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Detection of *CTLA4* Gene Polymorphism and Inflammatory Cytokine Profile Among Inflammation Bowel Disease Patients

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Abstract: Inflammatory bowel diseases (IBD) are chronic inflammatory conditions of the gastrointestinal tract driven by unsuitable immune responses to an changed gut microbiome in heritably susceptible individuals. The present study was designed to determined the *CTLA4* (rs231775) single nucleotide polymorphisms (SNPs) using sequencing analysis and assessment the serum levels of interleukin 11,15,18 and CTLA4 level using ELISA technique , that are related to etiology and pathogenesis of inflammation bowel disease (IBD) in two groups of Iraqi patients crohn**'**s disease (CD) and ulcerative colitis (UC). When comparing IBD patients to controls the results of *CTLA4* (rs231775) SNP revealed some variations, the G allele was demonstrated increased incidence in IBD patients than controls. The IL11 levels (mean ± SD; pg/mL) were significantly($p \leq 0.05$) increased (472.61±162.89 and 460.43 ± 144.65) in CD and UC patients, respectively compared with healthy control (160.15±160.56) pg/ml. Similarly, serum level of IL15 was significantly increased in crohn's disease patients and ulcerative colitis patients (451.18±197.33*vs.* 395.20 ± 183.54 pg/ml; $P > 0.05$) , respectively compared to controls (112.15±129.22 pg/ml; $p \leq 0.05$). Whereas, the IL18 levels (mean ± SD; pg/mL) were increased (43.68 ±16.52 and 41.56 ± 10.79) in CD and UC patients, respectively compared with healthy control (40.76 ± 13.27) pg/ml, though, the variation was not statistically significant. The CTLA4 levels (mean ± SD; pg/mL) were show significantly($p \leq 0.05$)decreased (0.64 ± 0.28 and 0.61 ± 0.29) in CD and UC patients, respectively compared with healthy control show a significant (0.80 ± 0.40) pg/ml .It was concluded that *CTLA4* SNP effected on CTLA4 protein expression, and cytokines 11,15 were appeared role in IBD than interleukins 18 and these results were indicated not significantly variation between CD and UC groups ($P>0.05$).

Keywords: Inflammation bowel disease, *CTLA4*, IL11, IL15, IL18.

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Introduction

Inflammatory bowel diseases (IBD) are chronic inflammatory conditions of the gastrointestinal tract driven by unsuitable immune responses to an changed gut microbiome in heritably susceptible individuals. Crohn's disease (CD) and ulcerative colitis (UC) are the most extensively recognized kind of IBD and have been the focus of attention reason to their rising frequency (1). The CD, one of the mainly common kinds of inflammatory disease universal, is characterized in the mucosa by the creation of fistulas, strictures, ulcers and granulomas (2). UC is a further kind of inflammation bowel disease

characterized by granularity, surface ulcerations and a vascular pattern. In difference with the inflammation found in CD occur throughout the entire GT but in UC is limited to the colon mucosal layer (3). The main symptoms of active inflammation bowel disease are diarrhea mixed with blood , fever, abdominal pain, weight loss, anemia can as well take place and rectal bleeding is less common in CD. Because the small intestine is responsible for the absorption of nutrients therefore malnutrition is very general in crohn's disease(4). While UC is commonly associated with rectal bleeding. Often, symptoms appear on gradually and can range from mild to severe. Symptoms typically happen from time to time with periods of no symptoms. Complications may involve abnormal dilation of the colon (megacolon), colon cancer, inflammation of the joints, eye and liver (5). The etiology is still not completely known, however, some factors that weakened immune system such as environmental factors such as a virus or bacteria, emotional distress, diet, smocking and others, as well as immunigical and genetic components which causes inflammation of the gastrointestinal tract which is more likely to develop this inappropriate immune response (6). As a candidate gene predisposed to IBD, *CTLA4* (rs231775) was attended, *CTLA4* is responsible for shutting off T cell responses against self-antigens in a process known as anergy (7). IL11,IL15, IL18 , IL17 and IL 23 is known as a cytokine with immunomodulatory effects and effective continued existence on cells of both the innate and adaptive immune systems that play a essential function in resistance mechanisms against pathogens (8, 9). Cytotoxic T-lymphocyte antigen 4 (CTLA4) also known as (co-inhibitory molecule) is cluster of differentiation 152 (CD152), a protein receptor that plays an significant regulatory function in the immune system involve immune tolerance and is an vital negative regulator of T cell-mediated immune response and inflammation prevention. CTLA4, which is expressed on the surface of T cells and it is a associate of the immunoglobulin superfamily (10). Therefore, this study aim the role *CTLA4* (rs231775) SNP in human immunity, and describe profiles of these cytokines, a major component in IBD etiology.

Materials and methods

Subjects

This study was approved by the Ethics Committee in College of Science, University of Baghdad (Ref.:CSES/0422/0063). One hundred and fifty seven subjects selected for this study which included patients under therapy (n=85, 39 male and 46 female) aged from 15 to 68 years who suffered from IBD diagnosed by consultant physician, depended on the clinical signs, laboratory diagnosis, colonoscopy and endoscopist, and well random subjects as control group (n=72, 35 male and 37 female). This work was completed in Biotechnology lab/ University of Baghdad and Gastroenterology and Hepatology Hospital.

Blood samples

A volume of 5-10 ml of blood was collected from each healthy control and patients by vein puncture using10ml disposable syringes. 2 ml

were added to EDTA tubes was stirred gently for few seconds to avoid clotting and then stored at -20ºC until for DNA extraction and detection of SNP *CTLA4* gene associated with IBD, the remaining 3ml of blood is distributed into the gel tubes, which was then left at room, temperature for 30 minutes, in order to initiate the clotting process, then separated the serum by centrifugation at for 10 minutes (3000 rpm) and stored sample at -20 ºC until the moment of immunologic assay interleukins 11, IL15, IL18 and cytotoxic T lymphocyte associated antigen 4(CTLA4).

DNA extraction

Extraction of genomic DNA from refrigerated peripheral blood samples were collected from the healthy control and IBD patients in EDTA tubes using Wizard® Genomic DNA Purification Kit (Promega, USA) following the manufacturer's guidelines.

PCR amplification of *CTLA4* (rs231775) SNP

In this study the (rs231775) SNPs, the152 bp a fragment, placed in chromosome 2 (2q33) in exon 1 of *CTLA4* gene. This gene was selected and amplified for investigation of association with autoimmune CD and UC (IBD) using the primers designed: F**: 5-**AAGGCTCAGCTGAACCTGGT-3 and R:5-CTGCTGAAACAAATGAAACCC-3 . PCR was performed in a 25µl a whole volume containing 1µl of both the F and R of *CTLA4* (rs231775) (10pmol/µL), 12.5 µl master ready mix (Go *Taq*® Green Master Mix 2X: Promega corporation, USA), nuclease free water (8.5 µl) and DNA (2µl) (100 ng). Compound tube was Mixed by Exispin system, after that amplification of DNA was carried out with the thermal cycler (Multigene TM Gradient Thermal Cycler, Labnet International, USA). It was programmed as follows: First denaturation at95°C for 5 minutes, followed by 35 cycles programmed as follows: First denaturation at 95°C for 30 seconds, annealing for 1min at 57°C, extension at 72°C for 30 seconds and followed by one cycle of a final extension 72°C for 10 minutes for *CTLA4*.Then separated products of PCR on 2% agarose gel electrophoresis at 5 v/cm^2 for 1 hour to confirm amplification in the existence of DNA ladder marker (100 bp) (Promega, USA) with 1 × Tris-borate-EDTA buffer and in the UV light was visualized subsequent to staining by ethidium bromide (11).

***CTLA4* (rs231775) SNP sequencing**

The PCR product were sent for sanger DNA sequencing (Macrogen Corporation; South Korea) to have an automated sequencing by Genetic Analyzer system ABI-310, which gave the identity of the genes comparison with the original genes in Gene Bank in NCBI.

Estimation of interleukins 11,15,18 and CTLA4 levels by ELISA

The test enzyme-linked immune sorbent assay (ELISA) was used for detection of interleukins (IL11), (IL15) (IL18) and (CTLA4) levels in human IBD patients and control, that was performed using producer's protocol ELISA Kits (YLbiont./ China)) based on the Biotin double antibody sandwich technology, then the samples optical density was compared to the standard curve.

Statistical analysis

Parametric variables were given as mean ± standard deviation (SD) using the IBM SPSS computer software version 24. A P value of ≤ 0.05 was regarded as statistically significant.

Results and discussion

***CTLA4* (rs231775) SNP PCR amplification**

The results of amplified *CTLA4* PCR products was visible in agarose gel electrophoresis as shown in Figure(1), demonstrated that a yield of 152 bp single band for the desired result for CD ,UC patients and control.

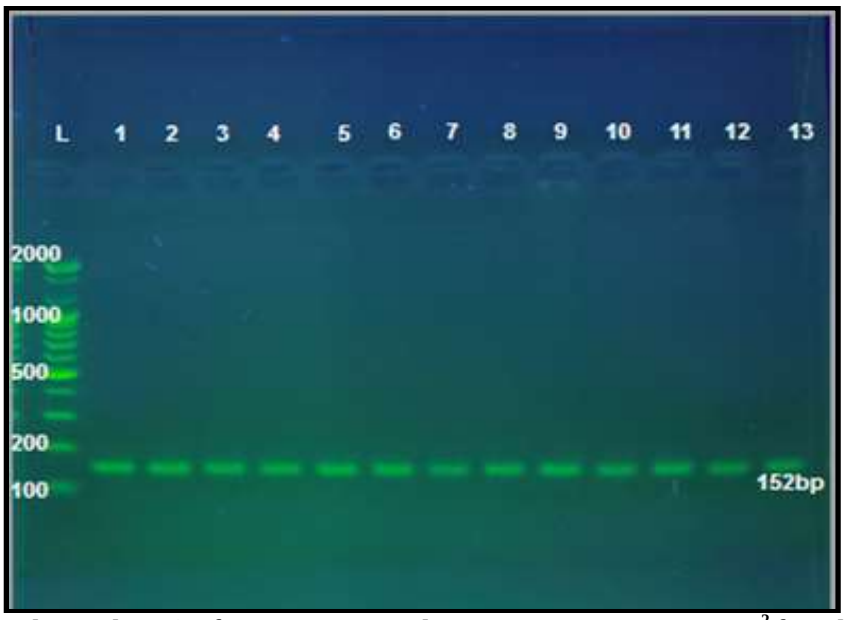

Figure (1): Electrophoresis of *CTLA4* gene products on 2% agarose at 5 v/cm^2 for 1 h. Lane L: 100bp DNA ladder; Lanes 1-5: CD patients ; Lanes 6-11: UC Lanes;12-13: control

***CTLA4* sequencing**

The result *CTLA4* gene sequence was investigated for samples were selected from CD patient comparison of the sequence with that in the database in Gene Bank by BLAST program, the gene was identified (96%) with no gaps (0%), there were four circles that illustrates these comparisons and their means, the first and second circle means at the subject 5293 there is 2 SNPs linked to *CTLA4* gene they are: (Replace A > T and addition A in sequence).While The third and fourth circles means at the subject 5233 there were 2 SNPs linked to same gene and they are (Replace A> G and Replace A> C). Genetic variations of *CTLA4* SNPs were investigated; rs231775 (changing *A* allele to *G* allele at the nucleotide position 5204 of the reverse DNA strand), as shown in figure (2). While result sequencing the sample that refer UC patient was identified (97%) with gaps (1%), there were three circles that illustrates these comparisons and their means, the first and second circle means at the subject 5301 there is 2 SNPs linked to *CTLA4* gene they are: (Addition C and A in sequence).While The third circles at the subject 5204 there were 1 SNPs linked to same gene and they are (A> G) as shown in

Figure(3). The present of *G* allele in patients *CTLA4* SNPs. That confirmed associations between this SNPs and IBD autoimmunity risk. In CD and UC manifestations, There was no variation between CD and UC IBD alleles of *CTLA4* gene (rs231775). This result agree with (12) who discovered that the *CTLA4* gene SNPs rs231775 G>A may raise the danger of developing IBD.

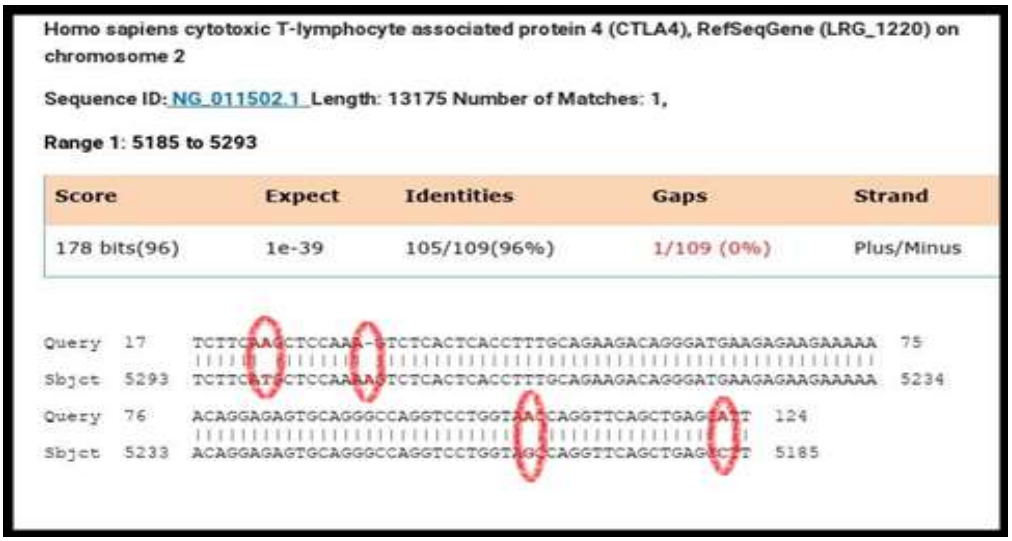

Figure (2): The automated sequencing of *CTLA4* gene from CD patient who carries the allele G SNP.

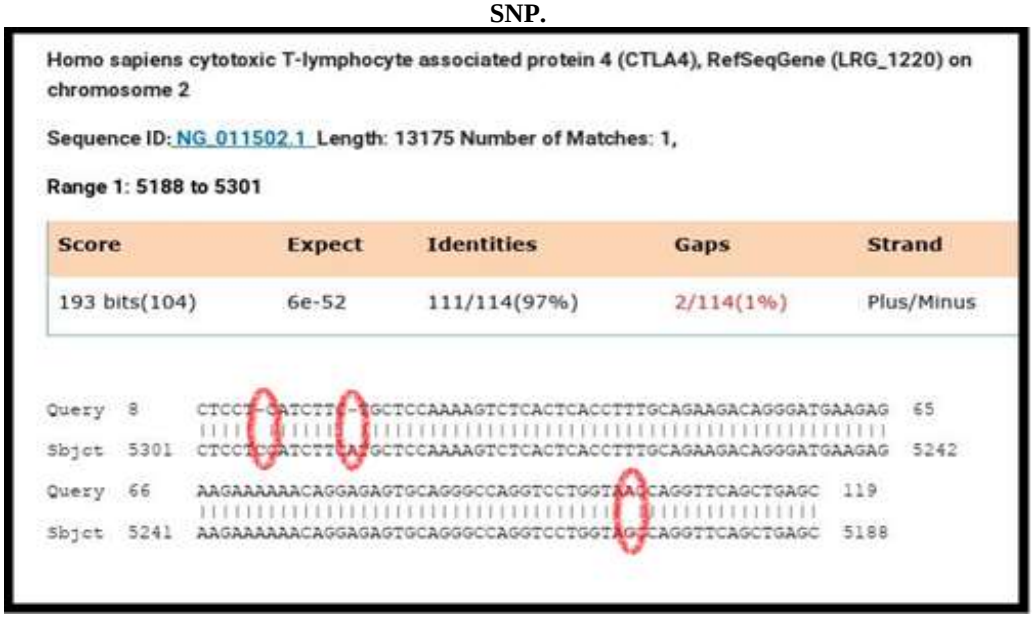

Figure (3): The automated sequencing of *CTLA4* gene from UC patient who carries the allele G SNP.

Determination of interleukin11 by ELISA

The IL11 levels (mean ± SD; pg/mL) were (472.61±162.89 and 460.43±144.65) in CD and UC patients, respectively, and these results were not significantly variation between two groups (*P*>0.05). The levels of IL11in IBD patients show significantly increase (468.17±155.72) pg/ml compared with healthy control (160.15 ± 160.56) pg/ml (Table 1). The present findings of increased serum IL11 levels in diagnosed IBD patients suggest the presence ofan inflammatory response by the imm une

system in IBD. This finding is consistent with previous studies that revealed that IL11 may contribute to the development of IBD due to their pro-inflammatory effects (13). Also, These results agreed with (14) that demonstrated the protein concentration of IL11 was increased in IBD patients compared to control group. When function of epithelial barrier was disrupted the autoimmune inflammation bowel disease was happened and, as a result, abnormal production of inflammatory cytokines. Among these, interleukin IL6, IL11, and IL22 are elevated in human IBD(15).

Table (1): The mean ± standard deviation of IL11 levels in IBD patients.

Interleukin11 Parameter	Crohn's disease pg/ml	Ulcerative colitis pg/ml	IBD Total pg/ml	Control pg/ml
Mean±SD	472.61 ± 162.89 ^{A}	460.43 ± 144.65 ^{A}	468.17 ±155.72 ^{A}	160.15 ±160.56 ^{B}
The different letters referred to significant variation (P < 0.05)				

Quantitative determination of interleukin15 by ELISA

The IL15 levels (mean ± SD; pg/mL) were (451.18 ± 197.33and 395.20 ± 183.54) in CD and UC patients, respectively, and these results were not significantly variation between two groups (P>0.05). The levels of IL15in IBD patients show significantly increase (430.77 ± 193.22) pg/ml compared with healthy control show a significant (112.15 ± 129.22) pg/ml (Table 2). These results agreed with (16**)** the ratio of IL15 in lamina propria of inflamed ileum from CD and of inflamed colon from UC was significantly higher than that in the ileum and colon from control patients (29.5 ± 4.2 in CD, 27.6 ± 4.5 in UC, 5.2 ± 1.7 in normal ileum, and 3.9 ± 1.1 in normal colon, p<0.001). These results indicate that IL15 is increased expression in IBD in the inflamed mucosa when stimulated with microbes or other etiology and explain that the source of IL15 by macrophages activation (17). The IL15 elevation activation of local T cell when T cell-macrophage contact-dependent way, in which CD40-CD40L interactions that lead to activation, proliferation of T cell and proinflammatory cytokine production by both such as TNF and IFN-γ. IL15 not directly cause T cell activation and proinflammatory cytokine creation, as well can synergize with IL12 to contribute immune mucosa injury. In addition, IL15 can too increase their cytotoxic activity against epithelial cells of intestinal. All these events may result in an abnormal immune response, chronic inflammation and tissue injure(18)(8).

Table (2): The mean ± standard deviation of IL15 levels in IBD patients

Interleukin15 Parameter	Crohn's disease pg/ml	Ulcerative colitis pg/ml	IBD Total pg/ml	Control pg/ml
Mean±SD	451.18 ± 197.33 ^{A}	395.20 ± 183.54 ^{A}	430.77 ±193.22 ^{A}	112.15 ±129.22 ^{B}
The different letters referred to significant variation (P < 0.05)				

Quantitative determination of interleukin18 by ELISA

The IL18 levels (mean ± SD; pg/mL) were (43.68 ±16.52 and 41.56 ± 10.79) in CD and UC patients, respectively, moreover these results were not significantly variation between two disease (P>0.05). The

levels of IL18 in IBD patients show increase (42.76±14.67)pg/ml compared with healthy control (40.76 ± 13.27) pg/ml, though, the variation was not statistically variation (Table 3). These results agreed with (19) who was indicated the IL18 mean concentration (422±88 pg/mL) in all patients doubled than the mean concentration in well controls (206±32 pg/mL); though, the variation was not statistically significant. A previous study (20) demonstrated that IL18 effecting by IL10, IFN-γ production , apoptosis and modulator role in the inflammatory flow of CD and UC. Another study(21) detected a likely relationship between two *IL18* gene SNPs promoter region (–607C/A and –137G/C) and susceptibility to IBD involve CD and UC in the population of Tunisian.

Table (3): The mean ± standard deviation of IL18 levels in IBD patients

Interleukin18 Parameter	Crohn's disease pg/ml	Ulcerative colitis pg/ml	IBD Total pg/ml	Control pg/ml
Mean±SD	43.68 ±16.52 [A]	41.56 ±10.79 [A]	42.76 ±14.67 [A]	40.76 ±13.27 [A]
The similar letters referred to non significant variation (P > 0.05)				

Estimation of CTLA4 protein by ELISA

The CTLA4 levels (mean±SD; pg/mL) were (0.64±0.28 and 0.61±0.29) in CD and UC patients, respectively, moreover these results were not significantly variation between two groups (*P*>0.05). The levels of CTLA4 in IBD patients show significantly decreased (0.63 ± 0.28) pg/ml compared with healthy control show a significant (0.80 ± 0.40) pg/ml (Table 4). As a result, decreased expression of CTLA4 protein in T regular cells in patients who carriers with SNPs *CTLA4*.Whilst high numbers of T regular cells were usually present, role suppressive. The labors were prepared to recognize SNPs that be able to change the structure of *CTLA4* gene, expression and role. at what time A is replaced with G, one of the mainly mutations occurs *CTLA4* gene major, resulting in an amino acid replacement from threonine to alanine, placed in the leading peptide segment found in humans, increased risk for IBD: rs231775. This order of this peptide is essential since it is recognized by other proteins and often produce the mature protein by cleaved off. That is introduced in the signal peptide, the new remains, vary in its properties from the original wild one. This mutation can perturb the identification of the signal peptide (22). Furthermore, the mutation introduces disturb correct folding , loss of hydrogen bonds more hydrophobic residue, variation in charge, geometry, size can too disrupt interactions the H bond with the nearby molecules (23). The stability of protein is important for functional activity and a conformational structural of protein. When a damaging that can influence the CTLA4 protein stability. Can creation degradation, or conglomeration of abnormal protein and misfolding when alter in protein stability. Furthermore, evolutionary conservancy in the sequence of protein is required to verify whether a mutation is unsafe to the host (24).The protein of CTLA4 in general immune regulation activity will be extensively reduced if a harmful mutation occurs in this protein, as a result, inactivating CTLA4 protein that rising the risk of autoimmune diseases like inflammation bowel disease (25).

Table (4): The mean ± standard deviation of CTLA4 protein in IBD patients.

CTLA4 Parameter	Crohn's disease pg/ml	Ulcerative colitis pg/ml	IBD Total pg/ml	Control pg/ml
Mean±SD	0.64 ± 0.28 [A]	0.61 ± 0.29 [A]	0.63 ± 0.28 [A]	0.80 ± 0.40 [B]
The different letters referred to significant variation (P < 0.05)				

Conclusions

The SNP *CTLA4* gene (rs231775) is associated with the subsequent development of chronic autoimmune CD and UC, on level of the allele, where allele *A* is a protective factor plus allele *G* might be a threat factor. Measurements of selected cytokines including IL11, IL15, IL18 and CTLA4 using ELISA, have shown elevated levels except CTLA4 decrease in IBD patients that demonstrated continuance homeostasis of intestinal by maintenance epithelial barrier integrity is vital. Respond to the intestinal microbiota and mutation leads to abnormal immune activation and, as a perturbation effect, outcome in autoimmune conditions, involve IBD.

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