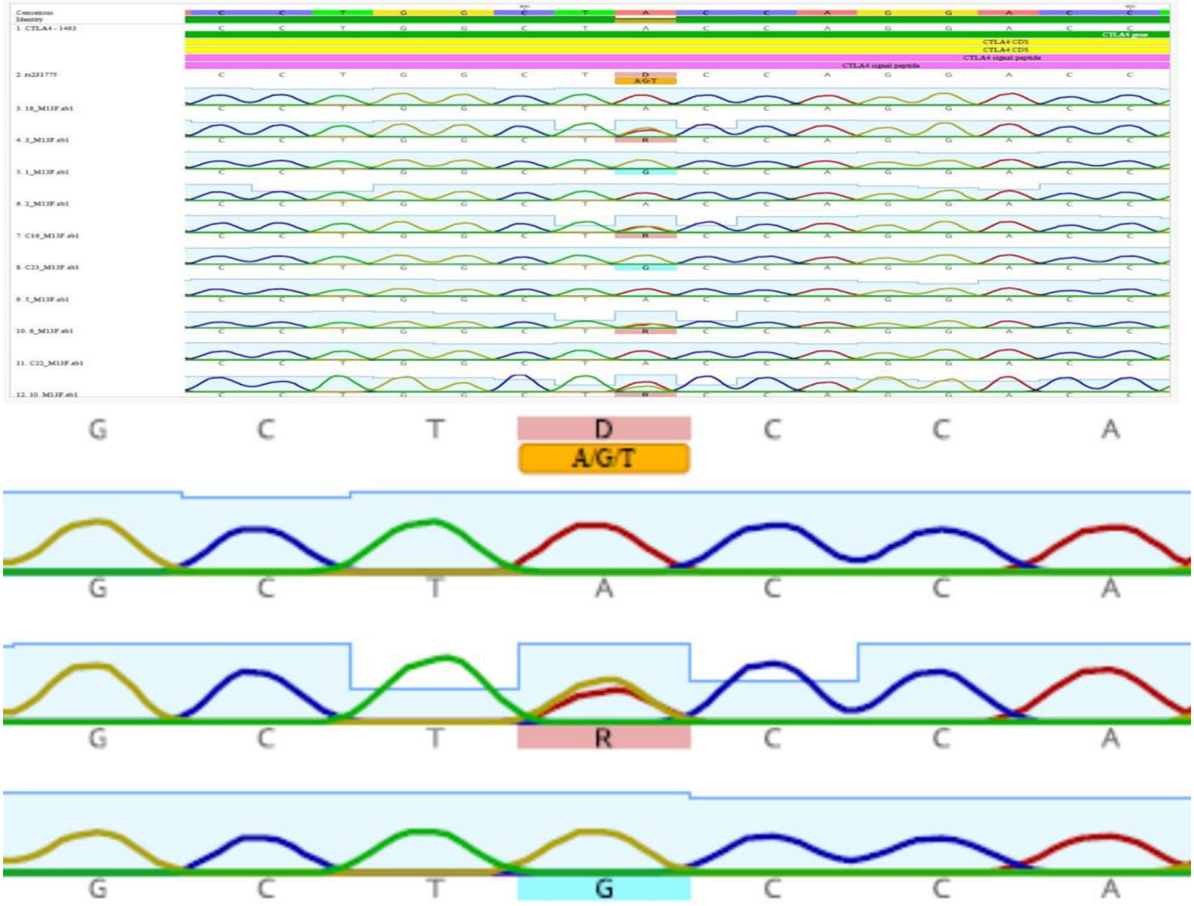

Figure (4): Sanger sequencing investigation of SNP rs231775 in the CTLA4 gene. A lone "A" peak indicates a homozygous allele. A single "A" peak indicates a G homozygous allele. If the "A" and "G" peaks are present, the allele is A/G heterozygous.

Genotype distributions and frequencies of the Paris SNP alleles for CTLA-4(+49A/G rs231775) among study groups:

The prevalence of homozygous wild genotype AA was lower in patient groups (non-gluten-free, gluten-free diet and newly diagnosed groups (14.7%, 8.8%, and 5.9%, respectively)) than in the control group (70.6%), with differed statistically significantly (P=<0.01). The prevalence of heterozygotes AG genotype distribution to the groups of CD (newly diagnosed) (was 36.0%, which is higher than in control groups (24.00%) and substantial differences between them (P<0.01). In addition to that, the distribution of the mutant GG genotype was higher in those newly diagnosed (100.0%) but (0.0%) in both (the diets that are gluten-free and those that are not), and control group) . According to the Chi square test, the P-value was statistically significant (<0.01). with two groups (patients' group and control group) As well as seen in table (2) and figure (4).

Table (2): Distribution of CTLA4 genotype (heterozygous and homozygous) among CD patients' group and control group

Status of patient	Count /%	SNP rs231775 (wild AA) Variation	Total	P-value

		homozygous wild AA	heterozygous AG	mutant GG		
newly diagnosed	**Count**	5	9	1	15	0.015
	%within SNP s231775	14.7%	36.0%	100.0%	25.0%	
Gluten-free diet	**Count**	3	5	0	8	0.015
	%within SNPrs231775	8.8%	20.0%	0.0%	13.3%	
non-gluten free diet	**Count**	2	5	0	7	0.015
	%within SNP s231775	5.9%	20.0%	0.0%	11.7%	
control	**Count**	24	6	0	30	0.015
	%within SNP s231775	70.6%	24.0%	0.0%	50.0%	
Total	**Count**	34	25	1	60	0.015
	%within SNP s231775	**100.0%**	**100.0%**	**100.0%**	**100.0%**	

The chi-squared test was applied. P = < 0.05 is statistically significant , (SNP : Single Nucleotide Polymorphism ,AA: Adenine , GG: Guanine, AG: Adenine Guanine)

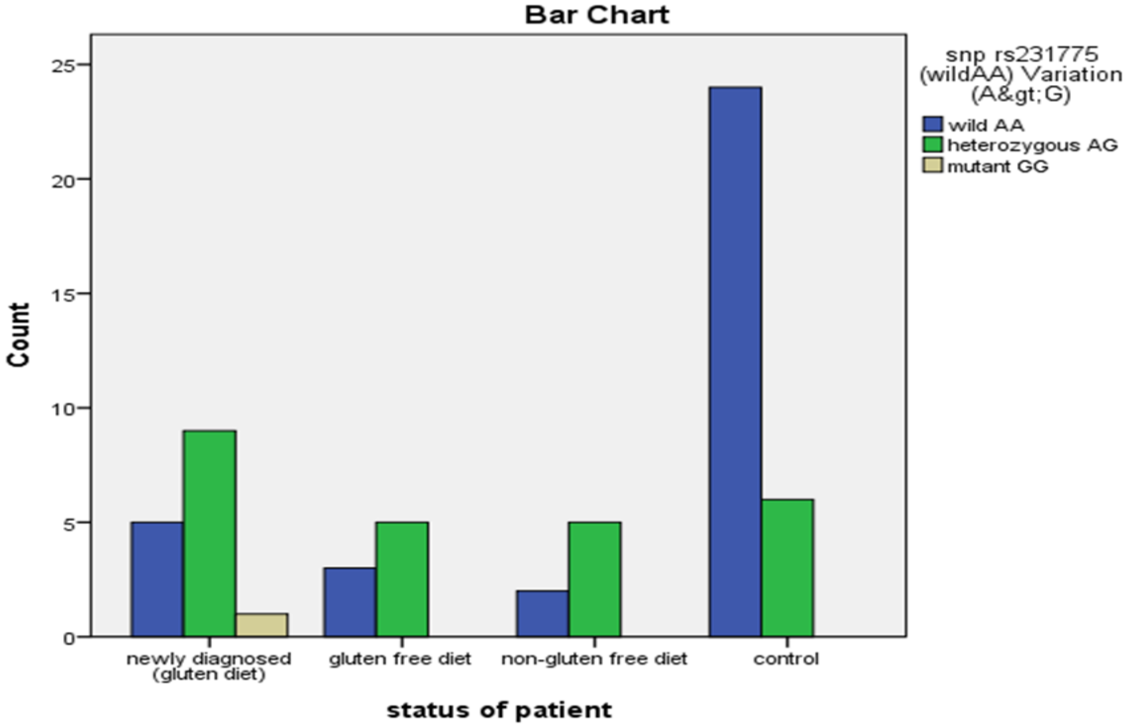

Figure .4: Distribution of CTLA4 genotype (heterozygous and homozygous) among CD patients' group and control group

Effects of CTLA4 rs231775 gene variations on sCTLA4 among study groups:

Based on genotypes and alleles of CTLA4(rs231775), data on the serum level of sCTLA4 varied Compared to those with Heterozygous (AG) and Homozygous mutant genotypes (GG), the means± SD of sCTLA-4 in those with homozygous wild (AA) were 49.51±34.80 ng/ml greater. There were notable statistical distinctions between CTLA-4 rs231775 genotypes and sCTLA-4 serum levels. According to allele analysis, those with the Wild allele (A) had a serum level of sCTLA-4 that was 62.2±13.1 ng/ml greater than people with the G allele, which was 45.01±16.7 ng/ml. According to the table(3), there was a very significant difference between the CTLA4 rs231775 alleles and the sCTLA4 serum value.

Table(3): The impact of the CTLA4 rs231775 polymorphism on the sCTLA-4 serum value among research groups

CTLA4 (+49A/G rs231775)		**sCTLA4 mean**	**Std. Deviation**
rs231775 Genotypes	**Homozygous wild AA**	**49.51**	**34.80**
	Heterozygous AG	**39.13**	**29.091**
	Homozygous mutant GG	**30.25**	**25.36**
	P-value	**0.039***	
rs231775 Alleles	**A wild**	**62.2**	**13.1**
	G mutant	**45.01**	**16.7**
P-value		**0.0001****	

The ANOVA was applied. *: statistically significant, **statistically highly

DISCUSSION

The results of the current investigation indicated that the occurrence of CD was more in women in both the control and patient groups. compared with males. This fact was demonstrated by several studies that found that CD is more common in women like most other autoimmune diseases **(11,12)**, additionally, another study found that Celiac illness is more common in women than in men ratio1.3 - 2.1 (13). on other hand Green and his group demonstrated that the ratio females than in males was 2.9:1(14). While in our study that Women to men ratio was (1.4:1). The reason for the difference in these proportions was due to precis male to female ratio may depend on the patient's race and ethnicity and geographic area. Different studies have shown that autoimmune disorders are more common in women than in males, women make up 65% of those with an autoimmune diagnosis. A number of theories have been proposed to explain why women are more susceptible than males, one of them is that female are genetically more likely to have abnormal autoimmune function because of the X chromosome, which can make them more prone to tolerance breakdown.. According to another idea, microchimerism results during organ transplantation, blood transfusion, twin-to-twin transmission in utero, and cell transfer between mother and fetus throughout pregnancy, may be crucial to the fetal semi-allograft's immunologic tolerance, and Women preponderance might be interpreted as a result of females' higher allogeneic cell flow. In light of increased exposure to cell traffic, female would be expected to pay a higher price, reflected in more autoimmune disease(15). On other hand a lot of people are prone to autoimmune diseases, which can be triggered by a variety of factors like the weather, bad food and lifestyle choices, inactivity, elevated stress levels, and/or insufficient sleep. Other causes, including hormonal changes in women throughout puberty and pregnancy, may often be inevitable (16). Our data clearly reveals that the mean serum sCTLA-4 level among individuals with newly diagnosed (gluten diet) and non-gluten-free diets is higher than the individuals with gluten-free diet and control group. These findings strongly imply that the presence or absence of dietary gluten regulates the synthesis of sCTLA-4 and suggests a potential immunomodulatory impact on the functioning of cytotoxic T lymphocytes. An elevated concentration of inhibitory immune checkpoints in people with a gluten diet may increase sCTLA-4 levels particularly prevent early activation of T- cells through preventing the CD80 and CD86 interaction and the CD28 Co stimulatory receptor. In actuality , these regulatory pathways trigger control immune hyperreaction, shielding the host against immunological dysregulation, tolerance loss, and autoimmunity (17).In our research, these results are compatible with Several previously studies (Saverino et al., 2010; Schiavo et al., 2013 , Simone, et al and Valencia M et al., 2014), which outlined that frequent exposure to elevated gluten levels led to the depletion of most or a significant portion of the Gluten specific CD4 T- Lymphocytes in blood and gut celiac illness patients but not in healthy individuals .On other hand, Gluten specific CD4 T -cell sinhabit the effector memory T-cell compartment; as a result these cells take on the characteristics of Gluten-specific cells in patients who are non-gluten-free diet. All of these are linked to the gradual upregulation of CTLA4 expression, and the gene for CTLA4, which produces a protein without a transmembrane region(sCTLA4), led to an increase of sCTLA4 sera in individuals with Autoimmune disorders (18,19,20,21). Regarding the functional SNP (CTLA-4 rs231775) and CD, the current research highlights the influence of CTLA-4 gene variants on CD susceptibility or disease development. The current study found that this SNP influence susceptibility or disease development, and this agree with several studies (Kristiansen, O P et al.,2000 and Abdullah et al.,2022), did detect a significant association between CTLA4 rs231775 polymorphisms and Celiac disease (22,23). Our investigation reveals the frequency of homozygous allele pairs (AA) genotype in patients Persons have lower percentile compared to control persons and it might serve as a protective genotype against CD occurrence, whereas frequency of Pairs of heterozygous alleles (AG) higher in those who are patients in contrast with control persons, and it might serve as a risk allele for CD incidence, with the difference between them being statistically significant P value =<0.05. The CTLA4 gene is a crucial T-cell response inhibitor and a guardian of tolerance, so any malfunction of CTLA4 is linked to a rise in risk of Autoimmune conditions. Thus, SNPs are significant determinants of illness risk because of their impact on gene expression and its products(24). It was also found that several SNPs or alleles were associated with different populations. For example, A Dutch study indicated that the Celiac disease was connected with the G allele of the same SNP, although a French group first identified the A allele of the CTLA4 SNP as being linked to Celiac disease (25). Research conducted on a population in Ireland found that CD was more closely associated with an extended haplotype (CTLA4-CD28-ICOS) than it was with the haplotypes of individual genes(26). Additionally, as was the case with the two Italian studies on CTLA4, one of which found a relationship with CD while the other did not, a polymorphism that was not linked to disease in one community but was in another that appears to be comparable to that population may still be linked to the same disease(27,28). Regarding Our results are compatible with previous studies from Iraq(Abdullah et al., (2022) demonstrated This polymorphism's G allele carriers (GG + GA), which is thought to be the dominant genetic model, have been linked to decreased T-cell proliferation as the consequence of this allele's diminished inhibitory activity and an elevated risk of celiac disease(23,29).

Due to the fact that the CTLA-4 gene is an essential immune response inhibitor, specific CTLA-4 polymorphisms may cause variations in a person's susceptibility to autoimmune disorders because of changing the protein's function(29). So, the results of this study revealed the levels of sCTLA-4 in the serum and CTLA4(rs231775)genotypes were significantly correlated. The ELISA results in this study demonstrated that people's serum levels of sCTLA4 with rs231775 A/A genotype higher than the individuals with rs231775 A/G and G/G . Individuals with elevated inhibitory immune checkpoint levels may induce T -cell suppression, which concurs with other research (Simone R, et al 2014and Talib AL, et al 2022) that showed association of the rs231775(A/A) with impaired proliferation of T cells, inhibiting T -cell activation or enhancing its apoptosis by increasing function CTLA-4 protein in contrast, The decreased function of CTLA-4 protein is linked to the rs231775(G/G) genotype, which therefore causes increasing proliferation of T cells and impaired function of regulatory T cells (30,31). On the other hand, according to our findings, those with the A allele had greater blood levels of sCTLA-4 than those with the G allele. and the difference between them is statistically high ($P< 0.\ 0.0001$) which agree with several studies (Naluai et al.,2000; Popat et al., 2002; Mora et al., 2003) Who proved that people with the **G** allele of (+49 G>A's) homozygotes had less control over T cell proliferation than those with the A /A allele, this might lead to the pathophysiology of CD and likely other autoimmune conditions . They also reported that sCTLA-4 is associated with the A allele. Similarly, compared to A/A homozygotes, Individuals with the G/G genotype exhibit a greater proliferative response of T cells with inadequate stimulation, which might be due to a decreased up-regulation of CTLA4 (32,33,34).This study had certain restrictions, including the challenge of obtaining medical data for the largest number of patients and the fact that only 60 people participated, representing patients from a limited number of hospitals in Baghdad, the capital.

Conclusion— The soluble inhibitory immune checkpoint(sCTLA-4) markers are significantly increased in individuals with gluten diet and control group while low levels in individuals with gluten free diet . These findings clearly imply a control of serum CTLA-4 production based on the existence or lack of gluten in the diet and suggest a potential immunomodulatory impact on the functioning of Cytotoxic T lymphocytes. The sCTLA-4 affected by CTLA-4 rs231775 polymorphism; the CTLA4 gene variations are Non-HLA the determinant that predisposes to CD. Regardless of whether it serves as a marker for a locus in linkage disequilibrium with CTLA4 or directly increases the risk of contracting an illness and finally, the homozygous AA genotype and The(G) allele of CTLA4 (+49 G / A rs231775) polymorphism was confirmed to be significantly associated with CD persistence.

Recommendations

1. Future study designs should be concentrated on investigation of other SNPs in CTLA-4 genes sing gene expression**s.**

2- Conducting more studies on a large number of Iraqi population to assess properly the relationship between CTLA-4 (+49G/ A rs231775) and progression of disease.

3- For more reliable results, further research with a larger sample size and a diverse ethnic population are required.

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Finding:

Self-funding

Institutional ethical approval:

The Institutional Review Board IRB at AL Nahrain University, College of Medicine and the Iraq Ministry of Health provided ethical approval for this investigation (No:2023.11.25).

Conflicts of Interest:

According to the authors, there is no conflict between their interests.

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