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Relationship between the tumor necrosis factor alpha polymorphism and the serum C-reactive protein levels in inflammatory bowel disease

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Abstract Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract, including ulcerative colitis (UC) and Crohn's disease (CD). The aim of the study was to determine the prevalence of the tumor necrosis factor alpha (TNF-α) promoter polymorphisms at positions −238 and −308, and to measure the serum CRP levels in CD and UC patients and in a healthy population. The TNF-α gene polymorphisms were determined by the PCR-RFLP method. Samples of 74 CD and 50 UC patients and 138 healthy Hungarian volunteers were examined. The G→A substitution at position −308 (designated the *TNF2* allele) was significantly less frequent among IBD patients than in the control group (P=0.0009); 15% of the CD patients and 18% of the UC patients carried the mentioned allele, which was significantly less frequent compared with the healthy population (33%, P=0.0035 and P=0.036, respectively). No difference in the G→A substitution at position −238 was observed. We found the median CRP levels to be significantly higher in the active phase of the disease than in the inactive phase among the *−308A* allele carriers (P=0.002), while this difference was not significant when the CRP levels in the active and inactive phases were compared among the −308GG homozygous patients (P=0.084). The decreased frequency of the *TNF2* allele (known to be associated with elevated TNF-α production) in IBD may determine the severity of IBD through its interaction with plasma CRP levels, and may modify the pathogenesis of this chronic inflammatory disease.

Keywords Inflammatory bowel disease · Crohn's disease · Ulcerative colitis · Tumor necrosis factor alpha · C-reactive protein

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Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract that includes ulcerative colitis (UC) and Crohn's disease (CD) (Podolsky 1991a, 1991b). IBD is thought to result from inappropriate and ongoing activation of the mucosal immune system driven by the presence of normal luminar flora (Podolsky 2002). Although its aetiology remains unclear, IBD is recognized as a multifactorial disease, arising from interactions of multiple genes with environmental factors.

Several clinical observations suggest that genetic factors contribute to susceptibility to IBD. These include wide variations in the incidence and prevalence of CD and UC among different populations, and familiar aggregation of IBD. Multiple studies have demonstrated that the absolute risk of IBD is approximately 7% among first-degree relatives, which is 4–20 times higher than that among the background population (Podolsky 2002).

Chromosome 6 was the first to be implicated in the aetiology of IBD. By a genome-wide approach, several other loci have been identified that may play a role in the development of IBD. These loci include the *IBD1* locus on Chr. 16; some other susceptibility loci have also been found on Chr. 1, 3, 4 and 7 (Hugot et al. 1999).

Chr. 6 influences the genetic predisposition and the progression to IBD. Associations have previously been reported with the major histocompatibility complex (MHC) class I, class II and class III alleles (Hampe et al. 1999). There have been numerous attempts to identify HLA antigens that are positively or negatively associated

with IBD. The results have been variable, both in CD and UC.

There are few reports of significant HLA associations in CD. Most studies conclude that CD is not associated with HLA (Wassmuth et al. 1993; Satsangi et al. 1996; Bouma et al 1997); others found positive associations with *DR7* and negative associations with *DR3* (Danzé et al. 1996; Reinshagen et al. 1996).

UC has been found to be positively associated with *DRB1*0103* and with *DR2 (DRB1*15*) but negatively associated with *DR4*, and *DR6* (*DRB1*13*) (Toyoda et al. 1993; Satsangi et al. 1996; Bouma et al. 1997; Concha et al. 1997).

The TNF-α gene is located within the MHC class III region on Chr. 6; its product, the TNF-α protein, is a key inflammatory mediator in the pathophysiology of IBD (Hampe et al. 1999). The expression of the TNF-α messenger RNA is increased in the intestinal mucosa of IBD, particularly in patients with disease in the inactive phase (Akazawa et al. 2002). Furthermore, therapeutic interventions targeting TNF-α with a monoclonal antibody have proved to be effective in clinical trials (Targan et al. 1997).

It has been previously described by Day and co-workers (Day et al. 1998) that a G→A substitution at position −308 in the promoter region of the TNF-α gene (designated the *TNF2* allele) increases TNF-α transcription (Wilson et al. 1997; Hajeer et al. 2001). Mitchell and co-workers reported that the *8.1* MHC ancestral haplotype (*HLA-A1*, *B8*, *TNFA−308(2)*, *TNFa2b3*, *DR3*, *DQ2*) is associated with elevated TNF-α production (Mitchell et al. 2001), and predisposes carriers to several autoimmune disorders. It is also well known that the TNF-α *−308A* allele shows strong linkage disequilibrium with *DR3/DQ2* (Wilson et al. 1993).

The relationship between IBD and the genes encoded in the MHC III region, such as the TNF-α gene, is not conclusive. Some authors have shown that the frequency of the *TNF2* allele was slightly but not significantly decreased in CD patients compared with healthy controls (Louis et al. 2000); others report that the *TNF2* allele was slightly but significantly less frequent among UC patients when compared with controls (Bouma et al. 1996), while others have found no significant difference between IBD and controls in the carrier frequencies of the *TNF2* allele (Papo et al. 1999; Koss et al. 2000). In the Japanese population the carrier frequency of the *TNF2* allele was four times higher in UC patients than in healthy controls (Sashio et al. 2002).

The aim of this study was to determine the frequency of alleles encoded in the TNF-α gene promoter region at positions −238 and −308, to measure the serum C-reactive protein (CRP) levels in patients with CD and UC, and to compare the results with the Hungarian healthy population. The measurement of plasma CRP level is an important tool for measuring disease activity. Since TNF-α is one of the major regulators of hepatic CRP production, we found it interesting to test whether a relationship exists between TNF-α promoter polymorphism and plasma CRP levels in IBD patients of different clinical status. Our results show a marked difference in the TNF-α gene between IBD patients and the healthy controls. Furthermore, a strong relationship between TNF-α polymorphism and plasma CRP levels was found in IBD patients stratified according to disease activity.

Materials and methods

Subjects

Seventy-four patients with CD (42 female/32 male, mean age 37.4±1.5 years) and 50 patients with UC (28 female/22 male, mean age 41.2±2.1 years) were examined. CD was divided into three subgroups according to Sachar and co-workers (1992): primarily fibrostenotic, fistulizing or mainly inflammatory CD. The activity of CD was determined according to the Best index (Best et al. 1976). Patients with an activity score below 150 were regarded as having inactive disease, whereas those with a Best index of 150 or higher were considered to suffer from active CD. In UC, disease activity was assessed by the method of Truelove and Witts (1954). The absence of symptoms, abdominal cramps, and weight loss, as well as normal temperature, ESR below 30, and a good appetite were considered to indicate inactive disease.

Among CD patients, the median follow-up was 6 years (3–10 years). As for the median follow-up in the subgroups of CD it was 9 years (6–12 years) in the fibrostenotic, 6 years (4–10.5 years) in the fistulizing, and 5 years (4–11 years) in the mainly inflammatory subgroup. Among UC patients the median follow-up was 8 years (3.5–12 years).

The existing extraintestinal manifestations (arthritis, arthralgia, primary sclerosing cholangitis, erythema nodosum), the number of surgical interventions (bowel resection, hemicholectomy, total cholectomy, abscess resection) were recorded in all examined IBD patients. Informed consent was obtained from all patients and the local Ethics Committee gave approval for the study.

As a control, data on 138 healthy Hungarian volunteers (96 female/42 male, mean age 44.4±1.8 years) were used.

There was no significant difference between the CD and UC patients and the healthy control population in respect of gender, mean age and length of follow-up.

Analytical techniques

Determination of TNF-α promoter polymorphism

Total genomic DNA was extracted using the method of Miller and co-workers (1988).

The TNF-α promoter polymorphisms at positions −238 and −308 were determined by PCR using the primers suggested by Day and co-workers (1998). The PCR products were digested at 37 °C with *Msp*I to detect the −238 polymorphism and *Nco*I to detect the −308 polymorphism, as described previously (Vatay et al. 2002).

CRP determination

CRP serum concentrations were measured by particle enhanced immunoturbidimetric assay (Roche, Cobas Integra 400). The detection limit of the assay is 0.07 mg/l, the coefficient of variation 3.9% at 108 mg/l mean value.

Statistical methods

Statistical analysis was performed using the GraphPad Prism V 3.00 for Windows software package (GraphPad Software, San Diego Calif., USA, www.graphad.com). Frequencies were com-

pared by the chi-square test, Fisher exact test or chi-square test for trend. The median CRP levels of the groups were compared by Mann-Whitney *t*-test. *P* values less than 0.05 were considered significant.

Results

Decreased frequency of A carriers in the TNF-α gene promoter region at position −308 in patients with IBD

The TNF-α promoter polymorphism at position −308 was determined in patients with IBD and healthy controls. As shown in Table 1, the number of carriers of the G→A substitution at position −308, designated the *TNF2* allele, was significantly lower in patients with IBD (all of the carriers were GA heterozygotes) (16%) when compared with the control group (both GA heterozygous and AA homozygous) (33%, *P*=0.0009). A similar difference was observed when the patients with CD (15%) were compared with the healthy population (*P*=0.0035). The same tendency was observed when UC patients (18%) and the healthy controls were compared (*P*=0.036). No significant difference was observed between CD and UC in the carrier frequency of the *TNF2* allele.

No difference in the TNF-α promoter region at position −238 in patients with IBD compared with the controls

We investigated the TNF-α promoter polymorphism at position −238 as well as in patients with IBD and healthy controls. However, as shown in Table 2, no difference was observed when the number of carriers of the G→A substitution at position −238 (designated the *TNFA* allele) in IBD (7.2%) were compared with healthy controls (3.6% *P*=0.191). Six patients with CD (8%) and three patients with UC (6%) carried the *A* allele (all of them were GA heterozygous) in the IBD group (*P* values were: *P*=0.153 and *P*=0.483 when compared with the controls, respectively).

Relationship between CRP level, clinical parameters and TNF-α carrier status

Next we investigated the association between the TNF-α gene −308A and G carrier status and the different clinical parameters, in order to determine whether the TNF-α promoter polymorphism has any effect on the disease course. We determined the extraintestinal manifestations (arthritis, arthralgia, primary sclerosing cholangitis, erythema nodosum), the number of surgical interventions (bowel resection, hemicholectomy, total cholectomy, abscess resection) and the disease activity (serum CRP levels) in all examined IBD patients.

We found that the median CRP level was significantly higher in the active phase of the disease (median CRP=18.46 mg/l) than in the inactive phase (median CRP=2.4 mg/l, *P*=0.002) among the *−308A* (*TNF2*) allele carriers, while this difference was not significant when the CRP levels in the active (median CRP=9.95 mg/l) and inactive phase (median CRP=6.1 mg/l, *P*=0.084) were compared among those who do not carry the mentioned allele (wild type, *−308GG* homozygous patients) (Table 3).

We found no significant difference in IBD, CD or UC between the TNFα gene *−308A* and *G* allele carriers in respect of the extraintestinal manifestations, number of surgical interventions or the disease activity overall (data not shown).

Table 1 TNF-α promoter polymorphism at position −308 in patients with inflammatory bowel disease (*IBD*). Analysed by χ^2 test for trend. The *P* value was 0.64 when Crohn's disease (*CD*) and ulcerative colitis (*UC*) were compared

TNF-α promoter region at position −308	IBD *n* (carrier frequency) No. 124	CD *n* (carrier frequency) No. 74	UC *n* (carrier frequency) No. 50	Controls *n* (carrier frequency) No. 138
AA homozygous	0 (0)	0 (0)	0 (0)	4 (0.03)
GA heterozygous	20 (0.16)	11 (0.15)	9 (0.18)	41 (0.3)
GG homozygous (wild type)	104 (0.84)	63 (0.85)	41 (0.82)	93 (0.67)
P value when compared with controls	0.0009	0.0035	0.036	

Table 2 TNF-α promoter polymorphism at position −238 in patients with IBD. Analysed by χ^2 test for trend. The *P* value was 0.657 when CD and UC were compared

TNF-α promoter region at position −238	IBD *n* (carrier frequency) No. 124	CD *n* (carrier frequency) No. 74	UC *n* (carrier frequency) No. 50	Controls *n* (carrier frequency) No. 138
AA homozygous	0 (0)	0 (0)	0 (0)	0 (0)
GA heterozygous	9 (0.072)	6 (0.08)	3 (0.06)	5 (0.036)
GG homozygous (wild type)	115 (0.928)	68 (0.92)	47 (0.94)	133 (0.964)
P value when compared with controls	0.191	0.153	0.483	

Table 3 Median serum CRP levels in IBD in the active and in the inactive phase of the disease, and in healthy controls according to the *TNF-α* promoter polymorphism

TNF-α *−308*	IBD					Controls	
	Active disease		Inactive disease		*P* value[b]		
	N	CRP (mg/l)[a]	N	CRP (mg/l)[a]		*n*	CRP (mg/l)[a]
GA heterozygous	11	18.46 [7.36–42.2]	9	2.4 [1.33–3.76]	0.002	46	1.93 [0.84–4.6]
GG homozygous (wild type)	62	9.95 [2.99–36.4]	42	6.1 [2.36–12.53]	0.084	92	1.725 [0.68–3.7]
All patients	73	11.84 [3.1–36.5]	51	4.32 [1.95–11.8]	0.0039	138	1.75 [0.73–3.9]

[a] Median [25th–75th percentile]
[b] *P* values when the active and the inactive disease were compared. Analysed by Mann-Whitney test

Table 4 Relationship between the TNF-α *−238A* allele carriers and the clinical features of the disease. Analysed by Fisher's exact test and χ^2 test

		Total number of patients	Number of extraintestinal manifestations	*P* values
IBD	GA heterozygous	9	3 (33.3%)	0.04
	GG homozygous (wild type)	115	9 (7.8%)	
CD	GA heterozygous	6	2 (33.3%)	0.133
	GG homozygous (wild type)	68	7 (10.29%)	
UC	GA heterozygous	3	1 (33.3%)	0.213
	GG homozygous (wild type)	47	2 (4.25%)	

We found, however, that the extraintestinal manifestations in IBD were significantly more frequent among the TNF-α *−238A* (*TNFA*) carriers (all of whem were GA heterozygous, 33%) compared with the TNF-α *−238G* carriers (GG wild type, 7.8%, *P*=0.04) (Table 4). No other significant differences were seen between the TNF-α *−238A* and *G* carriers in respect of the clinical parameters.

Determining the CD subgroups [primarily fibrostenotic, fistulizing or inflammatory (Sachar et al. 1992)], we did not find any significant difference in respect of the number of *TNF2* or the *TNFA* allele carriers in the different subgroups (*P* values were *P*=0.94, *P*=0.74 and *P*=0.61 when the subpopulations were compared in respect of *TNF2* carrier status, and *P*=0.44, *P*=0.506 and *P*=0.84 when the subpopulations were compared with respect to the *TNFA* carrier status, respectively).

Discussion

In the present study we found an association between IBD patients and the TNF-α −308 polymorphism in Hungarian Caucasian subjects. The frequency of the *−308A* allele was decreased in IBD patients and this association was more pronounced in CD patients. Our findings are very similar to those of Louis and co-workers (2000), who found that the frequency of the *−308A* allele was slightly but not significantly decreased in CD patients compared with healthy controls, and the findings of Bouma and co-workers (1996, 1997), who found a decreased frequency of the TNF-α *−308* allele in Dutch UC patients. In contrast to our finding, a 4 times increased frequency of the *−308A* allele was observed in Japanese UC patients (Sashio et al. 2002). This difference may be due to racial differences in disease association.

Most importantly we report in this study significantly higher median CRP levels in the active phase of the disease compared with the inactive phase among the *−308A* allele carriers, while this difference was not significant when comparing the CRP levels in the active and inactive phase among the *−308GG* homozygous patients. This observation indicates that *TNF2* carrier status may significantly influence disease course as reflected by plasma CRP levels. Expression of CRP is regulated mainly at the transcriptional level, but post-transcriptional mechanisms also play a significant role (Volanakis 2001). The CRP gene is found on human Chr. 1, between 1q21 and 1q23. Transcriptional regulation of the CRP gene has been studied extensively both in vitro and in vivo. The combined results have established that interleukin-6 (IL-6) is the principal inducer of the CRP gene, while IL-1, glucocorticoids and certain other factors, including complement activation products, act synergistically with IL-6 to enhance its effect (Szalai et al. 2000). IL-6 is tightly regulated at the level of expression by several hormones, cytokines and their transcription factors (Ferrari et al. 2003). Among them, IL-1 and TNF-α activate, whereas estradiol and glucocorticoids repress IL-6 gene transcription (Hirano et al. 1998). Hence, increased IL-1/TNF-α production and decreased estrogen synthesis enhance IL-6 expression and consequently CRP production. This could be one of the factors responsible for the observed differences.

We have not found any other association between disease characteristics (extraintestinal manifestations, the

number of surgical interventions and the disease activity) and the TNF-α promoter polymorphisms. Similarly, we observed no differences in the allele frequencies of polymorphisms between the CD subgroups classified according to Sachar and co-workers (1992).

In respect of the *–238* polymorphism, we did not find any associations between the *A* allele carriers and IBD patients overall. However, the number of extraintestinal manifestations was significantly higher among the *–238A* allele carriers than in the non-carriers. Although we studied only a relatively small number of patients with IBD, the associations seen are likely to be significant.

This study supports the hypothesis that one of the genes responsible for IBD may be the TNF gene or an adjacent gene. The G→A substitution at the promoter region at position *–308* is associated with elevated TNF-α secretion, which could lead to the persistence of the microbial agent in the luminar flora. As an important novel observation, we report here a possible modifying effect of TNF-α carrier status on disease course through the tight interaction with plasma CRP levels. It would be important to study if carriage of the *TNF2* gene modifies the effectiveness of therapeutic interventions monoclonal antibodies against TNF-α.

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