ACTA ABP BIOCHIMICA POLONICA

Vol. 58, No 4/2011
641–644
on-line at: www.actabp.pl

Regular paper

Clinical parameters of inflammatory bowel disease in children do not correlate with four common polymorphisms of the transforming growth factor β1 gene

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Transforming growth factor β1 (TGF-β1) is a cytokine affecting cell proliferation and development, which also has an immunomodulatory activity. Correlations between polymorphisms of the *TGF-β1* gene and clinical parameters of inflammatory bowel disease (IBD) were reported previously in adults. Here, we tested whether such correlations occur in pediatric patients suffering from IBD. One hundred and four pediatric IBD patients were involved in this study. Among them, 36 were diagnosed with Crohn's Disease (CD) and 68 were diagnosed with ulcerative colitis (UC). The control group consisted of 103 children, in which IBD was excluded. TGF-β1 levels were determined in plasma and intestinal mucosa samples. The presence of the TGF β1 protein and the amount of TGF β1 mRNA were estimated in intestinal mucosa by immunohistochemistry and reverse transcription Real-Time PCR, respectively. Four common polymorphisms of the *TGF-β1* gene were investigated: -800G/A, -509C/T, 869T/C and 915G/C. No significant correlation between *TGF-β1* genotypes and (i) TGF-β1 levels in plasma and tissue samples, (ii) *TGF-β1* gene expression efficiency in intestinal mucosa, (iii) IBD clinical parameters and (iv) inflammatory activity could be detected in children suffering from IBD. We conclude that, contrary to previous suggestions, the four common polymorphisms of the *TGF-β1* gene do not influence the susceptibility to or clinical parameters of IBD in the tested population of children.

Keywords: Transforming growth factor β1, gene polymorphism, inflammatory bowel disease, pediatric patients

Received: 14 November, 2011; revised: 30 November, 2011; accepted: 01 December, 2011; available on-line: 03 December, 2011

INTRODUCTION

Transforming growth factor β1 (TGF-β1) is a cytokine exhibiting a broad range of activities. It is involved in the control of cell differentiation and proliferation, activation of immune cells, wound healing and angiogenesis. Correlations between polymorphisms of the TGF-β1 gene and either susceptibility to various diseases or their clinical parameters were reported (Cotton *et al.*, 2002; Gewaltig *et al.*, 2002; Vidigal *et al.*, 2002; Park *et al.*, 2003; Coll *et al.*, 2004; Yang *et al.*, 2004; Drumm *et al.*, 2005; Cantor *et al.* 2005; Mak *et al.*, 2006). It appears that TGF-β1 plays an important role in inflammatory bowel disease (IBD) as significant increases in both efficiency of TGF-β1 gene transcription and levels of the TGF-β1 protein have been reported in patients suffering from Crohn's disease (CD) and ulcerative colitis (UC) (Powrie *et al.*, 1996; Fiocchi, 1998). It was postulated that the TT genotype at the –509 position of the *TGF-β1* locus was associated with the stricture forming type of the CD (Schulte *et al.*, 2001), and this type of the disease was also associated with a particular genotype at the 915 position (G/C polymorphism) (Hume *et al.*, 2006). Moreover, significant differences in allele frequencies and genotypes at the –800 position (G/A polymorphism) of the *TGF-β1* locus were found between patients with UC and control subjects (Tamizifar *et al.*, 2008). However, to our knowledge, all studies on the role of *TGF-β1* polymorphisms in IBD reported to date were performed in adult patients. Therefore, we have investigated whether these polymorphisms may play a role in IBD in children.

Eight common polymorphisms of the *TGF-β1* gene have been described to date (Derynck *et al.*, 1987; Cambien *et al.*, 1996; Langdahl *et al.*, 1997; Syrris *et al.*, 1998; Grainger *et al.*, 1999). Among them, the following four are the most frequent: –800G/A and –509C/T in the promoter region, and 869T/C and 915G/C in exon 1, as shown in Fig 1 (Bayat *et al.*, 2002; Kim *et al.*, 2003; Suzuki *et al.*, 2003; Lee-Chen *et al.*, 2004; Osterreicher *et al.*, 2005). Because of this, and due to the fact that some effects of *TGF-β1* polymorphism on IBD was reported to date only for –800G/A, –509C/T and 915G/C (Schulte *et al.*, 2001; Hume *et al.*, 2006; Tamizifar *et al.*, 2008), in our studies we have tested these four polymorphisms.

MATERIALS AND METHODS

Patients. One hundred and four pediatric IBD patients (36 diagnosed for CD and 68 diagnosed for UC), were tested. This group consisted of 67 males and 37 females, at the age between 1.5 and 18.5 years (mean: 13.0±4.5 y, median: 14.5 y).

One hundred and three children represented the control group, which consisted of 65 males and 38 females, 2.0–18.0 years old (mean 11.0±5.0, median 11.0), who underwent endoscopy, because of gastrointestinal bleed-

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Abbreviations: CD, Crohn's Disease; IBD, inflammatory bowel disease; TGF-β1, transforming growth factor β1; UC, ulcerative colitis.

ing events; in this group of patients inflammatory processes, immune disorders, malignancies and nutritional abnormalities were excluded. None of the patients received immunomodulating therapy at least 6 months prior to the study.

Endoscopic and histological classifications of intestinal mucosa were performed according to the Porto criteria (IBD Working Group of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition, 2005). Among IBD patients, severities of CD and UC were estimated according to the Hyams scale (Pediatric Crohn's Disease Activity Index, PCDAI) and the Truelove-Witts scale, respectively (Hyams *et al.*, 1991; Truelove and Witts, 1954).

Following parameters were determined for all subjects: full blood count, erythrocyte sedimentation rate (ESR), serum total protein, C-reactive protein (CRP), and iron.

Determination of TGF-β1 plasma concentration. Concentration of the TGF-β1 protein in plasma was determined by sandwich ELISA method (Quantikine kit, R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Detection of TGF-β1 in intestinal mucosa. TGF-β1 was detected in samples of intestinal mucosa by immunohistochemical methods, using Vactastain ABC Kit PK-4001 (Vector Laboratories, Burlingame, CA, USA).

Estimation of the level of mRNA of the *TGF-β1* gene. The mRNA level in the intestinal mucosa was estimated by the two-step reverse transcription quantitative real-time PCR method. Total RNA was isolated with Total RNA Prep Plus kit (A&A Biotechnology, Gdynia, Poland) and stored at –80 °C. Quality and concentration of RNA was determined spectrophotometrically, using Smart Spec 3000 apparatus (Bio-Rad Lab., Herts, UK). 1 μg of RNA, 0.25 μg of oligo T_{18} and M-MLV enzyme (Promega, Madison, WI, USA) were used for reverse transcription in a total volume of 10 μl. Quantitative real-time PCR reaction mixture contained 0.4 μl cDNA, 180 nM each primer and iQSybrGreen Supermix kit (Bio-Rad), and the reaction was performed in iCycler iQ (Bio-Rad) in a total volume of 20 μl. Primers for *TGF-β1* and *ACTB* were designed using Primer3Plus, based on BLAST, ENSMBL and AceView databases; TGF-β1: 5'-CAG CAA CAA TTC CTG GCG ATA CC and TGFβ-2, 5'-CGA AAG CCC TCA ATT TCC CCT C, bact1-1, 5'-TGT GCC CAT CTA CGA GGG GTA TGC and bact1-2, 5'-GGT ACA TGG TGG TGC CGC CAG ACA. The following primers were used in the reaction: TGF-β1, 5'-CAG CAA CAA TTC CTG GCG ATA CC and TGFβ-2, 5'-CGA AAG CCC TCA ATT TCC CCT C. The reactions were run in triplicate and the obtained data were averaged followed by data analysis calculated with iQ ver. 3.1 software (Bio-Rad). It was previously demonstrated that *ACTB* showed constant expression in IBD (Stanislawowski *et al.*, 2009; Wierzbicki *et al.*, 2009), thus, this gene was used to normalize the values in the common ΔΔCt quantification method.

Genotyping. The following polymorphisms of the *TGF-β1* gene were determined: –800G/A, 509C/T, 869T/C and 915G/C. DNA was isolated from blood samples by using QIAamp DNA Blood Mini Kit (QIAGEN, Germany). Appropriate regions of the *TGF-β1* locus were amplified by PCR using the following primers: TGF1 (5'-TTC CCT CGA GGC CCT CCT A) and TGF2 (5'-GCC GCA GCT TGG ACA GGA TC) for the exon 1 fragment, and TGF3 (5'-GGG GAC ACC ATC TAC AGT G) and TGF4 (5'-GGA GGA GGG GGC AAC AGG) for the promoter region. The amplified DNA fragments were treated with *Mae*III, *Eco*81I, *Msp*A1I or *Bgl*I restriction enzymes to determine specific polymorphisms, -800G/A, -509C/T, 869T/C or 915G/C, respectively. Restriction fragments were separated electrophoretically and particular genotypes were determined on the basis of patterns of bands in the gel.

Statistical analysis. Statistical analysis of the results was performed using non-parametric tests: the Mann-Whitney test for comparison of two groups, the ANOVA Kruskal-Wallis test for comparison of several groups, and the Spearman correlation test. For qualitative parameters, the Paerson's χ^2 test was used, with Yates correction if $n < 10$. The results were considered statistically significant when $p < 0.05$. The calculations were performed with the use of Statistica 7 software (StatSoft Inc., Tulsa, OK, USA).

Ethical considerations. This study was approved by the Independent Bio-Ethical Committee for Research at the Medical University of Gdańsk (NKEBN/13/2004). Informed consents of the patients' parents or legal guardians were obtained prior to the study.

RESULTS

The frequencies of particular genotypes at four polymorphic sites (-800G/A, -509C/T, 869T/C and

Table 1. Frequencies of particular genotypes at four polymorphic sites (–800G/A, –509C/T, 869T/C and 915G/C) of the TGF-β1 locus in the IBD group and control.
Statistically significant difference is marked by an asterisk.

TGFβ1 polymorphism	Number of cases (frequency in %)	
	IBD	control
C-509T		
CC	34 (40.5%)	37 (35.9%)
CT	30 (35.7%)	50 (48.5%) *
TT	20 (23.8%)	16 (15.5%)
Total number of cases	84	103
T869C		
TT	23 (27.4%)	32 (31.1%)
TC	41 (48.8%)	46 (44.7%)
CC	20 (23.8%)	25 (24.3%)
Total number of cases	84	103
G915C		
GG	72 (85.7%)	90 (87.4%)
CG	12 (14.3%)	12 (11.7%)
CC	0 (0%)	1 (1.0%)
Total number of cases	84	103
G-800A		
GG	76 (90.5%)	93 (90.3%)
GA	7 (8.3%)	10 (9.7%)
AA	1 (1.2%)	0 (0%)
Total number of cases	84	103

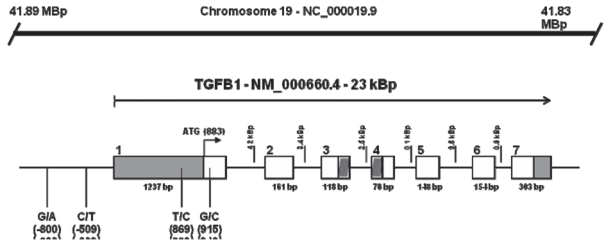

Figure 1. Schematic view of the genetic organization of *TGFB1*. The structure of the *TGFB1* gene region (with accession number) shows 7 exons (boxes) with sizes indicated below boxes. Sizes of introns are represented by vertical numbers. The scheme is shown not to scale. Grey areas mark 5′UTR and 3′UTR, respectively. Hatched boxes represent location of the exons considered in reverse transcriptase real-time PCR assay for *TGFB1* mRNA quantification. Location of specific sites are shown in brackets. The polymophic sites analyzed in this study are shown below the transcription outline.

915G/C) of the *TGF-β1* locus in pediatric patients suffering from IBD and in control subjects are presented in Table 1. There were no statistically significant differences in the frequencies of particular genotypes between tested groups, but one exception, namely, a higher number of –509CT heterozygotes was found in the control group relative to IBD patients ($p = 0.04$). Moreover, no significant differences were observed between control and CD and UC patients as well as between UC and CD patients (not shown).

No statistically significant differences among IBD patients, and within UC and CD subgroups, were found in relation to the clinical parameters of the disease, the immunohistochemically determined tissue content of TGF-β1, the levels of TGF-β1 in plasma, and levels of mRNA of the *TGF-β1* gene in intestinal mucosa samples (Table 1 and not shown). Moreover, there were no significant differences between *TGF-β1* polymorphisms and selected cytological and biochemical parameters (WBC, ESR, iron concentration and total protein concentration) measured in blood samples of IBD patients. The only two statistically significant differences were found when the –509C/T polymorphism was tested in relation to platelet count and CRP level. The platelet count was significantly higher in TT homozygotes than in CC homozygotes and heterozygotes ($p = 0.04$) (Fig. 2), and a significantly increased CRP level was found in –509CC homozygotes, relative to CT and TT genotypes ($p = 0.01$) (Fig. 3) in the study group.

DISCUSSION

Previous reports by other authors suggested a role for *TGF-β1* polymorphism in the clinical parameters of IBD. Particularly, –509C/T and 915G/C polymorphisms were reported to be associated with different frequencies of the stricture producing type of CD (Schulte *et al.*, 2001; Hume *et al.*, 2006) and differences in allele frequencies and genotypes of the –800G/A polymorphism were reported between patients with UC and control subjects (Tamizifar *et al.*, 2008). In our study, we could not find any statistically significant differences between three *TGF-β1* polymorphisms, namely –800G/A, 869T/C and 915G/C, and susceptibility to IBD. The only statistically significant difference detected was a higher number of –509CT heterozygotes in the control group relative to IBD patients ($p = 0.04$). However, such a difference seems to be of minor importance due to their relatively weak statistical support and the difficult to explain advantage of heterozygosity over two kinds of homozygosity in susceptibility to the disease. Moreover, no statistically significant differences were found when testing all four polymorphisms in relation to the clinical parameters of IBD.

An obvious question is why our results differ from those published earlier. One clear difference between our study and those reported by others (Schulte *et al.*, 2001; Hume *et al.*, 2006; Tamizifar *et al.*, 2008) is that we have investigated pediatric patients with IBD while those authors tested adult patients suffering from this disease. However, while the age of patients may influence the relationship between *TGF-β1* polymorphisms and susceptibility to and/or clinical parameters of IBD, it is worth mentioning that a lack of correlations between these items was indicated previously by some researchers who investigated adult IBD patients (Garcia-Gonzalez *et al.*, 2000; Tamizifar *et al.*, 2007). Thus, we speculate that other factors, apart from age, may be responsible for the discrepancy between results obtained by different authors. One factor is a population-specific genetic background, as it is worth noting that patients investigated by various authors came from as different geographical regions as The Netherlands (Garcia-Gonzalez *et al.*, 2000), Germany (Schulte *et al.*, 2001), Australia (Hume *et al.*, 2006), Iran (Tamizifar *et al.*, 2007, 2008), and Poland (this report). The second factor is a possible genetic coupling between *TGF-β1* locus and another locus, which is actually responsible for modulation of the disease susceptibility or clinical parameters; in such a case, the link between a particular *TGF-β1* polymorphism and IBD might be coincidental. It appears that the occurrence of such a possibility is often underestimated when testing

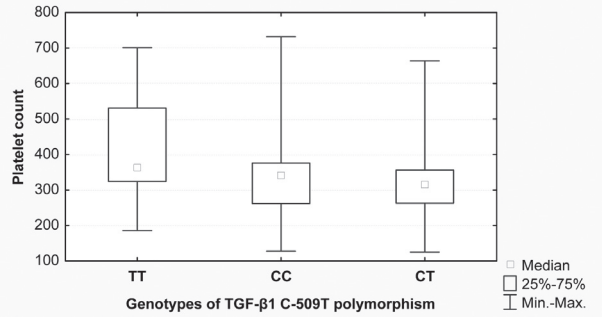

Figure 2. Platelet count in relation to genotypes of TGF-β1 C-509T polymorphism in the study group.

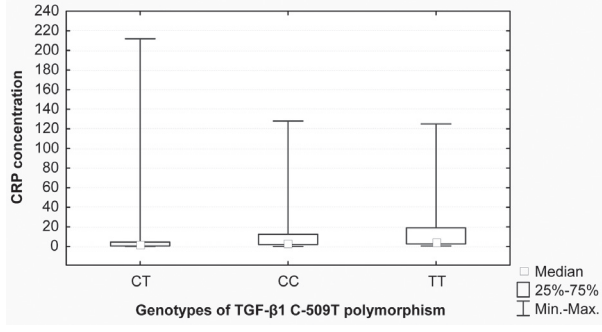

Figure 3. CRP concentration (mg/dl) in relation to genotypes of TGF-β1 C-509T polymorphism in the study group.

correlations between genetic polymorphisms and various diseases. Finally, one may assume that correlation between a single genetic polymorphism and a specific disease symptom or parameter can be influenced by so many other genetic factors that any definite conclusions must be postponed until the molecular mechanism of a putative genetic link is elucidated.

It is obvious that further studies, including those on larger and ethnically differentiated groups of patients, are necessary to solve the problem of different results of studies on correlations between *TGF-β1* polymorphisms and IBD. Moreover, further studies are also required to test whether correlations between the TT genotype at the -509 position of the *TGF-β1* locus and an increased platelet count in IBD pediatric patients, and between the -509CC genotype and an increased CRP level, described in this report, have any clinical importance. Nevertheless, in our opinion, no strong conclusions should be proposed until principles of molecular mechanisms of influence of any particular polymorphisms on certain biochemical or clinical effects are elucidated.

Financial support

Ministry of Science and Higher Education, Poland (project grant no. 2P05E 125 27) and University of Gdansk (task grant no. DS/L140-4-0114-11).

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