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Investigation of Interleukin-10 Promoter Polymorphisms and Interleukin-10 Levels in Children with Irritable Bowel Syndrome

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Background/Aims: The aim of this study was to investigate whether genetic variations at positions -1082, -819, and -592 in the interleukin (IL)-10 promoter affect IL-10 production in children with irritable bowel syndrome (IBS). **Methods:** Ninety-four children with IBS and 102 children as healthy controls (HCs) were enrolled. Genomic DNA was extracted, and IL-10 -1082, -819, and -592 polymorphisms were detected by direct sequencing from all participants. Peripheral blood mononuclear cells (PBMCs) from 46 IBS children and 38 HCs were isolated and cultured with and without 5 ng/mL *Escherichia coli* lipopolysaccharide (LPS). IL-10 levels in the culture supernatants were measured by enzyme-linked immunosorbent assay. **Results:** There were no significant differences in the distribution of IL-10 -1082, -819, and -592 polymorphisms or in the allele and haplotype frequencies between IBS children and HCs. PBMCs from children with IBS had significantly lower IL-10 levels after LPS stimulation than PBMCs from HCs (p=0.011); however, LPS-induced IL-10 levels in PBMCs with different genotypes of -819 and -592 polymorphisms were not significantly different between IBS patients and HCs. **Conclusions:** Although significantly lower LPS-induced IL-10 production by PBMCs was noted, it is unlikely that IL-10 production was fully genetically determined in our IBS children. ClinicalTrials.gov identifier: NCT01131442. **(Gut Liver 2013;7:430-436)**

Key Words: Irritable bowel syndrome; Child; Interleukin-10; Interleukin-10 gene polymorphisms

INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder that mainly affects children >5 years of age and adolescents.[1] It is defined as having abdominal pain, bloating, and changes in bowel habits (e.g., diarrhea, constipation, or mixed) in the absence of any organic or structural abnormality. Various etiologies like visceral hyperalgesia, disturbance of brain-gut interaction, autonomic and hormonal events, genetic and environmental factors, food sensitivity, postinfectious sequelae, and psychosocial disturbances have been implicated,[2-8] but the precise pathophysiology remains unclear.

Postinfectious IBS introduces a role for the immune activation in the development of IBS symptoms.[8] Postinflammatory changes in the gut may produce chronic alterations of the immune system, and altered cytokine profiles in IBS patients have been shown in some studies.[9-14] Our previous study also found that children with IBS tend to produce lower amounts of the anti-inflammatory cytokine interleukin (IL)-10 at baseline and after *Escherichia coli* lipopolysaccharide (LPS) stimulation, implying that defects in immune modulation may contribute to IBS in children.[15]

Evidences demonstrate that changes in the genetic make-up or expression of cytokines play a critical role in the inflammatory response in the gut.[10,16,17] Although a genetic component is suspected, unambiguous susceptibility genes have not been identified so far. Since the capacity for cytokine production can be genetically determined and is mainly related to genetic variations in the promoter region,[18] further study that measures cytokine profiles and explores cytokine gene polymorphisms in parallel will be beneficial in this field.

Several polymorphic sites within the IL-10 gene promoter region have been described, including three biallelic polymorphisms at positions -1082 (base G to A, db SNP. rs1800896), -819 (base C to T, db SNP. rs1800871), and -592 (base C to A,

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db SNP. rs 1800872) from the transcription start site, are known to regulate the capacity to produce IL-10.[19] The IL-10 -819 C and T alleles are completely in linkage disequilibrium with the IL-10 -592 C and A alleles, respectively.[19,20]

The aim of this study was to investigate if genetic variations at positions -1082, -819, and -592 in the IL-10 promoter affect IL-10 production and predispose to the development of IBS.

MATERIALS AND METHODS

1. Study population

Ninety-four children with IBS (49 females and 45 males; age, 5- to 18-year-old) and 102 healthy children as controls (61 females and 41 males; age, 2- to 18-year-old) were enrolled between November 2008 and February 2011. Patients were recruited consecutively from the outpatient Clinic of the Department of Pediatric Gastroenterology at Chang Gung Memorial Hospitals in Keelung and Taoyuan, whereas healthy volunteers were recruited through advertisements. All of the study participants were Han Chinese.

All patients had chronic or relapsing symptoms of IBS consistent with the Rome II criteria.[21] The symptoms were present for at least 3 months. Patients were further categorized based on their symptoms and predominant stool patterns.[22] Patients with more than three bowel movements per day and loose/watery stool consistency were categorized as diarrhea-predominant IBS (D-IBS, n=32), while those with fewer than three bowel movements per week and hard or lumpy stools were categorized as constipation-predominant IBS (C-IBS, n=33) and those with an alternating bowel pattern were categorized as mixed IBS (M-IBS, n=29).

A comprehensive diagnostic work-up, including hemogram, biochemistry, abdominal sonography, and serial stool testing, were conducted to exclude acute infections or any evidence of structural anomaly that may cause the symptoms.

The hospital's Human Ethics Committee (Institutional Review Board) approved the study and all of the participants provided informed consent.

2. DNA extraction

Genomic DNA was extracted from a 1 mL sample of whole blood from 94 IBS patients and 102 healthy controls and collected into tripotassium ethylenediaminetetraacetic acid sterile tubes. Extraction was performed using QIAamp DNA Blood Mini Kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's instructions.

3. IL-10 genotyping

The three biallelic IL-10 promoter polymorphisms (-1082, -819, and -592) were detected by polymerase chain reaction (PCR) using common primers (forward, 5'-ATC CAA GAC AAC ACT ACT AA-3'; reverse, 5'-TAA ATA TCC TCA AAG TTC C-3'). These primers yielded an amplicon 587 bp in size (-1115 to -528) containing the above polymorphisms. The amplification process was performed in 20 μL containing 1 μL of template DNA, PCR master mix 10 μL (RBC SensiZyme® HotstartTaq Premix; RBC Bioscience, Taipei, Taiwan), $MgCl_2$ (2.5 mmol/L) 1 μL, each primer 2 μL, and free water 4 μL. The parameters for thermocycling were as follows: denaturation at 95°C for 10 minutes, followed by 30 denaturation cycles at 95°C for 30 seconds; annealing at 55.5°C for 30 seconds; and extension at 72°C for 1 minute. This was followed by a final extension at 72°C for 5 minutes and then storage at 4°C. After confirming the final products by electrophoresis on agarose gels (3%), all of the PCR products were sequenced using ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA). The nucleotide polymorphisms at positions -1082, -819, and -592 were read directly.

4. Isolation of peripheral blood mononuclear cells

Blood samples (8 to 10 mL) from 46 (D-IBS, n=20; C-IBS, n=12; and M-IBS, n=14) of the 94 IBS children and 38 of the 102 healthy controls (HCs) were taken upon enrollment and those who used probiotics, antibiotics, analgesics, or immunosuppressive drugs within the past month were not included. In addition, subjects with recent infections, major allergic diseases, food intolerance, or psychiatric disorders were excluded. Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coats on Lymphoprep (Nycomed, Oslo, Norway) gradients by centrifugation. After washing, the cells were resuspended at a concentration of 1×10^6 cells/mL in Roswell Park Memorial Institute 1640 medium containing 10% heat-inactivated fetal bovine serum.

5. Cell cultures

The concentration of PBMCs was adjusted to 10^6 cells/mL in complete medium and then transferred to 24-well plates. Some were stimulated with *E. coli* LPS 5 ng/mL (SIGMA L4391; Sigma, St. Louis, MO, USA), while other were not. Duplicate cultures were prepared and incubated for 24 hours at 37°C in a humidified 5% CO_2 atmosphere. The supernatants were collected, pooled, and stored at -20°C until analysis by enzyme-linked immunosorbent assay (ELISA).

6. ELISA

Concentrations of IL-10 in the culture supernatants were determined using commercially available kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA: IL-10, D1000B). Optical density was measured at wavelength of 450 nm and reference wavelength of 590 nm. The values were correlated linearly with cytokine standards. The sensitivity limit of the assay was 5 pg/mL.

7. Statistical analysis

Genotype and allele frequencies of each IL-10 polymorphism

in the IBS patients and controls were compared by chi-square test. One-way analysis of variance (ANOVA) was performed to compare baseline and 5 ng/mL LPS-stimulated IL-10 levels between the IBS patients, subgroups, and HCs. Owing to the small numbers of subjects in different genotypes of IL-10 promoter polymorphisms and the sampling distribution was not normal, nonparametric test was used to compare baseline and 5 ng/mL LPS-stimulated IL-10 levels at each IL-10 genotype of polymorphisms at positions -1082, -819, and -592 in IBS patients, HCs, and between the two groups. A $p<0.05$ was considered statistically significant. Box plots indicated the median values at the 25th and 75th percentiles, and the error bars indicated the 10th and 90th percentiles. All statistical calculations were performed using the SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA).

Table 1. Demographic Data and Frequencies of IL-10 Promoter Genotype, Allele, and Haplotype in IBS Children and HCs

	IBS patients (n=94)	HCs (n=102)	p-value
Age, yr	14.2±3.1	12.3±5.1	0.082
Male:Female	45:49	41:61	0.314
IL-10 -1082			
Genotype			
A/A (L)	84 (89.5)	95 (93.1)	0.298
A/G (I)	8 (8.5)	7 (6.9)	
G/G (H)	2 (2)	0 (0)	
Allele			
A allele (L)	176 (93.6)	197 (96.6)	0.239
G allele (H)	12 (6.4)	7 (3.4)	
IL-10 -819 (-592)			
Genotype			
C/C (C/C) (H)	11 (11.7)	6 (5.9)	0.351
C/T (C/A) (I)	40 (42.6)	46 (45.1)	
T/T (A/A) (L)	43 (45.7)	50 (49)	
Allele			
C (C) allele (H)	62 (33)	58 (28.4)	0.380
T (A) allele (L)	126 (67)	146 (71.6)	
Haplotype			
GCC (H)	12 (6.4)	7 (3.4)	0.342
GTA (H)	0 (0)	0 (0)	
ACC (I)	50 (26.6)	51 (25)	
ATA (L)	126 (67)	146 (71.6)	
Diplotype			
GCC/GCC (H)	2 (2.1)	0 (0)	0.657
GCC/ATA (I)	5 (5.3)	5 (4.9)	
GCC/ACC (I)	3 (3.2)	2 (2)	
ATA/ATA (L)	43 (45.7)	50 (49.0)	
ACC/ATA (L)	35 (37.2)	41 (40.2)	
ACC/ACC (L)	6 (6.4)	4 (3.9)	

Data are presented as mean±SD or number (%).
IL, interleukin; IBS, irritable bowel syndrome; HC, healthy control; H, IL-10 high production; I, IL-10 intermediate production; L, IL-10 low production.

RESULTS

1. Demographic data and polymorphisms of the IL-10 promoter

Sex, mean age, genotype, and allele and haplotype frequencies in the IL-10 gene promoter were analyzed (Table 1). Ninety-four children with IBS (49 females and 45 males; age range, 5 to 18 years; mean age, 14.2±3.1 years) and 102 HCs (61 females and 41 males; age range, 2 to 18 years; mean age, 12.3±5.1 years) were enrolled. At the -1082 locus, the A/A genotype was highly predominant (89.5% in IBS; 93.1% in HCs), while the G allele (either A/G or G/G) was rare. The -819 T allele (IL-10 -819 C/T and T/T) and -592 A allele (IL-10 -592 C/A and A/A) were more prevalent (-819 and -592 in linkage disequilibrium) (Table 1) than the -819 C allele and -592 C allele, respectively, in both the IBS and HC groups (67% vs 33% and 71.6% vs 28.4%, respectively).

In this study, the ATA haplotype of IL-10 was more prevalent than the other haplotypes (67% in IBS and 71.6% in HCs). ATA/ATA was the most common diplotype (45.7% in IBS and 49% in HCs), followed by the ACC/ATA haplotype (37.2% in IBS and 40.2 % in HCs). There were no differences in genotype, allele, or haplotype frequencies in the three IL-10 polymorphisms between the IBS and control groups.

2. Baseline and *E. coli* LPS-induced cytokine production

IBS patients had lower baseline IL-10 levels compared with

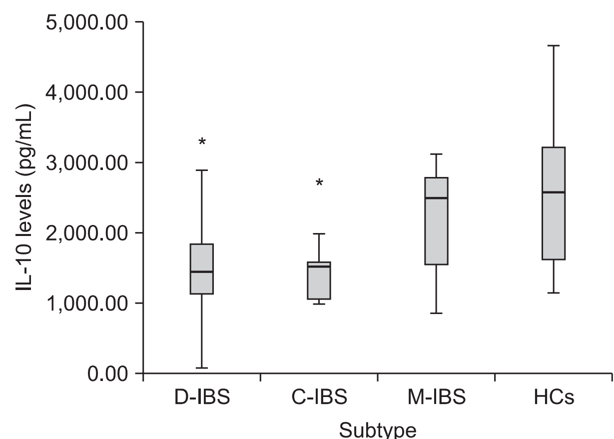

Fig. 1. Box plot of interleukin (IL)-10 levels following 5 ng/mL lipopolysaccharide stimulation in irritable bowel syndrome (IBS) subtype and healthy controls diarrhea-predominant IBS [D-IBS]: 1,532.68±870.22 vs. constipation-predominant IBS [C-IBS]: 1,428.39±413.66 vs. mixed IBS [M-IBS]: 2,159.44±265.48 vs. HCs [healthy controls]: 2,521.16±1,046.06 pg/mL).
*Statistically significant, $p<0.05$.

HCs (54.1±36.50 pg/mL vs 114.57±44.10 pg/mL, p=0.292). After stimulation with 5 ng/mL *E. coli* LPS, the IBS group had significantly lower IL-10 levels compared with the HC group (1,755.26±167.92 pg/mL vs 2,521.16±239.98 pg/mL, p=0.011). Analysis of the three IBS subgroups showed lower baseline IL-10 levels compared with HCs (data not shown). The IL-10 levels were significantly lower under LPS-stimulated condition (5 ng/mL) in the diarrhea and constipation subgroups than in the HCs (p<0.05) (Fig. 1). However, there was no difference among the patient subgroups.

3. Association of IL-10 promoter polymorphism with IL-10 production

To investigate whether genetic variations at positions -1082, -819, and -592 in the IL-10 promoter affected IL-10 production, LPS-induced IL-10 levels were analyzed in different genotypes of polymorphisms in the IBS patients and HCs separately (Table 2). Genotype -1082 A/A was highly predominant (91% in the IBS group and 100% in the HC group) whereas other genotypes at the -1082 loci were rare. Although there were significantly lower IL-10 levels at -1082 A/A in the IBS group compared with HCs (1,709.54±820.04 vs 2,521.16±239.98, respectively; p=0.011), whether IL-10 -1082 A/A affected IL-10 production was inconclusive because few -1082 A/G and G/G genotype were found both in the IBS and HCs group and the association with IL-10 production couldn't be well analyzed.

In genotype -819 T/T and -592 A/A, LPS induced IL-10 levels in IBS patients were much lower compared with HCs, approaching statistical significance (p=0.068) (Table 2). In general, PBMCs of the IBS patients tended to produce lower IL-10 levels after stimulation with 5 ng/mL LPS compared with the HC group in different genotypes of -819 and -592 polymorphisms (Fig. 2). However, there were no significantly different LPS-induced IL-10 levels between the IBS patients and HCs (p=0.101).

DISCUSSION

Cytokines are involved in the control of gastrointestinal motility and visceral sensitivity.[12] They are also important modulators of intestinal immune responses and inflammatory reactions.[23-25] Several studies have investigated systemic and mucosal cytokine profiles in IBS revealing evidence of disturbances in the balance of proinflammatory and anti-inflammatory cytokines.[9,14,15,26,27]

IL-10 is considered to be an anti-inflammatory cytokine that inhibits the production of several other cytokines including interferon-γ, IL-2, IL-4, IL-6, and tumor necrosis factor-α (TNF-α).[24] It also regulates B cell proliferation and differentiation and exhibits immunoregulatory activity.[28] Reduced IL-10 levels in IBS patients have been mentioned in several litera-

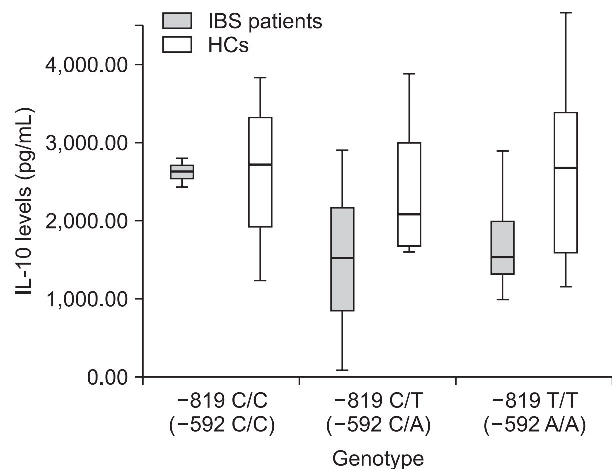

Fig. 2. Box plot showing that peripheral blood mononuclear cells (PBMCs) from irritable bowel syndrome (IBS) patients tend to produce lower interleukin (IL)-10 levels after stimulation with 5 ng/mL lipopolysaccharide compared to PBMCs from the healthy controls (HCs) group in different genotypes of -819 and -592 polymorphisms. There was no significant difference between the IBS patients and HC group (p=0.101).

Table 2. IL-10 Promoter Polymorphisms and LPS-Induced IL-10 Levels in Children with IBS and HCs

	Group (no.)	Mean age, yr	IL-10 post LPS 5 ng/mL stimulation, pg/mL	p-value
All genotypes	IBS (46)	13.5±2.44	1,755.26±167.92	0.011*
	HCs (38)	14.2±3.72	2,521.16±239.98	
-1082 A/A	IBS (42)	13.5±2.52	1,709.54±820.04	0.011*
	HCs (38)	14.2±3.72	2,521.16±239.98	
-819 C/C (-592 C/C)	IBS (4)	14.6±3.40	2,532.80±81.65	0.912
	HCs (4)	16.5±2.12	2,712.82±115.49	
-819 C/T (-592 C/A)	IBS (14)	12.4±2.92	1,500.25±375.59	0.121
	HCs (16)	12.2±4.10	2,370.44±297.66	
-819 T/T (-592 A/A)	IBS (28)	13.9±2.07	1,745.97±182.16	0.068
	HCs (18)	15.4±3.00	2,652.54±396.37	

IL, interleukin; LPS, lipopolysaccharide; IBS, irritable bowel syndrome; HC, healthy control.
*Statistically significant, p<0.05.

tures.[11,12,27,29] Those individuals predisposed to producing lower amounts of IL-10 may be at a higher risk of developing IBS symptoms.

To explore IL-10 production capacity, PBMCs from both IBS group and HCs were stimulated with 5 ng/mL *E. coli* LPS and determined the IL-10 production of these cells. PBMCs may exhibit similar responses with regard to proliferation and cytokine secretion as those by lamina propria mononuclear cells when they are exposed to bacterial antigens, such as *Helicobacter pylori*.[30] It is worth noting that *H. pylori* has a weaker stimulatory effect than other intestinal bacteria such as *E. coli* or *Salmonella*,[31] and it has been reported that the release of cytokines from PBMCs in response to a potent bacterial endotoxin can significantly exceed lamina propria mononuclear cell-mediated cytokine levels.[32]

In our study, PBMCs from the IBS group produced significantly lower IL-10 levels after stimulation with 5 ng/mL *E. coli* LPS compared with PBMCs from HCs (1,755.3±167.92 pg/mL vs 2,521.2±239.98 pg/mL; p=0.011). This suggested that defects in immune modulation might contribute to occurrence of IBS in children. Factors that regulate the expression of IL-10 may be involved in the pathogenesis of IBS. In this regard, genetic predisposition such as single nucleotide polymorphisms may be important and further study is required.

IL-10 possesses a highly polymorphic promoter, with variations at -1082, -819, and -592 that have been studied and implicated in regulating the rates of IL-10 gene transcription. The IL-10 A allele at -1082 and T allele at -819 have been associated with low IL-10 production.[29,33] The -1082 G, -819 C, and -592 C (GCC) alleles have also been associated with elevated levels of IL-10,[34] while ACC and ATA haplotypes exhibit intermediate and low IL-10 gene transcription, respectively.[33] In the present study, the prevalences of low production alleles (-1082 A allele and -819 T allele) and haplotype (ATA) are similar in both the IBS group and HCs without statistical significance.

Cytokine gene polymorphisms in individual susceptibility to certain diseases have been documented,[35-37] and have been evaluated in adult IBS patients. A genetic predisposition to lower anti-inflammatory cytokine production in IBS patients can mean that control of the inflammatory response may be compromised in some individuals. However, the results are inconsistent.[12] Gonsalkorale *et al*.[16] found that IBS patients had a significantly lower frequency of the high-producer genotype for IL-10 (-1082 G/G) compared to controls. Conversely, Barkhordari *et al*.[24,38] found that the low-producer IL-10 (-1082 A/A) had a lower frequency in IBS patients compared to controls, while van der Veek *et al*.[17] found that IL-10 genotypes were similarly distributed in the IBS patients and the controls. Combined high TNF-α and low IL-10 producer (-1082 A/A) genotypes are considerably more frequent in the IBS patients. Lee *et al*.[39] studied IL-10 (-1082 G/A) and TNF-α (-308 G/A) gene polymorphisms and found the genotype and allele distribution were similar in both IBS and control groups.

To date, there is no literature analyzing the association between IL-10 gene polymorphisms and IL-10 production in children with IBS. Whether decreased IL-10 levels in IBS children are primarily determined by cytokine genetic polymorphism or by other mechanisms is not clear and our study is probably a pilot study in this regard. However, LPS-induced IL-10 production by PBMCs is not different between the IBS patients and HCs in -819 and -592 polymorphisms, indicating that lower IL-10 production is not fully genetically determined.

The conflicting results between the current and previous studies regarding the role of IL-10 gene polymorphisms in IBS may be due to several factors. First, it is difficult to ascertain the magnitude of the effects of genetic polymorphisms on disease susceptibility because of the existence of IL-10 homologues. Different IL-10 binding receptors are likely to complicate the determination of IL-10 expression levels *in vitro*.[40] Moreover, insufficiently large sample sizes (especially the IBS subgroups), the younger ages of the study subjects (≤18 years of age), gene-gene and gene-environment interactions, and ethnic differences may contribute to these discrepancies. In this study, the lack of association between IL-10 promoter polymorphisms and lower IL-10 levels in children with IBS implies that different combinations of factors affect the regulation of cytokine production. Genetic variations may affect only a part of the cytokine profile changes that precipitate IBS.

In conclusion, children with IBS tend to produce lower amounts of the anti-inflammatory cytokine IL-10 at baseline and after LPS stimulation. However, there are no significant associations between IL-10 promoter polymorphisms and IL-10 levels in these children. The inherited component and genetic variations in children with IBS require further investigation.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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