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BRIEF COMMUNICATION

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TGFB1 gene polymorphisms and inflammatory bowel disease

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Transforming growth factor-β (TGF-β) is a regulatory protein that plays a key role in inflammatory, fibrotic, and immunological events in the intestinal mucosa (Dignass and Podolsky 1993; Kurokowa et al. 1987; Roberts and Sporn 1990). In mammals, the TGF-β superfamily encompasses over 40 proteins including three isoforms denoted TGF-β1, TGF-β2, and TGF-β3 (Massague 1990). Although TGF-β1 is the most abundantly expressed member of the TGF-β family, all three isoforms are produced within the gastrointestinal tract. Recent attention has focused on the role of TGF-β in the etiopathogenesis of inflammatory bowel diseases (IBD). Enhanced expression of *TGFB1* mRNA in the lamina propria and a disordered expression pattern of TGF-β receptors I and II in epithelial cells have been documented in the colonic mucosa of patients with ulcerative colitis (UC) and Crohn's disease (CD) (Babyatsky et al. 1996; McCabe et al. 1993; Ohtani et al. 1995). Furthermore, TGF-β1 is implicated in the fibrosis and stricture formation that occurs in IBD, especially in CD, due to its capacity to stimulate the synthesis and deposition of collagen and other extracellular matrix proteins (Graham 1995; Stallmach et al. 1992). Recently, the polymorphisms at positions +869 (T→C) and +915 (G→C) in the signal protein sequence of the *TGFB1* gene, which change codon 10 (Leu^{10}→Pro^{10}) and codon 25 (Arg^{25}→Pro^{25}), respectively, have been reported to be related to variations in the production of TGF-β1, both *in vitro* (Awad et al. 1998) and at the serum level (Yamada et al. 1998). In addition, the presence of arginine at codon 25 (Arg^{25}) is strongly associated with diseases such as fibrotic lung pathology before lung transplantation (Awad et al. 1998), development of fibrosis in the graft (El-Gamel et al. 1999), and high blood pressure (Cambien et al. 1996; Li et al. 1999). On the other hand, the frequency of the T^{10} allele has been described to be significantly higher in subjects with osteoporosis than in healthy Japanese individuals (Yamada et al. 1999). Based on these associations, we report in this study the genotype and allele frequencies of the codon 10 and the codon 25 *TGFB1* gene polymorphisms in a Dutch population of IBD patients and healthy controls and we analyze whether these two polymorphisms are involved in the susceptibility to and type of inflammatory response in IBD.

The subjects in this study comprised 104 unrelated Dutch Caucasian patients with CD (42 males, 62 females), and 87 patients with UC (43 males, 44 females) attending the Department of Gastroenterology, Academic Hospital Vrije Universiteit in Amsterdam. A total of 132 ethnically matched healthy volunteers (66 males, 66 females) served as controls (HC). Diagnosis of UC and CD was established on the basis of conventional clinical, radiological, endoscopic, and histological criteria (Lennard-Jones 1989). Since CD and UC are dynamic diseases (Bouma et al. 1999; Perri et al. 1996), and patients can fluctuate into a different phenotype during the course of the disease, we

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analyzed the clinical records of our patients at two time points: when patients visited the hospital for the first time (mean time after diagnosis of 5 years), and at their last visit (after a mean follow-up of 8 years). All patients were subclassified according to gender, age of onset, localization of the disease, need for steroid therapy, and need for surgical treatment. In UC patients, the localization of gut involvement was defined as proctitis, left-sided (up to the splenic flexure), or pancolitis (beyond the splenic flexure). In CD patients, the localization and extent of the disease was defined as small bowel, ileocolon, or colon. Based on the classification described by Sachar and co-workers (1992) concerning the clinical course of the disease, CD patients were categorized in three clinical subgroups defined as inflammatory, fistulizing, and fibrostenotic groups.

Genomic DNA was extracted from peripheral blood leukocytes according to a conventional proteinase K digestion and phenol/chloroform procedure. The region containing the codon 10 polymorphism at position +869 ($T^{10}\rightarrow C^{10}$) and the codon 25 polymorphism at position +915 ($G^{25}\rightarrow C^{25}$) in the first exon of the *TGFB1* gene was amplified by the polymerase chain reaction (PCR). PCR fragments spanning sequences from positions +798 to +1004 were generated using the oligonucleotides 5′ CCTGTTCGCGC TCTCGGCAGTG 3′ and 5′ GACAGGATCTGGCC GCGGATGG 3′ as primers. Reaction mixtures (25 µl) contained 500 ng of genomic DNA in 1×Tsp XI buffer (MRC Holland, The Netherlands) (50 mm KCl, 19 mm Trizma pH 8.5, 1.6 mm $MgCl_2$, 0.5% Nonidet P-40, and 0.5% Tween 20), 200 µm of each dNTP, 0.2 µm of each primer, and 0.2 units Tsp XI DNA polymerase (MRC Holland). Amplification was carried out according to the following parameters: 97°C for 90 s, 61°C for 90 s and 72°C for 60 s for three cycles followed by 32 cycles of 97°C for 30 s, 61°C for 60 s, and 72°C for 60 s, and a final elongation at 72°C for 10 min.

A single-stranded conformational polymorphism (SSCP) method was optimized for the simultaneous detection of the biallelic polymorphisms at both positions +869 ($T^{10}\rightarrow C^{10}$) and +915 ($G^{25}\rightarrow C^{25}$) in the *TGFB1* gene. PCR products were diluted twofold in a loading buffer containing 99% formamide and 0.05% bromophenol blue, denatured for 3 min in boiling water, placed on ice, and loaded onto a precast nondenaturing 20% polyacrylamide PhastGel. Horizontal electrophoresis at 20°C, and silver staining were performed automatically on the PhastSystem (Amersham Pharmacia, LKB Biotechnology AB, Uppsala, Sweden) (Fig. 1). PCR products presenting a different SSCP migration pattern were sequenced using a Big Dye Deoxy Terminator Cycle Sequencing RR Kit (Perkin-Elmer). Sequencing reactions were performed according to the conditions recommended by the manufacturer in both 5′ and 3′ directions, and analyzed using an ABI 310 A DNA sequencer system (Applied Biosystems, Perkin-Elmer). The migration of the single strands in the SSCP patterns coincided with the

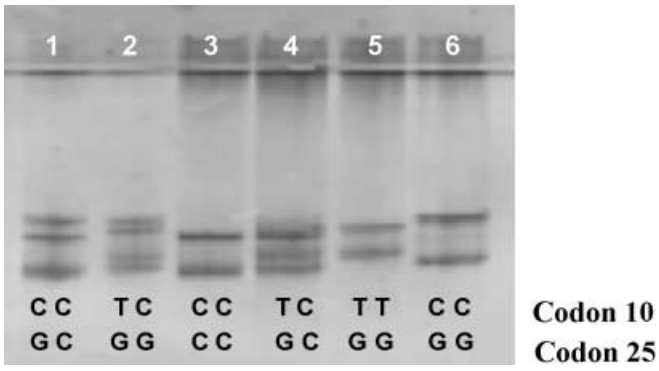

Fig. 1 All the banding patterns detected on a nondenaturing 20% polyacrylamide PhastGel with the codon 10 and codon 25 *TGFB1* genotypes indicated

nucleotides already described at the variant positions in codon 10 and in codon 25 by Cambien and coworkers (1996).

The genotypes and allele frequencies of the codon 10 and the codon 25 polymorphisms in cases and control subjects are shown in Table 1. Genotype frequencies of each polymorphism did not deviate significantly from Hardy-Weinberg expectation in control and patients groups. The strength of the association between *TGFB1* gene polymorphisms in each group was estimated by the odds ratio (OR), and the 95% confidence intervals (CI) after performing Fisher's exact test (2×2 contingency tables). A two-sided *P*-value <0.05 was considered statistically significant. In our study, alleles of the codon 10 and codon 25 polymorphisms were shown to exist as three haplotypes denoted as *TGFB1* haplotype 1 (T^{10}-G^{25}), haplotype 2 (C^{10}-G^{25}), and haplotype 3 (C^{10}-C^{25}). There were no significant differences in genotype and allele frequencies of *TGFB1* gene polymorphisms between UC, CD, and HC. Similarly, no significant differences in genotype, carriage, and frequencies of *TGFB1* haplotypes were found between IBD patients and controls (Table 2). However, individuals homozygous for the rare *TGFB1* haplotype 3 (C^{10}-C^{25}) were only detected in the group of patients with IBD (3 out of 191), whereas none of the 132 healthy individuals presented this haplotype. Our study confirms the finding already suggested by previous investigations (Awad et al. 1998; Lympany et al. 1998; Syrris et al. 1998) that alleles of the polymorphisms in the *TGFB1* gene occurred in a restricted number of haplotypes. Codon 10 and codon 25 polymorphisms are strongly associated with four polymorphic sites at positions –800, –509, +72, and at codon 263 in the *TGFB1* gene. Syrris and co-workers (1998) went even further, trying to identify a putative ancestral *TGFB1* haplotype in humans. Based on variations in the allele frequencies of these polymorphisms observed in different ethnic populations, they defined GCCGC (–800, –509, +72, codon 10, codon 25, and codon 263) as the ancestral *TGFB1* haplotype. Howev-

Table 1 *TGFB1* genotype and allele frequencies in healthy controls and inflammatory bowel disease (IBD) patients (*n* number of individuals, *AF* allele frequency)

Genotype	Healthy controls (*n*=132)		Crohn's disease (*n*=104)		Ulcerative colitis (*n*=87)	
	n (%)	AF	*n* (%)	AF	*n* (%)	AF
Codon 10						
T^{10}/T^{10} (Leu/Leu)	50 (37.9)	0.62	44 (42.3)	0.67	40 (46)	0.67
T^{10}/C^{10} (Leu/Pro)	64 (48.5)		51 (49)		36 (41.4)	
C^{10}/C^{10} (Pro/Pro)	18 (13.6)	0.38	9 (8.7)	0.33	11 (12.6)	0.33
Codon 25						
G^{25}/G^{25} (Arg/Arg)	114 (86.4)	0.93	91 (87.5)	0.93	73 (83.9)	0.91
G^{25}/C^{25} (Arg/Pro)	18 (13.6)		12 (11.5)		12 (13.8)	
C^{25}/C^{25} (Pro/Pro)	0 (0)	0.07	1 (0.96)	0.07	2 (2.3)	0.09

Table 2 Genotype, carriage, and frequencies of *TGFB1* haplotypes in healthy controls and IBD patients. Haplotype 1: T^{10}(Leu)-G^{25}(Arg); haplotype 2: C^{10}(Pro)-G^{25}(Arg); haplotype 3: C^{10}(Pro)-C^{25}(Pro) (*n* number of individuals. *HF* haplotype frequency)

TGFB1 haplotypes	Healthy controls (*n*=132)			Crohn's disease (*n*=104)			Ulcerative colitis (*n*=87)		
	n (%)	Carriage *n* *(%)*	HF (%)	*n* (%)	Carriage *n* *(%)*	HF (%)	*n* (%)	Carriage *n* *(%)*	HF (%)
1.1	50 (37.9)	114 (86.4)	62.1	44 (42.3)	95 (91.3)	66.8	39 (44.8)	75 (86.2)	65.5
1.2	53 (40.2)			42 (40.4)			29 (33.4)		
1.3	11 (8.3)			9 (8.6)			7 (8.1)		
2.2	11 (8.3)	71 (53.8)	31.1	5 (4.8)	50 (48.1)	26.5	5 (5.7)	39 (44.8)	25.3
2.3	7 (5.3)			3 (2.9)			5 (5.7)		
3.3	0	18 (13.6)	6.8	1 (1)	13 (12.6)	6.7	2 (2.3)	14 (16.1)9.2	

er, the precise role of these haplotypes and their significance in the variations of TGF-β1 levels are still a matter of speculation. Stimulated peripheral blood leukocytes from control individuals with the G^{25}/G^{25} genotype have been reported to produce significantly more TGF-β1 than individuals with the G^{25}/C^{25} genotype (Awad et al. 1998). The functional importance of the codon 10 polymorphism is more controversial. Yamada and co-workers (1998) found significantly higher TGF-β1 serum levels in healthy Japanese individuals with the C^{10}/C^{10} genotype than in age-matched subjects with the T^{10}/C^{10} or T^{10}/T^{10} genotypes. However, a previous study performed in a British population of patients suffering from cystic fibrosis described a positive association between the carriage of allele T^{10} and elevated circulation levels of TGF-β1 (Awad et al. 1998). These conflicting results do not allow elaboration of a hypothesis to correlate certain haplotypes with high or low producers of TGF-β1. Further work is therefore needed to clarify the functional importance of these two polymorphisms in relation to variations in TGF-β1 production.

In our study, a mean follow-up time of 8 years allowed us to categorize our patients into well-defined specific subgroups. However, no significant associations with any *TGFB1* allele or haplotype were found when CD and UC patients were classified according to gender, age of onset, localization of disease, clinical type, and need for steroid therapy or surgical treatment (data not shown). This lack of association suggests that these polymorphic sites do not constitute a genetic risk factor for the predisposition to and type of inflammatory response in IBD. However, this finding does not exclude a key role for this protein in the regulation of inflammatory events in these diseases. The importance of TGF-β in maintaining intestinal immune homeostasis has been shown recently in a study by Fuss and co-workers (1999), suggesting that intranasal administration of DNA encoding active TGF-β1 is a successful treatment in a mouse model of acute colitis. These anti-inflammatory and immunosuppressive effects of TGF-β1 offer important potential physiological and therapeutic applications, especially in those entities with a marked Th1 inflammatory response. Finally, genotype and allele frequencies of polymorphisms in codon 10 and codon 25 in our control population were similar to those reported by some European studies performed in healthy individuals from Northern Ireland and France (Cambien et al. 1996) and from the UK (Awad et al. 1998; Syrris et al. 1998). However, allele frequencies of the codon 10 polymorphism in our control group differed from those described by Lympany and coworkers (1998) in a UK Caucasoid population, although the differences did not reach statistical significance (see Table 3).

To date, many studies have been performed to elucidate the influence of genetic factors in IBD. However, this is the first research analyzing the possible link between *TGFB1* gene polymorphisms and IBD. We conclude that codon 10 and codon 25 *TGFB1* gene polymorphisms do not participate in defining the susceptibility to and the nature of the clinical course in IBD. Further studies are needed to establish whether

Table 3 *TGFB1 g*enotype and allele frequencies in different healthy control populations (*n* number of individuals, *AF* allele frequency)

Genotype	This study (*n*=132)		Cambien and co-workers (1996) (*n*=629)		Syrris and co-workers (1998) (*n*=244)		Awad and co-workers (1998) (*n*=107)		Lympany and co-workers (1998) (*n*=203)	
	n (%)	AF	*n* (%)	AF	*n* (%)	AF	*n* (%)	AF	*n* (%)	AF
Codon 10										
T^{10}/T^{10}	50 (37.9)	0.62	225 (35.8)	0.60	102 (41.8)	0.64	44 (41.1)	0.65	102 (50.2)	0.70
T^{10}/C^{10}	64 (48.5)		297 (47.2)		109 (44.7)		51 (47.7)		79 (39)	
C^{10}/C^{10}	18 (13.6)	0.38	107 (17)	0.40	33 (13.5)	0.36	12 (11.2)	0.35	22 (10.8)	0.30
Codon 25										
G^{25}/G^{25}	114 (86.4)	0.93	546 (86.8)	0.93	214 (87.7)	0.93	87 (81.3)	0.90	189 (93)	0.96
G^{25}/C^{25}	18 (13.6)		81 (12.9)		30 (12.3)		19 (17.8)		102 (5)	
C^{25}/C^{25}	0	0.07	2 (0.3)	0.07	0	0.07	1 (0.9)	0.10	2 (1)	0.04

these polymorphisms affect the function of the TGF-β1 protein or whether any of the *TGFB1* haplotypes are associated with variations in the serum levels of TGF-β1.

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