CARD15 Gene 3020insC Mutation with Inflammatory Bowel Diseases Patients in the Black Sea Region of Turkey

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OBJECTIVE: We investigated whether the CARD15/NOD2 3020insC frameshift mutation is a risk factor for patients with inflammatory bowel disease in Black Sea Region population in Turkey.

METHODS: We studied 3020insC mutation of CARD15/NOD2 gene by allele-specific multiplex PCR in 69 patients with IBD (18 Crohn’s disease [CD] and 51 ulcerative colitis [UC]) and 101 ethnically matched healthy controls.

RESULTS: CARD15/NOD2 3020insC frameshift mutation was positive in 7/18 (38.8 %), 13/51 (25.5 %), and 4/101 (4 %) of CD, UC, and healthy control groups, respectively. None of the controls or patients with Crohn’s disease and ulcerative colitis was homozygous for this mutations.

CONCLUSION: This study is to investigate a relation between CARD15/NOD2 3020insC frameshift mutation and in patients with IBD in the Turkish Population. C-insertion frameshift mutation is a major contributor to the susceptibility to both CD and UC, but it is not specific to patients with CD in Turkish population.

Introduction

Crohn’s disease (CD) and ulcerative colitis (UC) are two main forms of inflammatory bowel diseases (IBD) characterized by chronic inflammation of the digestive tract sometimes associated with an extra digestive inflammation [1]. The incidence of these diseases is higher in developed countries. The origin, etiology, and pathogenesis of IBD have not been fully understood [1, 2]. In recent years, certain genes were identified in the pericentromer region of human chromosome 16 (locus IBD1) that increase the susceptibility to inflammatory bowel diseases, both chronic UC and CD. However, the candidate genes did not show a definitive association with this pathology [1, 3]. Recent articles proposed a gene on chromosome 16q12, known as CARD15 (NOD2) is possibly responsible for CD [4-8]. Identified nucleotide oligomerisation domain (NOD2) as the IBD gene and recently, the nomenclature of NOD2 has been changed to caspase activating recruitment domain (CARD15). CARD15/NOD2 has a role in inflammatory response to bacterial triggers, especially lipopolysaccharides (LPS), and is expressed exclusively in monocytes and it has structural homology with R protein which is a class of plant disease resistance gene product [8]. CARD15/NOD2 comprises an amino terminal effector domain, a nucleotide-binding domain and leucine-rich repeats (RRs) and regulates apoptosis and/or nuclear factor (NF)-κB activation [9]. The C-insertion mutation at nucleotide 3020 (3020insC) in the LRR region results in a frameshift in the 10th LRR followed by a premature stop codon [10]. This truncation mutation is responsible for the inability to activate nuclear factor (NF)-κB in response to bacterial LPS [10, 11].
The frequency of CARD15/NOD2 3020insC frameshift mutation and relationships with IBD in various populations was investigated by researchers and the genetic heterogeneity of CD in different populations was established. Here we studied this frameshift mutation in 69 Turkish patients with IBD (18 CD, 51 UC) and 101 healthy control subjects, to identify the susceptible gene for Turkish population.

Materials and Methods

**Study patients and control subjects**

We studied 69 unrelated patients 18 CD and 51 UC. The study also included 101 healthy control subjects. These control subjects were without a personal or family history of inflammatory bowel disease. Blood samples were obtained from patients (n = 69) at Kocaeli University Hospital and Karadeniz Technical University Farabi Hospital in Black Sea Region of Turkey. Blood samples were obtained from healthy control subjects (n = 101) in the same region. The diagnosis of either ulcerative colitis or CD was confirmed by clinical, radiological, endoscopic and histological examination in accordance with Vienna classification (Gasche et al., 2000). Suspicious cases were excluded from the study.

**DNA extraction and 3020insC frameshift mutation analysis**

Blood samples were drawn from cases and controls in the fasting state and collected in EDTA-tubes (Vacutaine, Becton Dickinson, Meylon, France). DNA was extracted with salting out method from peripheral blood leukocytes using the Pure gene DNA extraction kit (GENTRA, Minneapolis, USA). DNA concentrations were measured spectrophotometrically at 260 nm and stored at −70°C. All tests were subsequently performed within 3 weeks of collection.

**Table 1: CARD15/NOD2 3020insC Frameshift Mutation Allele-Specific PCR Conditions.**

<table>
<thead>
<tr>
<th>Stock Concentrations</th>
<th>Final Conc.</th>
<th>Volume (25 μl)</th>
<th>Primer Sequences (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>10XPCR buffer</td>
<td>1X</td>
<td>5.0 μl</td>
<td></td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2.5 mM</td>
<td>1.5 μl</td>
<td></td>
</tr>
<tr>
<td>25 mM dNTP Mix</td>
<td>0.2 mM</td>
<td>1.5 μl</td>
<td></td>
</tr>
<tr>
<td>Control-F (100 μM)</td>
<td>25uM</td>
<td>1.5 μl</td>
<td>CTGAGCCCTTTGTTGATGAGC</td>
</tr>
<tr>
<td>Control-R (100 μM)</td>
<td>1 uM</td>
<td>1.5 μl</td>
<td>TCTCAACCCACATCCTGATT</td>
</tr>
<tr>
<td>Allele-specific(w)-F</td>
<td>1 uM</td>
<td>1.5μl</td>
<td>CAGAGCCTCTCTGACGCCCCT</td>
</tr>
<tr>
<td>Allele-specific(w)-R</td>
<td>0.25 uM</td>
<td>1.5 μl</td>
<td>CGGTGTAGTTCTTCATGGGCC</td>
</tr>
</tbody>
</table>

| Patient's DNA         | 30ng/μl     | 2 μl           |                             |
| qPCR (5 μl/u)         | 0.03IU/μl   | 1.0 μl         |                             |
| Sterile ddH₂O         | 13.0 μl     |                |                             |

The CAR15/NOD2 3020insC frameshift mutation was genotyped by using allele-specific multiplex polymerase chain reaction (PCR), in MJ PTC-100™ Thermocycler (MJ Research, Inc., USA). The primers required for this method were purchased from Promega. PCR conditions were as follows (Table 1): an initial denaturation for 2 min at 94 °C, then, 35 cycle at 94 °C for 30 s, at 60 °C for 30 s, at 72 °C for 1 min and final extension at 72 °C for 10 min (Ogura et al., 2001). The PCR products were detected by agarose gel electrophoresis (at 70V, 55 A for 1h) on 2% agarose gel containing ethidium bromide and the fluorescent intensity of each band was evaluated with a UV transilluminator. The three genotypes were defined as follows; 300 bp, normal homozygous; 200 bp and 300 bp (two bands in electrophoresis), heterozygous; and only 200 bp, mutant homozygous (Figure 1).

**Statistical Methods**

In this study, the statistical analyses were made with the Graphpad Prism 6.02 software. In the evaluation of the data, in addition to the descriptive statistical methods (mean, standard deviation), the χ² test was used in the comparison of the patient and control groups and the Fisher reality test was used in the comparisons of the qualitative data. The significance level of P = 0.05 was taken as basis in the evaluation of the results. In the calculation of the genotypes and allele frequencies, Hardy-Weinberg equality was taken as basis.

**Results**

The distribution of CARD15/NOD2 3020insC frameshift mutation in the two groups of patients and control subjects is shown in Table 2.

**Table 2: CARD15/NOD2 3020insC frameshift mutation in IBD (UC, CD) and healthy controls in Turkish population.**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Wild type</th>
<th>Heterozygote</th>
<th>Homozygote</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD (n = 69)</td>
<td>11 (61.1 %)</td>
<td>7 (38.9 %)</td>
<td>0</td>
<td>0.0001</td>
</tr>
<tr>
<td>UC (n = 51)</td>
<td>13 (25.4 %)</td>
<td>13 (25.4 %)</td>
<td>25 (49.0 %)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Total IBD (n = 69)</td>
<td>49 (71.1 %)</td>
<td>20 (29.2 %)</td>
<td>0</td>
<td>0.0001</td>
</tr>
<tr>
<td>Controls (n = 101)</td>
<td>97 (96.0 %)</td>
<td>4 (4.0 %)</td>
<td>0</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

According to allele-specific multiplex PCR results, the frequencies of this frameshift mutation in Turkish patients were 38.8 % (7 of 18), 25.4 % (13 of 51) and 4 % (4 of 101) in CD, UC and healthy subjects, respectively. None of the controls or patients showed the presence of this mutation.

http://www.mjms.mk/  
http://www.id-press.eu/mjms/
with CD and ulcerative colitis was homozygous for these mutations. Significant differences were found in the genotype and allele frequencies of the C-insertion mutation of CARD15/NOD2 gene among patients with IBD (Crohn’s disease and ulcerative colitis) and healthy controls (P < 0.05). In this study, prevalence of C-insertion mutation of CARD15/NOD2 gene was found to be higher in patients with CD than UC. However, the difference was not significant (P > 0.05).

Discussion

CD and UC are classified as chronic idiopathic inflammatory bowel diseases. The incidence of these diseases is higher in developed countries such as USA, Japan and East European countries and it has increased about twofold in these countries from 1970s to date [13]. Although its etiology and pathogenesis have not been fully understood, result of epidemiological and genetic linkage studies have suggested that IBD is predisposed by certain genetic and environmental factors [14]. The origin of CD is still an enigma in current medicine. In this study, we investigated the association between 3020insC variants and IBD disease using healthy controls. Genomic analyses and statistical association revealed that the mutation in the region is associated with CD and ulcerative colitis among the Black Sea Region population in Turkey. The previous literature has indicated that the rare CARD15/NOD2 3020insC is the risk factor for the CD among different populations [15, 16]. Among the Japanese population however, patients with the CD did not carry the mutants [17]. The frequency of the mutants in the African American patients of CD was found to be lower in comparison to the patients who are Caucasian Americans [18]. The literature indicates that the frequency of mutation varies in different ethnic populations. Therefore, association studies on the frequency of mutation in patients with the CD should target each population individually before any clinical implication [19]. This study is to our knowledge the first attempt to target the IBD patients in the Black Sea population of Turkey and has potential to be applied in the clinical diagnosis. The difference of the mutation frequency in various populations is an important research question. Hence, intensive research has been conducted to tackle the patterns of mutation among various ethnic groups in different continents. The incidence of 3020insC mutation of CARD15/NOD2 in European populations who carry the CD was found to be about 12% [20-24]. The more detailed analyses among European population revealed that the frequency of the mutation was 16% among Germans [18, 25], 11.6% among Italians [21], 9.4% among British [26, 27] and 10.9% among Dutch [23, 24]. In the North America, the reported results indicated that the frequency of the mutation was 8.4% for Caucasian Americans [25-28], 7.8-8.4% for Jewish [29] and 1.3-3.7% among Chinese [30-32]. The prevalence of 3020insC, a frameshift mutation of CARD15/NOD2 gene among the members of Jewish population in various regions who carry CD was found to be around 7.3% whereas the same mutation was found to be 8.4% among the members of non-Jewish population [23]. The frequency of the mutation among the Jews who have CD was 8.7% whereas the frequency was found to be 3.2% in control group. However, the occurrence of the mutation among non-Jewish populations who had CD was 4.3% and that of control group was about 1.6% [28]. The elevated number of Crohn alleles could be linked to the effect of prevalence of the mutants [33, 34]. In the present study, we found that the frequency of the 3020insC mutant was about 25.4%, which was found to be higher than European populations [30]. Our results are in agreement with the previous literature in which the prevalence of a mutation of 3020insC was found to be higher among Caucasians who have CD in comparison with European population. This is particularly important since the region we studied is very close to Caucasus. The previous studies have rarely described the mutation in the context of UC [35]. In CD, same mutation 3020insC was studied by some of the groups West of Turkey but their results suggested lower frquencies than in our results [36].

In this study, we found that in addition to CD, the frequency of the mutation is very high among the patients of UC. The 3020insC mutation of CARD15 gene has been suggested useful in clinic diagnosis of CD and UC both of which are IBD. However, we have found the prevalence of mutation to be 25.4% among patients of CD and %38.8 among those who are UC.. Based on this result we conclude that this particular mutation may not be suitable as a diagnostic marker to differentiate between the two IBD types at least for the Black Sea region. Investigation of a similar pattern among other populations will be an interesting research venue. Moreover, the genetic heterogeneity of the Anatolian population is not well understood due to paucity of research and more in-depth analyses of linkage between those mutations and IBD has potential for clinic applications.

Acknowledgments

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References

3. Crawford NP, Collier DW, Eichenberger MR, Funke A A,


