NOD2/CARD15 rs2066845 polymorphism in children with acute appendicitis.

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Abstract
Background: Innate immunity is extremely important in the antimicrobial defense mechanism of the human body. Innate immunity genes are known to effect the severity of Acute Appendicitis (AA). NOD2/CARD15 gene has a function in innate immune system, and it is expressed in large amounts in antigen-presenting macrophage-like cells, and intestinal Paneth cells. NOD2/CARD15 polymorphism can be a risk factor for other diseases of the gastrointestinal system. rs2066845 polymorphism on this gene has been correlated with Crohn's disease, and development of rejection after intestinal transplantation. This mechanism has the similar action in AA and this polymorphism may be effective on AA and provide a new data to stimulate future debate and genetic investigations of AA, particularly in accessible peripheral tissues for AA.

Methods: Study population consisted of 50 children with AA, and 49 healthy children. DNA was extracted from peripheral blood lymphocytes. The rs2066845 is localized on NOD2/CARD15 gene and was analyzed using PCR (Polymerase Chain Reaction), and RFLP (Restriction Fragment Length Polymorphism) techniques.

Results: A statistically significant difference did not exist between the patient and the control groups as for age, and gender of the study participants (p>0.05). Besides, a correlation was not detected between groups for age, gender, routine laboratory parameters, and allele-genotype frequencies. In the study group, allele, and genotype frequencies did not differ as for NOD2 polymorphism (p>0.05). The genotype frequencies of patient group were in Hardy-Weinberg equilibrium (HWE) but not in the patient group.

Conclusion: Although NOD2/CARD15 polymorphism is a biologically important SNP in inflammatory bowel diseases (IBDs), in our study, it was not determined as a risk factor for AA. Data retrieved from investigation of this SNP may a knowledge for genetic researches to be performed for AA. Besides, researchers may focus on new molecular alternatives, and investigate other SNPs of the NOD2 gene.

Keywords: Polymorphism, Inflammation, Appendicitis.

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and MAP (Mitogen-Activated Protein) which leads to synthesis of various proinflammatory cytokines and/or chemokines [7-10]. Also in cases with AA, binding activity of NF-κB increases [11]. NOD2 maintains homeostasis of intestinal mucosa. Especially in intracellular infections it is a major host defense mechanism, and assumes a task in the regulatory functions of the gastrointestinal system [12]. It is a positive regulator for the activation of NOD2, NF-κB and IL-1b [13]. Accordingly, aggravation of intestinal inflammation in Crohn’s disease has been observed. Gly908Arg [rs2066845] polymorphism on this gene is associated with Crohn’s disease [14,15]. NOD2 is the major component of innate immunity, and exerts its effects in many immunological and inflammatory diseases as Crohn’s Disease (CD) and graft versus host disease [6,16]. Just like Crohn’s disease and ulcerative colitis, inflammatory bowel disease (IBD) is characterized by abnormal mucosal response against bacteria-derived antigens in genetically sensitive bowel of the host [17]. Mutations observed in NOD2 gene in Crohn’s disease have not been fully understood yet. Indeed, signal from mutant NOD2 protein leads to defective activation of NF-κB. According to a hypothesis on this subject Th1 response associated with defective NF-κB increases susceptibility to intestinal infections. This viewpoint has been recently supported by findings which demonstrated the presence of wild-type NOD2. However in human beings the presence of mutant variants have not been demonstrated yet. One of the genetic risk factors which increase predisposition to IBD is rs2066845 localized on NOD-2 gene [14]. It has been found to be associated with psoriatic arthritis [18]. GC, and CC genotypes of rs2066845 also predispose to IBD. The rs2066845 increases development of post-appendectomy risk of CD [19]. Pathophysiology of CD can be thought as that of appendicitis [20]. Both of these diseases are characterized by cytokine release.

As is the case with AA, local inflammation can be also effected by polymorphisms of genes involved in innate immunity. Therefore, in our study, the contribution of innate immunity gene NOD2/CARD15 rs2066845 polymorphism which can be responsible from the pathogenesis of appendicitis to the detection of local inflammation or microbial conditions, and its risk factor in AA have been investigated.

Study Population and Epidemiologic Data

Our study group consisted of 50 children with AA ( male, n=31, and female, n=19) and 49 healthy children (male, n=21 and female, n=28) whose ages ranged between 6 and 16 years. All blood samples drawn from volunteers who applied to Gaziantep Cengiz Gökçek Obstetrics and Children’s Hospital between June 2015, and December 2015 were analyzed. Diagnosis of AA was based on medical history, physical examination, routine biochemical tests (WBC, CRP), ultrasonographic examinations, and histopathological sampling. Control subjects had not any history of AA. Definitive diagnosis was made on histopathological sampling. Then the correlations between allele and genotype frequencies of SNP, and CRP values were investigated. The approval of the Ethics Committee of Firat University Faculty of Medicine was obtained for the study protocol. All volunteered study participants undersigned an informed consent form which indicated their participation in the study. Local immune response in appendiceal tissue reflects immune response of blood. Thus studies on immune response in appendicitis justify use of peripheral blood samples for immunological studies [21]. In our study each blood sample was drawn into heparinized tubes, and examined together with other routine analyses.

Selection of Candidate Genes and Functional SNPs:

Variations in SNP can result in many disease states. SNP has been investigated in widely seen complex diseases such as obesity, stroke and Crohn’s disease, but it was not analyzed in pediatric appendicitis. We investigated NOD2/CARD15 (rs2066845) polymorphism so as to fill the knowledge gap on this subject and to be able to demonstrate the possible role of SNP in AA.

DNA Extraction: Genomic DNA was retrieved using QIAamp DNA Mini Kit and spin-column method (QIAGENE, Germany) according to the protocol in the product information recommended by the manufacturer. The samples were kept at -20°C till analysis.

Detection of Polymorphisms: Polymorphisms were determined using polymerase chain reaction (PCR) (Rotor-Gene Q, Qiagen). Primers were synthesized for amplification (F: 5'- AAG TCT GTA ATG TAA AGC CAC-3' R: 5'-CCC AGC TCC TTC CTC TTC-3') [22]. For PCR analyses, a final volume of 16µl containing 2 µg DNA, 2X SYBRGreen PCR Mix (SYBR® Green), in addition to 0.4 mM from each primer was prepared. PCR cycles were realized as follows: baseline denaturation; 10 min at 95°C, then 40 cycles of amplification (15 s at 94°C, 20 s at 62°C, 20 s at 72°C) and final extension: 5 min at 72°C. All PCR products were stained using standard method with ethidium bromide (final concentration 0.5 mg/ml) incorporated into 2% agarose gel. Bio-Rad PowerPac power supply system was used for PCR and product size was determined as 380 bp using Bio-Rad CheimiDoc gel imaging system. PCR products were cut with 16 unit- HhaI (exon 8; Thermo Scientific, Waltham, MA, USA) enzyme, and then the samples were analyzed in 2,5% agarose gel. Based on a reference marker with 100 bp (Biolab, #N3231L) sizes of G and C alleles were determined as 380 bp and 241 bp+139 bp, respectively.

Statistical Analyses

In the comparison of age, and WBC between groups, Independent Samples t test, and for the comparison of CRP, Mann-Whitney U test were used. Besides, for the intergroup comparison of WBC and CRP values Pearson chi-square test was used. Association between genotype, and the disease was investigated using one of the cross-
tab statistics, namely Fisher-Freeman-Halton exact test. OR values for variables considered to be significant were calculated separately in univariate analysis. These variables were included in combination in the multivariate logistic regression analysis, and their OR values were calculated. For statistical analysis SPSS v.22 package program was used, and level of statistical significance was considered as p=0.05.

Results
Age (p=0.957), and gender (p=0.071) analyses performed in AA, and healthy control groups demonstrated a homogenous distribution of these variables in both groups (Table 1). Mean ages of the patients, and control subjects were 10.60 ± 3.04, and 10.57 ± 2.15 years, respectively. As can be expected, CRP, and WBC levels in AA patients were statistically significantly higher than those of the control group (p<0.001). CRP data did not demonstrate a homogenous distribution between the patient and the control groups. Therefore, instead of means, quartiles, minimum and maximum values were taken into consideration. Mean WBCs were 14.77 ± 4.08/mm³ in the patient, and 8.96 ± 2.81/mm³ in the control groups. Univariate, and multivariate analyses revealed the presence of a correlation between WBC and CRP values which reinforces diagnostic accuracy of AA [(for WBC: univariate analysis; OR (95% CI)=6.429/mm³ (2.680-15.418/mm³), p=0.001 and multivariate analysis; OR (95% CI)=6.977/mm³ (2.425-20.075/mm³), p<0.001) and for CRP: univariate analysis; OR (95% CI)=11.389 mg/l (4.400-29.482 mg/l), p<0.001, Multivariate; OR (95% CI)=12.185 mg/l (4.167-35.633 mg/l), p<0.001)]. Genotypes were evaluated based on standard markers (Figure 1). A statistically significant

Table 1. Age and gender distribution in AA, and control groups

<table>
<thead>
<tr>
<th></th>
<th>AA (N=50)</th>
<th>Control (N=49)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>10.60 ± 3.04</td>
<td>10.57 ± 2.15</td>
<td>0.957</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>31 (62.0)</td>
<td>21 (42.9)</td>
</tr>
<tr>
<td>Female</td>
<td>19 (38.0)</td>
<td>28 (57.1)</td>
<td></td>
</tr>
</tbody>
</table>

N: Number of individuals; AA: Acute Appendicitis

Figure 1. Genotypes of NOD2/CARD15 of rs2066845 polymorphism
A) Samples numbered from 1 to 18 indicate data related to control subjects. Homozygous GG genotype product size: 380 bp
B) Samples numbered from 1 to 18 AA. Sample #11: Heterozygous GC phenotype, and product size: 380 bp, 241 bp+139 bp
C) Samples numbered from 19 to 37 indicate control subjects: Sample #30: Homozygous CC genotype: 241 bp+139 bp
difference was not observed between the patient (96, 4 and 0%, respectively) and the control (98, 0 and 2%, respectively) groups as for the distribution of GG, GC and CC genotypes (p=0.493) (Table 2). Frequencies of wild-type GG genotype were similar in the patient, and the control groups. GC heterozygous genotype was seen in the patient group, while it was not encountered in the control group. However, CC heterozygous polymorphic genotype was encountered in the control group, while it was not seen in the patient group. Contrary to the control group genotype frequencies in the patient group were in Hardy-Weinberg Equilibrium (HWE).

**Table 2.** Genotype distributions of rs2066845 in the AA and control groups

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Variant (M&gt;m)</th>
<th>Genotype</th>
<th>AA Control N=50 (%)</th>
<th>Control N=49 (%)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD2/CARD15</td>
<td>rs2066845</td>
<td>G&gt;C</td>
<td>GG</td>
<td>48 (96.0)</td>
<td>48 (98.0)</td>
<td>0.493</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GC</td>
<td>2 (4.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>0 (0.0)</td>
<td>1 (2.0)</td>
<td></td>
</tr>
</tbody>
</table>

p-Values<0.05 are indicated as bold marks

OR: Odds Ratio; CI: Confidence Interval; M: Major allele; m: minor allele; N: Number of individuals; AA: Acute Appendicitis

**Discussion**

Luminal obstruction in appendicitis causes luminal distension and consequently leads to increase in intraluminal, and intramural pressures [3,23]. Progression of this condition results in rapid bacterial proliferation, and invasion [24]. Appendix harbours both aerobic, and anaerobic bacteria found in normal gastrointestinal flora [25,26]. In appendicitis, neutrophilic infiltration in muscular layer of appendix is accompanied by bacterial invasion. Recognition of these microorganisms by immune system is realized by specific Pattern Recognition Receptors (PRRs) localized in cytosolic compartment or on the surface of the host cell [27]. Nucleotide-binding oligomerization domain-containing protein-2 (NOD2/CARD15) protein belongs to a pattern recognition receptor of the innate immune system and leads to inflammatory response [28].

Recent studies have distracted attention to the importance of NOD2 gene in intestinal immune health by demonstrating its significant role in the maintenance of functional integrity of intestinal mucosal barrier. NOD2 protein is a critical regulator of intestinal bacterial immunity [29]. It can recognize microbial components and stimulates inflammatory response mediated by activation of NF-κB [30]. NF-κB can be stimulated. It is considered as a prototypical proinflammatory signal pathway, and plays a very important role in immune regulation, and expression of inflammatory cytokine gene. NF-κB is activated by proinflammatory cytokines (IL-1α, IL-1β, TNF-α), and bacterial toxins (LPS and exotoxin B). NF-κB stimulates inflammatory cytokine response in inflammatory diseases [31]. The activity of NF-κB is not correlated with the number of white blood cells [32]. In cases with AA, NF-κB binding activity is increased which reaches baseline level within 18 h after appendectomy [32]. In a study, NF-κB was reported as a potential molecular indicator of inflammation in inflammatory conditions requiring surgery, and also in the prediction of prognosis [32]. NOD2 acts as an intracellular receptor for bacterial products in monocytes [12]. NOD2 expression was essentially discovered in monocytes, then it was detected in epithelial Paneth cells of the small bowel [33]. Besides it was also found in macrophages, dendritic cells and other intestinal epithelial cells. NOD2 gene defect impairs appropriate cytokine expression required for the coordination of the balance between activation and immune suppression which a must for healthy function of intestinal adaptive immune system [30].

NOD2, exerts a positive effect on the production of IL-10. IL-10 is an immune regulator cytokine. This cytokine effects antigen-presenting cells by inhibiting synthesis of proinflammatory and costulatory cytokines. Therefore, NOD2 polymorphisms impair the production of this anti-inflammatory cytokine. NOD2 deficiency leads to a decrease in IL10 response which leads to similar decreases in proinflammatory response [34]. IL-10 pathway is very important in the regulation of intestinal inflammation [35]. Patients with impaired production of IL-10 have serious UC and CD. It has been found to be effective in immunological and inflammatory diseases as NOD2 graft-versus-host, Crohn’s disease and sarcoidosis [36].

The importance of NOD2 gene in human innate immunity was related to its identification firstly in a locus associated with Crohn’s disease [36,37]. Various alleles on NOD2 locus have abnormal functions, and the risk of having Crohn’s disease increases 20-40-fold in individuals carrying NOD2 rs2066845 mutation depending on their genotypes [38]. NOD2 rs2066845 mutation is one of the genetic factors which predispose an individual to Crohn’s disease. This mutation also alters capacity of the receptor which will recognize bacterial component. NOD2, accumulates autophagy-related protein ATG16L1, in bacterial entry site of the plasma membrane [38]. On the contrary, CD-related mutants impair accumulation of ATG16L1 in plasma membrane and packaging of invaded bacteria by autophagosomes is disrupted [39]. NOD2 mutations are associated with decrease in the concentration of mucosal alpha-defensin in CD [40]. Besides NOD2 polymorphisms have been reported at a higher frequency in allograft recipient populations. Although the association between NOD2 gene mutations and gastrointestinal diseases in human beings has been reported, the relationship of this polymorphism with AA has not been completely ruled out [41,42]. Appendicitis, anal fissure, fistula and
abscess are causes of gastrointestinal tract inflammation. Association between these diseases and NOD2/CARD15 polymorphisms supports the hypothesis indicating important role of NOD2/CARD15 in innate immunity. In our study, we have demonstrated that in pediatric patients with AA, NOD2 (rs2066845-Gly908Arg) polymorphism is not a risk factor for the pathogenesis of the disease. Contrary to AA, this condition can be attributed to the fact that IBD is a chronic inflammatory process and appendicitis is a multifactorial entity. Loss of alleles and genotypes is related to the small sample size of the study. In a study with a large sample size, definitive effect of NOD2/CARD15 and these mutations in common gastrointestinal system diseases could not be demonstrated [42].

Conclusion

As a multifactorial disease AA whose pathogenesis has not been known for sure, is a clinical condition where hereditary changes, and the response of the body to inflammatory response is effective. In the development of AA, NOD2/CARD15 (SNP: rs2066845) is not a risk factor. The results obtained may be a guiding tool for further studies. Investigations may focus on new molecular alternatives as other SNPs of the NOD2 gene. Further studies in larger study populations should be performed to define the association between genotype and phenotype, and also to investigate polymorphisms in innate immunity genes in AA.

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References

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