J Gastroenterol 2006; 41:304–310
DOI 10.1007/s00535-005-1780-z

Journal of Gastroenterology

NOD2/CARD15, *NOD1/CARD4*, and *ICAM-1* gene polymorphisms in Turkish patients with inflammatory bowel disease

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Purpose. The genetic susceptibility of people with certain *NOD2/CARD15*, *NOD1/CARD4*, and *ICAM-1* gene variants to inflammatory bowel disease is still under investigation. The aim of this study was to investigate polymorphisms in the *NOD2/CARD15* (R702W, G908R, and 3020insC), *NOD1/CARD4* (E266K, D372N), and *ICAM-1* (G241R, K469E) genes, which are known to be associated with inflammation, in Turkish patients with inflammatory bowel disease and healthy control groups. ***Methods.*** The genotypes of 70 patients with endoscopically and histopathologically diagnosed Crohn's disease (38 men, 32 women; mean age, 38.8 ± 1.3), 120 patients with ulcerative colitis (67 men, 53 women; mean age, 41.7 ± 1.3) and 106 healthy control subjects (37 men, 69 women; mean age, 35.7 ± 1.4), who stated that they had never had any prior bowel disease history, were compared. A polymerase chain reaction-restriction fragment length polymorphism analysis was performed for two variants of the *ICAM-1* gene, the three main variants of the *NOD2/CARD15* gene, and the E266K variant of the *NOD1/CARD4* gene, and DNA sequencing was used for the D372N polymorphism of the *NOD1/CARD4* gene. ***Results.*** In this study, the three previously described Crohn's disease-predisposing variants of the *NOD2/CARD15* gene and the polymorphisms examined in the *NOD1/CARD4* and *ICAM-1* genes were not found to be associated with ulcerative colitis or Crohn's disease. ***Conclusions.*** These findings suggest that the polymorphisms observed in the *NOD2/CARD15*, *NOD1/CARD4*, and *ICAM-1* genes are not genetic susceptibility factors for Crohn's disease or ulcerative colitis in Turkey.

Received: September 2, 2005 / Accepted: November 17, 2005
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Key words: Crohn's disease, *ICAM*, inflammatory bowel disease, *NOD/CARD*, ulcerative colitis

Introduction

Crohn's disease (CD) and ulcerative colitis (UC), both classified as chronic inflammatory bowel disease (IBD), are characterized by a dysregulated mucosal immune response of the gut. Linkage and epidemiologic studies, including studies of racial and ethnic differences and familial aggregation, and twin studies, suggest that genetic factors play a significant role in IBD susceptibility.[1–5]

The IBD1 locus, located in the vicinity of the centromere region of chromosome 16, was first identified as the CD locus in a genome-wide scan study.[6] Recent studies have shown that single nucleotide polymorphisms in the coding region of the *NOD2/CARD15* (nucleotide oligomerization domain 2/caspase-activating recruitment domain 15) gene, overlapping with the IBD1 locus on chromosome 16q12,[7] are significantly associated with susceptibility to CD.[8–10] In DNA sequence analysis studies of CD patients, two missense mutations (Arg702Trp alteration in exon 4 due to a 2104C→T nucleotide exchange and Gly908Arg alteration in exon 8 due to a 2722G→C nucleotide exchange) and a frameshift mutation due to a cytosine insertion at position 3020 in exon 11 (3020insC/1007fs, Leu1007→Pro) resulting in premature stop codon formation have been found to show significant associations with CD.[9–11] The studies, conducted in various European and American populations, have shown that these three main variants, each as an independent risk factor, are associated with CD,[8,12,13] but not with UC.[11,12] However, in some Asian countries, such as Japan, China, and Korea, no significant association has been found between the *NOD2/CARD15* gene variants and IBD.[14–17]

The studies on *NOD2*/*CARD15*,[7] and *NOD1*/*CARD4* gene products,[18,19] sharing quite a number of structural and functional similarities, have shown that NOD2/CARD15 and NOD1/CARD4 proteins induce nuclear factor κB (NFκB) activation, an effective transcription factor in the secretion of proinflammatory cytokines. It has been reported in several functional studies that all three main variants of the *NOD2*/*CARD15* gene determine a reduced NFκB activation in response to bacterial components,[9,13,20,21] which may alter signaling pathways of the innate immune system.

The *NOD1*/*CARD4* gene has been considered as a candidate gene not only because of the structural and functional similarities of the gene product with NOD2/CARD15 protein but also because of its location on the 7p14-p15 chromosome band, which has been previously reported to contain an IBD susceptibility locus in British families.[22] Among the *NOD1*/*CARD4* gene polymorphisms, E266K (796G→A, in exon 3) and D372N (1114G→A, in exon 3) missense mutations cause amino acid changes in the nucleotide-binding domain of the protein.[23]

Despite the lack of complete etiologic understanding, there are important reasons to believe that defects formed during the regulation of the limited inflammatory response of the gut contribute to the pathogenesis of both UC and CD. Therefore, another important candidate in genetic studies is the intercellular adhesion molecule 1 (ICAM-1), which determines the level of inflammatory response by playing a crucial role in the activation of leukocytes. A genome-wide scan study, in which an association between IBD and chromosome 19p13 (IBD6) was put forward, contributed to the research on the *ICAM-1* gene as well.[24] The *ICAM-1* gene includes two polymorphisms, one at codon 241 (G/R241; Gly/Arg; exon 4) and the other at codon 469 (K/E469; Lys/Glu; exon 6).[25]

The polymorphisms identified in the *NOD1*/*CARD4*, *NOD2*/*CARD15*, and *ICAM-1* genes, which play important roles in the inflammatory pathway, have been studied in quite a large number of populations, and genetic differences among distinct populations have been found. We, therefore, decided to perform a detailed study on the variations of the candidate genes *NOD2*/*CARD15*, *NOD1*/*CARD4*, and *ICAM-1* in a Turkish population, where no such studies had been carried out before.

Methods

Patients and healthy controls

In this study, a total of 70 patients with Crohn's disease (38 men, 32 women; mean age, 38.8 ± 1.3), 120 patients with ulcerative colitis (67 men, 53 women; mean age, 41.7 ± 1.3) and 106 healthy control subjects (37 men, 69 women; mean age, 35.7 ± 1.4) were investigated. Diagnosis and classification of IBD was made according to previously established international criteria.[26] Blood samples were obtained from CD and UC patients, all of whom were recruited from the Gastroenterology Department of Medical Faculty of Ankara University and Gastroenterology Clinic of Türkiye Yüksek Ihtisas Hospital, Ankara. As for the healthy control group, we collected blood samples from people who stated that they had not had any bowel disease. The study protocol and all study documents conformed to the ethical guidelines of the World Association Helsinki Declaration, amended in 2000, and were approved by the Ethics Committee of the Medical School of Ankara University. Informed consent was obtained from all patients for the use of their samples for the study.

Genotyping methods

Samples of genomic DNA were obtained from peripheral blood leukocytes, by using the high salt concentration method for genomic DNA isolation.[27] We analyzed the obtained genomic DNA samples using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods for the three main variants of the *NOD2*/*CARD15* gene (R702W, G908R, 3020insC), two single nucleotide exchanges of the *ICAM-1* gene (R241G, K469E), and the single nucleotide polymorphism (SNP) at codon 266 of the *NOD1*/*CARD4* gene. DNA sequencing was performed to detect the single nucleotide polymorphism of the *NOD1*/*CARD4* gene at codon 372. The PCR primer pairs (Table 1) and the restriction enzymes described by Heliö et al.,[28] Gbadegesin et al.,[29] and Zouali et al.[23] were used for PCR amplification and the restriction enzyme cleavage of *NOD2*/*CARD15*, *ICAM-1*, and *NOD1*/*CARD4* genes, respectively. The restriction enzymes, the restriction fragment lengths, and the genotyping can be seen in Table 2. The PCR conditions were as follows: initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min annealing at 58°C for codon 702 of *NOD2*/*CARD15* and codon 266 of *NOD1*/*CARD4*, 60°C for codons 908 and 1007 (3020insC) of *NOD2*/*CARD15*, codon 372 of *NOD1*/*CARD4*, and codon 469 of *ICAM-1*, and 52°C for codon 241 of *ICAM-1* for 1 min and extension at 72°C for 2 min, followed by a final extension step at 72°C for 7 min. A total of seven target sequence regions were amplified by PCR using an Eppendorf MasterCycler Personal thermocycler (Eppendorf, Hamburg, Germany).

Restriction endonuclease digestions were carried out by providing convenient conditions for each region for a total of 15 h at appropriate temperatures. Subsequent

Table 1. Primers used for PCR and sequencing

Exon	Variants	Primers	Reference no.
4	R702W	F: 5′-AGATCACAGCAGCCTTCCTG-3′ R: 5′-CACGCTCTTGGCCTCTCACC-3′	28
8	G908R	F: 5′-CTCTTTTGGCCTTTTCAGATTCTG-3′ R: 5′-CAGCTCCTCCCTCTTCACCT-3′	28
11	1007fs	F: 5′-GGCAGAAGCCCTCCTGCAGGGCC-3′ R: 5′-CCTCAAAATTCTGCCATTCC-3′	28
3	E266K	F: 5′-TGAGACCATCTTCATCCTGG-3′ R: 5′-CTTCCCACTGAGCAGGTTG-3′	23
3	D372N	F: 5′-GTGCCTGACAGCTCCTGC-3′ R: 5′-CAGGGTCATCGTGCAGTCG-3′	23
4	G241R	F: 5′-CGTGGTCTGTTCCCTGT-3′ R: 5′-CTCCTGGCTCTGGTTCC-3′	29
6	K469E	F: 5′-TTCCCAGCAGACTCCAATGT-3′ R: 5′-GGATACAACAGGCGGTGAGG-3′	29

F, forward primer; R, reverse primer

Table 2. Genotyping procedures for the variants used for PCR-RFLP

Position	Restriction endonuclease enzyme	PCR product length (bp)	Digested fragment length (bp)	Genotype
NOD2/CARD15				
2014C → T (R702W)	*Msp*I (*Hpa*II) (Fermentas, Lithuania)	185	76, 54, 35 130, 76, 54 130, 35	C/C C/T T/T
2722G → C (G908R)	*Hin*6I (*Hha*I) (Fermentas, Lithuania)	163	163 163, 136 136, 27	G/G G/C C/C
3020insC (1007fs)	*Apa*I (Roche, Penzberg, Germany)	151	151 151, 131 131, 20	WT/WT WT/MT MT/MT
NOD1/CARD4				
796G → A (E266K)	*Eco*88I (*Ava*I) (Fermentas, Lithuania)	379	379 379, 209 209, 170	G/G G/A A/A
ICAM-1				
G241R	*Bsr*GI (BilLabs, Beverly, MA, USA)	199	199 199, 180 180, 19	G/G G/R R/R
K469E	*Bst*UI (BilLabs, Beverly, MA, USA)	268	268 268, 170 170, 98	K/K K/E E/E

WT, wild-type allele; MT, mutant-type allele (insertion of C nucleotide at position 3020); bp, base pairs; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism

analyses were performed using 2%–4% agarose gel electrophoresis. The PCR product of *NOD1/CARD4*, which was amplified to detect the SNP at codon 372, was purified by using a Nucleospin extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The same forward sequence primer used in the PCR amplification was utilized for the cycle-sequencing product in the BigDye Terminator v3.1 cycle sequencing system (PE Applied Biosystems, Foster City, CA, USA). After amplification, the products were purified by sodium acetate–ethanol precipitation. Then, the purified products were analyzed with an ABI Prism 310 genetic analyzer (PE Applied Biosystems).

Statistical analysis

The χ-squared and Fisher's exact tests were used to compare the differences between the frequencies of the *NOD1/CARD4*, *NOD2/CARD15*, and *ICAM-1* gene

polymorphisms in patients and controls. A P value of <0.05 was considered to be significant. Allele frequencies were calculated as the percentages of variant alleles.

Results

Comparisons of gene variants between UC or CD and controls

NOD2/CARD15 *gene variants*

The genotypes and the allele frequencies of the three main variants of the *NOD2/CARD15* gene in 70 CD and 120 UC patients and 106 healthy controls are given in Table 3. At codon 702, the frequency of the rare allele (T) was 1.4% in patients with CD, 1.7% in patients with UC, and 1.9% in the healthy control group. There were no significant differences between either patient group and the control group (Table 3). The homozygous mutant genotype for the codon 908 variant was found in only one of the CD patients, and the frequency of the rare allele (C) leading to the formation of arginine was not significantly different between the groups (Table 3).

In our study, the heterozygous mutant genotype for the insertion mutation at position 3020 was observed in only two of the 70 CD patients. However, the rare allele (3020insC) was not detected in either the UC or healthy control group. In previous studies of populations in Europe and the United States, the allele frequency of the 3020insC mutation has been reported to be 2%–4%,[8–10] but it was 1.4% in Turkish CD patients (Table 3). In addition, the genotype distributions of all three main *NOD2/CARD15* variants was not significantly different between either of the patient groups and the control group (Table 3). Moreover, when the genotypes carrying at least one rare allele for the polymorphisms studied in *NOD2/CARD15* were examined, no statistically significant difference between groups was found (Table 4).

NOD1/CARD4 *gene variants*

In the present study, we analyzed gene polymorphisms at codon 266 using PCR-RFLP. The genotype distributions of E266K variants were found to be similar in both patient and control groups (Table 3). In addition, the frequency of both alleles of E266K, showing a high degree of polymorphism, was similar in both CD and UC groups, and no statistical significance was found when the groups were compared with each other or with the control group (Table 3). The gene polymorphism at codon 372 (1114G→A) was analyzed using the DNA sequencing method. All 70 CD and all 120 UC patients carried allele G at position 1114. Only one healthy control was homozygous for allele A. No significant difference was observed for codon 372 between the patient and control groups (Table 3).

ICAM-1 *gene variants*

In our study, we performed PCR-RFLP to analyze both of the *ICAM-1* gene polymorphisms. The R241 allele at codon 241 was not detected in either patient group or in the control group; both patient and control groups had the G/G genotype (Table 3). At codon 469, the K469 allele was found to be more frequent than the E469 allele. The allele frequencies of K469 in both CD and UC patient groups were similar, 57.9% and 60.8%, respectively. However, the frequency was higher (65.6%) in the healthy control group (Table 3). The allele frequency of E469 was higher in both the CD and UC patient groups (42.1% and 39.2%, respectively) than in the healthy control group (34.4%) (Table 3). There was no statistical difference between patient and healthy control groups regarding either the genotype or the allele distributions of the two *ICAM-1* gene polymorphisms.

Discussion

In the present study, we tried to establish an association between the inflammatory bowel diseases CD and UC and the three main variants in the coding region of the *NOD2/CARD15* gene, the two polymorphisms in the nucleotide-binding domain of the *NOD1/CARD4* gene, and the two polymorphisms of the *ICAM-1* gene in a Turkish population. The polymorphic regions were not statistically different between CD or UC patients and healthy control subjects.

It has been suggested that *NOD2/CARD15* gene polymorphisms explain about 20% of the genetic predisposition to CD in United States and European populations.[30] However, several studies have shown that *NOD2/CARD15* gene polymorphisms are not disease factors in Asian populations. In some of these patient–control studies of Japanese, Chinese, and Korean populations, the main *NOD2/CARD15* polymorphisms were not encountered in patient or in control groups.[14–17] In this study, the R702W and G908R variants were observed in almost equal frequencies in both patient and healthy control groups, and the frequency of the 3020insC/1007fs rare variant allele was found to be 1.4% in CD patients. Although no significant association was found between these variations and CD or UC, the results of this study showed the presence of the *NOD2/CARD15* gene variants in the Turkish population, although they are absent from most Asian populations.

The association between IBD and the *NOD1/CARD4* gene has been studied by SNP identification

Table 3. Genotype and allele distributions of *NOD2/CARD15*, *NOD1/CARD4*, and *ICAM-1* genes

Gene variants	CD $n = 70$	UC $n = 120$	Healthy controls $n = 106$
NOD2/CARD15 gene			
2014C → T (R702W), no. (%)			
C/C	68 (97.1)	116 (96.7)	102 (96.2)
C/T	2 (2.9)	4 (3.3)	4 (3.8)
T/T	0	0	0
Allele frequency (%)			
C	98.6	98.3	98.1
T	1.4	1.7	1.9
2722G → C (G908R), no. (%)			
G/G	68 (97.2)	118 (98.3)	104 (98.1)
G/C	1 (1.4)	2 (1.7)	2 (1.9)
C/C	1 (1.4)	0	0
Allele frequency (%)			
G	97.9	99.2	99
C	2.1	0.8	1
3020insC (1007fs), no. (%)			
WT/WT	68 (97.1)	120 (100)	106 (100)
WT/MT	2 (2.9)	0	0
MT/MT	0	0	0
Allele frequency (%)			
WT	98.6	100	100
MT	1.4	0	0
NOD1/CARD4 gene			
796G → A (E266K), no. (%)			
G/G	9 (12.9)	16 (13.3)	3 (2.8)
G/A	54 (77.1)	84 (70)	89 (84)
A/A	7 (10)	20 (16.7)	14 (13.2)
Allele frequency (%)			
G	51.4	48.3	44.8
A	48.6	51.7	55.2
1114G → A (D372N), no. (%)			
G/G	70 (100)	120 (100)	105 (99.1)
G/A	0	0	0
A/A	0	0	1 (0.9)
Allele frequency (%)			
G	100	100	99.1
A	0	0	0.9
ICAM-1 gene			
G241R, no. (%)			
G/G	70 (100)	120 (100)	106 (100)
G/R	0	0	0
R/R	0	0	0
Allele frequency (%)			
G	100	100	100
R	0	0	0
K469E, no. (%)			
K/K	16 (22.9)	30 (25)	36 (34)
K/E	49 (70)	86 (71.7)	67 (63.2)
E/E	5 (7.1)	4 (3.3)	3 (2.8)
Allele frequency (%)			
K	57.9	60.8	65.6
E	42.1	39.2	34.4

WT, wild-type allele; MT, mutant-type allele (3020insC); CD, Crohn's disease; UC, ulcerative colitis

Table 4. People carrying at least one mutant allele in the *NOD2/CARD15* gene

Group	Number of people (*n*)	Wild-type genotype		Heterozygous/ homozygous	
		n	%	*n*	%
UC	120	114	95	6	5
CD	70	64	91.4	6	8.6
Control	106	100	94.3	6	5.7

and genotyping in two studies so far.[23,31] The methods used and the results obtained in our study for the *NOD1/CARD4* gene codon 266 and codon 372 variants share some similarities with those used by Zouali and his colleagues.[23] The codon 372 rare variant in Turkish patients and the healthy control group, searched for by DNA sequence analysis, was found to be homozygous in only one person in the healthy control group. The absence of the rare allele in CD and UC patients in our study shows that this variant might not be associated with IBD. The SNP at codon 266, the conserved region in the *NOD2/CARD15* gene, was identified as the most frequent polymorphism in a European study performed by Zouali and his colleagues.[23] However, it was not found to be associated with IBD. In our study, the frequent occurrence of an SNP in codon 266 in UC and CD patients and in healthy control subjects and the lack of an association with IBD support the findings of Zouali and his colleagues.[23] In a recent study of a Caucasian population in the U.K., the frequency of the SNP at codon 266 (E266K) was found to be less than 1%,[31] showing a difference compared with our study. In the study of McGovern and his colleagues,[31] an association between the deletion allele of a complex functional *NOD1* polymorphism (ND_1 + 32656*1), which affects the binding of an unknown nuclear factor,[32] and IBD has been identified. This study indicates the necessity of further investigations dealing with regulatory polymorphisms in the *NOD1/CARD4* gene in the Turkish population.

Leukocyte adhesion is the most crucial step in inflammation. Hence, a genetic defect in the intercellular adhesion molecule ICAM-1 gives rise to differences in leukocyte behavior. Two SNPs G241R and K469E, leading to amino acid exchange have so far been identified in the *ICAM-1* gene.[25] The former has been suggested to affect the binding function of ICAM-1, because of its location in the binding region of the macrophage antigen-1 (Mac-1) leukocyte integrin, which is the third immunoglobulin-like domain of Mac-1.[25] The latter, which leads to an amino acid exchange from glutamic acid to lysine in the fifth immunoglobulin-like domain of ICAM-1, was suggested to alter the interaction between the lymphocyte function antigen-1 (LFA-1) and B cells.[33] Therefore, *ICAM-1* gene polymorphisms have been extensively searched for in various genetic studies of patient–control groups in various populations.[34–36]

The R241 allele, which was found to be a risk factor for UC in a study of a German population,[36] was not observed at all in our study. Some studies have shown that the frequency of R241 allele is quite low in Mediterranean populations[37,38] compared with in some European[36,39,40] and North American countries.[34] R241 was not observed at all in a study of a Japanese population.[41] The results of our study, showing the absence of the R241 allele, support these results. This difference in the R241 allele in different geographical regions may be due to the different ethnicities of the populations studied.

In a study in Japan, Matsuzawa and his colleagues[35] found that the K469 allele was associated with both UC and CD. In our study, the K469 allele was more frequent (65.6%) in the control group than in the CD or UC patient groups (57.9% and 60.8%, respectively); however, the difference was not statistically significant. The homozygous E/E genotype, which was reported to be associated with both UC and CD in Italy,[42] also a Mediterranean country, was not found to be associated with IBD in our study.

In our study, there was no association between IBD and *NOD2/CARD15*, *NOD1/CARD4*, or *ICAM-1*. The genetic predisposing factors in IBD may vary among different populations because IBD is known to be a disease with multiple etiologies, including quite a number of different genetic abnormalities. Different genes in different individuals are thought to cause genetic susceptibility to the disease because of genetic heterogeneity. Therefore, because of this genetic heterogeneity, the effect of a single gene that has been reported to be associated with IBD in a particular population may not be observed in another, so the studies may not support each other.

Acknowledgments. We would like to acknowledge the financial support received from the Institute of Biotechnology, Ankara University, grant no 2004/134.

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