Effect of the interleukin 10 polymorphisms on interleukin 10 production and visceral hypersensitivity in Chinese patients with diarrhea-predominant irritable bowel syndrome

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Abstract

Background: Irritable bowel syndrome (IBS), a functional gastrointestinal disorder, is characterized by cytokine imbalance. Previously, decreased plasma interleukin 10 (IL-10) level was reported in patients with IBS, which may be due to genetic polymorphisms. However, there are no reports correlating the IL-10 polymorphisms with IL-10 production in patients with IBS. This study aimed to analyze the effect of IL-10 polymorphisms on IL-10 production and its correlation with the clinical symptoms in Chinese patients with diarrhea-predominant IBS (IBS-D).

Methods: Two IL-10 single nucleotide polymorphisms (rs1800871 and rs1800896) were detected in 120 patients with IBS-D and 144 healthy controls (HC) using SNaPshot. IBS symptom severity score, Bristol scale, hospital anxiety, and depressive scale (HADS) were used to evaluate the clinical symptoms, as well as the psychological status and visceral sensitivity of the subjects. IL-10 levels in the plasma and peripheral blood mononuclear cell (PBMC) culture supernatant were measured using enzyme-linked immunosorbent assay, while those in ileal and colonic mucosal biopsies were measured using immunohistochemistry.

Results: The frequency of rs1800896 C allele was significantly lower in the patients with IBS-D than that in the HC (odds ratio: 0.49, 95% confidence interval: 0.27-0.92, $P = 0.0240$). The IL-10 levels in the plasma ($P = 0.0030$) and PBMC culture supernatant ($P = 0.0500$) of the CT genotype subjects were significantly higher than those in the TT genotype subjects. The CT genotype subjects exhibited a higher pain threshold in the rectal distention test than the TT genotype subjects. Moreover, IL-10 rs1800871 GG genotype subjects showed an increase in the HADS score compared to other genotype subjects.

Conclusions: IL-10 rs1800896 C allele is correlated with higher IL-10 levels in the plasma and the PBMC culture supernatant, which is associated with a higher pain threshold in the Chinese patients with IBS-D. This study provides an explicit relationship of IL-10 polymorphisms with IL-10 production, which might help in understanding the pathogenesis of IBS-D.

Keywords: Irritable bowel syndrome; Interleukin 10; Polymorphisms; Visceral hypersensitivity; Depression

Introduction

Irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorder characterized by the presence of abdominal pain, bloating, and altered bowel habits. According to the Rome IV criteria, there are four subtypes of IBS: constipation-predominant IBS, diarrhea-predominant IBS (IBS-D), diarrhea and constipation mixed IBS, and unsubtypeed IBS. The pathophysiological mechanisms underlying IBS are unclear. The abnormalities of motility, visceral hypersensitivity (VH), gut microbial alteration, and psychological stress contribute to the clinical symptoms of IBS. Additionally, systemic and mucosal immune activation play an important role in IBS. Previous studies have confirmed that patients with IBS exhibited an imbalanced cytokine profile.$^{[1,2]}$ The infection after an acute gastroenteritis is a major trigger for IBS development, resulting in post-infectious IBS.$^{[3,4]}$ The plasma concentration of interleukin 6 (IL-6) and IL-8 tends to increase and that of interferon-γ tends to decrease in the patients with IBS. IL-10, an anti-inflammatory cytokine, is very important in the immune activation of the patients with IBS. However, there are conflicting reports on the role of IL-10 in IBS.$^{[1,3,6]}$ Some studies have reported that the IL-10 in plasma decreased in patients with IBS,$^{[7,8]}$ while others reported that there was no difference when compared to that in the healthy participants.$^{[1,9]}$ Several lines of evidences have demonstrated that there was a decrease in the level of IL-10 mRNA in the intestinal mucosa of patients with IBS.$^{[10,11]}$
IL-10 is synthesized in the immune cells such as T and B lymphocytes, monocytes, macrophages, and mast cells. The cytokine gene polymorphisms have been suggested to influence the cytokine production. The single nucleotide polymorphisms (SNPs) of IL-10 gene, such as IL-10-1082 G/A (rs1800896) and IL-10-819 C/T (rs1800871), are both associated with IBS-D. However, most of the studies were correlated with the risk of developing IBS-D, and rs1800871 polymorphisms of IL-10 were both correlated with the risk of developing IBS-D, which indicated the genetic susceptibility of patients with IBS-D.

Methods

Ethical approval

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Peking University Health Science Center (No. 2013-12). All the participants provided written informed consent.

Study design and participants recruitment

This study was conducted from 2013 to 2018. We recruited IBS-D patients according to Rome III criteria from the Outpatients of Gastroenterology Department of Peking University Third Hospital sequentially, aging from 18 to 65 years old. Healthy volunteers (aging from 18 to 65 years) were recruited from community. The participants were excluded if they met the following exclusion criteria: (1) histories of antibiotics, probiotics/prebiotics, or psychotropic medications intake during the previous 4 weeks; (2) systemic or gastrointestinal diseases, such as diabetes mellitus and inflammatory bowel disease; (3) current infectious diseases of the respiratory, digestive, or urinary system; (4) a history of abdominal surgery. Gastrointestinal symptom severity, daily bowel movement frequency and consistency were evaluated by IBS symptom severity scores and Bristol stool form scale. Visceral sensitivity was assessed by rectal distension test using BAROSTAT (Distender Series II; G&J Electronics, Ontario, Canada). Sensory thresholds for initial sensory, initial defecation, and defecation urgency were calculated for each individual by the lowest pressures. Psychological status was evaluated by hospital anxiety and depressive scale (HADS). All participants underwent colonoscopy or had colonoscopy/barium enema performed in the past 6 months to rule out organic colonic diseases. Participants underwent colonoscopy in Peking University Third Hospital with biopsies in distal ileal and sigmoid colonic mucosa. Each participant was routine to investigate a hemogram, plasma chemistry profile, blood test for hepatic virus B and C and HIV, stool microscopy and occult blood testing, liver function tests. Peripheral blood was collected for further analysis.

Cytokine gene polymorphisms evaluated through SNAPSHOT

DNA was isolated from cells of approximate 4 mL of the peripheral blood following a phenol/chloroform protocol. Each DNA sample was quantified twice using the DNA quantification NanoDrop (Thermo Scientific, Waltham, MA, USA). Samples were only accepted if the average DNA concentration was at least 0.25 ng/mL and the coefficient of variation between the two rounds of quantification was smaller than 0.1.

SNAPSHOT was used to genotype the IL-10 polymorphisms including rs1800896 and rs1800871. The amplification primers of the candidate SNPs as followed. **IL-10**

SNAPSHOT products (1 μL of GeneScan-120 LIZ size standard (ABI). Data were analyzed by GeneMapper 4.0 (ABI). In order to guarantee the quality of the data, approximately 3% of the samples were randomly selected and genotyped by direct sequencing.
The isolation and culture of peripheral blood mononuclear cells

The 4 mL of peripheral blood sample was collected in ethylene diamine tetraacetic acid vials and plasma was collected. Ficoll Histopaque was used for peripheral blood mononuclear cells (PBMCs) isolation according to the protocol. PBMCs were harvested and counted with a hemocytometer. Cell viability was assessed by trypan blue staining following which they were resuspended at 1 x 10⁶ cells/mL in complete media. Cells were transferred to plates and incubated, non-stimulated, for 72 h at 37°C in a 5% CO₂ humidified atmosphere. Cell-free supernatants were stored frozen at –80°C and analyzed for cytokine levels in batches.

Enzyme-linked immunosorbent assay

Systematic inflammatory tone was assessed by measuring in both plasma and supernatant of PBMCs culturing through enzyme-linked immunosorbent assay (ELISA; EBioscience (Barcelona, Spain), Human IL-10 Platinum ELISA Kit). All samples and standards were assayed in two duplicates. To start with the measurement, each well of the 96-well plate was pre-wet with 200 μL assay buffer, then covered with a foil plate sealer and incubated 10 min at room temperature on a shaker. A volume of 25 μL of standard, wash buffer (served as the blank) or sample and 25 μL microparticles was added to each well and incubated at 4°C overnight. The liquid in each well was removed and wells were washed with 200 μL wash buffer for two times. After wash buffer was removed thoroughly, 25 μL biotin antibody was added to each well and incubated at room temperature for 2 h. The liquid in wells was removed and wells were washed with 200 μL wash buffer for two times again. Afterwards, 25 μL streptavidin-phycocerythrin was added to each well and incubated at room temperature for 30 min followed by twice wash with the Wash Buffer. A volume of 150 μL wash buffer was added to each well to resuspend to microparticles and incubated for 5 min on the shaker. Then, the plate was placed into Luminex 200 to measure median fluorescence intensity of standards and samples.

Immunohistochemistry staining

Immunohistochemistry was carried out to assess IL-10 protein expression in the mucosa biopsy tissues from distal ileum and sigmoid. Sections were deparaffinized in xylene and rehydrated in decreasing concentration of ethanol (100%, 95%, 80%) and subjected to immunohistochemical technique using the ZSGB-BIO ALK system (ZK-9600; Origene; and ZSGB-BIO; MO BIO, Beijing, China) After antigen retrieved and endogenous peroxidases blocked, primary antibody against IL-10 (ab134742, 1:800; Abcam, Cambridge, MA, USA) was incubated for 12 h at 1:800 dilution. As a secondary antibody and for visualization, a peroxidase/3, 3-diaminobenzidine-positive was used according to the manufacturer’s protocol (ZSGB-BIO ALK Detection System Peroxidase 3, 3-diaminobenzidine-positive mice; PV-6000; Origene; and ZSGB-BIO; MO BIO). The IL-10 levels were evaluated based on integral optical density of positive stain sing Image Pro Plus 6.0.

Statistical analysis

Data were expressed as mean ± standard deviation or median (q25, q75) depending on data distribution. Comparisons of parametric data with normal distribution between two groups were performed by Student t test. Comparisons of no-normality data between two groups were performed by Mann-Whitney U test. Non-parametric data were compared by Chi-square test if the theoretical value was more than 5, otherwise, the Fisher exact test was used. A P < 0.05 was considered statistically significant. Both allele and genotype models (allele model; dominant model; recessive model; homozygote model; heterozygote model) were used. Genetic association analyses and odds ratio (OR) calculations were performed for minor alleles based on genotypes using IBM SPSS version 30.0 (IBM Corp., Armonk, NY, USA) and PLINK 1.0.7 (http://pngu.mgh.harvard.edu/purcell/plink), Hardy-Weinberg equilibrium (HWE) determination and Pearson correlation analysis were also performed.

Results

Clinical characteristics of the subjects

A total of 264 participants aged between 18 and 65 years old were enrolled in this study. Based on the Rome III criteria, we confirmed that there were 120 patients with IBS-D (IBS group). Totally 144 healthy volunteers (healthy controls [HC] group) without previous or current gastrointestinal symptoms and infection were recruited during the same time. The clinical characteristics such as the gender, age, and body mass index were similar between the IBS and HC groups [Table 1]. The IBS group exhibited a higher HADS score than the HC group (HC: 6.50 [2.00, 12.00] vs. IBS: 7.25 [11.50, 15.00]). The scores for abdominal pain, abdominal bloating, dissatisfaction with bowel habits and life disturbance were significantly higher in the IBS group than those in the HC group [all P < 0.05] [Table 1]. Moreover, the IBS group had a significantly looser stool consistency, based on the Bristol scale, than the HC group (P = 0.001). A total of 78 participants underwent rectal distention test (52 in the IBS group and 26 in the HC group). The IBS group exhibited a significantly lower visceral pain threshold in the initial sensory, initial defecation, and defecation urgency than the HC group (all P < 0.05) [Table 1], indicating that patients with IBS-D were more sensitive in the visceral nociception.

Genotyping of IL-10 gene polymorphisms

The genotype distribution of all studied polymorphisms in the HC group were consistent with the HWE (IL-10 rs1800871: P = 0.5400; IL-10 rs1800896: P = 0.500). The detective rate for rs1800871 and rs1800896 was 99.52% and 99.76%, respectively [Table 2]. The genotype of these two SNPs is shown in Figure 1. There was no correlation between rs1800871 and the risk for developing IBS-D in the allele or any other genotype model [Table 3]. The frequency of rs1800896 C allele was significantly lower in the IBS group than that in the HC group (OR: 0.49, 95% confidence interval [CI]: 0.27–0.92, P = 0.024).
Table 1: Clinical characteristics for patients with IBS-D and HC.

| Characteristics                  | HC group (n = 144) | IBS group (n = 120) | Z      | P       |
|----------------------------------|-------------------|---------------------|--------|---------|
| HADS†                            | 6.50 (2.00, 12.00) | 7.25 (11.50, 15.00) | 3.376  | 0.0010  |
| HA                               | 4.00 (1.00, 7.00)  | 7.00 (4.25, 9.00)   | 3.192  | 0.0010  |
| HD                               | 2.00 (1.00, 5.00)  | 5.00 (3.00, 7.00)   | 3.244  | 0.0010  |
| IBS-SSS                          |                   |                     |        |         |
| Abdominal pain                   | 0                 | 50.00 (30.00, 81.25) | 6.927  | 0.0001  |
| Abdominal bloating               | 0                 | 30.00 (0, 36.25)    | 5.464  | 0.0001  |
| Dissatisfaction with bowel habits | 20.00 (5.00, 50.00)| 30.00 (0, 36.25)    | 0.814  | 0.4160  |
| Life disturbance                 | 3.00 (0.00, 20.00) | 70.00 (40.00, 70.00)| 7.643  | 0.0001  |
| Bristol scale                    | 4.00 (4.00, 4.50)  | 6.00 (5.00, 6.00)   | 6.486  | 0.0001  |
| Rectal distension (mmHg)         |                   |                     |        |         |
| Initial sensory                  | 12.00 (9.00, 15.00)| 8.00 (6.00, 12.00)  | −2.705 | 0.0070  |
| Initial defecation               | 20.00 (18.00, 27.00)| 16.00 (14.00, 19.50)| −2.836 | 0.0050  |
| Defecation urgency               | 28.00 (25.00, 37.00)| 24.00 (20.50, 26.00)| −2.742 | 0.0060  |

Data are shown as median (q25, q75), compared using Mann-Whitney U test. *Participants in HC and IBS group underwent HADS were 135 and 87 separately. HA: Hospital anxiety scale; HADS: Hospital anxiety and depression scale; HC: Healthy controls; HD: Hospital depression scale; IBS: Irritable bowel syndrome; IBS-D: Diarrhea predominant IBS; IBS-SSS: IBS symptom severity scores.

Table 2: Genotyping of IL-10 gene polymorphisms.

| SNPs   | Ref | Alt | DR (%) | Genotype | HC (%) | IBS-D (%) | HC | P_HWE |
|--------|-----|-----|--------|----------|--------|-----------|----|-------|
| IL-10  |     |     |        |          |        |           |    |       |
| rs1800871 | A   | G   | 99.52  | AA       | 32.62  | 31.97     | 0.5400 |
|         | GA  |     |        |          | 50.35  | 54.92     |     |       |
|         | GG  |     |        |          | 17.02  | 13.11     |     |       |
| rs1800896 | T   | C   | 99.76  | TT       | 77.30  | 86.89     | 0.5000 |
|         | CT  |     |        |          | 20.57  | 13.11     |     |       |
|         | CC  |     |        |          | 2.13   | 0         |     |       |

Alt: Altered allele; DR: Detectable rate; HC: Healthy controls; IBS-D: Diarrhea-predominant irritable bowel syndrome; IL-10: Interleukin 10; P_HWE: Significance of Hardy-Weinberg equilibrium test; Ref: Reference allele; SNPs: Single nucleotide polymorphisms.

In genotype analysis, the frequency of rs1800896 CC + CT genotype in the IBS group was significantly lower (OR: 0.51, 95% CI: 0.27–0.99, P = 0.0450) than that in the HC group in the dominant model.

**Association of IL-10 polymorphisms and clinical symptoms**

The correlation between IL-10 polymorphisms (rs1800871 and rs1800896) and clinical symptoms was evaluated. IL-10 rs1800871 was significantly correlated to the HADS score (R = 0.234, P = 0.023). The subjects with GG genotype had a higher HADS score than the subjects with AA or AG genotype [Figure 4A]. There was a significant positive correlation between IL-10 rs1800896 polymorphism and the pain threshold for initial defecation (R = 0.310, P = 0.007) and the defecation urgency (R = 0.298, P = 0.010). The subjects with CT genotype presented a significantly higher pain threshold of initial defecation (P = 0.007) and defecation urgency (P = 0.010) than the subjects with TT genotype [Figure 4B and 4C]. There was no correlation between the IL-10 polymorphisms and other clinical symptoms.

**Discussion**

Patients with IBS-D present a particularly VH with a looser stool consistency, which may result by chronic systemic and mucosal inflammation. IL-10 plays an important role in the anti-inflammation response and is considered to be a potent suppressor of T lymphocytes or macrophages and their derived effector molecules, such as proinflammatory cytokines (IL-1β, tumor...
necrosis factor (TNF)-α and chemokines (monocyte chemotactic protein 1, macrophage inflammatory protein 1α). It is speculated that IL-10 gene polymorphisms might be involved in the IL-10 production. Our study suggested that the carriers with IL-10 rs1800871 C allele have a lower risk for developing IBS-D, which may be associated with higher production of IL-10.

The gene encoding IL-10 is located on the human chromosome 1q31–1q32. The two polymorphisms analyzed in this study were −1082 A/G and −819 C/T, which are located in the gene promoter region. According to the single nucleotide polymorphism database (http://www.ncbi.nlm.nih.gov/SNP/), they are annotated as rs1800871 (T > C) and rs1800896 (A > G), which represents the polymorphism in the complementary strands. Qin et al. Table 3: IL-10 gene polymorphisms analysis of IBS-D and HC in allele model and genotype model.

| Items | IL-10 rs1800871 | IL-10 rs1800896 |
|-------|----------------|----------------|
| AM    | G vs. A       | 0.7100*        | 0.0240*        |
| DM    | GG + AG vs. AA| 0.9100*        | 0.0450*        |
| RM    | GG vs. AG + AA| 0.3800*        | 0.2500*        |
| HoM   | GG vs. AA     | 0.5400*        | 0.5700*        |
| HeM   | AG vs. AA     | 0.7000*        | 0.2500*†       |

*Chi-square test; †Fisher exact test, if there is a theoretical value that is smaller than 5, use Fisher exact test, otherwise use Chi-square test. AM: Allele model; CI: Confidence interval; DM: Dominant model; HC: Healthy controls; HeM: Heterozygous model; HoM: Homozygous model; IBS-D: Diarrhea-predominant irritable bowel syndrome; IL-10: Interleukin 10; OR: Odds ratio; RM: Recessive model.

Figure 1: Genotyping of IL-10 rs1800871 (A) and rs1800896 (B). G: guanine; C: cytosine; T: thymine; A: adenine. IL-10: Interleukin 10.
investigated that the IL-10 rs1800871 polymorphism was associated with a decreased risk for developing IBS in the eastern population. Moreover, other studies have confirmed that the C allele of IL-10 rs1800896 was strongly associated with the decreased risk for developing IBS in the western population.\(^{1,7}\) However, there are studies which have reported no correlation between IL-10 polymorphisms and the risk for developing IBS.\(^{12,20}\) In this study, the frequency of IL-10 rs1800896 C allele was significantly (OR: 0.49, 95% CI: 0.27–0.92, \(P = 0.0240\)) lower in the patients with IBS-D and was associated with a decreased risk for developing IBS-D. While the polymorphism rs1800871 presented no association with developing IBS, which investigated in patients with IBS-D and the healthy subjects.

IL-10 was initially described as a T helper 2-type cytokine and was reported to be expressed in various cells of the adaptive immune system as well as the cells of the innate immune system. Patients with IBS exhibit cytokine imbalance characterized by decreased levels of IL-10 and increased levels of TNF-\(\alpha\).\(^{1,7}\) The lower levels of IL-10 can be a predictor for IBS development.\(^{21}\) The T allele of IL-10 rs1800896 has been reported to be associated with lower production of IL-10, while the C allele is associated

| Items                  | AA            | GA            | GG            | TT            | CT            |
|------------------------|---------------|---------------|---------------|---------------|---------------|
| Plasma (pg/mL)         | 1.46 (0.66, 2.15) | 1.48 (1.14, 1.84) | 1.38 (1.00, 2.56) | 1.37 (0.74, 2.02) | 1.82 (1.29, 3.22) |
| PBMC (pg/mL)           | 49.95 (16.71, 87.86) | 38.40 (19.02, 104.80) | 69.16 (29.52, 113.75) | 31.69 (12.09, 76.87) | 55.87 (23.65, 104.80) |
| Ileum (IOD)            | 0.91 (0.62, 1.68)  | 1.04 (0.72, 1.51)  | 1.44 (1.08, 4.32)   | 1.08 (0.69, 1.54)   | 1.33 (0.82, 2.35)   |
| Colon (IOD)            | 0.11 (0.10, 0.13)  | 0.10 (0.09, 0.12)  | 0.10 (0.09, 0.12)   | 1.04 (0.77, 1.79)   | 1.07 (0.66, 1.35)   |

Data are shown as median (q25, q75). *Significance of IL-10 expression when comparing IL-10 rs1800896 TT genotype with CT genotype, \(P \leq 0.05\), Mann-Whitney U test. IL-10: Interleukin 10; PBMC: Peripheral blood mononuclear cells.
with higher IL-10 production. The C allele is associated with IL-10 production in the low stimulated cell culture. In addition, there are very few studies that report a direct correlation between IL-10 polymorphisms and IL-10 production. Our data confirmed that the subjects carrying C allele of IL-10 rs1800896 exhibited a higher IL-10 concentration in the plasma and the PBMC culture supernatant. Additionally, we also demonstrated that the subjects with CT genotype were less sensitive in the rectal distention test than the subjects with TT genotype. Earlier studies demonstrated that IL-10 and other inflammatory cytokine concentration in the PBMC correlated with the severity of symptoms in IBS-D, including the intensity and frequency of painful events and motility-associated symptoms.

Some researchers hypothesize that IBS is on a spectrum with inflammation bowel disease (IBD), because they have largely overlapping pathophysiological mechanisms and clinical symptoms. For example, the abdominal pain and diarrhea are predominant symptoms for patients with IBS or IBD, and post-inflammatory abnormalities are important in IBD and IBS. The subjects without C allele are more likely to develop IBS after intestinal infection. Additionally, the polymorphisms of IL-10 gene can exert a protective effect on patients with IBD, ulcerative colitis or Crohn disease.

Our finding was intriguing as the IL-10 rs1800871 polymorphism exhibited a marginally positive correlation with the HADS score. The development of depressive disorder is considered to be associated with the activation of systemic inflammation as well. It has been reported that the genotypes IL-10 rs1800871 and IL-10 rs1800896 decrease the risk for developing depression and are correlated with the Hamilton depression rating scale. Globally, patients with IBS have a high comorbidity rate for depression or anxiety. Our study supports the correlation between high comorbidity in IBS and mental disorder in an aspect of genetics polymorphism.

To the best of our knowledge, this study took the lead in studying the correlates of IL-10 gene polymorphisms and the expression level of IL-10. However, this study has some limitations. Only two polymorphisms for IL-10 were analyzed in this study. There may be other SNPs in IL-10 corresponding to rs1800896 and/or rs1800871, which may contribute to IL-10 expression. On the other hand, further studies are required for evaluating the transcriptional and epigenetic effects in IL-10 polymorphism.

In summary, the IL-10 rs1800896 polymorphism has a correlation with a higher concentration of IL-10 in both
the plasma and the PBMC culture supernatant of Chinese patients with IBS-D. Additionally, this SNP is also associated with a higher visceral pain threshold. This study demonstrated a correlation between the IL-10 polymorphisms and IL-10 production, which might help in understanding the pathogenesis of IBS-D.

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Conflicts of interest
None.

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