

## Original Article

## Polymorphism of the Promoter Region of C-509T of Transforming Growth Factor-Beta1 Gene and Ulcerative Colitis

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**Background:** Transforming growth factor-beta is a regulatory protein that plays a key role in inflammatory, fibrotic, and immunological events in the intestinal mucosa. Recently, attention has been focused on the role of transforming growth factor-beta in the etiopathogenesis of inflammatory bowel disease. Enhanced expression of transforming growth factor-beta mRNA in the lamina propria and a disordered expression pattern of transforming growth factor-beta1 receptors I and II in epithelial cells have been documented in the colonic mucosa of patients with ulcerative colitis and Crohn's disease. Based on these associations, we report in this study, the restriction fragment length polymorphism-polymerase chain reaction and allele frequencies of the transforming growth factor-beta gene polymorphisms in a population of Iranian patients with ulcerative colitis and healthy controls. We analyzed whether these two polymorphisms are related with the disease characteristics.

**Methods:** One hundred fifty-seven (75 males and 82 females) unrelated patients with ulcerative colitis attending the Departments of Gastroenterology, Nemazi and Faghihi Hospitals, affiliated to Shiraz University of Medical Sciences, Shiraz, Iran were enrolled into this study. Ninety-four age- and sex-matched healthy volunteers with no history of chronic bloody diarrhea and abdominal pain (41 males and 53 females) served as the control group. The change at position -509 (C/T) of the transforming growth factor-beta1 gene was studied using restriction fragment length polymorphism-polymerase chain reaction in this study.

**Results:** The mean age of patients was 36.4 (range: 23 – 51) years. The genotype at position -509 (C/T) in 58 (37%) patients with ulcerative colitis, and 39 (41.5%) normal subjects, were homozygous as CC. In addition, 65 (41.5%) patients and 44 (47%) normal individuals were heterozygous as CT. Thirty-four (21.5%) of 157 patients, and 11 (11.5%) of 94 normal individuals, were homozygous as TT.

There was no statistically significant difference between patients and normal female individuals in respect to genotype distribution and allele frequency at the said position ( $P = 0.138$ ).

**Conclusion:** No association could be found between transforming growth factor-beta1 -509 (C/T) promoter gene polymorphism and patients with ulcerative colitis.

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**Keywords:** Gene • polymorphism • transforming growth factor-beta • ulcerative colitis

### Introduction

Transforming growth factor-beta (TGF- $\beta$ ) is a cytokine, which is produced by both immune and nonimmune cells. It exhibits a broad range of functions; first among equals, being the modulation of immune responses. TGF- $\beta$  controls the differentiation, proliferation, and state of activation of all immune cells, wound healing, angiogenesis, and is implicated in immune

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abnormalities linked to cancer, autoimmunity, opportunistic infections, and fibrotic complications.<sup>1, 2</sup> It has chemotactic properties and may stimulate cells to produce cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-alpha (TNF- $\alpha$ ) at sites of inflammation.<sup>1</sup>

Moreover, TGF- $\beta$ , which appears to play such a pivotal role in promoting restitution through enhanced migration and modulation of extracellular matrix, is paradoxically a potent inhibitor of intestinal epithelial proliferation.<sup>3</sup>

It is proposed that the production of TGF- $\beta$  is under genetic control.<sup>4</sup> In the human TGF- $\beta$  gene, which is located on chromosome 19q13,<sup>5-7</sup> eight polymorphisms are presently known. Three of them are located in the promoter region at positions -988C>A, -800G>A, and -509C>T from the first transcribed nucleotide.<sup>1, 8</sup>

Repair of intestinal mucosa after injury occurs through the aggregate effects of coordinated processes, the relative contributions of which are likely dependent on the depth rather than the extent of damage. Even the most superficial injury involves epithelial destruction. Thus, restitution, the process through which epithelial continuity is reestablished, is central to healing after any forms of injury, regardless of the underlying cause or severity.<sup>5, 6</sup> Restitution is achieved through rapid migration of the residual epithelium from the wound edge. Reestablishing continuity is clearly of paramount importance in preventing bacterial invasion and penetration to the vascular space and the intense stimulation of mucosal immune response and subsequent inflammation due to dietary and bacterial antigens. Cells become elongated and thin, enabling them to resurface surprisingly broad areas of denuded mucosal surface without actual proliferative replacement.<sup>7</sup>

Inflammatory bowel disease (IBD) comprises a group of idiopathic diseases of the intestine characterized by chronic inflammation of the bowel with periods of exacerbation and remission. The two major categories of IBD are ulcerative colitis (UC) and Crohn's disease (CD), which are distinguishable both clinically and histologically.<sup>8</sup>

Moreover, IBD is thought to result from inappropriate and ongoing activation of the mucosal immune system driven by the presence of normal luminal flora. This aberrant response is most likely facilitated by defects in both the barrier function of the intestinal epithelium and the mucosal immune system.<sup>8</sup>

The importance of TGF- $\beta$  in maintaining intestinal immune homeostasis has been shown recently in a study by Fuss and colleagues, suggesting that intranasal administration of DNA encoding active TGF- $\beta$  is a successful treatment in a mouse model of acute colitis.<sup>9</sup> These anti-inflammatory and immunosuppressive effects of TGF- $\beta$  offer important potential physiologic and therapeutic applications, especially in those entities with a marked Th-1 inflammatory response.

Here, we investigated whether C-509T TGF- $\beta$ 1 polymorphism was associated with UC in southern Iranian white patients with established disease.

## Materials and Methods

### Subjects

Those enrolled in this study included 157 (75 males and 82 females) unrelated patients with UC attending the Departments of Gastroenterology, Nemazi and Faghihi Hospitals, affiliated to Shiraz University of Medical Sciences, Shiraz, Iran. Meanwhile, some other cases were also referred from Motahhari Outpatient Clinics. The disease duration was at least two years. Patients were asked about acute phases of the disease since diagnosis. Each patient's chart was revisited to complete all the available information. Ninety-four (41 males and 53 females) age- and sex-matched healthy volunteers with no history of chronic bloody diarrhea and abdominal pain were included in the study and served as the control group. Diagnosis of UC was made based on the conventional clinical, radiographical, endoscopic, and histological criteria. Since CD and UC are both dynamic and patient's condition can fluctuate into different phenotypes during the course of the disease, we analyzed the clinical records of our patients at two time points: when patients visited the hospital and for the first time he/she referred for follow-up (the mean time after the diagnosis was one and a half years). All patients were subclassified according to gender, age of onset, localization of the disease, need for steroid therapy, and need for emergency surgical treatment. In patients with UC, the localization of gut involvement was defined as proctitis defined as involvement of left-sided (up to the splenic flexure), or pancolitis when involvement progressed beyond the splenic flexure.

### DNA extraction and TGF- $\beta$ 1 genotyping

Venous blood was collected in EDTA-coated

tubes. DNA was extracted from whole blood using the salting-out method. Specific oligonucleotide primers were used as previously described.<sup>5</sup> The following primers (MOLBIOL, Germany) were used for amplification of the promoter regions of -509. The primer pairs for delineating the polymorphism at nucleotide acid -509 were:

5'-CAGTAAATGTATGGGGTCGCAG-3'  
(sense) and

3'-GGTGTTCAGTGGGAGGAGGG-5'  
(antisense)

The polymerase chain reaction (PCR) product size from these primers was 300 bp. PCR amplification of each polymorphism was performed in a total reaction volume of 20  $\mu$ L with 300 ng DNA as template. Genotyping was performed by restriction fragment length polymorphism (RFLP) analysis. For digestion of PCR products containing position -509, the restriction enzyme Eco81 I (Fermantes, Letuvania) was used. The reaction mixture was preheated at 94°C for 4 min. Subsequently, 0.4 unit of Taq polymerase was added. The 30 cycles of PCR amplification were performed with a temperature profile consisting of denaturation at 94°C for 45 sec, annealing at 58°C and 61°C for 30 sec, extension at 72°C for 30 sec, and final extension at 72°C for 10 min. Other digestion conditions were in accordance to the manufacturer's procedures.

### Statistical analysis

The allele frequencies for each polymorphic site were calculated by allele counting method. Deviation of the genotype counts from the Hardy-Weinberg equilibrium was tested using  $\chi^2$  test. Differences between the patients with UC and the control with respect to the allele frequencies and genotype distributions were analyzed by  $\chi^2$  test or Fisher's exact test when necessary.

## Results

### Position -509 (C/T)

The genotype at position -509 (C/T) of TGF- $\beta$ 1 gene was studied in a group of patients with UC. Fifty-eight (37%) of those with UC, and 39 (41.5%) of normal subjects, were homozygous as CC at this position. In addition, 65 (41.5%) of cases and 44 (47%) of normal individuals were heterozygous as CT. Thirty-four (21.5%) of 157 UC, and 11 (11.5%) of 94 normal individuals, were homozygous as TT. There was no statistically significant differences between those with UC and

normal female individuals in respect to genotype distributions and allele frequencies at this position ( $P = 0.138$ ). The distribution of TGF- $\beta$ 1 genotype and allele frequency in cases and controls are summarized in Table 1. A typical genotyping at position -509 (C/T) is represented in Figure 1.

In the case of -509 polymorphism, while the frequency of CC in patients was lower while the frequency of CT genotype in the control group was lower (Table 1). On the other hand, the frequency of TT genotype was higher in cases. These differences, however, were not statistically significant.

A Hardy-Weinberg equilibrium test was performed for the two polymorphisms. The observed distributions of studied genotypes were not significantly different from the distributions expected from the Hardy-Weinberg equilibrium states in neither the patient nor the control groups.

### Relation between other factors and polymorphisms

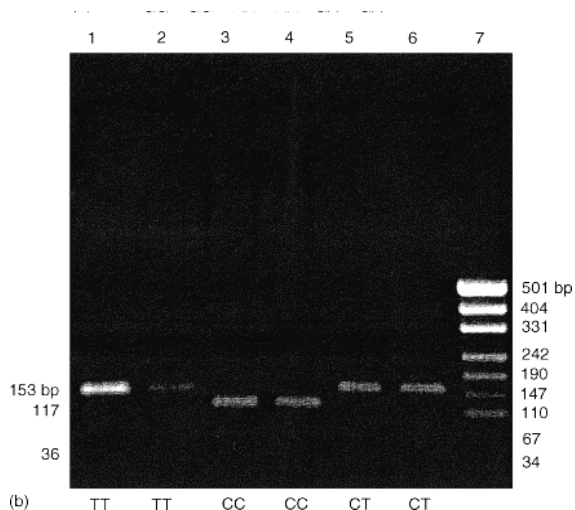
In our study, there was no significant correlation between the two mentioned polymorphisms and the frequency of factors such as gender, age of onset, cumulative dose of steroids administered the need for steroid therapy, localization of the disease, and the need for emergency surgical treatment.

## Discussion

IBD is a classic autoimmune disease involving either a reaction against a self-constituent or a gut constituent that cross reacts with a self-constituent. These autoantibodies then interact with nonspecific cytotoxic cells to cause tissue injury via an antibody-dependent cell-mediated cytotoxic mechanism. In autoimmune diseases, B cells secreting pathogenic auto-

**Table 1.** Distribution of TGF- $\beta$  genotype and alleles in patients with ulcerative colitis (UC) and healthy control groups.

TGF- $\beta$ genotype and alleles	UC patients <i>n</i> =157 (%)	Controls <i>n</i> = 94 (%)	<i>P</i> value
Genotype-509			
CC	58 (37)	39 (41.5)	0.138
CT	65 (41.5)	44 (47)	
TT	34 (21.5)	11 (11.5)	
Allele			
C	0.58	0.64	0.38
T	0.42	0.36	



**Figure 1.** Restriction fragment length polymorphism-polymerase chain reaction genotyping of three individuals (duplicate). Position -509 (C/T). PCR product (153 bp) was digested by Eco81 I restriction enzyme. Lane 1 – 2, homozygote TT; lane 3 – 4, homozygote CC; lane 5 – 6, heterozygote CT; and lane 7, DNA size marker.

reactive antibodies are supported by T helper cells, implying defects in the regulation of both types of cells. Defective central or peripheral tolerance, inadequate suppression of auto-reactive cells, and unusual activation of T and B cells may underlie the persistence of auto-antibodies that characterize autoimmune diseases.

The autoantibodies of interest in IBD include antineutrophilic cytoplasmic antibodies, gene product, colonic 40 kDa protein, antigoblet cell autoantibody, antiendothelial cell antibody, and anticardiolipin antibody.<sup>7</sup>

TGF- $\beta$ 1 is an immunoregulatory and predominantly an immunosuppressive cytokine.

Three polymorphisms have so far been reported in the TGF- $\beta$ 1 promoter region at positions -988, -800, and -509.<sup>6-7</sup> It has been suggested that the -509 (C/T) polymorphism is significantly associated with the plasma concentration of TGF- $\beta$ 1.<sup>4</sup> Some studies indicated that polymorphisms in the promoter region of this cytokine resulted in altered transcriptional regulation and thereby, might influence the development and severity of TGF- $\beta$ 1-related diseases. The -800 (G/A) substitution is thought to disrupt a consensus half-site for binding of the nuclear transcription factor CRE-binding protein, that consequently leads to a

lower production of total TGF- $\beta$ 1 in the circulation. On the other hand, the T allele of the -509 (C/T) polymorphism has been reported to be associated with a higher transcriptional activity and therefore, higher production of total and active TGF- $\beta$ 1.<sup>4,10</sup>

TGF- $\beta$  expression is associated with inflammation, irrespective of the nature of the underlying disease process, suggesting its role in the processes (though not identical) involving in both UC and CD. Determination of the actual role of TGF- $\beta$  in these disorders is more problematic than that for TGF- $\alpha$ . Although Northern blot analysis focused on TGF- $\beta$ 1, presumably other forms of TGF- $\beta$ , such as TGF- $\beta$ 2 and TGF- $\beta$ 3 found in rodent intestine, are also present in colonic mucosa. However, the functional distinctions and the difference in importance among these different TGF- $\beta$  species is still unclear. Bioassay showed that aggregate content of the collective species of TGF- $\beta$  was increased in association with inflammatory activity in IBD mucosa. The highest level of TGF- $\beta$  mRNA in the lamina propria cells, closest to the surface epithelium, suggests that TGF- $\beta$  may play a role in promoting healing of the overlying epithelium by enhancing the process of restitution, which serves to reestablish the surface continuity after mucosal injury.

In the present study, the genotype distribution and allele frequencies of polymorphisms at position -509 (C/T) ( $P = 0.138$ ) was not significantly different between patients with UC and controls. However, the frequency of -509T allele in patients was higher than controls, which was consistent with the finding of Schulte et al, who showed that those carrying T allele at this position produce higher amounts of TGF- $\beta$ 1 (Table 2).<sup>11</sup>

Previous studies of cytokine gene polymorphisms in patients with UC presented similar results. Garcia-Gonzalez and coworkers recently studied codons 10 and 25 TGF- $\beta$ 1 polymorphisms in a Dutch population of patients with IBD and healthy controls. They concluded that codons 10 and 25 TGF- $\beta$ 1 gene polymorphisms do not alter the susceptibility to and the clinical course in IBD.<sup>12</sup> However, as the authors stated, the analyzed polymorphisms are not known to affect the function of the TGF- $\beta$ 1 protein or serum levels of TGF- $\beta$ . The analysis given in Schulte et al's report contributes to an

**Table 2.** RFLP analysis of the C-509T polymorphism of the TGF- $\beta$ 1 promoter in healthy control and patients with ulcerative colitis (UC). The frequency of T allele and genotypes are given in absolute number (n) and percentage (in parenthesis).

Study group	Evaluated patients (n)	Frequency of T allele	Genotype CC	Genotype CT	Genotype TT
Twin study (Schulte)- UC group	42	30 (35)	14 (35)	26 (60)	2 (4.7)
Twin study (Schulte)- Crohn's patients	99	66 (42)	43(43)	46 (47)	10 (10)
Twin study(Schulte)-healthy control	88	46 (26)	50 (57)	30 (34)	8 (9)
Current study-UC group	157	133 (42)	58 (37)	65 (41.5)	34 (21.5)
Current study- healthy control	94	66 (35)	39 (41.5)	44 (47)	11 (11.5)

adaptation of experimental results obtained *in vitro* to the *in vivo* situation.<sup>11</sup> The data did not support moderate changes in TGF- $\beta$  availability for the genesis of fibrosing complications. This statement must be made cautiously, since several aspects interfere with data interpretation. There are a number of other polymorphisms with a known influence on differential TGF- $\beta$  expression.<sup>7, 8</sup> Since organ-specific gene expression control is relevant for paracrine growth regulation, the extent to which the genotype at the C-509T locus influences the local availability of TGF- $\beta$  in the intestinal mucosa, is not clear.

However, as we analyzed Schulte et al's results on their UC population, we found that there were significant differences between their UC groups and controls, which have not been reported.

Finally, considering the results of this investigation, it was concluded that there are no significant differences between the distribution of TGF- $\beta$ 1 polymorphisms at position -509 (C/T) in southern Iranian patients with UC and an age-matched normal population. This conclusion is based on using two known SNP regions of TGF- $\beta$ 1 gene promoter, which has been broadly investigated in other autoimmune diseases.

We are currently exploring other known SNPs of the TGF- $\beta$ 1 gene in UC, and also searching for possible TGF- $\beta$ 1 variants in a large Iranian normal population using direct sequencing.

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