Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

CTLA-4 (+49A/G) and NOD2/CARD15 (N852S) polymorphisms with inflammatory bowel disease in Turkish patients

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Received December 25, 2017; **Accepted** August 29, 2018; **Published** August 30, 2018

Doi: http://dx.doi.org/10.14715/cmb/2018.64.11.18

Abstract: Crohn's disease (CD) and ulcerative colitis (UC) are the major types of inflammatory bowel disease (IBD) and exhibit similar clinical features and epidemiology. The main objective of this study was to analyze the correlation between the CTLA-4 gene +49A/G polymorphism and the NOD2/CARD15 gene N852S polymorphism in Turkish patients with IBD using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) analysis. In this study, we evaluated the frequency of the CTLA-4 (+49A/G) and NOD2/CARD15 (N852S) polymorphisms in 62 patients with CD, 76 patients with UC, and 152 healthy individuals. The CTLA-4 and NOD2/CARD15 variants, rs231775 and rs104895467, were genotyped by PCR followed by RFLP. The results for the patients and the control group were statistically analyzed. According to our results, the CTLA-4 gene +49A/G polymorphism AA genotype was prevalent in CD patients and controls (29% vs 40%); the AG (56% vs 51%) and GG (15% vs 9%) genotypes were also observed. The prevalence of the of AA, AG and GG genotypes for the +49A/G polymorphism was 56%, 32% and 12%, respectively, in the UC patients, and 40%, 51% and 9%, respectively, in the healthy controls. In all subjects, just one band of 151 bp, corresponding to wild-type N852S, was found, and no other N852S mutant bands (151+129+22 and 129+22 bp) were detected using PCR-RFLP fragment electrophoresis.The CTLA-4 gene +49 A/G polymorphism and the NOD2/CARD15 gene N852S polymorphism were not associated with CD or UC in a Turkish population.

Key words: Inflammatory bowel disease; CTLA-4 gene; NOD2/CARD15 gene; PCR; RFLP.

Introduction

Inflammatory bowel disease (IBD) is a multifactorial (inflammatory, intestinal) disorder of the gastrointestinal tract that is characterized by immune dysregulation and leukocyte recruitment and seems to be related to microbial exposure (1-3). Approximately 25% of IBD patients are diagnosed in childhood (about 13-18 years) and the incidence is rapidly increasing (4). Crohn's disease (CD; MIM 26600) and ulcerative colitis (UC; MIM 191390) are the two major types of IBD. The clinical characteristics of CD and UC are quite different (5).

UC has a bimodal type of incidence, with the mean age diagnosis between ages 15 and 30 years and between ages 50 and 70 years. UC is one of the major IBD types that cause irregular immune responses in genetically predisposed individuals. UC is idiopathic and characterized by superficial, progressive mucosal inflammation, with ulcers limited to the colon. In contrast, CD is a segmental, transmural disorder that can include any part of the gastrointestinal tract (5,6). Europe has been reported to be the leading continent with a 24.3/100000 yearly incidence rate of UC, and North America follows it with a rate of 19.2/100000. In Asia and the Middle East, the rate is much lower at 6.3/100000 (5).

The cytotoxic T-lymphocyte antigen 4 (CTLA-4) gene is located in chromosome 2 (2q33) and encodes a protein of 223 amino acids called cytotoxic T-lymphocyte-associated protein 4. This protein is a member of the co-stimulatory family, has homology to CD28 and binds the B7 family of ligands. The CTLA-4 gene consists of three coding exons and two introns and spans approximately 6 kb of genomic DNA (6,7). Many genetic polymorphisms (>100 single nucleotide polymorphisms, or SNPs) have been reported in the human CTLA-4 gene. Of these polymorphisms, the 49A/G SNP (rs231775) is located in exon 1 (5,7). Because of its inhibitory effect on T-cell responses, polymorphisms in the CTLA-4 gene have been related to diabetes mellitus, Graves' disease, Hashimoto thyroiditis, celiac disease, systemic lupus erythematosus, thyroid-associated orbitopathy, other autoimmune diseases and cancer (7-12). One of the genes identified as causing susceptibility to CD is the NOD2/CARD15 gene. Three NOD2/CARD15 polymorphisms (R702W, G908R, and 1007fs) have been reported to be substantially associated with CD (13). Five novel rare variants, including N852S, have been identified in the NOD2/CARD15 gene. The N852S polymorphism has been identified only in Ashkenazi Jewish individuals. N852S has been reported in 15% of the evaluated Ashkenazi Jewish families with CD and in none of the Sephardi/Oriental Jewish families with CD (14).

The aim of the present research was to assess whether these known SNPs in the CTLA-4 and NOD2/CARD15 genes (+49A/G and N852S, respectively) determine susceptibility to CD and UC in the Turkish population.

Table 1. Demographic and clinical features of the patients with Crohn's disease (CD), ulcerative colitis (UC) and controls.

Characteristics		Crohn's disease n=62, Mean±SD, %	Ulcerative colitis n=76, Mean±SD, %	Controls n=152, Mean±SD, %
Age		44.2±13.36	46.9±15.80	43.2±15.84
Gender	Male	32 (41.5±11.56)	41 (47.9±15.29)	59 (41.8±14.88)
	Female	30 (47.1±14.71)	35 (45.7±16.51)	93 (44.0±16.43)
Smoking status	Yes	42 (42.1±12.56)	22 (50.5±18.04)	32 (41.3±14.32)
	No	20 (48.8±14.20)	54 (45.4±14.71)	120(43.7±16.24)
Localization	Ileitis	26 (42%)	-	-
	Colitis	7 (11%)		
	Ileocolitis	29 (47%)		
	Proctitis		26 (34%)	
	Left colitis		40 (53%)	
	Pancolitis		10 (13%)	

Materials and Methods

Subjects

The subjects were studied retrospectively and included 62 patients with CD (32 males and 30 females, mean age 44.2±13.36 years), 76 patients with UC (41 males and 35 females, mean age 46.9±15.80 years), and 152 unrelated healthy volunteers (59 males and 93 females, mean age 43.2 ±15.84 years) as controls (Table 1). All participants were Turkish and were randomly recruited from the gastroenterology clinic of Niğde State Hospital, Niğde, Turkey. This retrospective study protocol was approved by the Human Ethics Committee of the Erciyes University School of Medicine (Ethics number: KAEK 2016-349). A diagnosis of IBD was defined according to endoscopic, radiological, histological, and clinical criteria. The patients suffering from UC were classified considering the location and extent of disease (proctitis, left colitis, or pancolitis). Likewise, the patients with CD were divided into three subgroups, namely, ileitis, colitis and ileocolitis, according to the location and extent of disease. The control group included population-matched voluntary participants who did not have IBD or other autoimmune disease.

DNA extraction

Peripheral blood samples were collected from the participants in ethylenediaminetetraacetic acid (EDTA) tubes. Genomic DNA was extracted from 200 µL EDTA-anticoagulated peripheral blood leukocytes using a QIAGEN QIAamp DNA Blood Mini Kit (Maryland, USA) following the manufacturer's guidelines. The extracted DNA was stored at -20°C until analysis.

For genotyping of the IBD variants in the CTLA-4 (rs231775) and NOD2/CARD15 (rs104895467) genes, PCR-RFLP methods were implemented (12,14). The primers designed and used are stated in Table 2. The

Figure 1. The PCR products of the CTLA-4 gene +49 A/G polymorphic site (162 bp). Lanes 1-2 show PCR product; lane M shows DNA ladder (50bp).

PCR amplifications were performed on an Applied Biosystems Veriti 96-Well thermal cycler (USA) using the following conditions: initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing for 45 seconds at 60°C (+49A/G) or 55°C (N852S) (Table 2), extension at 72°C for 45 seconds and final extension 72°C for 5 minutes. All PCR products were electrophoresed on a 1.5% agarose gel with 1 × Tris-borate-EDTA buffer at 100 V for 30 minutes and then monitored under ultraviolet illumination (+49A/G, Fig. 1; N852S, Fig. 2). The amplicons were digested by the allele-specific restriction endonucleases BbvI (rs231775) and AluI (rs104895467) (Table 3). The restriction fragments were separated by electrophoresis on 3% agarose gels containing ethidium bromide and visualized by UV transillumination (BbvI,

Table 2. Polymerase chain reaction primers, annealing temperatures and polymerase chain reaction fragment sizes in genotyping of CTLA-4 and NOD2/CARD15 genetic variants.

Single nucleotide polymorphism		Primers	Annealing temperature (°C)	PCR fragment size (bp)
CTLA-4 +49A/G	rs231775	F: 5-GCTCTACTTCCTGAAGACCT-3 R: 5-AGTCTCACTCACCTTTGCAG-3	60	162
NOD2 CARD15 N852S	rs104895467	F: 5-CTGTTTGCATGATGGGGGG-3 R: 5-CAGCCGTCAGTCAATTTGTAG-3	55	151

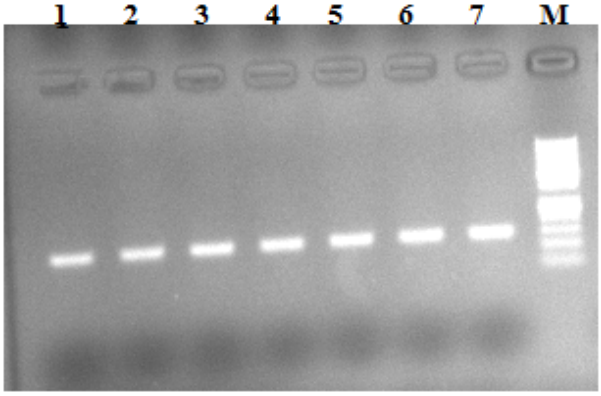

Figure 2. The PCR products of the NOD2/CARD15 gene N852S polymorphic site (151 bp). Lanes 1-7 show PCR product; lane M shows DNA ladder (50bp).

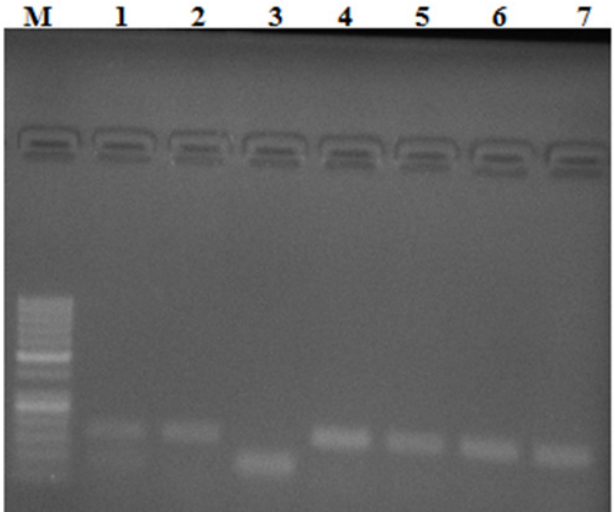

Figure 3. The BbvI restriction profiles of the CTLA-4 gene +49 A/G polymorphic site. Lane M shows DNA ladder (50bp); lane 1 shows AG genotype (heterozygous,162 bp, 88 bp, and 74 bp); lanes 2,4,5,6 and 7 show AA genotype (homozygous, 162 bp wild-type); lane 3 shows GG genotype (homozygous, polymorphic, 88 bp and 74 bp).

Fig. 3; AluI, Fig. 4).

Statistical analysis

We used the SPSS 15.0 package for Windows (SPSS Inc, Chicago, IL, USA) to perform the statistical analyses. The baseline characteristics were tested using Student's t-test for equal variance. These data are shown as the mean±SD. Pearson's $\chi 2$ test was applied to compare allele and genotype frequencies for the CTLA-4 (rs231775) and NOD2/CARD15 (rs104895467) variants between the patient and control groups. A P value of 0.05 or less was considered statistically significant. The Hardy-Weinberg equilibrium test was used to test the distributions of mutation genotype frequency. Deviations from Hardy-Weinberg equilibrium were analyzed using Michael H. Court's (2005-2008) online calculator. http://www.tufts.edu/~mcourt01/Documents/Court%20lab%20-%20HW%20calculator.xls.

Results

One variant in the CTLA-4 gene (rs231775) and one variant in the NOD2/CARD15 gene (rs104895467) were genotyped in Turkish CD and UC patients as well as in controls. No departures from Hardy–Weinberg equilibrium were identified in either the CD or UC pa-

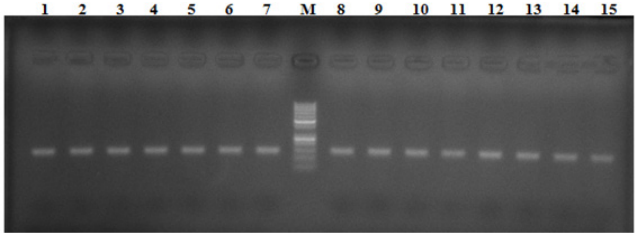

Figure 4. The AluI restriction profiles of the of the NOD2/CARD15 gene N852S polymorphic site. Lane M shows DNA ladder (50bp); lanes 1-15 show AA genotype (homozygous, wild-type). No other N852S mutant bands (151+129+22 and 129+22 bp) were detected using RFLP fragment electrophoresis.

Table 3. Enzymes and single nucleotide polymorphism analysis.

SNPs	Base change	Enzyme	Restriction fragment size (bp)	
			Wild-type	Mutant
+49A/G	A→G	BbvI	162	Heterozygote 162+88+74 Homozygote 88+74
N852S	A→G	AluI	151	Heterozygote 151+129+22 Homozygote 129+22

Table 4. Distribution of genotype and allele frequencies of the CTLA-4 gene +49A/G polymorphism and the NOD2/CARD15 gene N852S polymorphism in patients with CD and healthy controls.

Gene/Genotypes		Patients	Controls	P value	Odds ratio (OR)	95% (CI)
		n=62 n/%	n=152 n/%			
+49A/G	AA	18 (29)	61 (40)	-	1	-
	AG	35 (56)	78 (51)	0.212	0.658	0.340-1.272
	GG	9 (15)	13 (9)	0.089	0.426	0.157-1.158
	AG+GG	44 (71)	91 (60)	0.127	0.610	0.323-1.154
Alleles	A	71 (57)	200 (66)			
	G	53 (43)	104 (34)	0.097	0.697	0.454-1.068
N852S	AA	62 (100)	152 (100)	-	1	-
	AG	0 (0.0)	0 (0.0)	NS		
	GG	0 (0.0)	0 (0.0)	NS	NS	NS
Alleles	A	124 (100)	304 (100)			
	G	0 (0.0)	0 (0.0)	NS (Not significant)		

Table 5. Distribution of genotype and allele frequencies of the CTLA-4 gene +49A/G polymorphism and the NOD2/CARD15 gene N852S polymorphism in patients with UC and healthy controls.

Gene/Genotypes		Patients	Controls	P value	Odds ratio (OR)	95% (CI)
		n=76 n/%	n=152 n/%			
+49A/G	AA	43 (56)	61 (40)	-	1	-
	AG	24 (32)	78 (51)	0.006*	2.291	1.256-4.180
	GG	9 (12)	13 (9)	0.970	1.018	0.400-2.594
	AG+GG	33 (44)	91 (60)	0.019*	1.944	1.113-3.395
Alleles	A	110 (72)	200 (66)			
	G	42 (28)	104 (34)	0.156	1.362	0.888-2.088
N852S	AA	76 (100)	152 (100)	-	1	-
	AG	0 (0.0)	0 (0.0)	NS		
	GG	0 (0.0)	0 (0.0)	NS	NS	NS
Alleles	A	152 (100)	304 (100)			
	G	0 (0.0)	0 (0.0)	NS (Not significant)		

*In the current UC cohort, the AG genotype for the CTLA-4 gene +49A/G polymorphism was significant for the patient group; odds ratio: 2.291 (1.256–4.180), P<0.05. The AG+GG genotype for the CTLA-4 gene +49A/G polymorphism was also significant for the patient group; odds ratio: 1.944 (1.113–3.395), P<0.05.

tients or the controls. We determined the genotype distributions and allele frequencies of the analyzed variants in the CD patients and controls (Table 4). For the CTLA-4 +49A/G polymorphism, the AA genotype was prevalent in the CD patients and controls (29% vs 40%); the AG (56% vs 51%) and GG (15% vs 9%) genotypes were also observed. There were no significant differences in the frequency of any of the alleles between the CD patients and controls (p>0.05). Logistic regression analysis showed that neither the CTLA-4 gene +49A/G polymorphism nor the NOD2/CARD15 gene N852S polymorphism was significantly associated with the disease (Table 4).

The frequencies and distributions of genotypes among the UC patients and controls are shown in Table 5. We found that the CTLA-4 gene +49A/G polymorphism GG genotype did not significantly influence susceptibility to UC; however, the AG genotype of this polymorphism did confer risk (P=0.006, OR=2.291, 95% CI=1.256-4.180). Likewise, the frequency of the AG+GG genotype was significantly higher in the UC patients than in the control group (P=0.019, OR=1.944, 95% CI=1.113-3.395). There were no significant differences in allele frequency in the UC patients (Table 5). In all subjects, just one band of 151 bp, corresponding to wild-type N852S, was found, and no other N852S mutant bands (151+129+22 and 129+22 bp) were detected using PCR-RFLP fragment electrophoresis. The NOD2/CARD15 gene N852S polymorphism was not found to be associated with CD or UC in the Turkish population (Tables 4, 5).

Discussion

In recent years, genome-wide association studies have successfully identified nearly 30 genes involved in the development of IBD. Accordingly, numerous studies have investigated the CARD15, ATG16L1, IL23R, DLG5 and CTLA-4 genes (15). The CTLA-4 gene +49A/G polymorphism on chromosome 2q33 has been studied in the context of several chronic inflammatory diseases. In addition to type I diabetes, Grave's disease, rheumatoid arthritis and multiple sclerosis have all been found to be related to the +49A/G SNP in the CTLA-4 gene (16). Machida et al. (17) suggested that in the Japanese, CTLA-4, which is located at 2q33, is a determinant of UC and confers risk for the development of CD associated with fistula formation; the GG genotype was also more often detected in CD patients with fistula. Xia et al. (18) found no association of the CTLA-4 gene +49G polymorphism with IBD in Dutch Caucasian patients or with UC in Chinese patients.

In our study, we compared the frequency of the heterozygous AG genotype, homozygosity for the GG variant, and the G allele of the CTLA-4 gene +49A/G polymorphism and did not find any associations in patients with CD. However, we showed that the AG genotype for the CTLA-4 gene +49A/G polymorphism was associated with insusceptibility to UC. No associations for the GG genotype were found in the patients with UC, indicating that the G variant of the +49A/G allele of the CTLA-4 gene does not indicate an obligatory susceptibility factor for CD or UC. Consistently, Zhao et al. (6) revealed that the CTLA-4 gene SNPs rs3087243 G>A and rs231775 G>A may increase the risk of developing UC.

Csöngei et al. (15) found a possible association of the CTLA-4 gene +49A/G substitution and two observed IBD5 variants with disease risk. On the other hand, another study showed that carrying the +49G SNP in heterozygous or homozygous type does not confer risk for CD or UC in the Hungarian population (16). Likewise, Lozano et al. (19) showed that there was no association between the CTLA-4 SNPs at -318 and +49 and resistance or susceptibility to paracoccidioidomycosis. Some explanations have been proposed for the discrepancy between the negative and positive findings. The most reasonable is the known genetic diversity of the different populations at the haplotype level (16).

There were no substantial differences between the CD patients and healthy controls in the allele or genotype frequencies of CTLA-4 +49A/G. However, in a Tunisian IBD patients-controls study, Alaya et al. (1) found that the A allele and AA genotype of the CTLA-4

gene promoter (49A/G) polymorphism in exon 1 was associated with CD in the entire patient population. Another study, Wang et al. (20), suggested that the +49A allele was significantly related to tumor size in patients (P=0.0033). Farbod et al. (21) investigated the distribution of CTLA-4 SNP (1661A/G) in breast cancer patients and controls and found that the AA genotype was associated with breast cancer.

Furthermore, in this study, the N852S polymorphism of the NOD2/CARD15 gene was genotyped using PCR-RFLP and gene sequencing, and N852S was identified in Turkish patients. The N852S polymorphism of the NOD2/CARD15 gene was not found to be related to IBD susceptibility in these patients. Similarly, Long et al. (18) did not detect the N852S mutation of the NOD2/CARD15 gene in Guangxi Zhuang or Han patients with IBD. However, Tukel et al. (14) found that the N852S mutation of the NOD2/CARD15 gene was significantly related to CD in Ashkenazi Jewish populations. Nevertheless, we did not find any heterozygous or homozygous N852S mutations in the Turkish population. This locus has been reported in the Ashkenazi Jewish population (14), and further studies are necessary to explore this locus in a larger cohort in Turkey. The variation in these results may result from the differences in race, geography, environment, and population.

In conclusion, this is the first study in a Turkish population to report the CTLA-4 gene +49A/G polymorphism and NOD2/CARD15 gene N852S polymorphism in patients with IBD. In this study, the CTLA-4 gene +49A/G polymorphism and NOD2/CARD15 gene N852S polymorphism were investigated in Turkish IBD patients, and we did not find a statistically significant difference between the control and patient groups. In our study, as we studied relatively small samples, the power of this conclusion may be limited. Further studies researching risk factors and genetic susceptibility to IBD in a larger cohort of patients and in different ethnic groups are needed.

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