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Original Article

Genetic Polymorphisms of *NOD2* and *ATG16L1* in Different Types of Digestive Tract Inflammation

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Abstract
Inflammatory bowel diseases (IBD) result from genetic and environmental factors. The two clinical manifestations of inflammatory bowel disease are ulcerative colitis and Crohn's disease (IBD). Numerous studies have demonstrated a link between immune system molecules' single nucleotide polymorphisms (SNPs) and the incidence of IBD. The study aimed to examine the potential impact of the SNPs *NOD2* rs2066844 and *ATG16L1* rs2241880 in a group sample of Iraqi IBD patients. The AG genotype for rs2241880 was associated with an increased risk for CD (*P*=0.1) and a negative association with UC, whereas the AA genotype presents less in CD patients and a negative association with UC (*P*=0.89). For this SNP, the G allele was associated with the risk of CD but not for UC. For the rs2066844, there were no significant differences for NOD2 in both CD and UC, and associations between variation and diseases were not observed.
Keywords: NOD2, ATG16L1, Crohn's Disease, Ulcerative Colitis

1. Introduction

Chronic digestive tract conditions known as inflammatory bowel diseases (IBDs) are typically categorized into one of two groups: UC (ulcerative colitis) and CD (Crohn's disease) (1). In addition to causing ulcerations, severe hemorrhaging, toxic megacolon, and fulminant colitis, ulcerative colitis is confined to the colon and is characterized by surface mucosal inflammation that extends proximally in an adjacent manner. Contrarily, the CD is characterized by trans-mural inflammation, which can result in several problems such as abscesses, fistulas, and fibrotic strictures. CD may affect any part of the intestinal tract, frequently in a nonadjacent manner. Even though possibly essential differences among ulcerative colitis and CD have been distinguished, such as immune cell accumulation differentially enhanced (2) and genetic modifications (e.g., *NOD* and *ATG16L1*) that raise the possibility of CD infection but may can defensive to ulcerative colitis (3). Furthermore, heterogeneity over these IBD groups is probable; for example, colonic and ileal CD, colonic CD disease can be further categorized into different types depending on the origin of gene expression (4).

Previous studies recommended genes a risk that is much more for CD than for UC and a significant prevalence of inflammatory bowel diseases in first-degree families of patients compared with the general population (1, 5). So far, gene polymorphism revisions have recognized more than 240 possible alternates that disturb intracellular pathways that distinguish microbial particles (e.g., *NOD2*). The principal disease-

established gene primarily related to CD is called nucleotide-binding oligomerization domain-containing protein 2 (NOD2). NOD2 is located on the q arm of chromosome 16. Identification receptors of the type NOD2 are crucial for intestinal immunity (6, 7). Changes in NOD2 lead to aberrant control of the microbe-host interaction, which promotes CD-specific ileum infection (8). Three common SNPs (Single Nucleotide Polymorphisms) (Leu1007fsinsC, Arg702Trp, and Gly908Arg) have been connected explicitly with ileal inflammation, structural difficulties, and prior illness manifestation out of the over 60 variations of this gene that have been identified so far (9). Additionally, this aids in the autophagy pathway's reprocessing intracellular organelles and eradicating intracellular microbes (e.g., ATG16L1). There are many ways autophagy is involved in the pathogenesis of IBD, including Paneth cell excretion of antimicrobial chemicals, clearance of incoming bacteria, synthesis of antigen particles, and immune cell assembly of pro-inflammatory interleukins (10).

ATG16L1 and other genes may also be connected to IBD (autophagy-related gene 16 like 1). Patients with IBD may see changes in their bowel microbiota due to ATG16L1's impact on immune cells' cellular autophagy mechanisms and microbial removal (11). The most common and researched genetic SNP of ATG16L1 is rs2241880, which results in a T300A conversion and strongly correlates with the potential for rising CD infections. It contributes significantly to the removal of bacteria by producing excessive cytokines and is linked to other biotic processes, such as the endoplasmic reticulum pressure-unfolded protein response (12). Genome extensive profiling revisions have been dedicated to recognizing molecular properties, like gene express and epigenetic alterations that differentiate additional subtypes within the canonical CD or UC orderings, distinguish CD disease from UC, or classify between IBD and a healthy bowel.

Investigates of a particular gene or exon expression in samples of colonic cells have been applied to detect two molecular sub-kinds of CD that have alterations in cellular metabolic rate (e.g., lipid and glucose metabolism pathways) and immune signaling pathways (e.g., G protein-coupled receptors, interleukin receptors (13), and toll-like receptors) (4). Other revisions have recognized extra intensely expressed genes in cells from patients with IBD; for example, improved expression of the cytokine oncostatin-M was detected in swollen gut tissue from cases with IBD and was prognostic of the following: failure of anti-TNF treatment (14). A possible restriction of investigates using whole gut tissue, however, is the essential heterogeneity of tissue types contained within; hence, gene express measuring may especially identify the maximum remarkably expressed mRNA (messenger RNA) transcriptions in the most plentiful cells and cannot be connected to a precise cell form (15). Given the previous evidence, the main objective of the current study was to evaluate the effect of some auspicious SNPs in the IBDs at various levels.

2. Materials and Methods

A Blood sample was collected from patients and controls for the routine tests and molecular study. Biochemical routine tests such as CRP, B12, calcium, ESR, WBC, potassium, GPT, and GOT and stool sample test for calprotectin. Genetic material was obtained from 120 patients (70 male and 50 female) and 40 healthy participants (20 male and 20 female). All the patients within more than one year of IBDs. IBDs in all cases were identified through endoscopy (for Crohn's disease) and colonoscopy (for ulcerative colitis), and a histopathological examination in most cases. DNA was extracted from white blood cells using Genaid Kit as stated by the manufacturer's information. The purity of DNA and concentration were estimated by a NanoDrop, whereas the DNA integrity was tested via 0.8% agarose gel electrophoresis. The extracted DNA was used as a template for PCR.

IBD patients' blood samples were examined for the NOD2 SNP rs2066844 and the ATG16L1 SNP rs2241880. PCR (Polymerase chain reaction) was performed in accordance with the manufacturer's

instructions using the LightCycler® fast-start DNA Master HybProbe (Roche Diagnostics International AG, Rotkreuz, Switzerland). The following steps led to PCR: 45 cycles of 10 seconds at 95 °C, 10 seconds at 60 °C, and 15 seconds at 72 °C were performed after 10 minutes of denaturation at 95 °C. The particular primers listed in table 1 below were used to perform these reactions.

Table 1. Primers of NOD2 and ATG16L1

Wild Type Gene	Primers, 5'-3'	Ref.
NOD2 R702W rs2066844	F-CCAGACATCTGAGAAGGCCCTGCTC R-GGCGCCAGGCCTGTGCCCGCTGGTG	Hoffmann, Lamerz (16)
ATG16L1 T300A rs2241880	F-ACTTCTTTACCAGAACCAGGATGAG R-ATCCACATTGTCCTGGGGACTGGG	

2.1. DNA Sequencing

The PCR product of hundred twenty of IBDs cases was sequenced from their forward terminals depending on the instruction of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only obvious chromatographs achieved from ABI sequence files were further examined, confirming that the observation and alterations are not because of PCR or sequencing artifacts. The particular locations and other information of the retrieved PCR product fragments were recognized by matching the detected DNA sequences of native specimens with the registered DNA sequences of *NOD and ATG16L1* genes sequences. The sequencing results of the PCR fragments of all samples were processed via BioEdit-DNASTAR, Madison, WI, USA. The observed variant in each sequenced selected sample was enumerated in PCR amplicons and its corresponding location within the referring genome. The novelty of the detected SNPs was observed by the dbSNP server (https://www.ncbi.nlm.nih.gov/projects/SNP/).

2.2. Statistical Analysis

Statistical analysis of data was performed using SAS (Statistical Analysis System - version 9.1). One-way ANOVA and Least significant differences (LSD) post roc test were performed in case the variables were more than two and Independent T-test in case two to assess significant differences among means. $P\leq0.05$ was considered statistically significant.

3. Results

The study involved 120 patients. The median age at first diagnosis was 26 years, with 62.5 % of them being male and 37.5 % female. Out of 120 patients, ulcerative colitis affected 50 % and CD 50 %. 37.4 % of patients had first-degree relatives who had IBD, and 1.8% had relatives who had colon cancer. Immunomodulatory and anti-inflammatory medicines were formerly used in medicine. The findings of biochemical tests performed on CD patients and people with ulcerative colitis are shown in table 2.

Table 2. Results of biochemical tests

Variable	CD Patients	ulcerative colitis patients
n	120	
Male	70	
Female	50	
Disease duration at baseline (years), mean ± SD	16.9±10.4	13.5±11.9
Age at diagnosis (years), mean±SD	18.8±6.8	14.7±9.7
CRP, mean±SD	28.1±10.7	8.9±6.8
ESR, mean±SD	40.8±15.4	15.9±10.2
WBC, mean±SD	8600±4200	6400±3800
Potassium, mean±SD	3.9±1.4	3.0±1.1
GPT, mean±SD	28.5±15.9	19.8±12.6
GOT, mean±SD	20.6±14.4	21.1±14.7
Stool for calprotectin, mean±SD	17.6±8.5	8.1±5.3

3.1. Molecular Analysis

3.1.1. Genotypic and Alleles Analysis of *NOD2 R702W rs2066844 and ATG16L1 T300A*rs2241880*polymorphism* in CD Patients

In the present study, the *NOD2 and ATG16L1* genetic sequences were analyzed in 120 samples of patients alongside 40 samples of controls. The sequencing reactions indicated the exact identity of this genetic fragment after performing NCBI BLASTn. The NCBI BLASTn engine had shown more than 99% sequence

similarities between the sequenced samples and the intended reference target sequences, which entirely cover targeted genes.

The Hardy Weinberg equation was used to analyze the distribution of NOD2 R702W node genotypes (CC, CT, and TT) in the CD patient and control groups. The results are displayed in table 3. With a p-value of 0.86, the homozygous wild genotype CC was found in 42 out of 60 patient subjects, the heterozygous CT genotype in 8 out of 60 patient subjects, and the homozygous mutant TT genotype in 10 out of 60 patient subjects. 34 of the 40 control subjects had the homozygous wild genotype CC, while 6 of the 40 control subjects had the heterozygous genotype CT, with a p-value of 0.88. The genotype distribution for ATG16L1 for CD patients and the control group is also provided in table 3, along with the associated results. Among the 60 sick participants, 11 had the homozygous wild genotype AA, 26 had the heterozygous AG genotype, and 23 had the homozygous mutant GG genotype, with a *P*-value of 1.0 indicating a highly significant connection. With a p-value of 0.97, the heterozygous wild genotype AG was only found in 3 out of 40 control subjects, while 37 out of 40 control subjects had the homozygous wild genotype AA.

3.1.2. Genotypic and Alleles Analysis of *NOD2 R702W rs2066844 and ATG16L1 T300A*rs2241880*polymorphism* in Ulcerative Colitis Patients

The homozygous natural genotype CC was found in 50 out of 60 patient subjects with ulcerative colitis, the heterozygous CT genotype was discovered in 8 out of 60 patient subjects, and the homozygous mutant TT genotype was seen in 2 out of 60 patient subjects, with a p-value of 0.86. According to CD results, 6 out of 40 control subjects had the heterozygous CT genotype, while 34 out of 40 control subjects had the homozygous wild genotype CC. In addition, the homozygous wild genotype AA was found in 48 out of 60 patient subjects for the same group, the heterozygous AG genotype was found in 7 out of 60 patient subjects, and the homozygous mutant GG genotype was found in 5 out of 60 patient subjects, all with a p-value of 0.89. 37 out of 40 control subjects had the homozygous wild genotype AA, and 3 out of 40 had the heterozygous genotype AG, with a p-value of 0.97. Table 3 includes all of the results mentioned previously.

Table 3. Genetic models and genotypes distribution in patients and controls

Gene *SNP*	Group	1/2	Genotype, N (%)			*P*-value
			CC	CT	TT	
NOD2 rs2066844	CD	C/T	42 (70.0)	8 (13.3)	10 (16.6)	0.86
	UC		50 (83.3)	8 (13.3)	2 (3.3)	
	Controls		34 (85.0)	6 (15.0)	0 (0.0)	0.88
			AA	AG	GG	
ATG16L1 rs2241880	CD	G/A	11 (18.3)*	26 (43.3)*	23 (38.3)*	0.10*
	UC		48 (80.0)	7 (11.7)	5 (8.3)	0.89
	Controls		37 (92.5)	3 (7.5)	0	0.97

Means significant correlation if the *P*-value <0.5

4. Discussion

Inflammatory bowel disease (IBD) is a group of chronic multifactorial illnesses that includes CD, characterized by transmural swelling that can affect any part of the gut, and UC, which causes inflammation and ulcers in the colon and rectum. Inflammatory bowel disease's exact cause is unknown. However, it is hypothesized that in genetically predisposed hosts, an imbalanced interaction between gut luminal material and the mucosal immune system causes the condition (17). The NOD2 gene was the first to be revealed to be directly associated with CD risk due to three main mutations in the gene's leucine-rich repeat region. Because NOD2 is a pattern recognition receptor involved in host-microbe interactions, numerous studies have shown that NOD2 polymorphisms affect the microbiome in IBD patients (18). NOD-2, which recognizes muramyl-dipeptide and identifies internal bacteria, protects the intestinal mucosa's homeostasis (19). The SNPs under consideration, situated in the LRR coding area, modify how their ligand is recognized, enabling bacterial growth and lowering immunological tolerance to commensal microbes (20). IBD risk has been linked to the TT genotype of SNP rs2066844 (6). The homozygous TT genotype for rs2066844 was not significantly associated with IBD risk in this study's CD and UC patients, indicating that these molecules cannot be employed as genetic markers in the Iraqi population. The ATG16L1 gene contains the SNP rs2241880/GG, which causes a T300A mutation that interferes with the proper assembly of the autophagosome. If the autophagy gene exhibits SNPs, this could change how the body recognizes the microbiota and result in gastrointestinal changes that could contribute to the development of IBD (21). However, it was discovered that the study population was linked to this mutation. The main drawback of this study is that numerous other immune system SNPs have been investigated for associations with the risk of IBD; however, some are still individual reports, lack meta-analysis, or have been determined to be irrelevant. For example, in one study, two functional relevant polymorphisms in TLR2, the GTA microsatellite repeat polymorphism in intron 2 and the Arg753Gln variant, did not demonstrate associations with the susceptibility to CD or UC (22, 23). The aforementioned underscores the necessity to produce new data about the genetic mechanisms involved in the various IBD presentations. The study's findings show that the variant of ATG16L1 rs2241880 that appears to be strongly related to just CD in this cohort, rather than rs2066844 of NOD2, was not associated with IBD in the Iraqi population. This research may increase the understanding of the genetic variants involved in the pathogenic process that characterizes CD and/or UC.

Authors' Contribution

Study concept and design: N. M. H. A.
Acquisition of data: N. F. N. A.
Analysis and interpretation of data: M. J. A.
Drafting of the manuscript: S. M. H.
Critical revision of the manuscript for important intellectual content: A. M. A.
Statistical analysis: N. F. N. A.
Administrative, technical, and material support: N. M. H. A.

Ethics

The human study was approved by the Al-Qasim Green University, Babylon Province, Iraq Review Board.

Conflict of Interest

The authors declare that they have no conflict of interest.

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