Inflammatory bowel disease (IBD) is a chronic inflammation of the gastrointestinal tract, comprising Crohn’s disease (CD) and ulcerative colitis (UC), with combined prevalence of 0.2% in Caucasians. Though the prevalence in Iran is not known, it appears that the number of affected individuals is rising. Genetic factors contribute to susceptibility to IBD. Both CD and UC are considered complex genetic traits, as inheritance does not follow any simple Mendelian model. Since the first report in 1996, more than nine IBD susceptibility loci have been identified by wide genome scans, including loci on chromosomes 16q12 (IBD1), 12q13 (IBD2), 6p13 (IBD3), 14q11 (IBD4), 5q31-33 (IBD5), 19p13 (IBD6), 1p36 (IBD7), 16p (IBD8), 3p (IBD9) and 7q. Within these susceptibility loci, a wide variety of candidate genes have been studied. Hugot et al. and Ogura et al. independently reported the first CD-associated gene, NOD2 (nucleotide oligomerisation domain 2), located on chromosome 16q12 (IBD1) within the area of strongest linkage to CD. NOD2, also known as CARD15 (Caspase Activating Recruitment Domain 15), is related structurally to the R proteins in plants, which mediate host resistance to microbial pathogens. CARD15 protein is expressed in cytoplasm of peripheral blood monocytes, paneth cells and intestinal epithelial cells, and acts as an intracellular recognition protein for bacterial components such as peptidoglycans, and activates the nuclear factor kappa B pathway which regulates apoptosis and inflammation. More than 60 sequence variants have been identified in the CARD15 gene. Three major coding recognition polymorphisms (R702W, G908R, 1007fsinsC) are associated with susceptibility to CD in many Caucasian populations. European and North American patients with Crohn’s disease are more likely to have these major variants of CARD15 than healthy population. In many studies, 10% to 30% of CD patients are heterozygotes for one of these 3 common mutations, whereas 3% - 15% of patients are either homozygotes or compound heterozygotes. Recent studies have reported a variable association of CARD15 with CD in different populations. Particularly, no disease association with CARD15 common variants was

Frequency of three common mutations of CARD15/NOD2 gene in Iranian IBD patients


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Background: The CARD15/NOD2 gene, located on the pericentromeric region of chromosome 16 (IBD 1) has been reported to have an association with IBD, especially Crohn’s disease. Three common mutations of CARD15 are variably associated with Crohn’s disease in different ethnic groups. We evaluated the frequency of these mutations (R702W, G908R and 1007fsinsC) in Iranian IBD patients and compared it with the healthy control population.

Methods: One hundred patients with ulcerative colitis, 40 patients with Crohn’s disease, and 100 sex- and age-matched controls were enrolled from a tertiary center during a one-year period (2005-2006). The three mutations were assessed in DNA of leukocytes by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP).

Results: The frequency of R702W mutation was significantly higher in Iranian patients with Crohn’s disease (p<0.001; OR 19.21; 95% CI 4.23-87.32) compared to healthy controls. No association was observed between the other mutations and Crohn’s disease and none of these mutations was associated with ulcerative colitis.

Conclusion: The R702W mutation of CARD15 gene was associated with Crohn’s disease in the Iranian population.

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documented in Chinese, Japanese and Korean CD patients.\textsuperscript{13,14}

Considering the limited data on CARD15/NOD2 variants in Iranian population, our aim was to investigate the frequency of three common CARD15/NOD2 variants (R702W, G908R, 1007fsinsC) in Iranian patients with IBD and compare it with matched healthy controls.

**Methods**

One hundred UC patients and 40 CD patients referred to a tertiary center in a one-year period (2005-2006) were included; none of the patients were members of the same family. One hundred age- and sex-matched healthy individuals without any GI symptoms, or positive familial or personal history of GI disorders were also enrolled. All participants were of Iranian origin. The diagnosis of UC and CD was made by a gastroenterologist and based on endoscopic, radiological, and histopathologic examinations. Patients with a diagnosis of indeterminate colitis were excluded. Demographic data and disease information were recorded in a registry form at the time of enrollment. The study protocol was approved by the ethics committee of the Research Center for Gastroenterology and Liver Diseases, Tehran, Iran, and written informed consent was obtained from all participants.

Genomic DNA was extracted from peripheral blood leukocytes by standard salting out method. Each CARD15 mutation was assessed using initial amplification of the DNA sample by polymerase chain reaction (PCR) and subsequent analysis of the PCR products by restriction fragment length polymorphism (RFLP). DNA fragments were amplified with specific primers\textsuperscript{15} for each variant by a personal thermal cycler (Eppendorf, Germany). The PCR protocol for R702W and 1007fsinsC mutations was as follows: initial 5 min at 94°C followed by 35 cycles, consisting of denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C. The same protocol was used for G908R variant with annealing temperature of 61°C. DNA amplification was analyzed on 1.5% agarose gel stained with ethidium bromide and visualized under ultraviolet light. For assay of the R702W mutation, the PCR product (185 bp in size) was digested at 37 °C for 16 h with 2U \textit{Hha} I (Fermentase, Germany), the following fragments were obtained: 163 bp in G908R homozygotes; 27, 136, and 163 in G908R heterozygotes; and 27 and 136 bp in R908R homozygotes. In order to detect the 1007fs mutation, the amplified fragment was 151 bp in size. After digestion at 37°C for 16 h with 2U \textit{Apa} I (Fermentase, Germany), the following panel was obtained: 151 bp for Leu1007Leu homozygotes; 20, 131, and 151 bp in Leu1007Pro heterozygotes; and 20 and 131 bp in Pro1007Pro homozygotes. Digested fragments were separated using gel electrophoresis on 12% polyacrylamide. Twenty-four samples were randomly selected and sequenced with same primers to evaluate the accuracy of the RFLP method.

Genotype and allele frequencies for these polymorphisms were calculated from observed genotype counts. These frequencies were assessed for association with UC and CD, using the standard \(\chi^2\) test. Results were analyzed by the SPSS software (version 13, USA), odds ratio (OR) were given with 95% confidence intervals, and \(p<0.05\) was considered significant.

**Results**

The mean (SD) age of CD patients was 36.6 (14.1) years, of UC patients was 38.6 (14.3) years and of the control subjects was 38.6 (14.2) years. Fifty-six patients each in UC and control group, and 22 CD patients were women.

The genotype and allele frequencies of the three common CARD15 variants in patients and controls are shown in Table 1. They were all in Hardy-Weinberg

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Alleles</th>
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<tbody>
<tr>
<td>R702W</td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>C/C 27</td>
</tr>
<tr>
<td>UC</td>
<td>C/T 13</td>
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<tr>
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<tr>
<td>G908R</td>
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<tr>
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<td>1007fsinsC</td>
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<tr>
<td>CD</td>
<td><em>-</em> 38</td>
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<tr>
<td>UC</td>
<td>*/+ 100</td>
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<tr>
<td>Controls</td>
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Table 1. Genotype and allele frequencies of CARD15/NOD2 variants in Iranian patients with inflammatory bowel disease and healthy controls.
equilibrium. Only two mutant alleles were detected for each variant of CARD15 gene (total 6 mutant alleles) in controls. Among these three variants, a significantly higher frequency of R702W mutant allele was observed in CD patients (p<0.001; OR 19.21; 95% CI, 4.23-87.32). The frequency of R702W heterozygote genotype was significantly higher in CD patients compared to controls (p<0.001; OR 23.59, 95% CI, 5.02-110.98). In addition, none of 2 remaining variants (G908R and 1007fsinsC) were significantly more frequent in CD or UC patients compared to controls. Although the frequency of these variants (G908R, 1007fsinsC) was higher in CD patients than controls, no statistical significance was observed (p=0.056).

Less than 40% of CD patients (32.5%) were heterozygous carriers for one of these variants (Table 2). The number of CD patients carrying any variant was also significantly higher compared to controls (p<0.001; OR 10.44; 95% CI, 3.69-29.55). There was one combined heterozygote of R702W and G908R variants; one showed a combination of R702W and 1007fsinsC variants and one carried three different mutant alleles. No healthy subjects showed the compound heterozygous state.

Considering the 3 variants together, no difference was found in UC patients in comparison to controls.

**Discussion**

In our study, only the association of R702W variant with CD was statistically significant (Table 1). The frequency of mutant allele was significantly higher in CD patients compared to controls (16.25% vs. 1%, p<0.0001; OR 19.21; 95% CI, 4.23-87.32). These results were similar to studies in Hungary and Greece but no significant difference was reported in Fineland. Italy and Germany. The heterozygote genotype of this variant showed a significant association with CD compared to controls (p=0.0001; OR 47.67; 95% CI, 5.97-380.78), which was comparable to other European reports. Generally, R702W mutation allele and genotype frequencies in our study population were similar to European populations. The definite role of this mutation in susceptibility to CD is not clear yet, but it has been reported that R702W variant causes a significantly diminished ability of NOD2 protein to activate NFkB with LPS treatment. Therefore it exhibits a deficit in NFkB activation in response to bacterial components, providing a mechanism for susceptibility to CD.

The frequencies of two other mutant alleles (G908R, 1007fsinsC) were higher in CD patients compared to controls, but no significant association with CD was verified. These results were in agreement with some European studies, where no significant association was observed. G908 mutant allele frequency in CD patients covered a wide range in different ethnic groups (0.6% - 14.2%). The frequency of mutant allele and heterozygote genotype in our CD patients were similar to observations in Germany, Hungary and Italy, whereas their frequencies in healthy subjects in our study were lower than that reported in previous European population.

Mentioned dissimilarities in these two mutation frequencies confirm the genetic heterogeneity of CARD15 variants among different populations. This would be further supported by the demonstration that in contrast to many European studies, we did not find any association between 1007fsinsC variant and CD. The allele frequency of the 1007fsinsC mutation was significantly lower in our study population, both CD patients and healthy subjects in comparison with other Caucasian populations (4.8% - 17.9%). It is likely that the lack of association between these two mutations (G908R and 1007fsinsC) and CD reflect the relatively small sample size. It is, however, conceivable that this could probably be related to genetic heterogeneity among different populations. Particularly when the three common variants of CARD15 were analyzed together, our results were similar to previous reports from Caucasian studies. In this study 32.5% of CD patients were heterozygote for one of these mutations, and 7.5% had the compound heterozygote genotype, which is similar to studies in Europe.
The Iranian population is genetically diverse; thus there may be more CARD15 variants contributing to disease risk in Iran. Studies with larger sample size, using more sensitive methods like sequencing can identify other possible changes in this locus. According to genetic diversity, independent studies for each geographical region by wide genome scans or recently gene expression studies such as microarray techniques seem necessary to identify responsible candidate genes and SNPs in the disease risk.

References


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