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Gene polymorphism in transforming growth factor-beta codon 10 is associated with susceptibility to Giardiasis

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Summary

Secretory immunoglobulin A (S-IgA) antibodies have a central role in anti-Giardial defence. It has been demonstrated that transforming growth factor-beta1 (TGF-β1) stimulates B lymphocytes to produce and secrete S-IgA. We sought to determine the association between TGF-β1 polymorphism (T+869C) with susceptibility to Giardiasis. The TGF-β1 genotypes and levels of salivary (S-IgA) were analysed in individuals with Giardiasis (97 symptomatic and 57 asymptomatic) and controls (n = 92). Individuals with symptomatic Giardiasis had the lowest levels of S-IgA compared to individuals in asymptomatic Giardiasis and control groups (97%, 73% and 43%, <1 g L^{-1}, respectively, P = 0.002). The frequency of allele C and CC genotypes of TGF-β1 polymorphism was significantly higher among symptomatic patients than asymptomatic and control groups. Logistic regression analysis demonstrated that the individuals homozygous for allele C of TGF-β1 had a significantly higher risk for symptomatic Giardiasis with odds ratio of 2.76 (95% CI: 3.88, 1.71, P = 0.007). Among the participants with TT genotype per cent of individuals with S-IgA level of more than 1 g L^{-1} was almost twice the percentage in CC genotype individuals (14% versus 7% respectively P = 0.01). Our data suggest that CC genotype of TGF-β1 polymorphism at codon 10 is associated with occurrence of Giardiasis.

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Introduction

The protozoan parasite *Giardia lamblia* is an intestinal parasite of humans, which the World Health Organization (1992) estimates to have infected 250 million people worldwide. This protozoan pathogen colonizes small intestine and can attach to the epithelium but does not invade the mucosa. Infections are normally self-limiting, as immunocompetent hosts can control and typically eradicate *G. lamblia*, a process that involves CD4 T cells and the generation of secretory immunoglobulin A (S-IgA) and other, poorly understood effectors (Faubert, 2000; Singer & Nash, 2000; Langford *et al.*, 2002; Eckmann, 2003). Despite the clinical symptoms, diarrhoea, abdominal pain, malabsorption and weight loss, infection is not accompanied by significant mucosal inflammation (Oberhuber *et al.*, 1997). Role of transforming growth factor-beta1 (TGF-β1) in intestinal parasitic infections has attracted significant attention, as *in vitro* tests showed that TGF-β1 stimulates the isotype switch to IgA, as well as IgA secretion by LPS-stimulated $mIgA^{-}B$ cells from Peyer's patches and spleen (Sonoda *et al.*, 1989, Coffman *et al.*, 1998). To date, several TGF-β1 polymorphisms have been identified among these, two are located in exon 1 changing the amino acid sequence of the signal peptide (at positions +869 and +915) resulting in variations of codon 10 or codon 25, respectively. The third polymorphism in codon 263 (in exon 5) results in the amino acid substitution threonine to isoleucine in the precursor part of the protein. The TGF-ß1 polymorphism (+869 in codon 10) is a T to C transition at nucleotide 29 located in the hydrophobic core of the signal peptide, resulting in the replacement of a hydrophobic leucine with a small, neutral proline (Cambien *et al.*, 1996). Such a substitution would change the overall hydrophobicity of the core transport sequence and disrupt the alpha-helical structure of the region, therefore altering its ability to direct protein transport across the endoplasmic reticulum (Randall & Hardy, 1989). It has been shown that the production of TGF-β1 is associated with some polymorphisms of the gene (Awad *et al.*, 1998). A correlation between the polymorphism of C+915G and the concentration of TGF-β in human serum has been

shown (Grainger *et al.*, 1999). These findings indicate that the TGF-β1 polymorphisms may be involved in the modulation of expression of the TGF-β1 gene and therefore a predisposing factor for Giardiasis could be linked to the particular alleles of this gene. Data regarding the association of TGF-β1 T+869C polymorphism and S-IgA is controversial. In addition, no previous study investigated the role of TGF-β1 polymorphism in susceptibility to Giardiasis.

Giardiasis is quite common among Iranian population. A national survey of the prevalence of intestinal parasitic infections in Iran revealed that from 53 995 people aged 2+ years (12 495 families), 10.9% were infected with Giardia (Sayyari *et al.*, 2005) The infection rate was highest in the 2–14 years age group (25.5%) and in rural residents (23.7%).

The purpose of this study was to determine the association between polymorphism of TGF-β1 at codon 10 and susceptibility to Giardiasis in Iranian population. The association of levels of S-IgA and the genotypes of TGF-β1 at codon 10 were also assessed. In addition, the role of polymorphisms in TGF-β1 gene and levels of S-IgA in development of symptom among patients with Giardiasis were evaluated. We, for the first time, found an association between CC genotype of TGF-β1 T+869C polymorphism and susceptibility to Giardiasis.

Materials and methods

The population under study was 97 patients with symptomatic Giardiasis (49 men and 48 women) who referred to Sina and Ekbatan hospitals in Hamadan province, Southwest of Iran between the years of 2006 and 2008. A group of 51 individuals (27 men and 24 women) recruited from the hospitals personnel and their family members who had *G. lamblia* in their stool examination without any symptoms. This group was chosen from 543 individuals underwent annual check-up for parasitic intestinal infection. The control group consisted of 92 volunteer individuals (48 men and 44 women) recruited from the same hospitals with a negative test result for Giardiasis. Informed consent was obtained from each subject. The Ethical Committee of Hamadan University of Medical Sciences approved the study protocol. Diagnostic criteria were as follows: positive stool examination smears in direct smear or after formalin ether concentration (at least one of three times). Individuals with *G. lamblia* divided into two groups, symptomatic that had at least one of the symptoms (diarrhoea, steatorrhoea, gastrointestinal discomfort, malabsorption and weight loss) and asymptomatic that had only cyst in the stool. Blood samples were treated with sucrose lysis buffer and mononuclear layer of peripheral blood was harvested and the cell pellet stored at −80 °C until DNA extraction. Genomic DNA was extracted from EDTA-treated peripheral blood by the standard method (Miller *et al.*, 1998).

Genotyping

Amplification refractory mutation system–polymerase chain reaction (ARMS-PCR) method was used to genotype individual samples for the TGF-β1 polymorphisms under investigation using primers as already described (Perrey *et al.*, 1999). Fragments containing the single nucleotide polymorphism T+869C were amplified by ARMS-PCR. The primers for detection of polymorphism at nucleotide acid +869 were 5′-GCAGCGGTAGCAGCAGCG-3′ (C allele specific), 5′-AGCAGCGGTAGCAGCAGCA-3′ (T allele specific) and 5′-TCCGTGGGATACTGAGACAC-3′ (generic primer); and the generated PCR product size was 241 bp. We used internal control primer 1 (HGH forward), 5′-GCCTTCCCAACCATTCCCTTA-3′; and the internal control primer 2 (HGH reverse), 5′-TCACGGATTTCTGTTGTGTTTC-3′. The internal control primers were used to amplify a segment of human growth hormone gene to check for successful PCR amplification. Amplification was carried out using a PCR Techne Flexigene apparatus (Roche, Mannheim, Germany) in a total volume of 15 μL that contained 100 ng of genomic DNA, each primer pair consisting of 2 μmol of allele specific and 0.5 μmol of common primers, 200 μmol L^{-1} each dNTP; 10 mM Tris–HC1 (pH 8.3); 50 mM KCI, 1.5 mM $MgCl_2$ and 0.5 IU *Taq* DNA polymerase. PCR performed without DNA template represented the negative control. The reaction was carried out as follow; initial denaturation was 94 °C for 2 min, followed by ten cycles of amplification at 96 °C for 20 s and annealing at 62 °C for 50 s, followed by 20 cycles of denaturation at 96 °C for 20 s and annealing at 60 °C for 50 s, with extension for 40 s at 72 °C, and then with final soak at 10 °C. The amplified PCR products were analyses by 2% agarose gel electrophoresis followed by 0.5 μg mL^{-1} ethidium bromide staining and ultraviolet visualization.

Measurement of total salivary IgA

Saliva flow was stimulated by chewing a single Parafilm (5 cm^2) section for 1 min, which typically elicits 1–3 mL min^{-1} of flow. Subjects were encouraged to allow the saliva to accumulate in the mouth until the urge to swallow occurred. Without expectorating, the subjects allowed the saliva to flow into the collection tube and repeated the entire process until time elapsed. The sample was immediately placed on ice and then centrifuged to remove cellular debris. The supernatant fluid was frozen at −80 °C for later analysis of total IgA by an immunonephelometric technique (Minineph, The Binding Site, Oxford, UK) used as instructed by the manufacturer.

Briefly, total IgA in plasma were analyzed using enzyme-linked immunosorbent assays performed in 96-well plates. Plates were coated with anti-IgA (ICN, Costa Mesa, CA, USA) at 10 μg mL^{-1}. The reaction

was revealed anti-human IgA-peroxidase-conjugated (Sigma, St. Louis, MO, USA) (1/2000). IgA concentration was extrapolated from a reference curve generated by assaying dilutions of a pool of plasma specimens from blood donors whose subclass concentrations were determined by an immunonephelometric technique (Minineph).

Statistical analysis

The data were analysed by constructing contingency tables to determine differences in genotypic frequencies of the relevant polymorphisms in patients and controls. *P*-values for association studies were calculated using the chi-square test and one-way analysis of variances (ANOVA). Results of the logistic regression model were expressed as odds ratio (OR) and 95% CI. All *P*-values were evaluated in a two-sided model, and $P < 0.05$ was considered statistically significant. Chi-square (χ^2) test was used to determine the significance of differences from the Hardy–Weinberg equilibrium. Statistical analysis was performed using the SPSS 13.0 software package (SPSS, Chicago, IL, USA).

Results

There was no significant difference between the ages of symptomatic patients, asymptomatic individuals and the controls (Mean ± SD, 35 ± 4, 37 ± 6, 34 ± 5, respectively). The ratio of male/female also was not significantly different between the groups. The levels of total S-IgA in the patients and control groups are shown in Table 1. Because the frequency distribution of S-IgA was not a Gaussian distribution, we divided the levels into two groups to make the data more comparable. The individuals with symptomatic Giardiasis had a lower level of S-IgA compared to individuals in asymptomatic Giardiasis and control groups. The level of S-IgA in 97% of the symptomatic patients were <1 g L^{-1} in contrast to 73% and 42% in asymptomatic and control group ($P = 0.002$). The frequency of TGF-β1 polymorphism in codon 10 was assessed in patients and controls. The observed genotype counts were not significantly different from those expected under Hardy–Weinberg equilibrium (verified by chi-square test). As indicated in Table 2 prevalence of allele C was significantly higher in the symptomatic patients in comparison with the asymptomatic and the control groups ($P = 0.001$). The control and asymptomatic groups also had higher frequency of T allele than the symptomatic patients. The distribution of TGF-β1 genotypes for codon 10 polymorphism was also different among the groups. The CC genotype was significantly higher among the symptomatic patients than the other two groups ($P = 0.005$). While the distribution of TT genotype did not show significant difference, the control and asymptomatic patients had a higher frequency of CT genotypes in comparison with the symptomatic patients. Multiple logistic regression analysis controlled for sex and age demonstrated that homozygous genotype of allele C of TGF-β1 polymorphism is an independent risk factor for contracting Giardiasis (OR: 2.76, 95% CI: 3.88, 1.71, $P = 0.007$).

Table 3 compares the levels of total S-IgA among individuals with CC, CT and TT genotypes of TGF-β1 T+869C. The levels of S-IgA were <1 g L^{-1} in more than 74% of participants and only 26% showed a concentration of more than 1 g L^{-1}. Among the participants with TT genotype per cent of individuals with S-IgA level of more than 1 g L^{-1} was almost twice the percentage in CC genotype individuals (14% versus 7%, respectively, $P = 0.01$). This per cent appears to be 42% for individuals with the heterozygous genotype.

Discussion

Our study provides evidence for an association between TGF-β1 T+869C polymorphism and susceptibility to Giardiasis. We found that the prevalence of allele C and CC genotypes of TGF-β1 T+869C polymorphism was significantly higher in the patients with symptomatic Giardiasis and these patients had significantly lower levels of S-IgA compared to the

Table 1. Levels of salivary IgA in individuals with symptomatic and asymptomatic Giardiasis and control group

Salivary IgA g L^{-1}	Symptomatic infected	Asymptomatic infected	Control
0.19–0.99	94 (96.9%)	37 (72.5%)	39 (42.5%)
1–4	3 (3.1%)	14 (27.5%)	53 (57.5%)

Table 2. Alleles and genotype frequencies in control subjects and individuals with Giardiasis

TGF-β1 polymorphism	Symptomatic patients	Asymtomatic patients	Control	*P*-value
Alleles, *n* (%)				
C allele	103 (53.1)	44 (43.1)	80 (43.5)	0.001
T allele	91 (46.9)	58 (56.9)	104 (56.5)	
Genotype, *n* (%)				
CC	39 (40.2)	8 (15.7)	20 (21.7)	0.005
CT	25 (25.8)	28 (55)	40 (43.5)	
TT	33 (34)	15 (29.3)	32 (34.8)	

TGF-β1, transforming growth factor-beta1.

Table 3. Levels of salivary IgA among individuals with CC, TC and TT genotypes of TGF-β1 T+869C polymorphism

Salivary IgA g L^{-1}	CC genotype	TC genotype	TT genotype
0.19–0.99	53 (92.5%)	60 (58.2%)	69 (86.2%)
1–4	4 (7.5%)	43 (41.8%)	11 (13.8%)

asymptomatic and control groups. In addition, our data suggest that individuals with TC or TT genotypes are more likely to have high levels of total S-IgA. Several studies suggest an important role for B cells in clearing *Giardia* infection. For example, infections of humans with *G. lamblia* or of mice with *Giardia muris* result in the production of anti-Giardial antibodies of the IgA, IgM and IgG isotypes in mucosal secretions and serum. The specific antibody production correlates with *Giardial* clearance (Daniels & Belosevic, 1994; Faubert, 2000). Such antibodies reach their targets *in vivo*, since anti-Giardial IgA and IgG antibodies coat trophozoites in *Giardia*-infected mice. Mice depleted of B cells by treatment with anti-IgM antibodies and mice with X-linked immunodeficiency, which have a defect in B-cell development and function, are unable to clear *G. muris* infection (Snider *et al.*, 1985, 1988). In contrast, other data suggest that B cells have only a limited role in anti-Giardial immunity (Zhou *et al.*, 2003). For example, mice with X-linked immunodeficiency can develop acquired immunity against secondary challenge with *G. muris* (Skea & Underdown, 1991). These data suggest that B-cell-independent host defences against *Giardia* may play an important role in controlling and clearing infection.

Our study is the first that investigates the role of TGF-β1 polymorphism in susceptibility to Giardiasis. We demonstrated that the CC genotype of TGF-β1 was a susceptibility factor for contracting Giardiasis among Iranian population. The association between the C allele and scleroderm has been reported (Crilly *et al.*, 2002). The proline allele at codon 10 has also been linked to bone mineral density (Yamada *et al.*, 1998) regulation of blood pressure (Rivera *et al.*, 2001) and diabetic nephropathy (Wong *et al.*, 2003).

Currently, it is not clear whether a leucine or proline at amino acid position 10 of the signalling sequence of the TGF-β1 gene results in high active TGF-β1 concentrations *in vivo*. Awad *et al.* (1998) reported that patients with cystic fibrosis with a leucine residue at codon 10 (allele C) have elevated circulating levels of TGF-β1, whereas Yamada *et al.* (1998) showed that Japanese women with osteoporosis who have a Proline residue at this position (allele T) have the highest TGF-β1 serum levels. The association of proline allele at codon 10 with high level of TGF-β1 has been reported by other investigators (Suthanthiran *et al.*, 2000)

In our study the patients with symptomatic Giardiasis showed lower levels of total S-IgA. We did not measure the specific S-IgA against *Giardia*. However, a correlation between total S-IgA and specific anti-Giardia S-IgA has been reported in children infected with Giardiasis (Rodriguez *et al.*, 2004). It has been demonstrated that the levels of S-IgA are elevated in the mice infected with *Giardia*. The same group showed that IgA-deficient mice could not eradicate *Giardia* infection, demonstrating that IgA is required for the parasite clearance (Langford *et al.*, 2002).

It seems that the level of S-IgA in the person's first contact with the parasite is in a low level and after some time, with the activity of the immune system and other involved cytokines (including TGF-β1), the level of S-IgA increases (Faubert, 2000; Thompson, 2000). In our study, the symptomatic patients with lower levels of S-IgA might be in their early phases of the infection and thus produce lower levels of S-IgA. This weak reaction might be also related to a defect in symptomatic patients in their response to pathogen. In support of this notion, an impaired IgA response to Giardia antigen has been reported in children with persistent diarrhoea and Giardiasis (Char *et al.*, 1993). Furthermore, these individuals carry CC genotype of TGF-β1 and this genotype has been reported to be associated with lower levels of TGF-β (Yamada *et al.*, 1998; Suthanthiran *et al.*, 2000). Therefore, one can speculate that levels of S-IgA in the symptomatic patients might be lower than the other groups due to a reduced stimulatory effect of TGF-β1 on S-IgA secretion. As we did not measure serum levels of TGF-β in our patients, cautious interpretation of the data is needed. The future studies will help us to shed light in this issue. The lower levels of S-IgA in the symptomatic patients might also contribute to the severity of the disease by reducing the power of immune system for clearing the parasite. In support of this, in our study the levels of S-IgA in individuals with asymptomatic Giardiasis were close to those in the control group and not to those of symptomatic patients. In addition, like the control group, the frequency of T allele and CT genotype were also higher in the asymptomatic group than the symptomatic patients. However, the association between CC genotype of TGF-β1 and Giardiasis can be attributed to linkage disequilibrium. The CC genotype may lead to the lower levels of IgA and may also control symptomatic Giardiasis through association with other genes that control symptomatic Giardiasis.

The results of our study need to be replicated by others to ensure that the genetic association can be applied to several different populations of people exposed to *Giardia*.

Conclusion

Our findings suggest an association between TGF-β1 polymorphism and susceptibility to Giardiasis in Iranian patients.

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