Original Article

TNF-Alpha Gene Polymorphisms in Iranian Azari Turkish Patients with Inflammatory Bowel Diseases

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ABSTRACT

Context: Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory diseases of the bowel (IBD) whose causes are not fully known. Emerging data indicate that alterations in cytokine synthesis may play a role in IBD pathogenesis. **Aims:** We aimed to determine the association between tumor necrosis factor-alfa (TNFα) promoter polymorphisms (at positions − 308 and − 1031) and susceptibility to IBD among Iranian Azari Turkish patients. **Settings and Design:** One hundred and one patients with IBD and 100 healthy subjects were analyzed. **Materials and Methods:** Both polymorphisms in the promoter region of the TNFα gene at positions -1031T/C and -308G/A were detected by polymerase chain reaction–restriction fragment length polymorphism assay. All statistical analyses were calculated with SPSS for Windows 16.0. The Fisher's exact test was used to test for departure from Hardy–Weinberg equilibrium of the genotype frequencies ($P > 0.05$). **Results:** The allele frequency of the TNFα-308G and -1031T were higher in IBD patients but did not reach statistical significance. However, the homozygous TT genotype for the SNP-1031 T > C was significantly higher in UC patients than in healthy controls ($P = 0.01$) and the heterozygous CT genotype for the SNP -1031 T > C was significantly lower in UC patients than in healthy controls ($P = 0.03$). **Conclusions:** The TNFα-1031 T allele confers a significant risk for developing UC in Iranian Azeri Turkish patients. Also the frequency of TNFα-1031 C allele was considerably low among patients with UC and it may have protective role among them (OR = 0.43; $P = 0.01$).

Key Words: Genetics, inflammatory bowel disease, single nucleotide polymorphisms, tumor necrosis factor alfa

Received: 21.07.2013, Accepted: 14.09.2013

How to cite this article: Bonyadi M, Abdolmohammadi R, Jahanafrooz Z, Somy M, Khoshbaten M. TNF-alpha gene polymorphisms in Iranian Azari Turkish patients with inflammatory bowel diseases. Saudi J Gastroenterol 2014;20:108-12.

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is a chronic condition that involves inflammation of the digestive tract.[1,2] It has been the most common health problem worldwide, particularly in the developed countries. UC and CD affect different parts of the digestive tract. UC causes swelling and the formation of ulcers on the surface lining the top layer of the large intestine. Patients with CD experience thickening of the intestinal wall, most frequently in the last part of the small intestine and the first part of the large intestine.[2,3] Although IBD can appear at any age, it is rare in babies and very young children. It has been reported that incidence rates were 0.70 per 100,000 person-years for CD and 1.85 per 100 000 person-years for UC worldwide.[4]

The cause of the IBD-UC and CD- is not well known. Studies have suggested that IBD is a multifactorial disease that has arisen from both environmental risk factors and genetic susceptibility.[5-7] There is multiple evidence in favor of a genetic role in the pathogenesis of IBD. These include increased rates of IBD among identical twins compared with fraternal twins and among siblings compared with spouses of affected individuals, the differences in prevalence in diverse ethnic groups and the increased familial incidence. Although there is strong evidence to suggest a genetic basis for IBD, no specific gene has been identified thus far.[8,9] The main genetic associations in IBD can be divided

Access this article online

Quick Response Code:

Website: www.saudijgastro.com

DOI: 10.4103/1319-3767.129475

into genes that contribute to innate and adaptive immune responses.[10] TNFα is a multifunctional cytokine involved in the promotion of inflammatory responses and plays a critical role in the pathogenesis of inflammatory, autoimmune, and malignant diseases.[11-13] TNFα is also known to play a key role in cell-mediated immunity and elevated serum levels of TNFα have been demonstrated in IBD.[13] These facts suggest a direct role for TNFα in IBD pathogenesis. The TNFα has a large number of polymorphisms-most of them located in its promoter region-of which some of them have effect on the expression level of the gene.[14] We postulated that differences in cytokine levels as a result of gene polymorphisms may have an important role in the inflammatory response and thus influence the pathophysiology of IBD.

In this study, for the first time, the association between TNFα promoter polymorphisms (at positions -308 and -1031) and susceptibility to IBD among Iranian Azari Turkish patients was investigated.

MATERIALS AND METHODS

Our case–control study included 101 Iranian Azeri Turkish IBD patients (21 CD patients and 80 UC patients) and 100 healthy, unrelated, age- and gender-matched individuals from the same geographical area were selected as a control group. The diagnosis and extent of IBD was made on the basis of clinical symptoms, endoscopic, radiological, and histopathological findings, according to conventional criteria.[15] Genomic DNA was extracted from peripheral blood cells using simple salting out procedure.[16] Both polymorphisms in the promoter region of the TNFα gene at positions -1031T/C and -308G/A were detected by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) assay. For the amplification of -1031T/C polymorphism site via PCR we used the forward (5'-GGGGAGAACAAAAGGATAAG-3') and reverse (5'-CCCCATACTCGACTTTCATA-3') primer pair. Initially, the PCR reaction was subjected to denaturation for 5 min at 95°C, followed by 30 cycles of amplification (30 s at 95°C, 30 s at 55°C and 30 s at 72°C). A final elongation step (5 min at 72°C) was applied at the end of the 30 cycles. The PCR product is 270-bp. Then PCR followed by an overnight digestion with the restriction enzyme BbsI (C allele, 159 and 111 bp; T allele, 270 bp) at 37°C. Digested PCR fragments were separated by a 10% polyacrylamide gel electrophoresis and visualized by ethidium bromide staining. Primers forward (5'-AGGCAATAGGTTTTGAGGGCCAT-3') and reverse (5'-TCCTCCCTGCTCCGATTCCG-3') were used to amplify the 107-bp DNA fragment of the TNFα-308A/G polymorphism. PCR conditions were 5 min for initial denaturation at 95°C; 35 cycles at 95°C for 1 min for denaturation, 30 s at 65°C for annealing and 30 s at 72°C for extension, followed by 5 min at 72°C for final extension. After amplification, PCR products were digested (at 37°C) by restriction endonuclease NcoI (G allele, 87 and 20 bp; A allele, 107 bp) for 16 h. Digested PCR products were electrophoresed in a 10% polyacrylamide gel and visualized by ethidium bromide staining.

Statistical analysis

Allelic and genotypic association between patients and healthy controls was carried out using Chi-square test with Yates' correction or Fisher's exact test, where appropriate. The Fisher's exact test was used to test for departure from Hardy–Weinberg equilibrium of the genotype frequencies ($P > 0.05$). Allele frequencies were determined by gene counting. Odds ratios and corresponding 95% confidence intervals were estimated by cross-tabulation. Statistics were calculated using SPSS 16.0.

RESULTS

In this study, 101 patients with IBD, 21 patients with CD and 80 patients with UC (21-69 years old, 51 males (51%), 50 females (50%) as well as 100 healthy controls (16-74 years old, 49 males (49%), 51 females (51%) were included. No statistically significant differences were observed between patients with IBD and controls regarding age and gender. Clinical analysis of IBD patients showed that 20.8% (21/101) had fever, 34.7% (35/101) had diarrhea, and 18.8% (19/101) had vomiting. For TNFα we genotyped two SNPs in the promoter region: -1031 T > C and -308 A > G. The allelic and genotypic frequencies are shown in Table 1. We found no significant difference of -1031T/C and -308 G/A allele/carrier frequency between IBD patients and healthy controls. The frequency of the TNFα-308G and -1031T alleles were higher in IBD patients but did not reach statistical significance (at -308G allele frequency 92.58% vs 90.5%, $P = 1$ and at -1031T allele frequency 89.1% vs 83%, $P = 0.08$). However, when the UC patient groups were compared with controls separately, we observed significant difference for the SNP -1031 T > C with the allele C present in 8% of UC and 17% of controls and allele T present in 92% of UC and 83% of controls. The heterozygous CT genotype for the SNP-1031 T > C was significantly lower in UC patients (16.2%) than in healthy controls (30.0%), and reached statistical significance ($P = 0.03$; OR = 2.31; 95% CI = 1.11-4.93) [Table 2]. For the SNP-308 A > G, with the allele G present in 92.5% of UC and 90.5% of controls, was although not statistically significant. We also did not find homozygous CC genotype for the SNP-1031 T > C in UC patients.

For assessing the relationship between genotype and clinical parameters, the allelic and genotypic frequencies of these polymorphisms between controls and patients with/without symptoms were evaluated. These data showed no significant

Table 1: TNF-alfa genotype and allele frequencies at position -308 and position -1031 in patients with IBD and in healthy control

	Genotype	IBD (%)	Control (%)	*P* value	OR	95% CI
Position-308	A/A	2 (2)	2 (2)	0.99	0.98	0.13-7.16
	A/G	11 (10.9)	15 (15)	0.38	0.69	0.30-1.59
	G/G	88 (87.1)	83 (83)	0.41	1.38	0.63-3.03
	Allele "A"	15 (7.42)	19 (9.5)	0.45	0.76	0.37-1.54
	Allele "G"	187 (92.58)	181 (90.5)	0.454	1.30	0.64-2.65
Position-1031	C/C	1 (1)	2 (2)	0.62	0.49	0.04-5.49
	C/T	20 (19.8)	30 (30)	0.09	0.57	0.30-1.10
	T/T	80 (79.2)	68 (68)	0.07	1.79	0.94-3.39
	Allele “C”	22 (10.9)	34 (17)	0.07	0.59	0.33-1.06
	Allele "T"	180 (89.1)	166 (83)	0.08	1.67	0.94-2.98

IBD: Inflammatory bowel disease, OR: Odds ratio, CI: 95% confidence interval of the odds ratio, NS: No significant

Table 2: TNF-alfa genotype and allele frequencies at position -308 and position -1031 in patients with UC and in healthy controls

	Genotype	UC (%)	Control (%)	*P* value	OR	95% CI
Position-308	A/A	2 (2.5)	2 (2.0)	1	1.25	0.17-9.12
	G/A	8 (10.0)	15 (15.0)	0.31	0.62	0.25-1.57
	G/G	70 (87.5)	83 (83.0)	0.39	1.43	0.61-3.33
	"A" Allele	12 (7.5)	19 (9.5)	0.50	0.77	0.36-1.64
	"G" Allele	148 (92.5)	181 (90.5)	0.502	1.29	0.60-2.75
Position-1031	C/C	0 (0.0)	2 (2)	0.50	0	-
	C/T	13 (16.2)	30 (30)	0.03	0.45	0.21-0.94
	T/T	67 (83.8)	68 (68)	0.01	2.42	1.17-5.02
	"C" Allele	13 (8.1)	34 (17)	0.01	0.43	0.21-0.84
	"T" Allele	147 (91.9)	166 (83)	0.012	2.31	1.17-4.55

UC: Ulcerative colitis, OR: Odds ratio, CI: 95% confidence interval of the odds

difference in genotype distribution and allelic frequency among groups.

DISCUSSION

CD and UC are chronic inflammatory diseases of the bowel, whose causes are not fully known. Emerging data indicate that alterations in cytokine synthesis may play a role in IBD pathogenesis.[17] The single nucleotide polymorphisms in gene promoter regions result in differential production of cytokines and may influence disease activity as well as more intense inflammatory activity in both forms of IBD.[18] Tumor necrosis factor (TNF)-alfa is an important proinflammatory cytokine that has been implicated in the pathogenesis of IBD. Overexpression of TNFα have been observed in inflamed and normal intestinal mucosa and in the serum of patients with IBD.[19] In 2003, Gonzalez *et al.*, showed that the TNFα-308A polymorphism was associated with enhanced TNFα production and an increased risk for susceptibility to arthritis in CD patients.[20] In the present study, we determined the allelic and genotypic distribution of two SNPs (at positions -308 and -1031) in the TNFα gene among IBD patients and healthy controls in the Iranian Azari Turkish ethnic group. In our study, we found a nonstatistically significant reduction of -308A and -1031C allele frequencies in IBD patients compared with controls. However, the comparison of UC group with healthy controls showed a significant variation in TNFα-1031 polymorphism allele and genotype frequency [Table 2]. The homozygous TT genotype for the SNP-1031 T > C was significantly higher in UC patients (83.8%) than in healthy controls (68%) (P = 0.01; OR, 2.42; 95%CI, 1.17-5.02). Also, the heterozygous CT genotype for the SNP -1031 T > C was significantly lower in UC patients (16.2%) than in healthy controls (30.0%), and reached statistical significance (P = 0.03; OR, 0.45; 95%CI, 0.21-0.94). These observations suggest that the TNFα-1031 T allele confers a significant risk for developing UC and the SNP-1031 T > C polymorphism may have a functional effect in IBD pathogenicity and could explain the reason for high levels of TNF-α production observed in IBD patients. Nevertheless, until now, there has been no available molecular genetic mechanism to explain how the -1031T > C allele influences TNFα production. The polymorphic -1031T > C site may

either bind to unknown regulatory elements or alter the secondary structure of DNA to affect accession of cis-acting transcription factors to the promoter/enhancer region of the TNFα gene.[20] Previous studies regarding the association of TNFα gene polymorphism and IBD susceptibility have reported conflicting results. Depending on studied populations, some reports show positive association and the others failed to show any significant association with TNFα gene polymorphisms and IBD.[12,19,21-24] Bouma *et al*. reported small but statistically significant association between TNFα-308 polymorphism and UC. They found a lower frequency of the TNFα-308 A allele in UC patients compared with healthy controls.[8] Also Vatay *et al*. reported the frequency of TNFα-308 A allele to be less in IBD patients (both UC and CD) compared with controls.[25] The results of our study are consistent with some previous reports.[20,26] In the study by Zipperlen *et al*., no association between TNFα promoter polymorphisms and CD was observed.[21] Conversely, in a study carried out by Balding and colleagues, there was a significant variation in TNFα-308 polymorphism genotype frequency when comparing the control group with the UC group. The UC group had a lower frequency of the TNFα-308A allele.[2] A meta-analysis by Lu *et al*. showed that the TNFα-308 A allele confers a significant risk for developing UC in East Asians but there is no association between the polymorphism of TNFα-308 gene promoter and UC in Europeans.[27] The associations between IBD and the TNFα polymorphism have already been discussed. Genetic variations among different populations and ethnic variability might be responsible for these discordant results in the frequency of -308 and -1031 TNFα gene polymorphisms between the present study and others' studies.[2,8,25]

In conclusion, the homozygous TT genotype for the TNFα-1031 T > C was significantly associated with UC and the TNFα-1031 T allele confers a significant risk for developing UC in Iranian Azeri Turkish patients. Also the frequency of TNFα-1031 C allele was considerably low among patients with UC and may have a protective role among them (OR = 0.43; P = 0.01). Finally, one of the limitations of our study is that a relatively small number of patients are available for study. Because of the small sample size, this report should be considered as exploratory and further studies are required to confirm these genetic associations with IBD.

ACKNOWLEDGMNTS

The authors express their appreciation to all the participants for their contribution to this study. This work is financially supported by Liver and Gastrointestinal Disease Research Center, Tabriz University of Medical Sciences.

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Source of Support: This study was supported by the Centre of Liver and Gastrointestinal Disease Research Centre, University of Medical Sciences, Tabriz, Iran. **Conflict of Interest:** None declared.