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Full Length Research paper

Evaluation of the association of NOD2/CARD15 gene polymorphisms with clinical course of Turkish Crohn's disease patients

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NOD2/CARD15 gene variants may be associated with distinct phenotypic expressions of Crohn's disease, however, this association may change according to the ethnic and regional variation. The aim of this study was to analyze the impact of NOD2/CARD15 gene mutations on disease phenotype in Turkish Crohn's disease patients. Fourty-five Crohn's disease patients (32 males, 13 females) with a mean age of 38.7 ± 12.1 (range: 19-78) were enrolled into this prospective study. The three major polymorphisms (R702W, G908R, 3020insC) on NOD2/CARD15 gene were studied from the peripheral blood genomic DNA. R702W and G908R mutations were studied by PCR-RFLP method, and 3020insC mutation was studied by DNA sequencing. No homozygous mutation was detected. Heterozygous R702W, G908R, and 3020insC mutations were detected in 4, 3, and 4 patients, respectively. The frequency of R702W, G908R, and 3020insC mutations was found to be 4.4, 3.3, and 4.4%, respectively. The overall mutation frequency was found to be 12.2%. There was no statistically difference between the clinical course of the patients with (n = 11) and without (n = 34) mutations (p>0.05). NOD2/CARD15 gene polymorphisms do not have impact on disease phenotype in Turkish Crohn's disease patients.

Key words: NOD2/CARD15 gene, Crohn's disease, phenotype.

INTRODUCTION

NOD2/CARD15 mutations are associated with susceptibility to Crohn's disease (CD) but not ulcerative colitis (Hampe et al., 2001; Hugot et al., 2001). Three major mutations (R702W, G908R, and 1007fs) were confirmed to be independently associated with susceptibility to CD (Lesage et al., 2002). However, this impact changes according to the ethnic and even regional variation. Frequencies of the NOD2/CARD15 gene mutations have been reported up to 50% of central Europeans with CD (Lesage et al., 2002), however, a considerable regional variation occurs even in Europe (Heliö et al., 2003; Hampe et al., 2002; Bairead et al., 2003; Arnott et al., 2004). On the other hand, no association between the NOD2/CARD15 gene variants and CD have been reported from Asian countries (Inoue et al., 2002; Leong et al., 2003). To our knowledge, three studies with Turkish CD patients (Ozen et al., 2006; Uyar et al., 2006; Ince et al., 2008) and one study with Turkish Behcet's disease patients (Uyar et al., 2004) have been previously performed to investigate the polymorphisms in the NOD2/CARD15 gene. However, it seems likely that lack of data exists about the association of NOD2/CARD15 genotype with clinical course of Turkish CD patients. The aim of this study was to define the impact of the NOD2/CARD15 gene mutations on Turkish CD patients phenotype.

MATERIALS AND METHODS

Patients

Fourty five sporadic CD patients were enrolled into the study. The diagnosis of CD, disease behaviour and locations were established on clinical, radiological, endoscopical and histopathological findings. Written, signed consent was obtained from all participants.

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Table 1. NOD2/CARD15 gene mutations in 45 sporadic Crohn's disease patients.

Mutation	Patients (n=45) (n, %)	Frequency of mutant genes (n, %)
No mutation	34 (75.6%)	
Homozygous mutation	0 (0%)	
Heterozygous mutation	11 (24.4%)	11 / 90 (12.2%)
R702W mutation	4 (8.8.%)	4 / 90 (4.4%)
G908R mutation	3 (6.7%)	3 / 90 (3.3%)
3020insC mutation	4 (8.8.%)	4 / 90 (4.4%)

Genotyping methods

2 ml of whole blood samples was collected into EDTA–anticoagulated tubes by standard venipuncture method. Genomic DNA was extracted from EDTA–anticoagulated whole blood samples employing the QIAmp Blood DNA mini-kit (Qiagen, Hilden, Germany) following manufacturer's instructions. DNA concentration was determined by the PicoGreen dsDNA quantitation kit (Molecular Probes Inc., Eugene, OR) according to the manufacturer's instructions and diluted as 100 ng/µl.

R702W and G908R mutations were studied by PCR-RFLP method, and 3020insC mutation was studied by DNA sequencing (Hugot et al., 2001; Lesage et al., 2002; Ogura et al., 2001). Briefly, G908R polymorphism was assayed by PCR-RFLP method. For investigation of missense mutation G908R (GenBank accession No. G67951) PCR amplifications for each sample were carried out with the following primer pairs: 5'forward-CCCAGCTCC-TCCCTCTTC-3' and 5'reverse-AAGTCTGTAATGTAAAGCCAC-3' which were synthesized by Invitrogen, Life Technologies (Inchinnana, Paisley, UK). Amplification was carried out on a Gene-Amp 9700 PCR System (PE Applied Biosystems, Foster City, CA) in a 25 µl reaction mixture in 0.2 ml thin-wall PCR strip tubes (Axygen Scientific, Inc., CA) containing 1 µl genomic DNA solution, GeneAmp Gold Buffer (15 mmol/l Tris-HCl, pH 8.0, 50 mmol/l KCl; PE Applied Biosystems), 2.0 mmol MgCl2, 50 µmol/l each of the dGTP, dATP, dTTP and dCTP (Promega, Madison, WI), 5 pmol each forward and reverse primers and 1.0 U AmpliTaq Gold Polymerase (PE Applied Bio-systems). The cycling conditions comprised a hot start at 95℃ for 10 min, followed by 35 amplification cycles at 95℃ for 30 s, 55℃ for 30 s, and 72℃ for 25 s, followed by one elongation step at 72℃ for 5 min.

The missense mutation R702W (GenBank accession No. G67950) was genotyped by amplification refractory mutation system with primers 5'-ATCTGAGAAGGCCCTGCTCC-3' (wild type sense), 5'-ATCTGAGAAGGCCCTGCTCT-3' (mutated sense) and 5'-CCCACACTTAGCCTTGATG-3' (antisense). The annealing temperature was 58℃. The PCR products were loaded on a 2% agarose gel with internal control. Genotypes were directly deduced from the migration profiles. For 3020insC mutation (GenBank accession No. G67955) at exon 11 of the CARD15 gene was amplified using the following primers: primer 5'-GGGACAGGTGGGCT-TCAGTA-3' (sense), primer 5'-CCATTCCTCTCTCCCG-TCAC-3' (antisense) (3). The annealing temperature was 62℃. Before sequencing, the PCR products were purified using QIAquick PCR Purification Kit (Qiagen). DNA sequencing of the amplified exon was performed by cycle sequencing with fluorescent dye terminators. Analysis was performed using an ABI 310 auto-matic sequencer (Applied Biosystems). The analysis was confirmed by sequencing in both directions. For sequence evaluation, the pro-gram Sequencher was used.

Statistical analysis

Fisher's exact test was used to compare the clinical course of the patients with and without mutations. Comparison of the age at diagnosis of the patients with and without mutation was performed by Mann-Whitney U test. A p-value less than 0.05 was accepted as statistically significant.

RESULTS

There were 32 males and 13 females CD patients with a mean age of 38.7 ± 12.1 (range: 19 - 78) years. None of the patients had a family history for CD. The involvement sites of the disease were: ileocolon (n = 20, 44%), ileum (n = 17, 38%), and colon (n = 8, 18%). Inflammatory, penetrating and stricturing disease behaviours were determined in 30 (67%), 9 (20%) and 6 (13%) patients, respectively.

Overall, 11 (12.2%) heterozygous mutations were detected, and there was no homozygous mutation (Table 1). No patient had double mutations. There was no statistically difference between the clinical course (age at diagnosis, sex, smoking, location and behaviour of the disease, extraintestinal manifestation, history of bowel operation and appendectomy) of the patients with (n = 11) and without (n = 34) mutations ($p>0.05$) (Table 2).

DISCUSSION

There was no control group in this study since we did not aim to identify the association between frequency of NOD2/CARD15 gene mutations and Turkish CD patients. The frequencies of NOD2/CARD15 gene mutations in Turkish CD patients have already been investigated in other studies (Ozen et al., 2006; Uyar et al., 2006; Ince et al., 2008). These data are summarized in Table 3. Among these studies, Uyar et al. (2006) found that only the frequency of the G908R variant allele was statistically higher than the controls (8% vs 0%, p = 0.0002), but there was no difference in terms of the frequency of the R702W and 1007fs mutant alleles between the CD patients and the controls. Other two studies (Ozen et al., 2006; Ince et al., 2008) also have found no association between the CD patients and the major three (R702W, G908R, and 1007fs) variant alleles. When considering all the three studies, we can clearly state that NOD2/-CARD15 genes are not genetic suspectibility factors for Turkish CD patients.

On the other hand, we believe that there is insufficient

Table 2. Comparison of the clinical course of 45 sporadic Crohn's disease patients with and without mutations.

Clinical properties	Mutation negative (n = 34)	Mutation positive (n = 11)	p
Age at diagnosis (mean ± SD, years)	30,1 ± 13,7	28,5 ± 16,7	ns
Sex (male / female)	24 / 10	8 / 3	ns
Smokers (n, %)	19 (55.9%)	7 (63.6%)	ns
Location of the disease			
Ileocolon (n, %)	15 (44.1%)	5 (45.4%)	ns
Ileum (n, %)	13 (38.2%)	4 (36.4%)	ns
Colon (n, %)	6 (17.7%)	2 (18.2%)	ns
Behaviour of the disease			
Inflammatory (n, %)	23 (67.6%)	7 (63.6%)	ns
Penetrating (n, %)	7 (20.6%)	2 (18.2%)	ns
Stricturing (n, %)	4 (11.8%)	2 (18.2%)	ns
Diagnosed during appendectomy (n, %)	3 (8.8%)	1 (9.1%)	ns
History of bowel operation (n, %)	12 (35.3%)	4 (36.4%)	ns
Extraintestinal manifestation (n, %)	3 (8.8%)*	1 (9.1%)**	ns

Ns, not significant; *, Seronegative spondyloarthropathy (2 patients), erythema nodosum (1 patient); **, Seronegative spondyloarthropathy.

Table 3. Summary of the studies investigating NOD2/CARD15 gene mutations in Turkish Crohn's disease patients.

Reference (author)	Mutation type	Allele frequency (%)		P	Genotype-phenotype analyses
		Patients	Control		
10 (Ozen et al.)		n = 70	n = 106		No
	R702W allele	1.4	1.9	ns	
	G908R allele	2.1	1	ns	
	3020insC allele	1.4	0	ns	
	Overall allele	4.9	2.9	ns	
11 (Uyar et al.)		n = 56	n = 100		Location and behaviour
	R702W allele	0.9	0.5	ns	
	G908R allele	8	0	0.0002	
	3020insC allele	1.8	1	ns	
	Overall allele	10.7	1.5	0.0005	
12 (Ince et al.)		n = 67	n = 87		Disease- related surgery
	R702W allele	0.7	0.6	ns	
	G908R allele	0.7	1.2	ns	
	3020insC allele	0.7	4.8	ns	
	Overall allele	2.2	6.6	ns	
This study		n = 45	n = 0		Detailed (Table 2)
	R702W allele	4.4	-	-	
	G908R allele	3.3	-	-	
	3020insC allele	4.4	-	-	
	Overall allele	12.2	-	-	

data about the impact of NOD2/CARD15 mutations on the phenotype of the disease in Turkish CD patients. The study by Ozen et al. (2006) had no analyses on genotype-phenotype association. The other study by Uyar et al. (Uyar et al., 2006) concluded that there was the absence of the significant association between the disease location and behaviour. The last study by Ince et al. (2008) has analyzed the impact of the NOD2-/CARD15 mutations on disease-related surgery, and it revealed that there was no association. In this study we have investigated the association of the NOD2/CARD15 mutations and detailed clinical course (age at diagnosis, sex, smok-

ing, location and behaviour of the disease, extraintestinal manifestation, history of bowel operation and appendectomy) of the patients; no significant association was found.

Many studies, especially from Europe, concluded that NOD2/CARD15 variants are associated with early onset disease (Lesage et al., 2002; Zhou et al., 2002; Bonen et al., 2003), involvement of the ileum (Heliö et al., 2003; Ahmad et al., 2002; Cuthbert et al., 2002; Mendoza et al., 2003; Heresbach et al., 2004; Annese et al., 2005; Törkvist et al., 2006; Laghi et al., 2005), stricturing (Lesage et al., 2002; Heliö et al., 2003; Mendoza et al., 2003; Heresbach et al., 2004; Annese et al., 2005; Abreu et al., 2002) and penetrating disease (Heliö et al., 2003; van der Linde et al., 2007), previous appendectomy (Mendoza et al., 2003; Bianchi et al., 2007), history of bowel surgery (Heliö et al., 2003; Mendoza et al., 2003; Annese et al., 2005; Laghi et al., 2005), and higher rate of surgical recurrence (Alvarez-Lobos et al., 2005) suggesting more aggressive behaviour. However, this impact may show diversity according to the ethnic and regional variation. In addition to the studies with Asian (Inoue et al., 2002; Leong et al., 2003) and Afro-American (Bonen et al., 2003) patients documenting no association between the NOD2/CARD15 gene variants and CD, a study from Israel revealed no effect of NOD2/CARD15 gene mutations on phenotype in both Israeli Arabs and Israeli Jewish patients (Karban et al., 2005). Previous two studies from Turkey also documented the lack of impact of NOD2/CARD15 gene mutations on disease location and behaviour (Uyar et al., 2006) and disease-related surgery (Ince et al., 2008). The present study clearly documents the absence of association between the NOD2/CARD15 gene mutations and detailed disease phenotype.

In conclusion, genetic polymorphism of NOD2/CARD15 gene does not effect the clinical course of the Turkish CD patients. We suggest that further genetic characterizations may be needed for Turkish CD patients.

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