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Evaluation of tumor necrosis factor (TNF)-α mRNA expression level and the rs1799964 polymorphism of the TNF-α gene in peripheral mononuclear cells of patients with inflammatory bowel diseases

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Abstract. Crohn's disease (CD) and ulcerative colitis (UC) are types of chronic inflammatory bowel disease (IBD) of which the actual causes remain unknown. Emerging data indicate that alterations in cytokine synthesis may be involved in IBD pathogenesis. The aim of the present study was to determine whether the tumor necrosis factor (TNF)-α mRNA expression level and rs1799964 polymorphism are the genetic susceptibility component of IBD development. The TNF-α mRNA expression level of peripheral blood mononuclear cells (PBMCs) was measured using comparative reverse-transcription quantitative polymerase chain reaction (PCR). Genomic DNA from 201 individuals (CD: n=15; UC: n=86; control subjects: n=100) was analyzed for the presence of the *TNF-α*-1031 polymorphism by PCR-restriction fragment length polymorphism. An increased TNF-α mRNA expression level was additionally observed in the CC genotype of the -1031 TNF-α gene polymorphism compared with the TC and TT genotypes (P<0.05). Furthermore, the present results revealed that there was no significant difference in the genotype/allele frequencies of the -1031 *TNF-α* gene polymorphism in Iranian IBD patients. By comparison, the TNF-α mRNA expression level was evaluated in patients with a history of taking medications and demonstrated a significant association in the group that received the 5-ASA + Pred + AZA,5. 5-ASA + Pred + AZA + IFX when compared with the other groups (P<0.05). Thus, these results support the hypothesis that overexpression of the *TNF-α* gene, which correlated with the CC genotype, may represent a genetic risk factor for Iranian IBD.

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Introduction

Human inflammatory bowel diseases (IBD), ulcerative colitis (UC) and Crohn's disease (CD) are a group of chronic inflammatory conditions of the gastrointestinal tract (1). UC and CD are differentiated by the location of inflammation in the digestive tract (2).

Incidence and prevalence of IBD in the Nordic countries, North America and developing countries is steadily increasing (3,4). New Zealand has the highest rates of IBD in the world (5), and the exact rates of IBD in Iran are not clear, but it appears that the frequency of IBD is increasing (6,7). The exact causes of this disease remains unknown, but studies have proposed that it may be the result of complex interactions between multiple factors, such as genetic, environmental and immunological factors (8,9). There is direct evidence for the role of genetics in IBD, associated with the family history of patients, as well as increased rates of IBD in monozygotic twins (10,11). The predominant genetic association in IBD is divided into genes that contribute to innate and adaptive immune responses (12). Tumor necrosis factor (*TNF*)-*α* is a multifunctional cytokine involved in the advancement of inflammatory responses and is critical in the pathogenesis of inflammatory, autoimmune and malignant diseases (13). *TNF-α* is key in cell-mediated immunity, as demonstrated by high serum levels of *TNF-α* in IBD blood and tissue samples (14,15). *TNF-α* has numerous polymorphisms, the

majority of which are located in its promoter region and certain polymorphisms affect the expression level of the gene (16,17). The present study hypothesized that differences in cytokine levels are due to gene polymorphisms, which may have an important role in the inflammatory response and influence the pathophysiology of IBD.

No association between the TNF-α promoter polymorphisms at position-1031 and susceptibility to IBD in Iranian patients was demonstrated in our previous findings (18). Subsequently, studies have proposed a direct correlation between the single nucleotide polymorphism (SNP) genotype with disease; an important reason for regulatory SNPs to cause human genetic disease is that SNPs are located in regulatory sequences, for example promoter regions or UTRs, that could effected the expression of the gene. Tt is therefore important to investigate the association between the TNF-α gene expression pattern and its association with the -1031 polymorphism. The present study investigated the association between the *TNF-α* gene expression pattern and its association with the -1031 polymorphism in an Iranian population of patients with IBD.

Materials and methods

Patients. Between 2011 and 2014, 101 IBD patients (15 CD and 86 UC) and 100 healthy control subjects, who had been referred to the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences (Tehran, Iran), were included in the present study. The control group individuals did not have a family history of gastrointestinal disorders. Furthermore, control group subjects were selected based on an absence of any evidence, personal or family history of IBD or inflammatory diseases, such as gastritis, ulcerative colitis and CD. The patients and healthy individuals were all Iranian. Colonoscopy was performed by a gastroenterologists and the diagnosis of IBD was confirmed by a pathologist. Patients with a history of using anti-inflammatory drugs, such as 5-aminosalicylic acid (5ASA), prednisone (Pred), azathioprine (AZA) and infliximab (IFX) were evaluated. Written informed consent was obtained from each patient and healthy volunteer, and peripheral blood samples (6 ml) were collected in EDTA tubes and stored at 4˚C until DNA/RNA extraction. The Ethics Committee of the Research Institute for Gastroenterology and Liver diseases, Shahid Beheshti University of Medical Sciences provided approval for the present study.

Genotyping. Genomic DNA was isolated from 5 ml peripheral blood mononuclear cells (PBMCs) using the standard salting out method (19). The quality and quantity of DNA was determined using a spectrophotometer and electrophoresis on agarose gel. Genotype determination was performed by polymerase chain reaction (PCR); the promoter -1031 *TNF-α* gene polymorphism was PCR amplified in a 186-bp fragment using the following primers: Forward, 5-CTTCAGGGATATGTG ATGGACTC-3 and reverse, 5-GGAGACCTCTGGGGAGAT GT-3. Followed by restriction fragment length polymorphism (RFLP). The PCR reaction was performed on a Mastercycler (Eppendorf; Hamburg, Germany) as follows: Denaturation for 5 min at 94˚C, followed by 36 cycles of amplification (40 sec at 94˚C, 35 sec at 62˚C and 40 sec at 72˚C). A final elongation step (5 min at 72˚C) was applied at the end of the 36 cycles. The PCR product was 186 bp. Following amplification, PCR products were digested (at 37˚C) by restriction endonuclease *Bbs*I (Thermo Fisher Scientific, Inc.; C allele, 106 and 80 bp; T allele, 186 bp) overnight. The digested PCR products were visualized by electrophoresis in a 2.5% agarose gel (120 V for 40 min; Fig. 1).

Sequencing. To confirm the results of genotyping, 10% of the PCR products were sequenced using the ABI PRISM 3130xL Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) and the results were fully concordant.

RNA isolation with reverse transcription-PCR. Total RNA was extracted from PBMCs using a YTA RNA Extraction kit (Yekta Tajhiz Azma, Tehran, Iran) according to the manufacturer's instructions. The RNA quantity and quality was determined from spectrophotometric optical density measurement (wavelength, 260 and 280 nm). The cDNA was synthesized using a Revert Aid RT Reverse Transcription kit (cat. no. K1691; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The expression rate of the TNF-α gene was analyzed using an ABI 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and SYBR Master Mix (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. Primers were designed using Genscript and Primer3 online programs (http://primer3.ut.ee/). The following primer pairs were used: Forward, 5-CTGAACTTCGGGGTGATCG-3 and reverse, 5-GCTTGGTGGTTTGCTACGAC-3 for TNF-α; forward, 5-ACAACCTTCTTGCAGCTCCTC-3 and reverse, 5-TGACCCATACCCACCATCAC-3 for β-actin (ACTB). The expression levels of all target genes were normalized against the expression of ACTB, which served as the endogenous control (20). The first stage, initial denaturation at 95˚C for 30 sec, was followed by a second 40-replication cycle (two-step cycling at 95˚C for 5 sec and 60˚C for 34 sec) and the third step (performed following creation of the PCR melting curve) was as follows: 95˚C for 15 sec, 60˚C for 60 sec and 95˚C for 15 sec. The melting curve differentiates between the different products, and demonstrates contamination and the absorption peak. Melting curve analysis detects non-specific and primer dimer products. The ΔΔCq method was used according to Livak and Schmittgen (21).

Statistical analysis. SPSS statistical software version 21 (IBM Corp., Armonk, NY, USA) was used to perform statistical analysis of the genotyping. The χ^2 test was used to evaluate the distribution of the allele and genotype frequencies. Furthermore, the Hardy-Weinberg equilibrium was performed along with the χ^2 test to compare the observed genotype frequencies among the investigated cases and control subjects 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 gender. $P<0.05$ was considered to indicate a statistically significant difference. GraphPad prism 5 software (https://www.graphpad.com/scientific-software/prism/) was used to perform statistical analysis of the TNF-α mRNA

Table I. Demographic characteristics of the study population, including inflammatory bowel disease patients and control subjects.

Variable	Patient (n=101)	Control (n=100)	P-value
Age (mean ± standard deviation), years	33.99±10.86	42.00±13.52	<0.001
Body mass index (kg/m^2)[b]	25.19±4.99	25.49±5.85	0.698
Gender, n (%)[b]			0.138
Female	42 (41.6)	42 (42)	
Male	59 (58.4)	58 (58)	
Smoking, n (%)[b]			0.462
Smoker	16 (15.8)	15 (15)	
Non-smoker	85 (84.2)	85 (85)	

[a]Student's t-test; [b]χ^2 test.

Table II. Genotype distribution of -1031 polymorphism among inflammatory bowel disease patients and healthy control subjects.

Genotype	Patients, n (%)	Controls, n (%)	Odds ratio (95% confidence interval)	P-value
TT	65 (64.4)	63 (63.0)	1.00 (Ref.)	
TC	29 (28.7)	29 (29.0)	0.986 (0.509-1.912)	0.968
CC	7 (6.9)	8 (8.0)	1.308 (0.419-4.088)	0.644

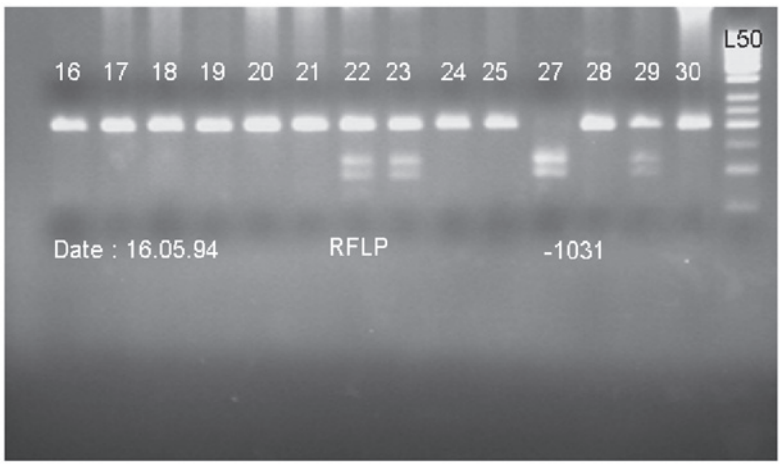

Figure 1. Polymorphism fragments on agarose gel following digestion with *BbsI* restriction enzyme. Ladder 16-21, 24, 25, 30: TT; ladder 22, 23, 29: TC; ladder 27: CC; L50, ladder 50 bp. RFLP, restriction fragment length polymorphism.

expression levels. One-way ANOVA was used to examine the TNF-α mRNA expression level between groups.

Results

Demographics. A total 101 patients with the diagnosis of IBD were investigated, including 59 males (58.4%) and 42 females (41.6%). The control group consisted of 100 non-IBD subjects, 58 males (58%) and 42 females (42%). The percentage of male and female subjects in the two groups was not significantly different (P>0.05). The mean age of the IBD group was significantly higher than that in the healthy control subjects group (P<0.05). Furthermore, no significant differences were identified between the IBD group and healthy control subjects with regard to the body mass index and smoking behavior (P>0.05; Table I).

Lack of associations of the TNF-α-1031T>C polymorphism with IBD in the Iranian population. According to our previous study (18), the frequency of TT, TC and CC genotypes in patients were 64.4, 28.7 and 6.9%, respectively and 63, 29 and 8%, respectively in the control groups. No statistically significant differences were identified between genotypes during the observation and study groups (18) (Table II).

TNF-α gene expression patterns were associated with pathological IBD subtypes. The extent of *TNF-α* gene expression

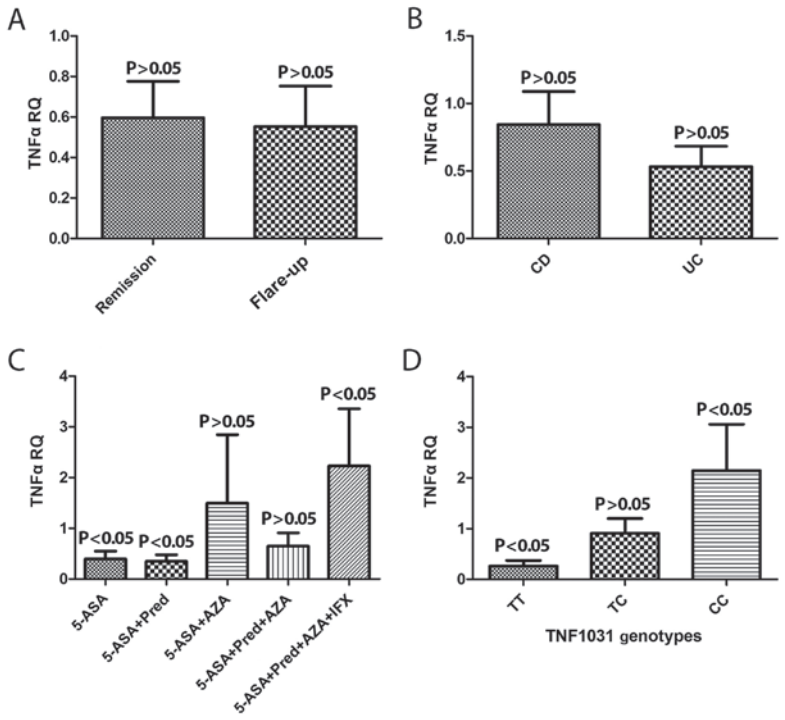

Figure 2. TNF-α mRNA expression level (A) at different IBD pathological phases, (B) according to the type of IBD (UC or CD) and (C) according to drug history. (D) Interaction between the -1031 genotype and TNF-α expression level. TNF-α, tumor necrosis factor-α; IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease; RQ, relative quantification; 5-ASA, 5-aminosalicylic acid; Pred, prednisone; AZA, azathioprine; IFX, infliximab.

in two disease phases, remission and flare up, were compared. The expression level of the *TNF-α* gene remained the same (Fig. 2A). In addition, the expression of *TNFα* mRNA levels between the CD and UC type of IBD patient were analyzed and were not statistically different (Fig. 2B).

An assessment of patients who had a history of taking medication was also performed and patients were classified into five different groups as follows: i) 5-ASA; ii) 5-ASA + Pred; iii) 5-ASA + AZA; iv) 5-ASA + Pred + AZA; v) 5-ASA + Pred + AZA + IFX according to medication usage. *TNF-α* gene upregulation of mRNA exhibited a significant association in the group that received all four types of drug when compared with the other groups (P<0.05; Fig. 2C).

Interaction between the -1031 T>C polymorphism genotype, phenotype and TNF-α expression levels. *TNF-α* gene expression was screened for the polymorphism -1031 T>C genotypes TT, TC and CC. Overexpression of *TNF-α* mRNA was observed in genotype CC when compared with CT and TT (P<0.05; Fig. 2D).

Discussion

IBD is complex multifactorial disease, the pathogenesis of which is not well understood (22). Allelic variants in cytokine genes have been shown to influence gene expression and subsequently the susceptibility to and severity of inflammation diseases (23). There is increasing evidence that TNF-α is key in the pathogenesis of IBD and may represent novel therapeutic targets (12). Studies indicate the TNF-α gene as an appropriate and functional candidate for IBD treatment (24,25). In the present study, the allele frequency of SNP-1031 T>C was greater in patients when compared with the control group, although the difference between the patients and control group was not significant. Similar studies to the current study, performed in Canada (26) and Japan (27) and Iran (28), demonstrated that the allele frequency between the control group and CD group in polymorphism -1031 was significant, but no significant association was observed between the UC and the control groups. This mutant allele frequency in certain studies, for example in the UK (24,29) and a Canadian study (30) was not significantly different. In another study, the T allele was shown to confer a significant risk for developing UC in Iranian Azeri Turkish patients (31). Previous studies have reported conflicting results in the TNF-α gene polymorphism susceptibility to IBD. The reasons for these contradictory findings remain unknown. Different ethnicities, distribution of genotype, environment-genetic interactions and sample size, may be the cause of the inconsistent results in the frequency of the -1031 TNF-α gene polymorphisms in the present study.

In the current study, a correlation between polymorphism -1031 T> C and the TNF-α mRNA expression level was significant. The results demonstrated that the level of TNF-α gene mRNA expression was reduced in genotype TT when compared with the other genotypes. A recent independent study by Rad *et al* (32) in 2004 demonstrated similar results with regard to the polymorphisms of cytokines and their effect on gene expression, which was increased in patients with the *Helicobacter pylori* infection. The results showed that variations in SNPs may change gene expression levels, leading to a significant association with IBD. Another study by Chen *et al* (33) in 1995, examined the effect of TNF-α gene polymorphisms with mRNA expression in patients with

pre-eclampsia, and indicated that TNF-α gene mRNA expression may be associated with its gene polymorphism.

The findings of the current study provide evidence that patients who had taken the four drugs (5-ASA + Pred + AZA and 5. 5-ASA + Pred + AZA + IFX) for the treatment of IBD exhibit overexpression of TNF-α mRNA. To the best of our knowledge, no study has determined the correlation between drug use and TNF-α gene expression in IBD patients. However, it may be that the TNF-α gene expression may serve as a biomarker for the drug-resistant inflammation in IBD initiation. Therefore, it is proposed that, in future studies, an improved understanding of the association with other TNF-α gene promoter polymorphisms and the underlying mechanism of functional polymorphisms may provide novel therapeutic target gene expression for the treatment of IBD patients.

In conclusion, the present study examined the association between the TNF-α gene expression pattern and its association with the -1031 polymorphism, which demonstrated an increased expression of the CC genotype and a decreased expression of the TT genotype. The overexpression of the CC genotype may represent a genetic risk factor for IBD in an Iranian population. In addition, overexpression of TNF-α was observed in patients who had a history of taking the four types of drug (5-ASA, Pred, AZA or IFX). Thus, these analyses may be used in the prognosis, diagnosis and treatment of IBD.

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