OCTN1 variant L503F is associated with familial and sporadic inflammatory bowel disease

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Organic cation transporter;
OCTN;
L503F

Abstract

Purpose: A two-allele haplotype of TC (OCTN1 rs1050152 and OCTN2 -207G→C) is associated with Crohn’s disease (CD). The association has been replicated in different populations, but also failed in some studies. The present study is to replicate the association of OCTN1 rs1050152 and examine another variant rs272879 with familial and sporadic inflammatory bowel disease (IBD) in a cohort from central Pennsylvania, USA.

Methods: The study samples (n=465) included 212 inflammatory bowel disease patients (CD=115, UC=97), including 103 familial (CD=55, UC=46) and 111 sporadic (CD=60, UC=51) IBD, 139 non-IBD family members from a familial IBD registry, and 114 unrelated healthy controls. A total of 12 OCTN1 variants within exonic sequences were examined. Two nonsynonymous SNPs, rs1050152 (L503F) and rs272879 (L395V) were genotyped by a PCR-based RFLP/cRFLP method and statistically analyzed. These samples with an additional 141 unrelated healthy samples were also genotyped for rs1050152 using the SNPlex™ Genotyping System.

Results: The OCTN1 rs1050152 is associated with CD (OR=1.745, 95% CI=1.019–2.990, \(\chi^2=4.129, p=0.042\)) and with IBD (OR=1.68, 95% CI=1.052–2.676, \(\chi^2=4.732, p=0.030\)); while
1. Introduction

Inflammatory bowel disease (IBD) is a complex immune-mediated gastrointestinal disease classified in two major forms; Crohn’s Disease (CD) and Ulcerative Colitis (UC). The large mucosal surface of the gastrointestinal tract is in direct contact with the external environment including an estimated 100 trillion bacterial microbes. The abnormal immune response associated with IBD is probably triggered by one or more environmental signals and determined by genetic and epigenetic factors. More than 30 distinct IBD susceptibility loci have thus far been identified, including genes associated with innate immunity, such as NOD2 that binds to muramyl dipeptide from bacterial cell walls and activates the host response to microbes; IL23R involved in regulation of T cell proliferation and inflammation; ATG16L1 and IRGM which play an important role in the maintenance of gastrointestinal health and prevention of gut inflammation. The gene OCTN1 encodes a 551 amino acid protein containing 11 transmembrane domains and one nucleotide site motif. Recently evidence showed that OCTN2 also plays a role in host-bacterial interaction in response to intestinal tract bacteria.

The association of a two-allele risk haplotype TC of OCTN1 rs1050152 (L503F) and OCTN2 (-207G→C) with CD patients was identified in 2004. This association has been widely replicated in different populations, however, the results from some studies have shown no significant association. The OCTN genes are part of the IBD5 locus that is linked to IBD. Their role in IBD pathogenesis is currently unknown. In this study we examined a total of 12 SNPs in the exonic sequences of the OCTN1 gene, and studied two nonsynonymous SNPs, rs1050152 (L503F) and rs272879 (L395V), with IBD in a cohort of familial and sporadic IBD from the central Pennsylvania, USA area. This area is largely populated by immigrants from Germany, the Netherlands, and the United Kingdom, and thus presents itself as having a patient population with a relatively homogeneous genetic background. The registry included clinical characterization of disease and also self-reported information from participants regarding demographics, disease characteristics, or intestinal activity. There were 122 males and 119 females ranging in age from 16 to 92 (mean 51) years old. Blood was collected from the participants and used to derive B cell lines by Epstein Barr (EB) virus transformation.

2. Materials and methods

2.1. Study samples

A total of 606 samples were studied. These included:

1) Familial IBD patients from a familial IBD registry. At the time of this study, there were 58 families with 241 individuals in this registry: 102 IBD (CD = 56, UC = 46) and 139 non-IBD individuals. This familial IBD registry was established in 1998 at the Milton S. Hershey Medical Center. The participating families had at least two members affected with IBD, from a relatively unique rural/suburban area. A total of 606 samples, including 241 samples from the familial IBD registry, 110 sporadic IBD, and 114 controls as described above in 1), 2) and 3) respectively, were genotyped with PCR-based RFLP/cRFLP method and statistically analyzed for the association study. These 465 samples with an additional 141 unrelated healthy controls were also genotyped with SNPlex™ method.

All the human tissues, blood, blood-derived B cell lines, intestinal tissues, and lung tissues, used as the source of DNA for this study, were collected from the Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania, USA, except lung tissues which were obtained from the Gift of Life Donation Program. Human tissue collection and preparation for this study were under protocols approved by the Human Subjects Protection Offices, The Pennsylvania State University, College of Medicine.

2.2. DNA isolation

Genomic DNA was isolated from B cell lines with the Gentra Systems kit (Minneapolis, MN, USA), and from blood, intestinal and lung tissue with the QiAamp DNA Mini Kit (Qiagen Inc. Valencia, CA., USA), according to manufacturer’s instructions. DNA was stored in 10 mM Tris buffer (pH8.0, the Gentra Systems) or distilled water (the Qiagen kit). DNA concentrations were measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, DE., USA), and the DNA was stored at –80 °C until use.
2.3. PCR amplification

PCR reaction was performed in a 30 µl volume containing 1 X mixture of buffers 1 and 2 (Roche, Indianapolis, IN), 50 – 100 ng DNA or 1 µl of PCR products, 5 µM of each dNTPs, 50 ng of each primer, and 1 unit of Taq DNA polymerase (Roche, Indianapolis, IN). The PCR profile included a denaturing step at 95 °C for 2 min, 35 cycles of 95 °C for 30 sec, 58 °C for 1 min, and 72 °C for 1 min, followed by a final extension step at 72 °C for 4 min. For the second round of PCR in SNP rs272879 analysis, the PCR cycling profile was 5 cycles of 95 °C for 30 sec, 50 °C for 1 min, and 72 °C for 1 min, followed by 30 cycles of 95 °C for 30 sec, 58 °C for 1 min, and 72 °C for 1 min.

2.4. Genotype analysis

Two methods were used for genotyping, PCR-based Restriction Fragment Length Polymorphism (RFLP) or converted RFLP (cRFLP) method and ABI SNPlex™ Genotyping System 48-plex.

A total of 12 SNPs within OCTN1 exonic sequences were examined. These SNPs were from DNA database including those in the coding sequences L148V, V159M, D165G, Q180H, M205I, R282stop, I306T, L395V, G462E, and L503F, in the 5'-UTR 136 A→T, and in the 3' UTR 1824A→G. The 95 samples used were the IBD patients and their relatives from the families of the familial IBD registry. Of these SNPs tested, two nonsynonymous SNPs, L395V and L503F, were selected for further study of their genetic association with IBD. The method used was RFLP/cRFLP method,32 and only for genotyping the last two SNPs was briefly described here. All the information of PCR primers, restriction enzyme and allelic fragments are listed in Table 1. The DNA segments containing SNPs rs1050152 (L503F) and rs272879 (L395V) were amplified from 1 µl (100 ng) of genomic DNA with at least one OCTN1 gene specific primer of the two PCR primers.

For SNP rs1050152 (L503F), the DNA fragments were directly amplified from genomic DNA with primers OCTN1M48f and OCTN1M50r. For SNP rs272879, the fragments were first amplified from genomic DNA with primers OCTNIM43f and OCTN1100r, and then nested with primers OCTN1107f and OCTN144r. The PCR products were digested with appropriate restriction enzymes (Scr F1 for rs272879 and Mnl I for L503F, NE Biolab, Beverly, MA, USA), and the allelic fragment in the digested PCR products was then separated by polyacrylamide gel electrophoresis (PAGE, 10% for rs272879, 8% for rs1050152).

The allelic fragments on PAGE were shown in Fig. 1A for rs272879 and in Fig. 1B for rs1050152. The alleles C and G of rs272879 and T and C of rs1050152 are indicated by arrows.

For each SNP genotyping, at least 10% of the samples were randomly selected and repeated for genotype analysis at the same conditions.

The OCTN1 variant L503F was repeatedly genotyped with the SNPlex™ Genotyping System. The method was described previously.10,33,34 Briefly, 100 ng of genome DNA was used for genotyping with SNPlex™ chemistry (Applied Biosystems, Foster City, USA) on an automated platform with TECAN Freedom EVO and 384well TEMO liquid handling robots (TECAN, Männedorf, Switzerland). Genotype assignments were confirmed by visual inspection with the Genemapper® software v4.0 (Applied Biosystems). The samples used for SNPlex™ Genotyping were the same as for RFLP–cRFLP methods, but without sporadic CD samples, and adding more unrelated healthy controls.

2.5. Statistical analysis

In genotyping with SNPlex™ Genotyping system only rs1050152 (L503F) was included for confirm the genotyping result with RFLP. In order to compare the association of rs1050152 (L503F) with IBD to the association of rs272879 with IBD in the same study samples, the final statistical analysis of genetic association was based on the genotyping results with the PCR-based RFLP/cRFLP method. In statistical analysis for genetic association, we calculated χ² values, and related p values for determining the overall significance of the observed association findings as outlined. We also calculated Genotype-based Odds Ratio (OR) using the Fisher's contingency tables. The difference was considered as significant when p<0.05.

3. Results

3.1. Genetic association of OCTN1 variants with IBD

In the present study, we aimed to investigate the effect of the OCTN1 gene on IBD. We examined 12 SNPs in the 10 exonic

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Table 1  Primers, restriction enzymes, and RFLP for SNPs rs1050152 (L503F) and rs272879.

<table>
<thead>
<tr>
<th>Prime I.D.</th>
<th>Primer sequence (5’ to 3’)</th>
<th>RE</th>
<th>RFLP</th>
<th>Fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>For rs272879</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First round of PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OCTN1M43f</td>
<td>GCTCCTAATTTCATAGGAGATG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OCTN1100r</td>
<td>GAAGACAAATGGCAGAGATGCGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second round of PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OCTN1107f</td>
<td>TCCAGCTTACATTGAAGCTTG</td>
<td>ScrF1</td>
<td>cRFLP</td>
<td>G allele: 59</td>
</tr>
<tr>
<td>OCTN144r</td>
<td>CTATGATATAACGCCTGGCCA</td>
<td></td>
<td></td>
<td>C allele: 39+20</td>
</tr>
<tr>
<td>For rs1050152 (L503F)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OCTN1M48f</td>
<td>TAGCTGCTGCTGTCGATTGGA</td>
<td>Mnl I</td>
<td>RFLP</td>
<td>T allele: 99</td>
</tr>
<tr>
<td>OCTN1M50r</td>
<td>CACCTTCTGCATCTGCTCAA</td>
<td></td>
<td></td>
<td>C allele: 63+36</td>
</tr>
</tbody>
</table>

a RE restriction enzyme.

b RFLP: restriction fragment length polymorphism; cRFLP: converted RFLP.
sequences of OCTN1 (from genomic data base) in 95 individuals of the familial IBD registry in our preliminary study. Ten SNPs were dropped because they were not polymorphic or the frequency of the minor allele was very low (~5%) in the studied population (data not shown). The remaining one SNPs, rs1050152 (L503F) in exon 9 (cDNA 1672 C→T) and rs272879 in exon 6 (cDNA 1348 C→G), were studied here. The T allele of rs1050152 (L503F) was one of the two-allele haplotype that was previously studied.15–23

The OCTN1 gene shares high similarity (about 60% of nucleotides in coding exons and 88% of amino acids in proteins) with OCTN2. For accurate genotype analysis, gene specific PCR amplification was critical. We designed and used OCTN1 gene specific primers (at least one of the two PCR primers), and optimized PCR conditions (such as high annealing temperature) to eliminate the amplification of non OCTN1 sequences. The genotyping results of all the samples were identical between the two repeats of PCR, 1 µl of the first round PCR products was used as template with PCR primers OCTN1107f and OCTN144r. A DNA fragment containing the rs272879 was amplified directly from genomic DNA with PCR primers OCTN1M48f and OCTN1M50r (all primers are listed in Table 1). Five microliters of PCR products were incubated with Scr FI (for rs272879) and Mnl I (for rs1050152) at 37 °C for 4 h. The restriction enzyme digested PCR products were loaded on polyacrylamide gels (10% for rs1050152) at 37 °C for 4 h. The restriction enzyme digested PCR products were loaded on polyacrylamide gels (10% for rs1050152, 8% for rs1050152) to separate allelic products, and the gel was stained with ethidium bromide. The patterns of allelic products for rs1050152 (L503F) are indicated by arrows.

3.2. The OCTN1 rs1050152 (L503F) is significantly associated with CD and IBD overall, but not UC

Table 2 shows that the OCTN1 rs1050152 (L503F) is significantly associated with all the IBD study patients, which includes 102 familial IBD patients and 110 sporadic IBD patients (OR=1.68, 95% CI=1.052–2.676, \( \chi^2=4.732, p=0.030 \)). Of these IBD patients (CD=115, UC=97), the rs1050152 (L503F) variant is associated with CD (OR=1.745, 95% CI=1.019–2.990, \( \chi^2=4.129, p=0.042 \)), but not with UC (OR=1.554, 95% CI=0.891–2.711, \( \chi^2=2.410, p=0.121 \)).

Comparison of unrelated healthy controls with all members of the familial IBD registry, including both IBD and non-IBD members, showed no significant difference of OCTN1 rs1050152 (L503F) variant with CD (OR=1.32, 95% CI=0.842–2.071, \( \chi^2=1.464, p=0.226 \)). However, in the same population, we have observed an elevated level of allele A of DLG5 R30Q in all members of the same familial IBD registry (OR=1.778, \( \chi^2=4.372, p=0.037 \)).

3.3. No association of the OCTN1 rs272879 variant with IBD was observed

Statistic analysis indicated that the rs272879 variant was not associated with IBD overall, and with neither IBD subgroup (CD nor UC). There was no difference in the distribution of the rs272879 variant in the 241 individuals of the familial IBD registry compared with healthy controls (Table 2).
3.4. Distribution of the OCTN1 variants in men and women

We further analyzed the distribution of the two OCTN1 variants in men and women. As shown in Table 3, there was no gender difference in the distribution of the OCTN rs272879 variant in the patients with IBD, nor with IBD subgroups CD and UC. However, a gender difference was observed for the rs1050152 (L503F) variant. In male IBD patients, the rs1050152 (L503F) variant was associated with UC (OR = 2.585, 95% CI = 1.139–5.869, p = 0.023) and IBD overall (OR = 2.039, 95% CI = 1.024–4.059, p = 0.042), but not CD. In female IBD patients, the OCTN rs1050152 (L503F) variant was associated with CD (OR = 2.329, 95% CI = 1.038–5.226, p = 0.039) but not in UC and IBD overall. As these associations were weak and the sample size studied small, further studies with a larger number of IBD patients will be needed.

### Table 3 Distribution of OCTN1 SNPs rs1050152 (L503F) and rs272879 in men and women.

<table>
<thead>
<tr>
<th>SNP</th>
<th>All</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
<td>p value</td>
</tr>
<tr>
<td>-------</td>
<td>---</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>For IBD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L503F</td>
<td>1.677</td>
<td>1.052–2.676</td>
<td>0.030</td>
</tr>
<tr>
<td>rs272879</td>
<td>0.594</td>
<td>0.287–1.229</td>
<td>0.157</td>
</tr>
<tr>
<td>For CD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L503F</td>
<td>1.754</td>
<td>1.019–2.990</td>
<td>0.042</td>
</tr>
<tr>
<td>rs272879</td>
<td>0.570</td>
<td>0.256–1.268</td>
<td>0.165</td>
</tr>
<tr>
<td>For UC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L503F</td>
<td>1.554</td>
<td>0.891–2.711</td>
<td>0.121</td>
</tr>
<tr>
<td>rs171879</td>
<td>0.584</td>
<td>0.255–1.340</td>
<td>0.201</td>
</tr>
</tbody>
</table>

4. Discussion

In this study, we confirmed that the OCTN1 rs1050152 (L503F) variant was associated with CD and IBD overall, but not UC, in a central Pennsylvania Caucasian population. We also showed that no association was observed for the rs272879 variant with IBD, neither with IBD subgroup CD nor UC.

The association of the TC haplotype of OCTN1 and OCTN2 genes with IBD has been widely replicated by different groups in different populations, but it was not confirmed in others. This may reflect that IBD is a heterogeneous disease that is determined by multiple genes, imply that OCTN1 is a moderate or minor IBD gene, and/or is subject to modification by other factors. Supporting this notion, the OCTN1-TC risk haplotype has been associated with penetrating disease. To reduce the difference in ethnic and environmental background in the present study, we used a familial IBD registry pedigree. The familial registry used here is 99% Caucasian and 73% of individuals are descendants or immigrants from the UK, Germany or the Netherlands. Using this familial IBD registry in the present study, we observed a significant association of the OCTN1 rs1050152 (L503F) variant, with CD and IBD. The present study supports the previous finding of that two-allele haplotype TC of OCTN1 and OCTN2 is associated with CD, and further showed that the OCTN1 variant rs1050152 (L503F) alone is associated with CD.

Using the same population, we observed a high frequency (approximately 50%) of NOD2 mutations, and a significant association of the DLG5 R30Q, and of the IL23R R381Q and L310P with CD and IBD overall. All the results from our studies indicate that multiple genes are involved in the pathogenesis of IBD.

Interestingly, we also observed a gender-specific association of the OCTN1 rs1050152 (L503F) variant with IBD and IBD subgroups: female specific for CD and male specific for UC and IBD overall. In a previous study from Babusukumar et al., no gender difference was observed for OCTN TC haplotype. In the samples studied here, we have found a male specific association for the DLG5R30Q variant with CD, UC and IBD overall, and a female specific protective effect against CD for the IL23R variants. These observations, together with others, support the early epidemiological characterization of gender effect on IBD from the molecular level.

Moreover, the results show a different association between the nonsynonymous SNPs rs1050152 (L503F) and rs272879 with IBD. The distance between the locations of these two SNPs is just within 5744 bp in the coding region of the OCTN1 gene. The different association of these two SNPs with IBD may reflect their potential different impact on OCTN1 protein function. rs1050152 (L503F) is located in the 11th OCTN1 transmembrane domain, while rs272879 is located in the middle of the region between transmembrane domains and no information is available for its impact on OCTN1 function. The function of L503F of OCTN1 as well as OCTN1 gene in IBD pathogenesis is currently unknown.

OCTN1 and OCTN2 are novel organic cation transporters expressed in the intestine, and likely play a role in innate immunity response to pathogens and potentially play a role in intestinal physiology and IBD pathology. The recent study of OCTN2-mediated transport of the competence and sporulation factor (CSF) suggested a role of OCTN2 as a mediator of host-microbial interaction. Once CSF is taken up by intestinal epithelial cells, CSF activates p38MAP kinase and protein kinase B (Akt) and includes cytoprotective heat shock proteins, the latter preventing oxidant-induced intestine epithelial cell injury and loss of barrier function. Bacteria use quorum-sensing molecules (QSMs) to
communicate and coordinate population behavior in response to environmental changes. Many gut bacteria species are known to express QSMs. The human intestinal tract is home of an estimated 100 trillion bacteria and an abnormal response to the normal gut flora may trigger IBD pathogenesis. Further study on the effect of OCTN in response to gut flora may help to understand the roles of innate immunity and epithelial barrier in IBD pathogenesis.

In summary, from a familial and sporadic IBD population of central Pennsylvania, we replicated the association of OCTN1 L503F with CD and IBD overall, but not UC, while no association for another OCTN1 variant rs272879 was found. We also observed a weak gender-specific effect of OCTN1 L503F on male UC and female CD.

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