ISSN: 0067-2904

Association of *CTLA4* and *NOD2/CARD15* (N852S) Genes Single Nucleotide Polymorphisms with Inflammatory Bowel Disease in Iraqi Patients

Rand Manaf Abd Al-Rhman*, Jinan Mohammed Jawad Alsaffar
Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq

Received: 27/9/2023 Accepted: 22/1/2024 Published: 30/1/2025

Abstract

The present study was designed to examine single nucleotide polymorphisms (SNPs) of *CTLA4* (rs231775) and *NOD2/CARD15* (rs104895467) genes profiles that are related to etiology and pathogenesis of inflammatory bowel disease (IBD) in two groups of Iraqi patients (Crohn's disease (CD) and ulcerative colitis (UC)). The frequency of the allele and genotypes were determined using polymerase chain reaction (PCR-RFLP) and then confirmed by sequence analysis. For *CTLA4* (rs231775) SNP comparing IBD patients to controls revealed some significant variations. The GG genotype demonstrated a significant increased incidence in IBD patients than controls (62.4*vs.* 0.0%; OR = 238.69; *pc* = $5.7x10^{-9}$). In contrast, the AA genotype (14.1*vs.* 77.8%; OR = 0.05; *pc*= $8.5x10^{-9}$) appeared to have a decreased frequency in patients. Such as the A allele observed significantly decreased frequency in IBD patients compared to controls (25.9*vs.* 89.0%; OR = 0.04; *pc* = $6.6x10^{-9}$). While the G allele showed a significantly increased frequency in patients (74.1*vs.* 11.0%; OR = 22.91; *pc*=$6.6x10^{-9}$). And for the *NOD2/CARD15* (rs104895467) SNP indicated no significant association with the IBD. The genotype frequencies of the *CTLA4* and *NOD2/CARD15* SNPs also showed no significant variation between CD and UC patients.

Keywords: Inflammatory bowel disease, Crohn's disease, Ulcerative colitis, PCR-RFLP.

علاقة تعدد أشكال النيوكليوتيدة الأحادية لمورثات*CTLA4* و(N852S) *NOD2/CARD15* مع داء الأمعاء الإلتهابي

رند مناف عبد الرحمن *، جنان محمد جواد الصفار
قسم التقنيات الإحيائية ، كلية العلوم، جامعه بغداد، بغداد، العراق

الخلاصة

صممت الدراسة الحالية لفحص تعدد أشكال احادي النوكليوتيدات (SNPs) لجينات *CTLA4* (rs231775)و *NOD2 / CARD15* (rs104895467)المرتبطة بامراضية ومسببات داء الأمعاء الإلتهابي لدى مجموعتين من المرضى العراقيين (مرضى داء كرون والتهاب القولون التقرحي). تم تحديد تردد الأليل والطرز الوراثية باستخدام تفاعل البوليميراز المتسلسل (PCR–RFLP) وتم تأكيده عن طريق تحليل التسلسل. بالنسبة لـ*CTLA4* (rs231775) SNP كشفت مقارنة مرضى داء الأمعاء الإلتهابي مع

*Email: rand.manaf@sc.uobaghdad.edu.iq

السيطرة عن بعض الفروقات المعنوية ، أظهر الطراز الوراثي GG زيادة كبيرة في الإصابة في مرضى داء الأمعاء الإلتهابي مقارنة بالسيطرة (62.4 مقابل 0.0٪ ؛OR = 238.69؛ pc = X5.7 10^{-9}).في المقابل ، الطراز الوراثي AA (14.1 مقابل 77.8٪ ؛ OR = 0.05؛ PC = 8.5x10^{-9}) أظهر انخفاض التردد في المرضى. على هذا النحو ، لوحظ أن الأليل A قد اظهر انخفاضا معنويا في التردد في مرضى داء الأمعاء الإلتهابي مقارنة بالسيطرة (25.9 مقابل 89.0٪ ؛ OR = 0.04 ؛ PC = 6.6X10^{-9}). بينما أظهر الأليل G زيادة معنوية في التردد لدى المرضى (74.1 مقابل 11.0٪ ؛ OR = 22.91؛ PC = 6.6x10^{-9}).لكن بالنسبة لـ *NOD2 / CARD15* SNP (rs104895467) ، تم الكشف عن عدم وجود علاقة معنوية مع مرضى داء الأمعاء الإلتهابي ولم تظهر ترددات الطرز الوراثية *CTLA4* و *NOD2 / CARD15* SNPs أي فروقات معنوية بين مرضى داء كرون والتهاب القولون التقرحي.

Introduction

Inflammatory bowel diseases (IBD) are chronic inflammatory conditions of the gastrointestinal tract driven by inappropriate immune responses to an altered gut microbiome in genetically susceptible individuals. Crohn's disease (CD) and ulcerative colitis (UC) are the most extensively recognized kind of IBDs and have been the focus of attention reason to their rising frequency [1]. Crohn's disease, one of the most frequent forms of inflammatory disease worldwide, is characterized by the formation of strictures, fistulas, ulcer, and granulomas in the mucosa [2]. UC is a further kind of inflammatory bowel disease characterized by granularity, surface ulcerations and a vascular pattern. In difference with the inflammation found in CD occurs throughout the entire GT, In UC it is limited to the colon mucosal layer [3]. The main symptoms of active inflammatory bowel disease are diarrhea mixed with blood, fever, abdominal pain and weight loss. Anemia can as well take place and rectal bleeding is less common in CD. Since the small intestine is responsible for the absorption of nutrients, malnutrition is very general in Crohn's disease [4]. While UC is commonly associated with rectal bleeding, often symptoms appear gradually and can range from mild to severe. Symptoms typically happen from time to time with periods of no symptoms in between. Complications may involve abnormal dilation of the colon (megacolon), colon cancer, inflammation of the joints, eye and liver [5]. The prevalence of IBD has been progressively rising worldwide, particularly in developed countries. In Asia, the highest incidence was found in China [6]. In Iraq, the greater part of patients was from urban areas or inside Mosul city center. Additional research has indicated that the greater frequency of patients had severe conditions [7]. Few epidemiological studies are available regarding the diversity factors that cause incidence IBD [8]. This, however, requires deeper research into the variety of factors leading the frequency of IBD in the specific population, especially when taking into consideration the increasing rate of IBD, alongside the peculiar socioeconomic variables in the local population [9]. The etiology is still not completely known, however, there are certain factors that weaken immune system such as environmental factors a virus or bacteria [10], emotional distress, diet, smocking, antibiotics and others [11], as well as immunological and genetic components which cause inflammation of the gastrointestinal tract which is more likely to develop this inappropriate immune response [12]. As candidate genes predisposed to IBD, we paid attention to *CTLA4* (rs231775) and *NOD2/CARD15* (rs104895467), *CTLA4* is responsible for shutting off T cell responses against self-antigens in a process known as anergy and can lead to several autoimmune diseases [13;14]. *NOD2/CARD15* gene encodes the NOD2 protein which is mainly expressed in dendritic cells, phagocytic immune cells, and enterocytes, monocytes and Paneth cells also NOD2 protein, playing an essential role in innate immune response of intestinal against cell wall of bacteria [15]. Therefore, this study aimed to explore the status of *CTLA4* (rs231775) and *NOD2/CARD15* (rs104895467) SNP, and their relationship with autoimmune IBD in Iraq patients.

Materials and Methods

Subjects

This study was approved by the Ethics Committee in the College of Science, University of Baghdad (Ref.: CSES/0422/0063). One hundred and fifty-seven subjects were selected for this study which included patients tacked therapy (N=85, 39 males and 46 females) aged from 15 to 68 years who suffered from inflammatory bowel disease. Also, the diagnosis depended on the endoscopist, clinical signs, colonoscopy and laboratory diagnosis by consultant physician, in addition to well random subjects as control group (N=72, 35 males and 37 females). This study was conducted in private lab and Gastroenterology Hepatology Hospital.

Blood Samples

Blood was collected from each healthy control and patients by vein puncture using 10ml disposable syringes. A volume of 4 ml was added to EDTA tubes which was stirred gently for few seconds to avoid clotting and then stored at -20ºC until DNA extraction and detection of SNP *CTLA4* (rs231775) and *NOD2/CARD15* (rs104895467) genes associated with IBD.

DNA Extraction

Isolation 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 which was followed by purity and concentration assessment. The extracted DNA was then stored at -20°C until analysis for genotyping.

PCR Amplification and Genotyping of CTLA4 (rs231775) and NOD2/CARD15 (rs104895467) SNPs

In this study two SNPs, the first a fragment 152 bp containing the (rs231775) SNPs placed in chromosome 2 (2q33) in exon 1 of *CTLA4* gene is also the second a fragment 151 bp containing the (rs104895467) SNPs in *NOD2/CARD15* gene is located in chromosome 16, were 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 for *CTLA4* gene and were used F: 5-CTGTTTGCATGATGGGGGG-3 and R: 5-CAGCCGTCAGTCAATTTGTAG-3 for *NOD2/CARD15* gene [16]. Then 100 pmole of stock solution forward and reverse primers was prepared by dissolving lyophilized products in nuclease free water which were then used to prepare working solution. The primers, supplied by Alpha DNA Corporation, Canada, and designed using NCBI Primer-BLAST, were used in the PCR reaction. The PCR reaction was performed in a volume of 25 µl, comprising 1 µl each of forward (F) and reverse (R) primers for CTLA4 (rs231775) at a concentration of 10 pmol/µL, as well as 1 µl of each primer for NOD2/CARD15 (rs104895467). The reaction mixture also contained 12.5 µl of master ready mix (Go Taq® Green Master Mix 2X: A reaction mixture containing DNA (2 µl, 100 ng), nuclease-free water (8.5 µl), and reagents from Promega Corporation, USA, was prepared. The mixed compound tube was processed using the Exispin system. The DNA amplification was then carried out using the thermal cycler (Multigene TM Gradient Thermal Cycler, Labnet International, USA). It was programmed as follows: First denaturationat 95°C for 5 minutes, followed by 35 cycles programmed as follows: First denaturation of denaturation at 95°C for 30 seconds, annealing for 1min at 57°C (rs231775), extension at 72°C for 30 seconds and followed by one cycle of a final extension 72°C for 10 minutes for *CTLA4*. Those for *NOD2/CARD15* were programmed as follows: initial denaturation of 5 min at 95°C and then 35 cycles of the following three steps: denaturation at 95 °C for 1min,

annealing 57°C for one minute (rs104895467) and extension at 72 °C for 1min and final incubation at 72 °C for ten minute. PCR products were then separated on 2% agarose gel electrophoresis at 5 v/cm^2 for 1 hour to confirm amplification in the existence DNA ladder marker (100 bp) (Promega, USA) with 1 × Tris-borate-EDTA buffer and in the UV light (302nm) was visualized subsequent to staining by ethidium bromide [17]. The amplicons were digested by the allele-specific restriction endonucleases BstEII (rs231775) (Takara/ Japan) for *CTLA4* (Wild-type 152 bp; Mutant Heterozygote152+130+22bp, Homozygote 130+22bp) and AluI (rs104895467) (supplied by Promega corporation, USA) for *NOD2/CARD15* (Wild-type 151 bp; Mutant Heterozygote151+129+22bp, Homozygote 129+22bp). For *CTLA4* gene, the substitution A to G allele produced a BstEII restriction site G^GTNACC site and was established when incubated at 60^{0}C overnight the amplified products of PCR(5µl) with BstEII (2 µl) and buffer (3µl). Also, for *NOD2/CARD15* gene, the substitution A to G allele produced a AluI restriction site which was established when incubated 5 µl of amplified products of PCR with 1 µl of AluI, 1 µl BSA and 3µl buffer at 37^{0}C overnight. Digestion reactions were performed on ice in a total volume of 10 µl. Digestion products were then run on 8% PAGE gel that was used for checking the SNPs. The gel was run at10-12.5 volts/cm for 2h-5h., using 0.5 X Tris borate buffer and stained with ethidium bromide. The resulting fragments were visualized under the UV florescence. Rest of the PCR products were also 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 compared with the original genes in Gene Bank in NCBI and confirmed PCR-RFLP results [16].

Statistical Analysis

Allele and genotype frequencies, as well as differences, were assessed by Pearson's Chi-square test. The association between *CTLA4* and *NOD2/CARD15* single nucleotide polymorphism and autoimmune inflammatory bowel disease was determined as odds ratio (OR) with the confidence of interval (CI estimate at 95%).

Results and Discussion

The genomic DNA from 157 subjects were extracted by the Wizard® Genomic DNA Purification Kit before PCR-RFLP and sequencing steps. Nanodrop results showed that, depending on white blood cell count of the subject, the recoded rang of DNA concentration was(20-787)ng/µl and depending on the freshness of blood samples,the DNA purity was (1.7-1.9). The results of amplified *CTLA4* PCR products, which were visible in agarose gel electrophoresis (Figure 1), demonstrated that a yield of 152 bp single band for the desired result and lane L: 100bp DNA ladder; lanes 1-10: CD patients ; lanes 11-20: UC patients; lanes 20-25: control. And successful amplification of *NOD2/CARD15* gene was indicated by agarose gel electrophoresis when single and sharp bands with 151 bp molecular sizes (Figure 2): Lane L: 100bp DNA ladder; Lanes 1-13(CD and UC patients); Lanes 14-17(controls).

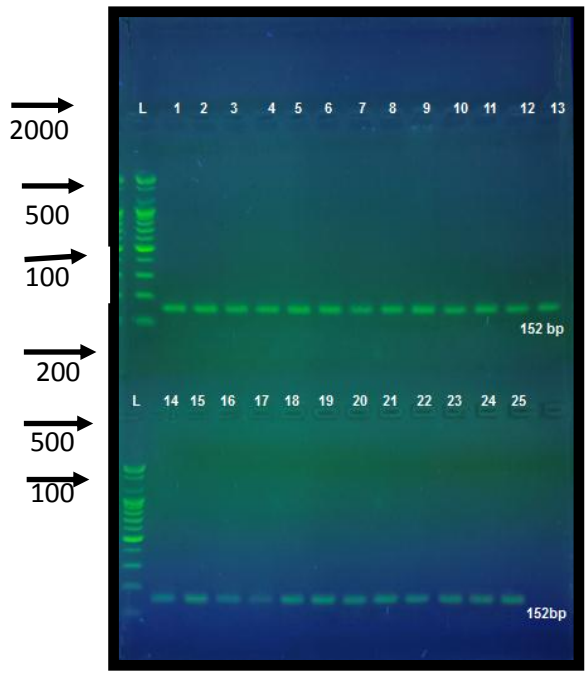

Figure 1: Electrophoresis of *CTLA4* gene

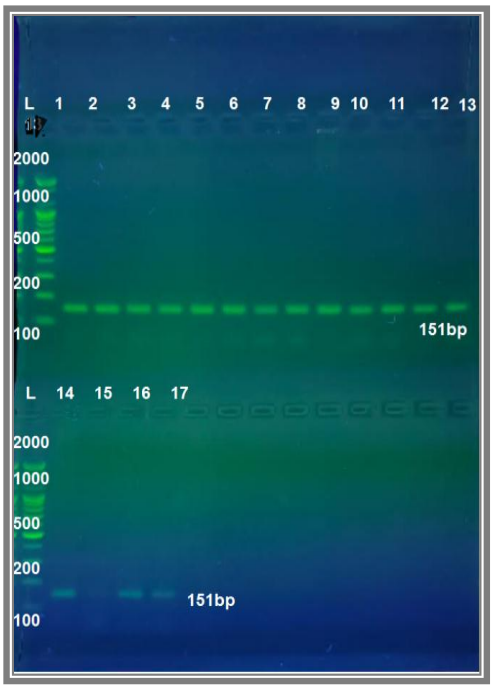

Figure 2: Electrophoresis of *NOD2/CARD15*gene

The results of genotypes distributions and frequencies among the CD and UC patients using PCR-RFLP fragment electrophoresis are as following: *CTLA4* gene (rs231775) polymorphisms are shown in Figure 3 where lanes 4, 7 and 8 show AG genotype (polymorphic, heterozygous 152 bp, 130 bp and 22 bp), lanes 1, 2, 3, 5, 6, 9 and10 show GG genotype (homozygous, 130 bp, 22bp). Due to its small size fragment ,which was 22 bp, it did not appear in the gel, indicating presence of G allele in CD and UC patients and that samples had substitution mutation A to G at nucleotide. The study showed that the G allele, AG and GG genotype for the *CTLA4* gene polymorphism were associated with susceptibility to CD and UC (IBD) compared to control subjects. Consistently, H. Turpeinen *et al*. [18] instituted a likely connection of the *CTLA-4* gene +49A/G substitution with IBD disease threat. Mutations and polymorphisms that alter *CTLA4* activity are thought to be critical factors in the risk of developing autoimmunity [19]. In addition, environmental factors are also involved in IBD. The resulting distributions and frequencies genotypes among the CD and UC patients for *NOD2/CARD15* gene polymorphisms are shown in Figure 4. Lanes 1-10 indicated no restriction fragment except only in one band (151 bp), AA genotype (wild-type, homozygous) and no other N852S mutant bands 151+129+22 and 129+22 bp exhibited. Also, Long [20] did not notice *NOD2/ CARD15* gene mutation in Han IBD patients. However, Tukel *et al*. [21] found that mutation of the *NOD2/CARD15* gene was significantly related to CD in Ashkenazi Jewish populations.

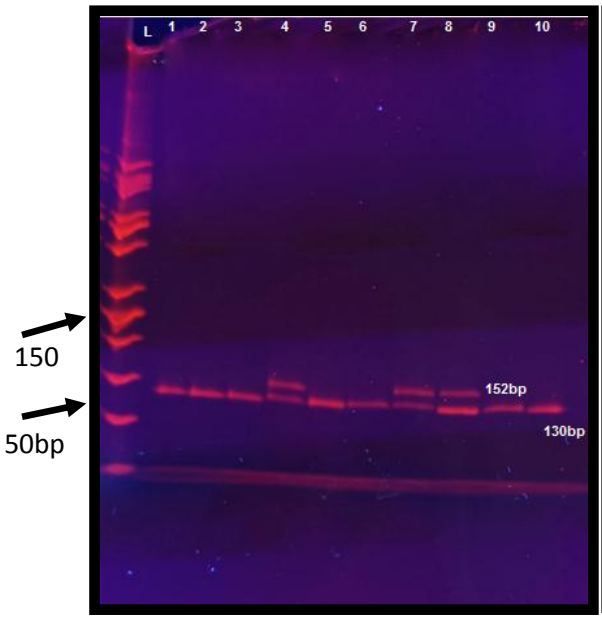

Figure 3: The BstEII restriction enzyme profiles.

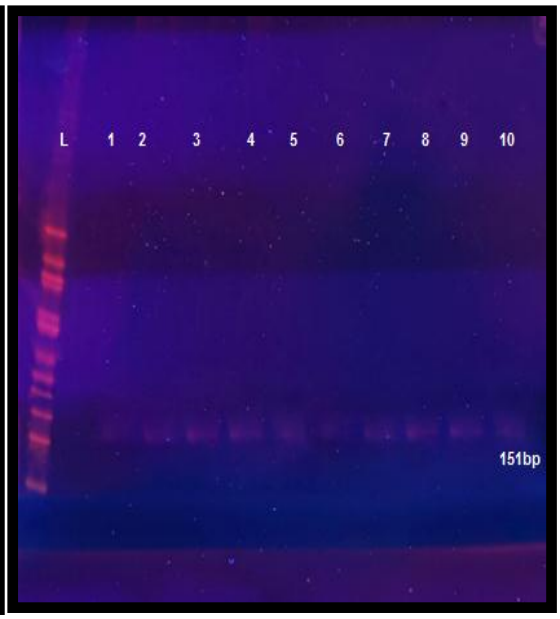

Figure 4: The AluI restriction enzyme profiles.

Genotype Distribution and Allele Frequencies of the *CTLA4* (rs231775) and *NOD2/CARD15*(rs104895467) SNP

The results of *CTLA4* and *NOD2/CARD15* genes were also sequenced and confirmed. PCR-RFLP results were investigated for samples selected from IBD patients, comparing the sequences with those in the Gene Bank database using the BLAST program**.** The comparison between IBD patients and controls revealed some significant variations for *CTLA4* gene. The GG genotype showed a corrected significantly increased frequency in IBD patients compared to controls (62.4*vs.* 0.0%; OR = 238.69; *pc* = $5.7x10^{-9}$). In contrast, the AA genotype (14.1*vs.* 77.8%; OR = 0.05; *pc*= $8.5x10^{-9}$) exhibited a decreased frequency in patients. The A alleles observed a corrected significantly decreased frequency in IBD patients compared to controls (25.9*vs.* 89.0%; OR = 0.04; *pc* = $6.6x10^{-9}$). Whereas the G allele showed a corrected significantly increased frequency in patients (74.1*vs.* 11.0%; OR = 22.91; *pc*=$6.6x10^{-9}$). The distribution of allele and genotype frequencies of *NOD2/CARD15* (rs104895467) SNP showed no significant variation between IBD patients and controls (Table 1).

Table 1: Genotype distribution and allele frequencies of the *CTLA4*and *NOD2/CARD15* SNP in IBD patient.

Allele or Genotype		Patients IBD n=85 (%)	Controls n=72 %	Odds Ratio (OR)	EF or PF	95% C.I.	*P*-value	PC-Value
CTLA4	**A**	44 (25.9)	120(83.3)	0.04	85.0 %	0.02-0.08	$1.1x10^{-8}$	$6.6x10^{-9}$
	G	126 (74.1)	24(16.7)	22.91	70.9%	12.31-42.62	$1.1x10^{-8}$	$6.6x10^{-9}$
	AA	12 (14.1)	56 (77.8)	0.05	74.1%	0.02-0.11	$2.3x10^{-9}$	$8.5x10^{-9}$
	AG	20 (23.5)	16 (22.2)	1.08	1.7%	0.51-2.26	1.0	0.851 NS
	GG	53 (62.4)	0 (0.0)	238.69	61.9%	14.57-3909.62	$1.4x10^{-9}$	$5.7x10^{-9}$
NOD2/CARD15	**A**	170 (100)	144(100)	-	-	-	-	-
	G	0 (0)	0(0)	-	-	-	-	-
	AA	85 (100)	72(100)	-	-	-	-	-
	AG	0 (0)	0(0)	-	-	-	-	-
	GG	0 (0)	0(0)	-	-	-	-	-

IBD: Inflammatory bowel disease; C.I.: Confidence interval; PF: Preventive fraction; EF: Etiological fraction; *p*: Fisher's exact probability; *pc*: Corrected *p*; NS: $p > 0.05$.

The *CTLA4* (rs231775) polymorphism genotype distributions and frequencies of allele in the CD patients and controls are indicated in Table 2. AA genotype was prevalent in the CD patients and controls (13% vs 77.8%); OR = 0.04; *pc*= $9.3x10^{-14}$) but appeared with decreased frequency in patients. The AG genotype (18.5% vs 22.2%); OR = 0.80; *pc*= 0.519) had no significant differences in the frequency between the CD patients and controls (p>0.05), and the GG genotypes also showed significantly increased occurrence in patients than controls (68.5% vs 0%); OR = 310.71; *pc*= $2.7x10^{-9}$). *A* alleles had a corrected significantly decreased frequency in CD patients compared to controls (22.2*vs.* 83.3%; OR = 0.06; *pc* = $3.1x10^{-9}$). The *G* allele showed a corrected significantly increased frequency in patients (77.8*vs.* 16.7%; OR = 17.50; *pc*=$3.1x10^{-9}$). Logistic regression analysis demonstrated that the *CTLA4* gene SNP was significantly associated with the CD disease. While *NOD2/CARD15* (rs104895467) SNP found no association to IBD susceptibility in CD patients.

Table 2: Genotype distribution and allele frequencies of the *CTLA4*and *NOD2/CARD15* SNP in CD patient.

Allele or Genotype		Patients CD n=54 (%)	Controls n=72 %	Odds Ratio (OR)	EF or PF	95% C.I.	*P*-value	PC-Value
CTLA4	**A**	24 (22.2)	120(83.3)	0.06	78.6%	0.03-0.11	$5.8x10^{-9}$	$3.1x10^{-9}$
	G	84 (77.8)	24(16.7)	17.50	73.3%	9.34-32.80	$5.8x10^{-9}$	$3.1x10^{-9}$
	AA	7 (13)	56(77.8)	0.04	74.5%	0.02-0.11	$2.5x10^{-13}$	$9.3x10^{-14}$
	AG	10 (18.5)	16(22.2)	0.80	4.5%	0.33-1.91	0.662	0.519 NS
	GG	37 (68.5)	0 (0)	310.71	68.0%	18.54-5206.92	$6.1x10^{-9}$	$2.7x10^{-9}$
NOD2/CARD15	**A**	108 (100)	144(100)	-	-	-	-	-
	G	0 (0)	0(0)	-	-	-	-	-
	AA	54 (100)	72(100)	-	-	-	-	-
	AG	0 (0)	0(0)	-	-	-	-	-
	GG	0 (0)	0(0)	-	-	-	-	-

CD: Crohn's disease**;** C.I.: Confidence interval; PF: Preventive fraction; EF: Etiological fraction; *p*: Fisher's exact probability; *pc*: Corrected *p*; NS: $p > 0.05$.

The *CTLA4* gene polymorphism genotypes distributions and frequencies allele in the ulcerative colitis (UC) patients and controls are revealed in Table 3. The AA genotype was prevalent in the UC patients and controls (16.1% vs 77.8%); OR = 0.05; *pc*= $2.5x10^{-9}$), appeared with decreased frequency in patients. There were AG genotype (32.3% vs 22.2%); OR = 1.67; *pc*= 0.226) which had no significant differences in the frequency between the UC patients and controls (*p*>0.05), and the GG genotypes also showed significant increased incidence in patients than controls (51.6% vs 0%); OR = 154.35; *pc*= $3.5x10^{-11}$). *A* alleles displayed a corrected significantly decreased incidence in UC patients than control (32.3%*vs.* 83.3%; OR = 0.10; *pc* = $1.3x10^{-9}$). The *G* allele showed a corrected significantly increased frequency in patients (67.7%*vs.* 16.7%; OR = 10.50; *pc*=$1.3x10^{-9}$). While the distribution of allele and genotype frequencies of *NOD2/CARD15* (rs104895467) SNP indicated no significant difference in UC patients than controls, and were also found to be not associated with IBD susceptibility in UC patients.

Table 3: Genotype distribution and allele frequencies of the *CTLA4*and *NOD2/CARD15* SNP in UC patient.

Allele or Genotype		Patients UC n=31 (%)	Controls n=72 (%)	Odds Ratio (OR)	EF or PF	95% C.I.	*P*-value	PC-Value
CTLA4	**A**	20 (32.3)	120(83.3)	0.10	75.4%	0.05-0.19	$2.5x10^{-9}$	$1.3x10^{-9}$
	G	42 (67.7)	24(16.7)	10.50	61.3%	5.29-20.84	$2.5x10^{-9}$	$1.3x10^{-9}$
	AA	5 (16.1)	56(77.8)	0.05	73.5%	0.02-0.16	$5.6x10^{-9}$	$2.5x10^{-9}$
	AG	10 (32.3.)	16(22.2)	1.67	12.9%	0.66-4.20	0.326	0.226 NS
	GG	16 (51.6)	0 (0)	154.35	51.2%	8.97-2655.86	$1.3x10^{-10}$	$3.5x10^{-11}$
NOD2/CARD15	**A**	62 (100)	144(100)	-	-	-	-	-
	G	0 (0)	0(0)	-	-	-	-	-
	AA	31 (100)	72(100)	-	-	-	-	-
	AG	0 (0)	0(0)	-	-	-	-	-
	GG	0 (0)	0(0)	-	-	-	-	-

UC: Ulcerative colitis; C.I.: Confidence interval; PF: Preventive fraction; EF: Etiological fraction; *p*: Fisher's exact probability; *pc*: Corrected *p*; NS: $p > 0.05$.

Similar observations were observed in CD and UC manifestations (Table 2 and 3). There was no significant variation between CD and UC IBD alleles and genotype frequencies of *CTLA4* gene (rs231775) and *NOD2/CARD15* (rs104895467) polymorphism SNP (Table 4)

Table 4: Genotype distribution and allele frequencies of the *CTLA4*and *NOD2/CARD15* SNP in two patients.

Allele or Genotype		Patients CD n=54 (%)	Patients UC n=31 (%)	Odds Ratio (OR)	EF or PF	95% C.I.	*P*-value	PC-Value
CTLA4	**A**	24 (22.2)	20 (32.3)	0.60	12.9%	0.30-1.20	0.202	0.109 NS
	G	84 (77.8)	42 (67.7)	1.67	31.1%	0.83-3.34	0.202	0.109 NS
	AA	7 (13)	5 (16.1)	0.77	3.6%	0.23-2.64	0.751	0.537 NS
	AG	10 (18.5)	10 (32.3.)	0.48	16.9%	0.17-1.30	0.187	0.120 NS
	GG	37 (68.5)	16 (51.6)	2.04	34.9%	0.83-5.0	0.163	0.109 NS
NOD2/CARD15	**A**	108 (100)	62 (100)	-	-	-	-	-
	G	0 (0)	0 (0)	-	-	-	-	-
	AA	54 (100)	31 (100)	-	-	-	-	-
	AG	0 (0)	0 (0)	-	-	-	-	-
	GG	0 (0)	0 (0)	-	-	-	-	-

CD: Crohn's disease; UC: Ulcerative colitis; C.I.: Confidence interval; PF: Preventive fraction; EF: Etiological fraction; *p*: Fisher's exact probability; *pc*: Corrected *p*; NS: $p > 0.05$.

W. Alaya *et al*. [22] discovered that the *CTLA4* gene SNPs rs231775 G>A may raise the danger of developing IBD. Though, in a Tunisian IBD patients-controls study found that *CTLA4* A allele and AA genotype were related with Crohn's disease in the patient population [23]. CTLA4′s structural conformation is vital for its functional role in controlling immune homeostasis [24]. As a result *CTLA4* polymorphism, the substitution of adenine (A) with

guanine (G) results in alanine (Ala) in the signal peptide instead of the wild-type threonine (Thr) resulted in ineffective CTLA4 glycosylation and reduced cell surface expression that disrupted the balance of CD28 and CTLA4 interactions with B7-1/2 and would product inflammatory bowel disease via preventing apoptosis or downregulating activated self-reaction T-lymphocyte [25; 26]. Previous studies of *NOD2/CARD15* regarding IBD mainly focused on the relevance of *NOD2/CARD15* mutations and loss within the disease onset and progression, more studies are exploring other functional roles that *NOD2/CARD15* gene encodes the NOD2 protein which is mainly expressed in phagocytic immune cells, playing a vital role in the intestinal innate immune response against the bacteria cell wall [27]. Thus, Liu *et al.* [28] demonstrated that the *NOD2/CARD15* gene plays a central role in the innate immune system, specifically in the maintenance of the intestinal barrier, the transport of gut derived toxins, and the sensing of intestinal microbes.

Conclusion

Based on the results of the current study, it can be concluded that 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, in addition to a *CTLA4* gene SNP that have a paramount role to effect to the stability of the CTLA4 protein via preventing apoptosis that result IBD. Whereas *NOD2/CARD15* gene N852S SNP (rs104895467) is not significantly related with the illness in Iraq population, this is the initial study of these SNPs in Iraq.

Conflict of Interest: There are no conflicts of interest.

References

[1] J. E. Axelrad and S. C. Shah, "Diagnosis and management of inflammatory bowel disease-associated neoplasia: considerations in the modern era," *Therapeutic Advance in Gastroenterology*, vol.13, pp. 1-14, 2020. https://doi.org/10.1177/1756284820920779

[2] B. R.R. De Mattos, M. P. G. Garcia, J. B. Nogueira, L. N. Paiatto, C. G. Albuquerque, C. L. Souza, L.G. R. Fernandes, W.M. d Tamashiro and P. U Simioni, "Inflammatory Bowel Disease: An Overview of Immune Mechanisms and Biological Treatments, " *Mediators Inflammation* ,vol.2015, pp. 1-11,2015. 493012.https://doi.org/10.1155/2015/493012

[3] L. M Spekhorst, M. C. Visschedijk, R. Alberts, E. A Festen, E. Wouden, G. Dijkstra, R. K. Weersma, and Dutch Initiative on Crohn and Colitis (ICC), "Performance of the Montreal classification for inflammatory bowel diseases, " *World Journal of Gastroenterology*, vol. 20, no.41 pp. 15374-15381, 2014.https://doi.org/10.3748/wjg.v20.i41.15374

[4] J. P. Hugot, A. Corinne, B. Dominique, B. Edouard and J.P. Cezard, "Crohn's disease: the cold chain hypothesis," *Lancet*, vol. 362, no. 9400,pp. 2012-2015, 2003. https://doi.org/10.1016/S0140-6736(03)15024-6.

[5] M. H. Wanderås, B. A. Moum, M.L. Høivik and Ø. Hovde, "Predictive factors for a severe clinical course in ulcerative colitis: Results from population-based studies," *World Journal of Gastrointestinal Pharmacology and Therapeutics*, vol. 7, no. 2, pp. 235-241, 2016. https://doi.org /10.4292/wjgpt.v7.i2.235

[6] B. Rupa, P. Partha, H. Susan, G. Girish, and R. Nageshwar, " Familial aggregation of inflammatory bowel disease in India: prevalence, risks and impact on disease behavior," *Intestinal Research*, vol.17, no. 4,pp. 486-495, 2019.https://doi.org/10.5217/ir.2018.00174

[7] B.I. Mohammed and B.K. Amin, "Sociodemographic characteristics, smoking, and family history of patients with inflammatory bowel disease, northern part of Iraq, " *Medical Journal of Babylon*, vol. 19, no. 4 ,pp.615-619, 2022.https://doi.org/10.4103/MJBL.MJBL_162_22

[8] B.M. Muhammed, " Epidemiological and Clinical Aspects of Ulcerative Colitis in Mosul city, Iraq," in *3rdInternational Conference on Health and Medical Sciences*: Insight into Advanced Medical Research, 2019.https://doi.org/10.24017/science.2019.ICHMS.6

[9] M. Mahmoud , A. Sameer , H. Fuad , A. R. Antoine , S.Faisal , and D. Silvio, " Incidence, Prevalence, and Clinical Epidemiology of Inflammatory Bowel Disease in the Arab World: A Systematic Review and Meta-Analysis," *Inflammatory Intestinal Diseases*, Vol. 6, no. 3, pp.123-131, 2021.https://doi.org/10.1159/000518003

[10] R. F. Hassen and H. A. Alsalim, " The Influence of Entamoeba histolytica Against Some Gut Microbiota in Children with Acute Amoebic," *Iraqi Journal of Science*, vol. 64, no. 1, pp.138-148, 2023.https://doi.org/10.24996/ijs.2023.64.1.14

[11] S. H. Kashan, M. G. Shahnaz, and A.M. Muhshin, " Prevalence of Oral Manifestations of Inflammatory Bowel Disease in Patients Admitted to Sulaymaniyah teaching hospital – Iraq" *AL-Kindy College Medical Journal*, vol.16, no. 1, pp. 47-53, 2020.https://doi.org /10.47723/kcmj.v16i1.190

[12] A.N. Ananthakrishnan, " Epidemiology and risk factors for IBD," *Natura Reviews. Gastroenterology and Hepatology*, vol. 12, no.4, pp.205-217, 2015.https://doi.org/10.1038/nrgastro.2015.34

[13] A. Abbas, *Basic Immunology*: *Functions and Disorders of the Immune System, 6e: e-E-Book. Elsevier India*. Chapter 9, 2011, p. 178.

[14] A. H. Yassin, A. A. Al-Kazaz, A. M. Rahmah and T.Y. Ibrahim, " Association of *CTLA-4* Single Nucleotide Polymorphisms with Autoimmune Hypothyroidism in Iraqi Patients, " *Iraqi Journal of Science*, vol.63 no.7 , pp.2891-2899, 2022.https://doi.org/10.24996/ijs.2022.63.7.13

[15] J.M. Allaire, S.M. Crowley, H.T. Law, S. Chang, H. Ko and B. Vallance, " The intestinal epithelium: Central coordinator of mucosal immunity," *Trends in Immunology*, vol.39, no.9, pp.677-696,2018. https://doi.org/10.1016/j.it.2018.04.002

[16] S. B. Diler and S. Yaraş, " *CTLA-4* (+49A/G) and *NOD2/CARD15 (N852S)* polymorphisms with inflammatory bowel disease in Turkish patients," *Cellular and Molecular Biology*, vol. 64, no.11, pp. 97-101, 2018. https://pubmed.ncbi.nlm.nih.gov/30213296/

[17] I. A. Dvortsov, N. A. Lunina, L. A. Chekanovskaya, E. N. Shedova, L. V. Gening and G. A. Velikodvorskaya, " Ethidium bromide is good not only for staining of nucleic acids but also for staining of proteins after polyacrylamide gel soaking in trichloroacetic acid solution," *Analytical Biochemistry*, vol.353, no.2, pp,293-295,2006.https://doi.org/10.1016/j.ab.2006.03.001

[18] V. Csöngei, L. Járomi, E. Sáfrány, C. Sipeky, L. Magyari and N. Polgár, " Interaction between *CTLA4* gene and IBD5 locus in Hungarian Crohn's disease patients," *International Journal of Colorectal Disease*, vol. 26, no. 9, pp.1119-1125, 2011.https://doi.org/10.1007/s00384-011-1202-z

[19] H. Turpeinen, A. P. Laine and R. Hermann, " A linkageanalysis of the *CTLA4* gene region in Finnish patients with type 1 diabetes," *Eurobean Journal of Immunogenetic*, vol.30, no.4, pp.289-293, 2003.https://doi.org/10.1046/j.1365-2370.2003.00407.x.

[20] W-Y. Long, " Association between *NOD2/CARD15* gene polymorphisms and Crohn's disease in Chinese Zhuang patients," *World Journal Gastroenterology*, vol. 20, no.16, pp.4737-4744, 2014. https://doi.org/10.3748/wjg.v20.i16.4737

[21] T. Tukel, A. Shalata and D. Present, " Crohn Disease: Frequency and Nature of *CARD15* Mutations in Ashkenazi and Sephardi/Oriental Jewish Families," *American Journal of Human Genetics*,vol. 74,no.4 ,pp. 623-636, 2004.https://doi.org/10.1086/382226

[22] J. J. Zhao, D. Wang, H. Yao, D. W. Sun and H. Y. Li, "*CTLA-4* and *MDR1* polymorphisms increase the risk for ulcerative colitis: A metaanalysis," *World Journal of Gastroenteroogyl*, vol. 21, no. 34, pp.10025-10040,2015.https://doi.org/10.3748/wjg.v21.i34.10025

[23] W. Alaya, I. Sfar, Aouadi, H. Jendoubi, T. Najjar and A. Filali, "Association between *CTLA-4* gene promoter (49 A/G) in exon 1 polymorphisms and inflammatory bowel disease in the Tunisian population," *Saudi Journal Gastroenterology*, vol. 15, no.1, pp. 29-34, 2009.https://doi.org/10.4103/1319-3767.43285

[24] X. Xiaozheng, D. Preston, Z. Jibin, S. Alice, Z. Yunlong, M. Takeya, D. Jack, C. Xu and H. Enfu, " CTLA4 depletes T cell endogenous and trogocytosed B7 ligands via cis-endocytosis," *Journal Experimental Medicine*, vol. 220, no. 7, pp.1-23, 2023.https://doi.org/10.1084/jem.20221391

[25] E. Ebrahim, T. Teklu, F. Tajebe, T. Wondmagegn, Y. Akelew and Mesfin Fiseha, "Association of Cytotoxic T-Lymphocyte Antigen-4 Gene Polymorphism with Type 1 Diabetes Mellitus: In

silico Analysis of Biological Features of CTLA-4 Protein on Ethiopian Population," *Diabetes, Metabolic Syndrome and Obesity*, vol. 2022 ,no.15,pp.2733-2751, 2022.https://doi.org/10.2147/DMSO.S375023

[26] T. V. Hviid, S. Hylenius, A. Hoegh, C. Kruse and O. Christiansen, " HLA-G polymorphism in couples with recurrent spontaneous abortion," *Tissue Antigens*, vol. 60, no.2 ,pp. 122-132, 2002. https://doi.org/10.1034/j.1399-0039.2002.600202.x

[27] A. Ferrand, Z. Al Nabhani,N. S. Tapias, E. Mas, J. Hugotand and F. Barreau, "NOD2 Expression in Intestinal Epithelial Cells Protects Toward the Development of Inflammation and Associated Carcinogenesis," *Cellular and Molecular Gastroenterology and Hepatology*, vol.7,no. 2, pp. 357–369, 2019. https://doi.org/10.1016/j.jcmgh.2018.10.009

[28] Z. Liu, Y. Zhang, T. Jin, C. Yi , D. K. Ocansey and F. Mao, "The role of NOD2 in intestinal immune response and microbiota modulation: A therapeutic target in inflammatory bowel disease, " *International Immunopharmacology*, vol. 113, Part B, pp.1-12, 2022. https://doi.org/10.1016/j.intimp.2022.109466