Towards an integrated psychoneurophysiological approach of irritable bowel syndrome

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ROLE OF TUMOR NECROSIS FACTOR-α AND INTERLEUKIN-10 GENE POLYMORPHISMS IN IRRITABLE BOWEL SYNDROME

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ABSTRACT

Background: Imbalances in the genetically controlled pro- and anti-inflammatory cytokine production may promote ongoing low-grade inflammation after an acute gastroenteritis, and, subsequently, IBS (post-infectious IBS, PI-IBS). We studied gene promoter single nucleotide polymorphisms (SNPs) of tumor necrosis factor α (TNF-α, pro-inflammatory) and interleukin 10 (IL-10, anti-inflammatory) in IBS patients and controls.

Methods: DNA was extracted from peripheral blood leucocytes of 111 IBS patients and 162 healthy controls. Genotype and allele frequencies were assessed by analyzing SNPs at position –308 (TNF-α) and -1082 and -819 (IL-10).

Results: Homozygous high producers for TNF-α (A/A) were rare (overall prevalence 2.6%). The heterozygous TNF-α genotype (G/A, high producer) was significantly more prevalent in IBS compared to controls (41% versus 26%, *P*=0.02). More patients (41%) than controls (30%) were positive for the A allele (*P*=0.044; OR 1.68, 95% CI 1.01-2.79), with a similar trend for diarrhoea (54%) versus constipation and alternating subtypes (<33%, *P*=0.079), but not for subgroups according to a history of acute gastroenteritis. IL-10 genotypes were similarly distributed in patients and controls for both SNPs. Possession of a high producer TNF-α *and* a low producer IL-10 genotype was significantly more prevalent in IBS (9%) versus controls (3%, *P*=0.035; OR 3.11, 95% CI 1.03-9.36) and in diarrhoea (20%) compared to other IBS subtypes (<4%, *P*=0.026).

Conclusion: Our results support the emerging hypothesis that genetically determined immune activity plays a role in the pathophysiology of IBS. Future studies in larger, clinically relevant, IBS subgroups are warranted to establish definite associations with cytokine gene polymorphisms.

INTRODUCTION

Irritable Bowel Syndrome (IBS) is a common functional bowel disorder characterized by recurrent abdominal pain and altered bowel habits[1,2]. Several mechanisms have been proposed in the pathophysiology of IBS, including visceral hypersensitivity[3,4], altered gut motility[5,6] and psychosocial factors[7,8]. In addition, inflammation and mucosal immune system activation may be important[9]. Recent studies demonstrated an increased risk for developing IBS after dysenteric illness[10-12] and increased numbers of immunocompetent cells in rectal mucosa of patients with post-infectious IBS (PI-IBS) up to 1 year after infection[13], implying that low-grade inflammation may contribute to symptom generation.

Pro- and anti-inflammatory cytokines are important modulators of the immune response and play a role in intestinal inflammation[14]. Cytokine production is under genetic control and imbalances in cytokine secretion may affect disease susceptibility and clinical outcome of various conditions. For instance, secretion of tumor necrosis factor alpha (TNF-α), a pro-inflammatory cytokine[15], is associated with a single nucleotide polymorphism (SNP) in the promoter region of the TNF-α gene (G→A substitution at position –308)[16,17]. Possession of the A allele (A/A or G/A) is associated with increased TNF-α production[18]. Homozygotes for the A allele have worse outcome of cerebral malaria[19] and virus-induced renal failure[20]. Likewise, production of the counter-inflammatory cytokine interleukin 10 (IL-10)[21] is associated with SNPs at positions –1082 (G→A) and –819 (C→T)[22]. Genetic predisposition for low IL-10 production (A/A for the –1082 and T/T for the –819 SNP)[22] is associated with inflammatory bowel disease, particularly ulcerative colitis[23], and acute rejection after liver transplantation[24]. IL-10 knock-out mice spontaneously develop chronic enterocolitis[25]. A recent study by Gonsalkorale et al.[26] showed that the high producer IL-10 genotype (-1082 G/G) is less prevalent in IBS patients compared to healthy controls. However, persisting low-grade inflammation may result from decreased production of anti-inflammatory cytokines, e.g. IL-10, as well as from high levels of pro-inflammatory cytokines such as TNF-α[27] or IL-1beta[28], or from imbalance between these cytokines. Our primary aim was therefore to study gene promoter SNPs of IL-10 and TNF-α in IBS patients and in healthy controls. In addition, we aimed to explore the frequencies of these SNPs in IBS subgroups based on post-infectious symptom onset and predominant bowel habit.

METHODS

Subjects

Patients were recruited through the outpatient department of Gastroenterology and Hepatology of the Leiden University Medical Center (LUMC) and through advertisement in a local newspaper. Healthy control subjects were recruited among spouses of non-IBS patients who attended our department and through advertisement. All participants were screened by one of the investigators (PvdV) and all patients met Rome II criteria for IBS[1]. Exclusion criteria for both groups were: presence of organic disease, previous abdominal surgery (cholecystectomy and appendectomy excluded), pregnancy and dependence on analgesics. Although the presence of immunological (astma, celiac disease) or other disorders was not excluded by means of physical, radiological or laboratory investigations, patients were explicitly requested to report the presence of any disease, now or in the past, and to specify any GI disorder in particular. Informed consent was obtained from each participant. The LUMC ethics committee had approved the study protocol.

Study design

Each subject completed a questionnaire concerning medical history and current abdominal symptoms and bowel habits. In a separate item, we explored whether symptom onset was associated with an episode of acute diarrhoea, fever and vomiting. Subsequently, blood samples were obtained.

Genotype assessment

Blood samples were collected in ice-chilled tubes containing EDTA and transported to the laboratory on ice. All samples were centrifuged at 1000 g for 10 min at 4°C. DNA was extracted from peripheral blood leucocytes according to the salting out procedure[29]. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was used to determine the TNF-α G-308A, IL-10 G-1082A and C-819T SNPs. Genotype assessment was done as previously described[30-32]. Briefly, gene specific primers were used to generate 147 bp (TNF-α) and 360 bp (IL-10) products. Restriction enzyme digestion yielded fragments, which were analyzed by electrophoresis on a 4% agarose gel and visualized under UV light (Fig 1A and 1B).

Statistical analysis

We aimed to enroll at least 100 subjects in both groups, based on 1) a 24% prevalence of the high producer IL-10 genotype (G/G) in the Dutch population[31], 2) a power of 0.80, and 3) 11% difference in genotype prevalence between IBS patients and controls[26]. Genotype frequencies were compared between groups by Pearson's

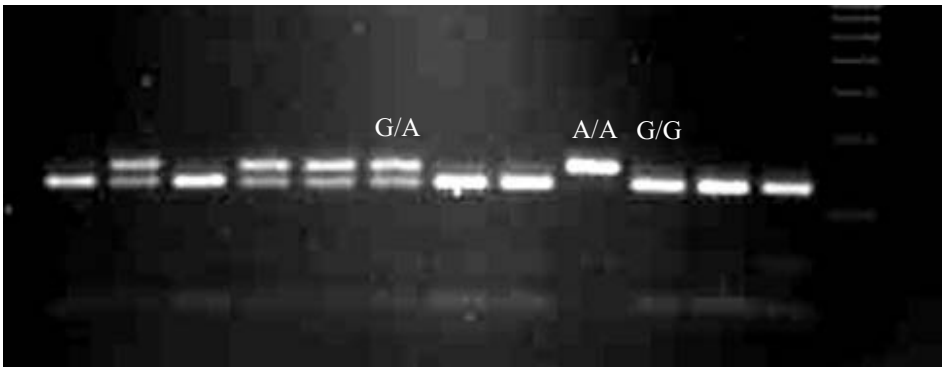

Figure 1A. Example of the TNF-α genotyping method using PCR-RFLP. A/A and G/A, high producer; G/G, low producer.

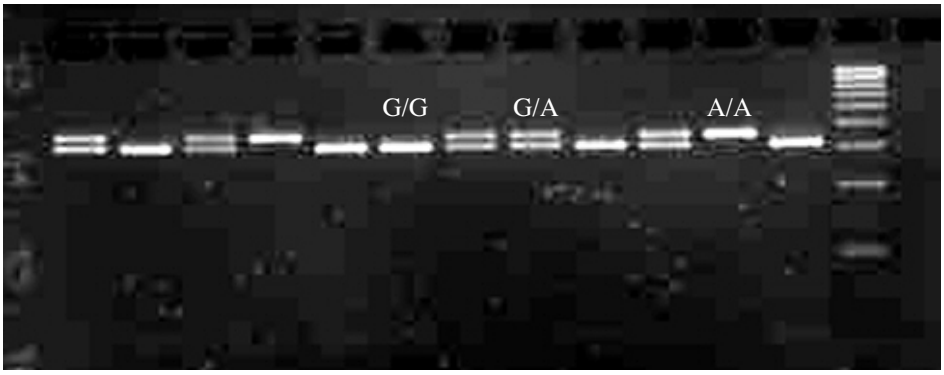

Figure 1B. Example of the IL-10 genotyping method using PCR-RFLP. G/G, high producer; G/A, intermediate producer; A/A, low producer.

chi-square analysis for each polymorphism. Allele and high/low producer genotype frequencies were compared by calculation of odds ratios. Data are expressed as mean (SD) or as number of cases (percentage) where appropriate. The level of significance is set at $P \leq 0.05$.

RESULTS

Subject characteristics

A total of 111 IBS patients and 162 healthy control subjects were eligible and included in the study. Table 1 displays patient and control group characteristics.

Twenty-three patients (21%) reported symptom onset after an episode of acute diarrhoea, vomiting and fever, and were marked as PI-IBS. Fifteen patients (13%) did not report their current bowel habit. Normal bowel habits were reported by 139 controls, and occasional occurrence of diarrhoea or constipation (less than 1 time

Table 1. Characteristics of study participants

Characteristic	IBS patients (n=111)	Controls (n=162)
Females	76 (84)	61 (98)
Age	48.6 (12.9)	37.6 (15.6)
Bowel habit		
diarrhoea	32 (35)	6 (10)
constipation	24 (27)	4 (6)
alternating	31 (34)	4 (7)
currently unknown	13 (15)	-
normal	-	86 (139)

Numbers without parentheses show percentages, numbers within parentheses show absolute numbers or SD (Age). IBS, irritable bowel syndrome; n, number of patients or controls.

per month) without abdominal pain was reported by 23 controls (Rome II negative). In both groups, more than 95% of participants were of Caucasian origin.

TNF-α and IL-10 genotype and allele frequencies

Genotype and allele frequencies for TNF-α are shown in Table 2. Homozygote high producers were rare (overall prevalence 2.6%). The heterozygous genotype (G/A) was significantly more prevalent in IBS patients compared to controls (41% versus 26%, *P*=0.02), with more patients than controls being positive for the A allele (A/A or G/A; 41% versus 30%, *P*=0.044; odds ratio (OR) 1.68, 95% confidence interval (CI)

Table 2. TNF-α G-308A genotype and allele distribution in IBS patients and controls

	IBS patients (n=111)		Controls (n=162)	
	n	%	n	%
Genotype				
A/A (high)	1	1	6	4
A/G (high)	45	41†	42	26
G/G (low)	65	59	114	70
Genotype				
A+ (A/A or A/G)	46	41‡	48	30
A- (G/G)	65	59	114	70
Allele frequency				
-308A (high)	47	21	54	17
-308G (low)	175	79	270	83

† $\chi 2=7.83$, $P=0.020$ versus controls; ‡ $\chi 2=4.07$, $P=0.044$ versus controls; odds ratio (OR) 1.68, 95% CI 1.01 - 2.79.

Table 3. IL-10 G-1082A genotype and allele distribution in IBS patients and controls

	IBS patients (n=111)		Controls (n=162)	
	n	%	n	%
Genotype				
G/G (high)	29	26	45	28
G/A (intermediate)	57	51	83	51
A/A (low)	25	23	34	21
Genotype				
G+ (G/G or G/A)	86	77	128	79
G- (A/A)	25	23	34	21
Allele frequency				
-1082G (high)	115	52	173	53
-1082A (low)	107	48	151	47

1.01 - 2.79). A allele frequencies were not different between patients and controls (21% versus 17%, *P*=0.18; OR 1.34, 95% CI 0.87 - 2.07).

Table 3 shows genotype and allele frequencies for the IL-10 G-1082A SNP. The low producer genotype (A/A) was similarly distributed in patients and controls (23% versus 21%, *P*=0.93). Likewise, frequencies of the A allele (low IL-10 production) were comparable between IBS patients and controls (48% versus 47%, *P*=0.71; OR 1.07, 95% CI 0.76 - 1.50). Similar results were obtained for the IL-10 C-819T SNP. Frequencies of the low-producer genotype (T/T) did not differ between patients and controls (6% versus 7%, *P*=0.73), nor did T allele frequencies (24% versus 27%, respectively, *P*=0.43; OR=0.85, 95% CI 0.58 – 1.27).

Combined high TNF-α and low IL-10 producer genotypes

Possession of both a low producer IL-10 genotype (–1082 A/A) and a high producer TNF-α genotype (-308 A/A or G/A) may make an individual particularly susceptible to an exaggerated inflammatory response or prolonged low-grade inflammation. Therefore we explored the frequencies of the presence of both genotypes in patients and controls. This combination was considerably more prevalent in IBS patients compared to controls (9% versus 3%, *P*=0.035; OR 3.11, 95% CI 1.03 - 9.36) (Table 4). The frequencies of the other genotype combinations were similar in patients and controls (Table 4). The combination of a high producer TNF-α genotype (A/A or G/A) and the other low producer IL-10 genotype (-819 T/T) was not significantly different between patients (3%) and controls (1%) (*P*=0.16; OR 4.47, 95% CI 0.46 – 43.56; other combinations not shown).

Table 4. Combined TNF-α G-308A and IL-10 G-1082A genotypes in IBS patients and controls

	IBS patients (n=111)		Controls (n=162)	
	n	%	n	%
Combination				
high TNF-α / low IL-10	10	9†	5	3
low TNF-α / high IL-10	50	45	85	53
high TNF-α / high IL-10	36	32	43	27
low TNF-α / low IL-10	15	14	29	18

† $\chi 2=4.45$, $P=0.035$ versus controls; OR 3.11, 95% CI 1.03 - 9.36.

IBS subgroups

Exact statistical comparisons between some subgroups according to reported post-infectious symptom onset or predominant bowel habit were not feasible due to small numbers in these groups. Yet, explorative analysis indicated a trend for the high producer TNF-α genotypes (A/A or G/A) to be more prevalent in IBS-D (54%) patients compared to IBS-C (33%) and IBS-A patients (29%) (P=0.079) (Table 5), but was found to be present in 48% of PI-IBS patients compared to 40% of non-PI-IBS patients (P=0.49) (Table 5). No differences were found regarding the IL-10 genotypes. Furthermore, the prevalence of a combined high producer TNF-α and low producer IL-10 genotype (-1082 A/A) appeared remarkably higher in IBS-D (20%) compared to IBS-C (4%) and IBS-A (3%) (P=0.026), but was similar in the PI-IBS and non-PI-IBS subgroups (9% versus 9%, P=0.95) (Table 5).

Table 5. TNF- and IL-10 genotype distributions and combinations in PI-IBS and non-PI-IBS patients, and in IBS subgroups according to predominant bowel habit

	PI-IBS (n=23)		non-PI-IBS (n=88)		diarrhea (n=35)		constipation (n=27)		alternating (n=34)	
	n	%	n	%	n	%	n	%	n	%
TNF-α G-308A										
high (A+)	11	48	35	40	19	54†	9	33	10	29
low (A-)	12	52	53	60	16	46	18	67	24	71
IL-10 G-1082A										
high (G+)	19	83	67	76	24	69	24	89	26	77
low (G-)	4	17	21	24	11	31	3	11	8	24
Combined										
high TNF-α / low IL-10	2	9	8	9	7	20‡	1	4	1	3
low TNF-α / high IL-10	10	44	40	46	12	34	16	59	17	50
high TNF-α / high IL-10	9	39	27	31	12	34	8	30	9	27
low TNF-α / low IL-10	2	9	13	15	4	11	2	7	7	21

† $\chi 2=5.08$, $P=0.079$ compared to IBS-C and IBS-A; ‡ $\chi 2=7.33$, $P=0.026$ compared to IBS-C and IBS-A.

DISCUSSION

This study demonstrates that the high producer TNF-α genotype is more prevalent in IBS patients compared to healthy controls. Although homozygous high producers were rare in both groups, the heterozygous genotype, which is also associated with a high TNF-α production phenotype[17], was present in 41% of patients versus only 26% of controls.

TNF-α is produced by monocyte-derived activated macrophages, which have a crucial role in chronic inflammatory states such as Inflammatory Bowel Disease[33] and rheumatoid arthritis[34]. It has been shown that patients with persisting symptoms after an acute infectious gastroenteritis have a fivefold increase in the number of these activated macrophages in the rectal lamina propria[13]. Macrophage TNF-α production can be stimulated by enteric pathogens such as Campylobacter jejuni, Salmonella and Shigella[35], which are important in the onset of PI-IBS[13,36,37]. Increased macrophage TNF-α production in patients carrying the A allele may contribute to the ongoing low-grade inflammation that is demonstrable in a subgroup of patients after an infectious enteritis[13,28]. The largest proportion of individuals positive for the A allele was indeed found in the PI-IBS group (48%) relative to the non-PI-IBS (40%), although this did not reach statistical significance. This does, however, not account for individuals carrying the A allele in the non-PI-IBS group. It is possible that low-grade inflammation can be provoked by unknown non-infectious stimuli, especially in patients who are genetically predisposed to an enhanced pro-inflammatory response. In addition, several other pro- and anti-inflammatory cytokines apart from TNF-α play a role in the regulation of the inflammatory process and may be involved in persistent low-grade inflammation. Finally, recall bias may have affected the composition of the PI-IBS and non-PI-IBS groups, as some patients had symptoms for more than 15 years.

Genotype frequencies for IL-10 at positions –1082 and –819 were not different between IBS patients and controls. We found that the high producer genotype (-1082 G/G) was present in 26% of patients and 28% of control subjects. These findings are in contrast with the recent preliminary observations by Gonsalkorale et al., showing a significant reduction in the high producer IL-10 genotype frequency in IBS patients compared to controls (21% versus 32%)[26]. When comparing these and our data, it is important to recognize that genotype frequencies vary according to ethnicity[31,38]. For instance, a recent study showed that the frequency of the high producer IL-10 genotype is much higher in the Irish population (34%) than in Africans (9.5%) or Singapore Chinese (0%)[39]. In our patient and control groups, more than 95% of individuals were of Caucasian origin, and the IL-10 –1082 high producer genotype frequencies that we found in controls (28%) are similar to those previously reported

in the Dutch population (24%)[31]. Although the study by Gonsalkorale et al.[26] provides no information on the ethnic origin of patients and controls, this may well explain the disparity between their study and ours.

The role of the C–819T SNP in IL-10 production is incompletely understood. This polymorphism is in linkage disequilibrium with C–592A, another SNP in the promoter region of the IL-10 gene[40]. Three haplotypes for the G-1082A, C-819T and C-592A SNPs are common in Caucasians, i.e. GCC, ACC, and ATA, respectively. Although a direct link between the C-819T SNP and levels of IL-10 production has not yet been established, the GCC/GCC genotype is more common in IL-10 high producers, whereas ATA/ATA is associated with low IL-10 production[22]. In our study, the –819 SNP was similarly distributed in patients and controls, supporting our observation that the genetic make-up for IL-10 production levels does not differ between these groups. However, other SNPs in the promoter region of the IL-10 gene may also be associated with increased or decreased IL-10 production. For instance, recent studies indicate that T-3575A, G-2849A, and C-2763A SNPs are associated with susceptibility to systemic lupus erythematosus[41] and leprosy[42] and disease severity in leprosy[42]. It may therefore be important to address these and other SNPs and haplotypes in IBS in future studies.

The combined presence of a high producer TNF-α and low producer IL-10 (-1082 A/A) genotype within one individual was 3 times more prevalent in IBS patients compared to controls. This finding is clinically relevant, since IL-10 is known to inhibit TNF-α synthesis as well as the initial inflammatory response[21]. Individuals with an inherited predisposition to produce high levels of TNF-α, which are not adequately counterbalanced due to a genetically determined low IL-10 secretion, may be particularly at risk to develop ongoing low-grade inflammation and IBS-like symptoms. However, only 1 in 10 patients had this genotype combination, implying that other mechanisms are also important in the pathogenesis of IBS.

Our study was not primarily designed to compare patient subgroups based on post-infectious symptom onset or predominant bowel habit. Patient numbers in these subgroups were small and therefore these results should be interpreted with caution. However, our data indicated that the proportion of individuals positive for the high producer TNF-α A-allele was relatively large in IBS patients with a diarrhoea predominant bowel habit (54%) compared to patients with constipation (33%) or alternating bowel habits (29%). Moreover, the combination of a high producer TNF-α genotype and a low producer IL-10 genotype appeared more prevalent in IBS-D compared to IBS-C and IBS-A (20% versus 4% and 3%, respectively). These are potentially interesting results, as several studies indicate that TNF-α is associated with the occurrence of diarrhoea. For instance, TNF-α is an important mediator of distal colonic secretion[43,44] and stool TNF-α concentrations are elevated in IBD[45] and

infectious HIV-related diarrhoea[46]. Decreased IL-10 mediated inhibition of TNF-α may further add to its biological actions in patients with this specific genotype combination. Our data indicate that IBS subgroups may exhibit different cytokine producer genotypes that might be involved in disease expression, and further studies in larger populations are warranted to confirm these preliminary results.

In conclusion, we have demonstrated that the high producer TNF-α genotype is more prevalent in IBS patients compared to healthy controls. Whereas the low producer IL-10 genotype is similarly distributed, the combination of a high producer TNF-α genotype and a low producer IL-10 genotype is also more prevalent in IBS. Our study contributes to the growing body of evidence that altered immune activation may be important in at least a subset of IBS patients. Future studies should further address the role of cytokine production in the pathophysiology of IBS and focus on clinically relevant subgroups.

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