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Interleukin 12B mRNA level and rs3212227 genotyping in peripheral blood mononuclear cells of inflammatory bowel disease patients

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Background/aim: Inflammatory bowel disease (IBD) is a multifactorial disorder. Single nucleotide polymorphisms (SNPs) in the IL-12 gene, which are the main factors to regulate the immune reaction, play an important role in the production of IL-12 molecules. The aim of this study was to evaluate the correlation between the SNP on position +1188 of the 3′UTR region of the IL-12 p40 subunit gene and expression of the IL-12 p40 gene.

Materials and methods: This case-control study was performed with 102 patients with IBD and 107 healthy people. PCR-RFLP and comparative real-time PCR were performed to assess the association between genotype and IL-12 gene expression.

Results: The frequency of AA, CA, and CC genotypes of this gene at position +1188 was calculated to be 58.8%, 32.4%, and 8.8% in patients and 61.7%, 26.2%, and 12.1% in the control group, respectively, with no significant difference between the two groups (IL-12B rs3212227: AA (Reference 1), CA (P = 0.407); OR (95% CI) 0.771 (0.418–1.424), CC (P = 0.561); OR (95% CI) 1.313 (0.524–3.292)). Also, the IL-12B mRNA expression level was compared between IBD patients and healthy controls and demonstrated a significant association (R^2 0.136, 95% CI 1.892–3.872, P < 0.0001).

Conclusion: Our results show that IL-12B expression in IBD may be associated with altered immune and inflammatory responses.

Keyword: Inflammatory bowel disease, interleukin-12 subunit p40, single nucleotide polymorphisms, Crohn's disease, ulcerative colitis

1. Introduction

Inflammatory bowel disease (IBD) is a general term for a group of diseases that cause inflammation in gastrointestinal tract, including Crohn's disease (CD) and ulcerative colitis (UC) (1–4). The disease is a multifactorial disorder with genetic factors, environmental factors, and the immune system playing vital roles in its development (5–7). Dysfunction of mucosal immune system activity in response to normal intestinal bacteria might also have a role in the development of IBD (8–10). Epidemiological studies across geographic regions, different races, twins, and family members suggested a crucial role of genetic factors in the pathogenesis of IBD (11–13). Currently, IBD is one of the most common causes of gastrointestinal diseases in developed countries (14). Although Scandinavian countries, Canada, and the United States have the highest rates of IBD, prevalence of this disease has significantly increased in Iran (13,15). Since 1950, IBD has dramatically increased in Northern Europe and North America (16). Cytokines are glycoproteins that are produced by T and APC cells. The cytokine receptors have semidedicated functions (17). Interleukin 12, which is a heterodimer molecule consisting of two subunits ([P35] IL-12A and [P40] IL-12B), is a member of the cytokines (18,19). In addition, interleukin 12 (IL-12) plays an important role in the initial inherent response to intracellular microbes and is the main cell and acquired immune response stimulator against microbes (20). It originates from macrophages and dendritic cells and is produced from Th1 due to antigen stimulation (21). IL-12 affects natural killer cells and T cells, highly activates cellular immunity, and stimulates the subunits of the receiver connected to JAK2 to dimerize (22).

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Phosphorylation of tyrosine kinase-2 causes induction of a binding site to STAT4, which initiates gene transcription subsequently (23,24). Genome-wide association studies indicate that polymorphisms of IL-12B and components of its signaling pathways such as TKY2 and STAT4 are considered as sensitivity and susceptibility factors for IBD (25). We aimed to investigate the IL-12B gene expression in peripheral blood mononuclear cells and genotyping of Il-12B gene rs3212227 polymorphism. We also evaluated the association between the Il-12B gene expression pattern and its association with the rs3212227 polymorphism in an Iranian population of patients with IBD.

2. Materials and methods

2.1. Study population

This study included 102 IBD patients (15 patients with CD and 87 patients with UC) who were referred to the Research Institute for Gastroenterology and Liver Diseases of Shahid Beheshti University of Medical Sciences (Tehran, Iran) with positive colonoscopy and pathologic results for IBD. The patients and healthy individuals were all Iranian. Patients had a mean age of 47.96 ± 10.11 years. A total of 107 healthy individuals without family history of gastrointestinal disorders participated as controls. The mean age of the controls was 42.01 ± 12.51 years. People with no personal or family history of cancer or inflammatory diseases including gastritis, ulcerative colitis, and/or Crohn's colitis were considered and included as controls. This study was approved by the Ethics Committee of the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

2.2. Genotyping

Genomic DNA was isolated from 5 mL of peripheral blood leukocytes using the standard salting-out method (26). The quality of the extracted DNA was then assessed using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Genotype determination was performed by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP). Characteristics of restriction enzymes used for RFLP and PCR primer sequences are summarized in Table 1. The digested PCR products were electrophoresed on a 2.5% agarose gel and stained with DNA Green Viewer (Parstous Biotechnology, Iran) to visualize the fragments using a UV gel documentation instrument. To confirm the genotyping results with the RFLP method, selected PCR products were examined by DNA sequencing randomly and the results were 100% compatible, as shown in Figure 1.

2.3. RT-PCR and real-time PCR analysis

The total RNAs were extracted from 5 mL of peripheral blood leukocytes using the YTA Total RNA Purification Mini Kit (Yekta Tajhiz Azma Co., Iran) according to the manufacturer's suggested protocol. RNA integrity, quantity, and quality were verified with agarose gel electrophoresis and the NanoDrop system (NanoDrop Technologies). RNA was stored at –70 °C until use. cDNA was synthesized using 1 μg of total RNAs with the Revert Aid RT Reverse Transcription Kit (Catalog Number K1691; Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The qPCR was carried out on a 7500 Real-Time PCR System (Applied Biosystems, USA). The cDNA fragments were used as the templates to amplify the IL-12 gene using SYBR Premix Ex Taq (Takara Bio Inc., Japan) according to the manufacturer's instructions. All real-time PCR reactions were performed in a total reaction volume of 20 mL. The experimental protocol consisted of the following: 1) 95 °C for 30 s; 2) 40 cycles of amplification at 95 °C for 5 s, and 60 °C for 34 s; 3) melting curve analysis; and 4) cooling at 40 °C for 30 s. Analysis was performed using ACTB as an endogenous control and results were expressed as $2^{-\Delta Ct}$. The qPCR primers are shown in Table 2.

2.4. Statistical analysis

SPSS 21 (IBM Corp., USA) was used for the statistical analysis. The chi-square test was used to evaluate the distribution of the allele and genotype frequencies. Hardy–Weinberg equilibrium was tested along with the chi-square test to compare the observed genotype frequencies among studied cases and controls with the expected genotype frequencies. Logistic regression was applied to calculate odds ratio (OR) and 95% confidence intervals and to adjust the data for confounding factors such as age and sex. $P < 0.05$ was considered as significant. GraphPad Prism software version 5 (GraphPad Inc., USA) was used for statistical analysis of interleukin 12B mRNA expression. ANOVA was used to examine the interleukin 12B mRNA levels between groups.

Table 1. Primer sequence and resulting fragment length for the rs3212227 PCR.

SNP reference ID	Primer sequence	Location (base change)	PCR product size (bp)	Restriction enzyme	RFLP fragments size (bp)
rs3212227	F: 5'-TACATCCTGGCAGACAAACG-3' R: 5'-GGCATGAAATCCCTGAAACC-3'	C/A	421 bp	TaqI	AA: 421 CA: 421 + 153 + 268 CC: 153 + 268

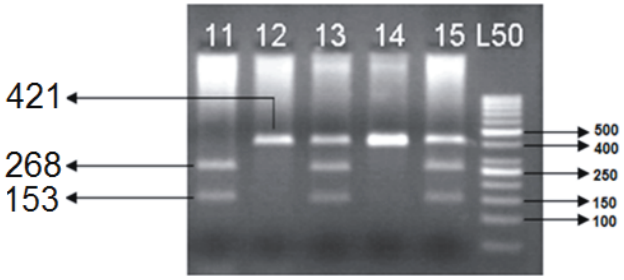

Figure 1. The fragments of polymorphism on agarose gel after digestion with TaqI restriction enzyme. L50: ladder, 50 bp.

3. Results

3.1. Demographics

A total of 102 patients with IBD, including 43 males (42.16%) and 59 females (57.84%), were evaluated. The control group consisted of 107 non-IBD patients: 54 males (50.47%) and 53 females (49.53%), with no statistically significant difference between the cases and controls. There were no differences between the cases and controls regarding their age, body mass index, and smoking behavior, either, as shown in Table 3.

3.2. Evaluation of IL-12 rs3212227 polymorphism in inflammatory bowel disease

Frequencies of alleles and genotypes were analyzed between two groups of patients and controls. We also analyzed the association between CD and UC compared with the control group. The results showed no statistically significant difference in the polymorphism for the frequency of allele and genotype between these groups, as shown in Table 4.

3.3. IL-12 gene expression patterns

In both groups of patients and the control group, a decreased level of IL-12B mRNA was observed, but evaluation of IL-12B gene expression between patients and healthy controls revealed that the IL-12B mRNA level was lower than in healthy controls (R^2 0.136, 95% CI 1.892–3.872, $P < 0.0001$), as shown in Figure 2A. Additionally, no correlation was seen between IL-12B mRNA level and type of IBD (CD, UC) ($P = 0.9$) or disease phase (flare-up, remission) ($P = 0.1$), as shown in Figures 2B and 2C.

Analysis of IL-B among three allele genotype (AA, CA, and CC) showed reduced expression between the two groups of patients and controls. Likewise, genotype CA had higher expression than genotypes AA and CC. No

Table 2. Primers used for quantification.

Gene symbol	Primer sequence	Length	GC (%)	TM
IL-23R Forward Reverse	 5'-TGCCCATTGAGGTCATGGTG-3' 5'-CTTGGGTGGGTCAGGTTTGA-3'	 20 20	 55 55	 60.32 59.82
ACTB Forward Reverse	 5'-CACTCTTCCAGCCTTCCTTCC-3' 5'-AGGTCTTTGCGGATGTCCAC-3'	 21 20	 57 55	 60.34 60.32

Table 3. Demographic characteristics of the IBD population.

Variable	Patient (n = 102)	Controls (n = 107)	P-value
Age (mean ± SD)	47.96 ± 10.11	42.01 ± 12.51	>0.05
BMI [a]	3.65 ± 24.96	5.89 ± 25.39	>0.05
Sex, n (%) [b]			>0.05
Female	59 (57.84%)	53)49.53 %(	
Male	43 (42.16%)	54 (50.47%)	
Smoking, n (%) [b]			>0.05
Smoker	17 (16.6%)	11 (10.28%)	
Nonsmoker	85 (83.4%)	96 (89.72%)	

[a] According to Student's t-test results; [b] According to chi-square test results.

Table 4. Allele and genotype distribution of studied rs3212227 SNP between IBD patients and healthy controls.

SNP rs3212227	[a]CD (n = 15) n (%)	[b]UC (n = 87) n (%)	[c]IBD (n = 102) n (%)	Controls (n = 107) n (%)		[d]CD (n = 30) n (%)	[e]UC (n = 174) n (%)	[f]IBD (n = 204) n (%)	Controls (n = 214) n (%)
Genotypes					Alleles				
AA	9 (60)	51 (58.6)	60 (58.8)	66 (61.7)	A	23 (76.7)	130 (74.7)	153 (75)	160 (74.8)
CA	5 (33.3)	28 (32.2)	33 (32.4)	28 (26.2)	C	7 (23.3)	44 (25.3)	51 (25)	54 (25.2)
CC	1 (6.7)	9 (9.2)	9 (8.8)	13 (12.1)					

[a]CD vs. controls: AA (Reference 1), CA (P = 0.654); OR (95% CI) 0.764 (0.235–2.483), CC (P = 0.602); OR (95% CI) 1.773 (0.207–15.217).
[b]UC vs. controls: AA (Reference 1), CA (P = 0.429); OR (95% CI) 0.773 (0.408–1.464), CC (P = 0.640); OR (95% CI) 1.256 (0.484–3.258).
[c]IBD vs. controls: AA (Reference 1), CA (P = 0.407); OR (95% CI) 0.771 (0.418–1.424), CC (P = 0.561); OR (95% CI) 1.313 (0.524–3.292).
[d]CD vs. controls: A (Reference 1), C (P = 0.822); OR (95% CI) 1.109 (0.451–2.729).
[e]UC vs. controls: A (Reference 1), C (P = 0.990); OR (95% CI) 0.997 (0.629–1.580).
[f]IBD vs. controls: A (Reference 1), C (P = 0.956); OR (95% CI) 1.012 (0.651–1.576).
Adjusted for age and sex as confounder variables.

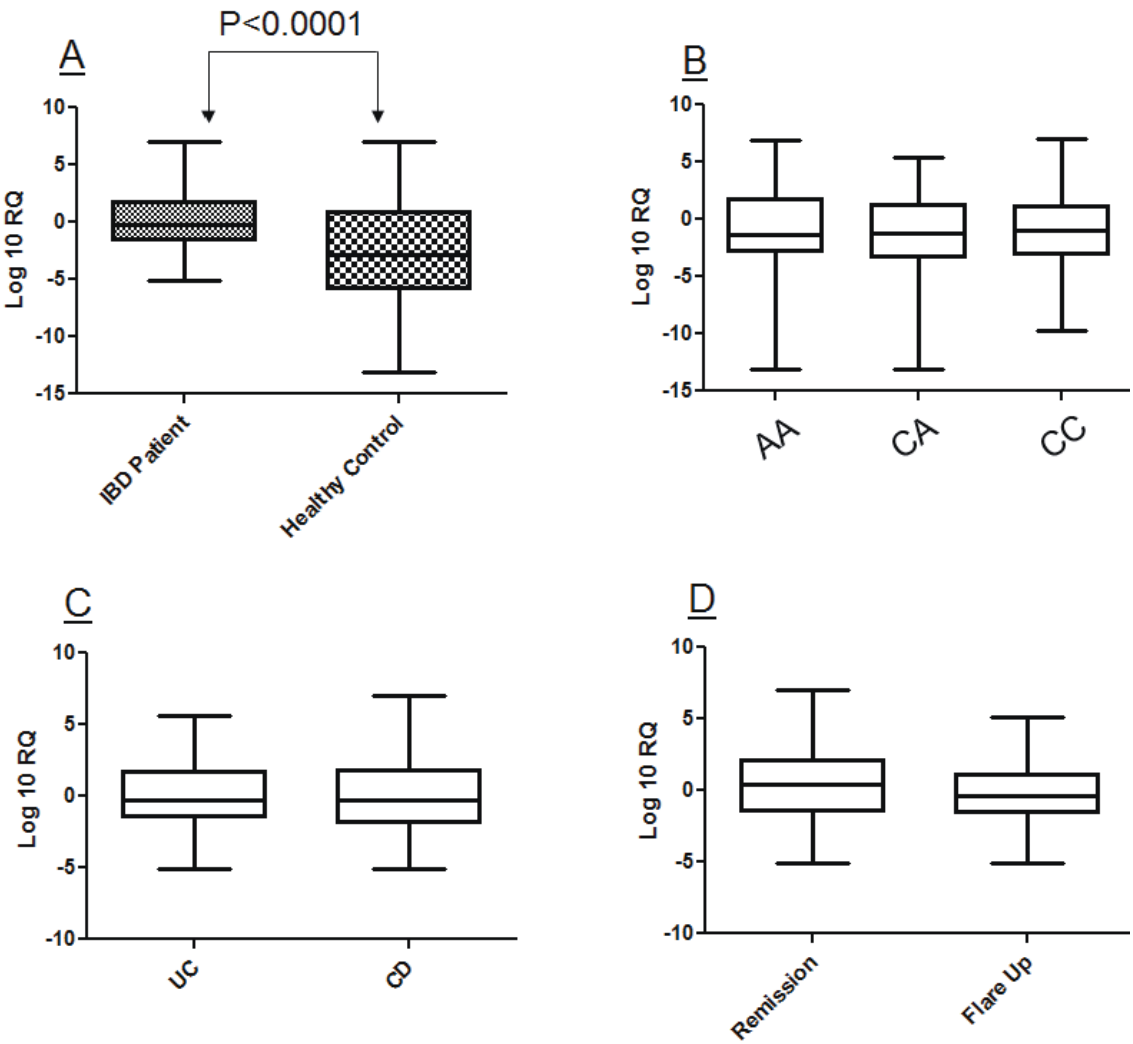

Figure 2. A) IL-12B mRNA levels between two groups of patients and controls; B) IL-12B mRNA levels according to the IBD type; C) IL-12B mRNA levels among different IBD pathological phases; D) interaction between genotype and IL-12B expression.

significant correlation was observed between genotypes in patients and controls (P = 0.3), as shown in Figure 2D.

4. Discussion

Decreased production of IL-12 could disrupt the intracellular Th1 response and increase the risk of infection with pathogens (27). The rs3212227 polymorphism in the IL-12 gene has been investigated for the first time in an Iranian population with IBD and expression of this gene was evaluated simultaneously. This analysis shows that there is no statistical difference between the polymorphism and genotypes (P > 0.05). According to our investigation, the expression of the IL-12B gene was dramatically decreased between patients and healthy individuals (P < 0.0001). In a study conducted in 2005 on expression of IL-23 and IL-27, which are subgroups of the IL-12 family, an increased expression of IL-23 and IL-27 was demonstrated in IBD patients (28,29). IL-12 contains two subunits of P40 and P35 and its receptor is composed of two subunits of IL-12Rβ1 and IL-12Rβ2 (29). However, due to the similarity of the IL-12 and IL-23 subunits, a crucial effect on inflammation has been proved with an increased activity of IL-23 or in combination with IL-12 (30).

The results of this study indicate that the AA and CA genotypes had higher frequency in patients compared to controls. The difference between the two groups of patients and controls (P = 0.929; CI 95% 0.617–1.554) was not significant. In two concurrent studies in Spain and Japan in 2008 and 2009 with patients and healthy controls, sensitivity and relation between two groups of CD and UC was observed in Japanese patients but not the Spanish ones (31,32). A similar study was conducted in Germany in 2012 and four polymorphisms of IL-12B were investigated. The results illustrated a significant increase in SNP rs6887695 of IL-12B in IBD patients (P = 0.035) (33). Various studies on SNPs of the IL-12B gene indicated the vital role of this gene in IBD. However, in the current study, rs3212227 polymorphism in IL-12 may not represent a genetic risk factor for Iranians. Due to the fact that genetic factors, environmental factors, and diet are different in different countries, results may be different in polymorphism investigations (34,35). Genetic comparison of IL-12B expression shows that in the Iranian population rs3212227 genotype AA has been accompanied with higher frequency of disease and increased expression of IL-12B (36).

In conclusion, no correlation exists between gene expressions of rs3212227 polymorphism in the IL-12B gene in IBD. This polymorphism may not be considered as a genetic factor associated with IBD. It seems that other genetic factors excluding IL-12 are involved in inflammation processes in IBD. Moreover, IL-12B gene expression in healthy individuals was significantly less than in patients.

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