Prevalence of SLC22A4, SLC22A5 and CARD15 gene mutations in Hungarian pediatric patients with Crohn’s disease

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Abstract
AIM: To investigate the frequency of the common NOD2/CARD15 susceptibility variants and two functional polymorphisms of OCTN cation transporter genes in Hungarian pediatric patients with Crohn’s disease (CD).

METHODS: A cohort of 19 unrelated pediatric and 55 unrelated adult patients with Crohn’s disease and 49 healthy controls were studied. Genotyping of the three common CD-associated CARD15 variants (Arg702Trp, Gly908Arg and 1007insC changes) with the SLC22A4 1672C→T, and SLC22A5-207G→C mutations was performed by direct sequencing of the specific regions of these genes.

RESULTS: At least one CARD15 mutation was present in 52.6% of the children and in 34.5% of the adults compared to 14.3% in controls. Surprisingly, strongly different mutation profile was detected in the pediatric versus adult patients. While the G908R and 1007insC variants were 18.4% and 21.1% in the pediatric group, they were 1.82% and 11.8% in the adults, and were 1.02% and 3.06% in the controls, respectively. The R702W allele was increased approximately two-fold in the adult subjects, while in the pediatric group it was only approximately 64% of the controls (9.09% in the adults, 2.63% in pediatric patients, and 4.08% in the controls). No accumulation of the OCTN variants was observed in any patient group versus the controls.

CONCLUSION: The frequency of the NOD2/CARD15 susceptibility variants in the Hungarian pediatric CD population is high and the profile differs from the adult CD patients, whereas the results for SLC22A4 and SLC22A5 mutation screening do not confirm the assumption that the carriage of these genotypes means an obligate susceptibility to CD.

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INTRODUCTION
Crohn’s disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract. Although the peak onset of the disease typically occurs in the second and third decades of life[15], the incidence of pediatric cases has been strongly increasing recently[16]. Despite the comprehensive research that has been made to discover the background of the disease, the etiology is still unknown. Besides the environmental effects, it is supposed that genetic susceptibility plays a crucial role in the development of the disease[17].

Genome-wide linkage analyses have resulted in identification of several loci of potential CD susceptibility genes[18-21]. CARD15 (NOD2) gene, which is located at the pericentromeric region of chromosome 16, was the first gene to be identified as CD gene[22-25]. NOD2 is an intracellular protein expressed in peripheral blood monocytes, Paneth and intestinal epithelial cells; and it is important for inflammatory signal transduction via activation of the transcription factor, nuclear factor kappa-B (NF-κB)[26]. Several studies on Caucasian populations have reported an association between CARD mutations and CD. Three coding variants (R702W, G908R
and 1007insC) have been identified as independent risk factors for development of CD.

Recently two polymorphisms in the carnitine/organic cation transporter gene cluster (SLC22A4 and SLC22A5, encoding OCTN1 and OCTN2, respectively) have been found to confer risk for CD[3]. The aim of the present study was to investigate the prevalence of these two functional variants of the SLC22A4 and SLC22A5 genes and the three CARD15 mutations in Hungarian pediatric population with CD.

MATERIALS AND METHODS

Patients

We examined 19 pediatric (14 males and 5 females; mean age: 13.4 years) and 55 adult (27 male and 28 female with real maturity onset disease; mean age: 42.3 years) patients with CD. This cohort was compared with 49 age- and sex-matched healthy controls (28 males and 21 females; mean age: 14.4 years). Both the pediatric and adult CD patients exhibited different clinical manifestations, therefore they represented mixed clinical CD populations. The diagnosis was confirmed by clinical, radiological, endoscopic and histological findings. Informed consent was obtained from each participant of the study and the study design was approved by the Local Ethics Committee.

Methods

Genomic DNA from the patients and the controls was isolated from peripheral blood using standard desalting procedure.

The presence of the NOD2 and OCTN variants was detected by direct sequencing using the primers designed in our laboratory. The primers’ sequences for the PCR amplification as well as for the sequencing and annealing temperatures are listed in Table 1. The PCR was carried out in a final volume of 50 µL containing 200 µmol/L of each dNTP, 2 units of Taq polymerase, 5 µL of reaction buffer [100 mmol/L Tris HCl (pH 9.0), 500 mmol/L KCl, 15 mmol/L MgCl2, 0.2 µmol/L of each primer and 1 µg of DNA to be amplified. The amplification was performed for a total of 35 cycles in an MJ Research PTC-200 thermal cycler. The amplification conditions were: pre-denaturation at 95°C for 2 min, denaturation at 95°C for 30 s, annealing for 30 s at the temperatures listed in Table 1 for the different SNP, primer extension at 72°C for 30 s, and the final extension at 72°C for 5 min. For DNA sequencing, a BigDye Terminator labeling was used and the analysis was performed in an ABI 3100 automatic sequencer.

Statistical analysis

Chi-square test (cross-table analysis) was used to analyze the possible associations with mutations in comparison of either the susceptibility variants and/or the normal haplotypes. P < 0.05 was considered statistically significant.

RESULTS

The allele frequencies are shown in Table 2. A total of 52.6% of pediatric patients with Crohn’s disease carried at least one NOD2 mutation compared to 34.5% of adult patients and to 14.3% of the controls (pediatric patients vs controls, P < 0.05).

While the T allele frequency, leading to heterozygous and homozygous R702W mutation, was increased approximately two-fold in the adult CD population (9.05%) compared to the controls (4.08%), it was only 2.63% in the pediatric CD patients (Table 2, P < 0.05 comparing the pediatric susceptibility and/or normal variants versus...
the same values of the adult patients or the controls). By contrast, the C allele frequency, encoding the G908R variant, was found highly elevated (18.4%) in pediatric patients, and was only 1.82% in adult CD patients, and 1.02% in the controls \( (P < 0.05) \). For the 1007T>C variant, a significantly increased prevalence was found both in pediatric (21.1%) and in adult CD (11.8%) patients as compared with the controls (3.06%) \( (P < 0.05) \).

There were no significant differences in the allele frequencies of SLC22A4 C1672T and SLC22A5 G-207C mutations when compared either the results of the pediatric or the adult CD populations to the results of the controls (Table 2).

**DISCUSSION**

The carriage rate for the three common CD-associated CARD15 mutations was reported 31% in a pediatric CD population in North America \(^{17}\), 60% in Germany \(^{19}\), 51.5% in the Israeli Jewish patients \(^{30}\), and 40.7% in an Italian cohort \(^{31}\). In two studies, the cystine insertion mutation 3020insC was significantly more common in the pediatric CD population \(^{32,33}\), whereas among the Jewish patients G908R missense mutation was the most frequent variant \(^{34,35}\).

An association of the three CARD15 mutations R702W, G908R and 1007T>C with CD has been confirmed in several studies \(^{17,18,20}\), although different allele frequencies have been observed. While the allele frequencies of the three mutations were almost the same (8.3%, 8.3% and 7.4%) in the Italian pediatric patients \(^{31}\), the G908R variant was the most frequent among Jewish children \(^{30}\) and 1007T>C was more common in children \(^{17,18,20}\) and in the USA \(^{34}\). An earlier onset of disease was found in the presence of a CARD15 mutation in three additional studies \(^{19,24}\). These findings suggest that CARD15 mutations may be more frequent in pediatric CD.

To our surprise, in our study groups, two mutations, G908R and 1007T>C, were significantly more frequent in the pediatric population with the allele frequencies of 18.42% in children \( \text{versus} 1.02\% \) in controls and of 21.05% in children \( \text{versus} 3.06\% \) in controls, respectively. The genotyping results for the adult population are in agreement with previous Hungarian findings \(^{2,25}\).

The OCTN1 and OCTN2 transporters mediate the transport of carnitine and a wide range of organic cations \(^{36,37}\) and have an important role in the energy supply of epithelial cells. Recently, it has become clear that the OCTN1 also transports the ergothioneine \(^{38}\), and the affinity parameters make it almost unquestionable that the carnitine transport function is secondary. A C1672T missense substitution in exon 9 of the SLC22A4 gene results in marked changes in OCTN1 transporter activity, whereas G-207C transversion in the SLC22A5 promoter region causes OCTN2 promoter function impairment.

By resequencing the five genes in the IBD interval, which harbors the cytokine gene cluster, and, therefore, is an attractive candidate region for IBD, Peltoketo et al. \(^{30}\) identified 2 novel polymorphisms in the SLC22A4 and SLC22A5 genes. These two mutations (SLC22A4 C1672T and SLC22A5 G-207C) form a two-allele risk haplotype (OCTN-T/C) which was associated with CD and showed significant interactions with CD-associated CARD15 mutations. This observation has been repeatedly confirmed \(^{32,34}\), although, in the absence of the IBD5 risk haplotype, no association of OCTN1/2 variants with CD was reported in two studies \(^{33,34}\). While a Belgian group \(^{35}\) found that the OCTN did not play a role in the susceptibility to CD, the two functional variants in the SLC22A4 and SLC22A5 genes were completely absent in Japanese \(^{36}\).

In our study, which is probably the first in the international literature for pediatric population, we could not find accumulation of any of the susceptibility haplotypes either in the pediatric or in the adult CD subjects, thereby not supporting the susceptibility role of the above haplotypes in the development of CD.

In conclusion, we observed an accumulation of CARD15 mutations in pediatric cases, whereas the results for SLC22A4 and SLC22A5 mutation screening do not confirm the assumption that the carriage of these genotypes means significant susceptibility to CD. However, for genotype-phenotype correlations, further studies are needed with larger study populations.

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