Association between polymorphisms in the Toll-like receptor 4, CD14, and CARD15/NOD2 and inflammatory bowel disease in the Greek population

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AIM: Crohn’s disease (CD) and ulcerative colitis (UC) are multifactorial diseases with a significant genetic background. Apart from CARD15/NOD2 gene, evidence is accumulating that molecules related to the innate immune response such as CD14 or Toll-like receptor 4 (TLR4), are involved in their pathogenesis. In further exploring the genetic background of these diseases, we investigated the variations in the CARD15/NOD2 gene (Arg702Trp, Gly908Arg, and Leu1007fsinsC), and polymorphisms in the TLR4 gene (Asp299Gly and Thr399Ile) as well as in the promoter of the CD14 gene (T/C at position -159) in Greek patients with CD and UC. METHODS: DNA was obtained from 120 patients with CD, UC, and 100 healthy individuals. Genotyping was performed by allele specific PCR or by PCR-RFLP analysis. RESULTS: The 299Gly allele frequency of the TLR4 gene and the T allele and TT genotype frequencies of the CD14 promoter were significantly higher in CD patients only compared to healthy individuals (P = 0.026<0.05; P = 0.0048<0.01 and P = 0.047<0.05 respectively). Concerning the NOD2/CARD15 mutations the overall presence in CD patients was significantly higher than that in UC patients or in controls. Additionally, 51.67% of the CD patients were carriers of a TLR4 and/or CD14 polymorphic allele and at least one variant of the NOD2/CARD15, compared to 27% of the UC patients. It should be pointed out that both frequencies significantly increased as compared with the 10% frequency of multiple carriers found in healthy controls. A possible interaction of the NOD2/CARD15 with TLR4 and especially CD14, increased the risk of developing inflammatory bowel disease (IBD).

CONCLUSION: Our results indicate that co-existence of a mutation in either the TLR4 or CD14 gene, and in NOD2/CARD15 is associated with an increased susceptibility to developing CD compared to UC, and to developing either CD or UC compared to healthy individuals.

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INTRODUCTION

Inflammatory bowel diseases (IBD), Crohn’s disease (CD) and ulcerative colitis (UC) are multifactorial disorders characterized by failure to limit the inflammatory response to luminal antigens. Genetic predisposition to IBD has been well established through epidemiological studies and genome wide linkage analyses, but little is known about the accountable genes[1]. Animal models have demonstrated that genes involved in the regulation of the immune response are likely to play a crucial role in the genetic predisposition to IBD[2].

The innate immune response represents the first defense line preventing systemic infection with bacteria. Several host receptors interact with endotoxins and mediate cytokine production of macrophages. Lipopolysaccharides (LPS) are the main endotoxins derived from Gram-negative bacteria, and their pivotal role in the pathogenesis of a variety of infectious and allergic diseases has been suggested[3,4]. CARD15/NOD2, a cytosolic protein expressed in monocytes, is involved in the innate immune response to LPS and peptidoglycans (PGN)[5].

The association between mutations in the CARD15/NOD2 gene and CD has been described recently[6,7]. The 3 major variants Gly908Arg, Arg702Trp, and Leu1007fsinsC are associated with a deficit in NF-κB activation in response to bacterial components, providing a unifying mechanism for the major CD-associated CARD15/NOD2 variants[8].

The question arises as to how CARD15/NOD2 mutations and impaired NF-κB activation confer susceptibility to CD. It has been suggested that the answer most likely lies within the leucine-rich repeats (LRR) of the CARD15/NOD2 gene and the family of Toll-like receptors. These receptors recognize pathogen-associated molecular patterns and activate signal transduction pathways of the innate immune response genes including inflammatory cytokines and the NF-κB signaling...
pathway. Therefore, one could speculate that extracellular Toll-like receptors and intracellular CARD15/NOD2 participate as pattern-recognition receptors in the regulation of mucosal innate immune responses to intestinal microbes. Among the Toll-like receptors, Toll-like receptor 4 (TLR4) was found to be strongly up-regulated in both UC and CD. TLR4 binds to LPS together with CD14 and internalization prevents inappropriate NF-kB activation.

Very recently, Arbour et al. reported that the Asp299Gly and Thr399Ile polymorphisms of human TLR4 determine, in concert with other genetic changes, the airway responsiveness to inhaled LPS in humans. In addition, Klein et al. demonstrated an association of CD with a functional relevant single nucleotide polymorphism in the promoter of the CD14 gene (T/C at position -159) and suggested that the interaction of the CARD15/NOD2 and CD14 genes increases the risk for developing CD.

In order to evaluate whether the above mentioned polymorphisms in TLR4 and CD14 genes contributed to the predisposition to IBD, as well as whether the interaction of CARD15/NOD2, TLR4 and CD14 genes could increase the risk for IBD in a Greek population, we genotyped 120 patients with CD, 85 patients with UC and 100 healthy controls for the Asp299Gly and Thr399Ile polymorphisms of the TLR4 gene and the promoter of the CD14 gene (T/C at position -159).

MATERIALS AND METHODS

Materials

Blood samples from 120 patients with CD, 85 patients with UC and 100 age and sex-matched healthy individuals were collected at the IBD Outpatient Clinic between September 2002 and February 2003. The diagnosis of either CD or UC was based on standard clinical, endoscopic, radiological, and histological criteria. Before commencement of the study, the Ethics Committee at the participating centers approved the recruitment protocols. All participants were informed of the study. DNA was isolated from blood with the NucleoSpin Blood Kit (Macherey-Nagel, Germany).

Methods

Genotyping for the TLR4 Asp299Gly and TLR4 Thr399Ile polymorphisms was performed using PCR-RFLP as previously described. Specifically, primers for TLR4 Asp299Gly were forward (5’ GATTGAGATTTAGACTACACCCCTCAG 3’) and reverse (5’ GATCACTTCTGAAAAACGATTCCAC 3’). Primers for TLR4 Thr399Ile were forward (5’ GGTGCTGTTCTCAATTCACCCAGCCTC 3’) and reverse (5’ CCTGAGATTTTGGGAG 3’). The underlined bases in both forward primers indicate the altered nucleotide to create a restriction site, respectively. PCR reactions were run at 95 °C for 5 min followed by 35 cycles at 95 °C 30 s, at 55 °C for 30 s, at 72 °C for 30 s, and a final incubation at 72 °C for 5 min. A 15-μL aliquot of the product was digested with the appropriate restriction enzyme and electrophoresed in a 3% agarose gel to identify the TLR4 alleles on the basis of the respective allele size. After digestion, fragment sizes for carriers of the polymorphic allele decreased from 249 bp (wild-type) to 223 bp for the 299 residue, from 406 bp (wild-type) to 377 bp for the 399 residue.

Genotyping for -159(C/T) of the CD14 gene was performed using the method described by Hubacek et al. In brief, the promoter of the CD14 receptor gene was amplified by the primers CDP-1 (5’ TTTGGTGCCACACAGATGAGTTCCAC 3’) and CDP-2 (5’ TTCTTTCTCTACACAGCGGCCAC 3’) under the following conditions: an initial denaturation at 95 °C for 5 min, followed by 35 cycles at 92 °C for 40 s, at 62 °C for 35 s, and at 72 °C for 50 s. The final extension step was prolonged to 5 min. The 561 bp PCR product was digested with the restriction enzyme HaeIII, into the fragments of 204, 201 and 156 bp in length in the presence of the wild-type allele. The variant allele showed a loss of one HaeIII cleavage site, resulting in the presence of fragments 360 and 201 bp in length.

The cytosine insertion mutation was genotyped by a PCR amplification of specific allele assay using two allele-specific forward primers L1007fsinsCWTF: 5’ CAGAAGGCTCTCGTGA GGCGCT 3’ for the wild-type allele and L1007fsinsCMUTF: 5’ CAGAAGGCTCTCGTGAAGCGCCT 3’ for the L1007fsinsC mutant allele, in combination with a common primer L1007fsinsCR: 5’ TCTTCAACACACATCCCCATT 3’, in two separate PCR reactions. The 3’-ends of the forward primers, were able to anneal to regions that differed between the two alleles. The PCR profile was as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturing at 94 °C for 45 s, annealing at 65 °C for 40 s and extension at 72 °C for 30 s and a final incubation at 72 °C for 10 min. The missense mutation R702W was genotyped by a PCR amplification of specific allele assay using two allele-specific forward primers R702WWTF: 5’ ATCTGAGAGGCGCTCGTCTC 3’ for the wild-type allele and R702WWMTF: 5’ ATCTGAGAGGCGCTCGTCTC 3’ for the R702W mutant allele, in combination with a common primer R702W: 5’ CCCACATCAGCTCGTATG 3’, in two separate PCR reactions. The 3’-ends of the forward primers, were able to anneal to regions that differed between the two alleles. The PCR profile was as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturing at 94 °C for 45 s, annealing at 53 °C for 40 s and extension at 72 °C for 30 s and a final incubation at 72 °C for 10 min. The missense mutation G908R created a restriction site for HhaI and was genotyped by a PCR-RFLP method (5’ CCCAGCTCCTCTCTCCTC 3’ and 5’ AAGTCTGTAA TGTAAAGCCAC 3’). The presence of a wild-type allele resulted in an intact 380 bp band, whereas the RFLP profile of the G908R variant was characterized by two bands of 138 bp and 242 bp. The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturing at 94 °C for 45 s, annealing at 53 °C for 40 s, extension at 72 °C for 30 s, and a final incubation at 72 °C for 10 min. All PCR assays were performed in a 50 μL volume reaction containing 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 2 mmol/L MgCl2, 250 μmol/L dNTPs, 0.20 μmol/L concentration of each primer, 200 ng of genomic DNA and 2.5 U of Taq DNA polymerase (Promega). PCR products were electrophoresed on an agarose gel and visualized by ethidium bromide staining.

Statistical analysis

Odds ratios (OR) were calculated with the corresponding 95% confidence intervals (CI95%). Frequencies and susceptibilities of mutations among CD, UC and controls were compared based on χ2 distribution. All tests were 2-tailed with significance at P<0.05. Inference was aided by GraphPad InStat (version 3.00, GraphPad Software Inc., San Diego, CA).

RESULTS

TLR4 Asp299Gly and Thr399Ile genotype carrier frequencies are summarized in Table 1. The 299Gly allele frequencies were 7.92%, 3.53%, and 3% in CD, UC and healthy controls, respectively. The frequency of the 299Gly allele was significantly higher in CD patients than in controls (P = 0.026–0.05, OR = 2.78 CI95%: 1.088–7.103). The 299Gly allele was not found to be significantly associated with UC (P = 0.77, OR = 1.18 CI95%: 0.37–3.74). No significant difference was found in the frequencies of the 399Ile polymorphism among CD or UC.
patients and controls.

Allele and genotype frequencies of the polymorphism -159 (C/T) of the CD14 gene are presented in Table 2. T allele and TT genotype frequencies were increased in CD patients only compared to controls \((P = 0.0048<0.01, \text{OR} = 1.73, \text{CI}_{95\%}: 1.18-2.54)\) and \(P = 0.047<0.05, \text{OR} = 1.93, \text{CI}_{95\%}: 1.00-3.72, \text{respectively}\).

Concerning the NOD2/CARD15 mutations, the overall presence in CD patients \((81.7\%; 98/120)\) was significantly higher than that in UC patients \((47\%; 40/85)\) \((P<0.001<0.01, \text{OR} = 5.01, \text{CI}_{95\%}: 2.67-9.38)\) or in healthy control individuals \((21\%; 21/100)\) \((P<0.0001<0.01, \text{OR} = 16.76 \text{CI}_{95\%}: 8.60-32.67)\) (Table 3). A significant association was found between ileal disease and possession of one or more variant alleles. For each NOD2/CARD15 variant, allele frequencies for overall ileal involvement (ileal disease and ileocolitis) were significantly different from non-ileal diseases \((R702W\text{ ileal} 8.3\%, \text{non-ileal} 1.7\%\), \(P = 0.014<0.05; \text{G908R ileal} 12.1\%, \text{non-ileal} 2\%\), \(P<0.0001<0.01\); and \(L007f\text{insC ileal} 17.1\%, \text{non-ileal} 0.83\% P<0.0001<0.01\)).

Co-existence of the TLR4 polymorphic allele and the T allele for overall ileal involvement (ileal disease and ileocolitis) were significantly different from non-ileal diseases \((R702W\text{ ileal} 8.3\%, \text{non-ileal} 1.7\%\), \(P = 0.014<0.05; \text{G908R ileal} 12.1\%, \text{non-ileal} 2\%\), \(P<0.0001<0.01\); and \(L007f\text{insC ileal} 17.1\%, \text{non-ileal} 0.83\% P<0.0001<0.01\)).

Four of the CD patients \((3.3\%)\), 3 of the UC patients \((3.5\%)\) and none of the healthy controls were found to carry simultaneously a polymorphic allele of all the genes tested. Additionally, 62 out of 120 \((51.67\%)\) of the CD patients were carriers of a TLR4 and/or CD14 polymorphic allele and at least one variant of the NOD2/CARD15, compared to UC patients \((27\%)\).

As indicated in Table 4, the TT genotype and T allele frequencies of the -159(C/T) polymorphism in the CD14 gene increased in CD patients harboring at least one variant of the NOD2/CARD15, compared to controls. The odds of developing CD significantly increased in either case \((P = 0.012<0.05, \text{OR} = 9.4 \text{CI}_{95\%}: 1.18-74.40, \text{and} P = 0.003<0.01, \text{OR} = 1.45 \text{CI}_{95\%}: 1.48-6.81, \text{for the T genotype and TT allele respectively})

Table 3 NOD2/CARD15 mutant allele frequencies in Crohn’s disease (CD) patients, in ulcerative colitis (UC) patients and controls

| Samples   | Genotype | Allele frequency (%) | OR |
|-----------|----------|----------------------|----|
| R702W     | CD       | 96                   | 10 | 11.05<0.01 |
|           | UC       | 73                   | 7.1| 7.52<0.01 |
|           | Control  | 98                   | 1  |          |
| G908R     | CD       | 87                   | 14.2| 4.51<0.01 |
|           | UC       | 63                   | 13.5| 4.31<0.01 |
|           | Control  | 93                   | 3.5 |         |
| L007finsC | CD       | 79                   | 17.9| 3.42<0.01 |
|           | UC       | 79                   | 3.5 | 0.57    |
|           | Control  | 88                   | 6   |         |

1: homozygous wild-type; 2: heterozygous; 3: homozygous mutant, \(b P<0.01\) vs UC group and control group.
CD14 Alleles

represent another innate immune system for the recognition of bacterial antigens. It could detect LPS in the cytoplasm of infected cells. In addition to the cell surface TLR4 complex, there is evidence that mammalian cells have an intracellular receptor complex has been demonstrated to serve as a surface receptor of NF-B, disturbed activation of the innate immune system by inflammatory response directed against the enteric microflora. It may be caused by an exaggerated host defense reaction of the inflammatory bowel disease. Concerning the CD14 gene, functional relevance between TLR4 and in combination, and IBD in a Greek population. Our findings indicate that the frequency of the mutations varies in different populations.

**DISCUSSION**

Crohn’s disease and ulcerative colitis are multifactorial diseases with a polygenic nature. Despite both being chronic disorders of the gastrointestinal tract with unknown etiology, an abnormal inflammatory response directed against the enteric microflora in a genetically susceptible host has been postulated as a possible explanation[9]. In human system, the TLR4-MD2.CD14 complex has been demonstrated to serve as a surface receptor for LPS[20]. In addition to the cell surface TLR4 complex, there is evidence that mammalian cells have an intracellular receptor that could detect LPS in the cytoplasm of infected cells[19]. These data suggest that TLRs and members of the NOD family represent another innate immune system for the recognition of a wide array of pathogen products[20].

Our study dealt with the relationship between the major mutations in TLR4, CD14 and CARD15/NOD2 genes singularly and in combination, and IBD in a Greek population. Regarding the TLR4, Cario and Podolsky[27] recently showed that TLR4 was strongly up-regulated in CD and UC, which may be caused by an exaggerated host defense reaction of the intestinal epithelium to endogenous luminal bacterial flora. It was intriguing to hypothesize that the Asp299Gly allele of the TLR4 gene could be related to such an imbalanced reaction. Our findings indicate that the frequency of the 299Gly allele was significantly higher in CD patients compared to UC patients and controls and support the hypothesis that innate immunity may play a role in Crohn’s disease pathogenesis. Our results differ from those of a recent study in 86 UC patients of Japanese population in whom this mutation could not be detected[21]; however, our results are in accordance with those of recent studies in European patients[22,23]. Nevertheless, it is well known that the frequency of the mutations varies in different populations[22,23].

Concerning the CD14 gene, functional relevance between T allele and increased expression of CD14, has been demonstrated previously[26]. A significant increase of the T allele and the TT genotype was found exclusively among CD patients. Our results are in agreement with those from a recent study by Klein et al[22] but differ from those of a cohort in a Japanese population[27]. However, they demonstrated a significant association of the T allele and TT genotype frequencies with UC[27].

Regarding NOD2/CARD15, all the three risk alleles were more common in patients with IBD than in the control Greek population. The frequencies of the R702W and L1007fsinsC mutations were significantly higher in CD patients compared to UC patients and controls, whereas the frequency of the G908R mutation was similar in CD and UC patients but significantly higher compared to controls. Our results concerning the presence of L1007fsinsC in Greek population differ from those of a recent study in a Cretan population whose incidence was only 5.3%[28]. This inconsistency may be attributed to the fact that Crete is an isolated geographic region with a homogenous population dispersed over a small geographic area where this mutation does not seem to predispose to the disease or to the relatively small size of the examined sample[28]. Collectively our study confirmed previous studies, which reported, increased mutation carrier frequencies of one of the three variant alleles in CD patients compared to UC patients or healthy controls[6,29]. However, in contrary to the previously mentioned European studies, which reported that mutation frequencies in UC patients are comparable with those found in healthy controls. The allelic frequencies of R702W and G908R appeared to be higher in our UC patients than those found in healthy individuals. Interestingly, very recently Andriuilli et al[29] reported a significant association between the L1007fsinsC mutation and UC, although at a lower frequency in comparison with that observed in CD patients, suggesting a possible involvement of the NOD2/CARD15 also in UC patients[30]. Our findings may indicate the contribution of NOD2/CARD15 variants to the pathogenesis of UC in our population as well, and may reflect the well known difficulty to classify correctly from the onset in all the patients with inflammatory bowel disease.

Co-existence of TLR4 and CD14 mutated alleles was higher in CD patients compared to UC or healthy subjects, but this association was not was significant. Additionally, there was no significantly increased risk of IBD related to the coexistence of mutations in TLR4 and in CARD15/NOD2. On the contrary, it is of interest that the T allele in the TT genotype appeared to increase the relative risk for developing CD in combination with at least one variation in the CARD15/NOD2 gene. These results, are in accordance with a recent study by Klein et al[31], suggesting that as both CD14 and CARD15/NOD2 genes are involved in the recognition of LPS and subsequent activation of NFkB, disturbed activation of the innate immune system by bacterial antigens may be crucial in some patients with CD.

Co-existence of mutations in all the three genes was found in only a small fraction of the CD or UC patients. But as mentioned earlier, the extracellular complex TLR4.MD2.CD14

### Table 4  Allele and genotype frequencies of the promoter polymorphism at position -159 of the CD14 gene

| CD14 genotypes | Genotyped for the CARD15/NOD2 gene | Controls |
|----------------|-----------------------------------|----------|
|                | No variant | At least one variant | No variant | At least one variant | No variant | At least one variant |
| CC             | 17         | 16                    | 18         | 14                   | 34         | 9                    |
| CT             | 21         | 32                    | 23         | 15                   | 31         | 9                    |
| TT, (%)        | 9 (19.1)   | 25 (34.2) (P = 0.012) | 8 (16.3)   | 7 (19.4) (P = 0.156) | 16 (19.7)  | 1 (5.3)              |
| C, (%)         | 55 (58.5)  | 64 (43.8)             | 59 (60.2)  | 43 (59.7)            | 99 (61.1)  | 27 (71)              |
| T, (%)         | 39 (41.5)  | 82 (56.2) (P = 0.003) | 39 (39.8)  | 29 (40.3) (P = 0.24) | 63 (38.8)  | 11 (28.9)            |

*P<0.05, **P<0.01 vs control group.

1.88-5.24). It should be pointed out that both frequencies significantly increased in CD and UC as compared to the 10% frequency of multiple carriers found in healthy controls (\(P<0.0001-0.01, \text{OR}=9.62, 4.56-20.27\) and \(P=0.002<0.01, \text{OR}=3.34, 1.48-7.50\), for CD and UC respectively). The allelic frequencies of the promoter polymorphism at position -159 of the CD14 gene were significantly higher in CD and UC as compared to the 10% frequency of multiple carriers found in healthy controls (\(P=0.012\)). Our results are in agreement with those from a recent study in a Cretan population whose frequency in comparison with that observed in CD patients, was significantly higher compared to controls. Our results demonstrating previously that the frequency of the mutations varies in different populations. A significant increase of the T allele and the TT genotype was found exclusively among CD patients. Our results are in agreement with those from a recent study by Klein et al but differ from those of a cohort in a Japanese population. However, they demonstrated a
and the intracellular CARD15/NOD2 might participate in the regulation of innate immune responses to intestinal microflora. This seemed to point to the danger of IBD development in patients with at least one mutated allele in TLR4 and/or CD14 and at least one variant of CARD15/NOD2. Our results indicate that co-existence of a mutation in either the TLR4 or CD14 gene and in NOD2/CARD15 is associated with an increased susceptibility to CD or UC.

In conclusion, the Greek population suffering from IBD most likely carry polymorphisms in one or more of the genes related to the innate immune system. However, further research in larger and diverse populations is needed to elucidate the biological mechanism behind IBD susceptibility. Understanding the influence of predisposing genes can lead to a more precise diagnosis and permit the development of personalized medicines.

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