Single nucleotide polymorphisms of OCTN1, OCTN2, and DLG5 genes in Greek patients with Crohn’s disease

Maria Gazouli, Gerassimos Mantzaris, Athanassios J Archimandritis, George Nasioulas, Nicholas P Anagnou

Abstract
AIM: To validate novel single nucleotide polymorphisms (SNPs) in Greek patients with Crohn’s disease (CD).

METHODS: A total of 120 patients with CD, 85 patients with UC, and 100 unrelated healthy controls were genotyped. Genotyping was performed by allele-specific PCR or by PCR-RFLP analysis.

RESULTS: Our results showed that the 1672T and -207C alleles were obviously over-represented in CD patients only (P<0.01 and P<0.05, respectively) compared to the control population. The G113A polymorphism was completely absent in our studied population. The odds ratio for the carriage of the TC haplotype was 2.21 for CD patients as compared with controls. Additionally, the frequency of the TC haplotype was increased in patients with ileocolitis or ileitis, and was mainly associated with the fibrostenotic phenotype of the disease. Furthermore, when the TC haplotype was compared jointly with the carriage of at least one mutation of the NOD2/CARD15 gene, there was an increased risk for CD, but not for UC, compared to controls. Regarding the location of the disease, the concomitant presence of the TC haplotype and NOD2/CARD15 mutations was mainly associated with ileocolitis or ileitis.

CONCLUSION: Collectively, our results suggest that the 1672T variant of the OCTN1 gene and the -207C variant of the OCTN2 gene represent risk factors for CD in the Greek population.

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Key words: Crohn’s disease; SNPs; OCTN1; OCTN2; DLG5
To investigate whether the above mentioned SNPs in OCTN1, OCTN2, and DLG5 genes contribute to the predisposition to IBD, as well as whether the interaction of specific haplotypes of the NOD2/CARD15, OCTN1, OCTN2, and DLG5 genes could increase the risk for IBD in the Greek population, we genotyped 120 patients with CD, 85 patients with UC, and 100 healthy controls. Our studies documented that mutations of the OCTN1 and OCTN2 genes were obviously associated with CD. Furthermore, the combination of the OCTN-TC haplotype was found to be significantly associated with ileocolitis or colitis and the fibrostenotic phenotype, while the combination of the TC haplotype with the NOD2/CARD15 variants was associated with ileocolitis or ileitis.

### MATERIALS AND METHODS

**Subjects**

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 of the Evangelismos Hospital, between September 2002 and February 2003. The vast majority of these patients had been diagnosed at our institutions (open-access visit to the IBD Outpatients Clinic or as emergency cases), but there were also some referrals by other physicians. All groups were matched with regard to sex and age, and all subjects were of Greek origin. The diagnosis of either CD or UC was based on standard clinical, endoscopic, radiological, and histological criteria\(^1\),\(^2\). For CD, the vast majority of the patients (102, 85%) had newly diagnosed disease that was classified according to the Vienna System. The records of CD patients were systematically reviewed for the following demographic and clinical characteristics: age, sex, smoking habits, age at diagnosis, disease localization (ileal, colonic, ileocolonic or upper gastroenteric), disease behavior (inflammatory, fibrostenotic or fistulizing), presence of extra-intestinal clinical manifestations (e.g., arthritis, erythema nodosum), and familial IBD (Table 1). Before the commencement of the study, the ethics committee at the participating centers had approved the recruitment protocols. Informed consent was obtained from all the participants.

### Genotyping

DNA was isolated from blood with the NucleoSpin blood kit (Macherey-Nagel, Germany). To confirm the integrity of DNA, initially a 430-bp sequence in the human glyceraldehyde-3-phosphate dehydrogenase gene was amplified.

The genotyping for the three casual NOD2/CARD15 variants (L1007fsinsC, R702W, and G908R) in the studied group of patients and controls has been previously performed\(^3\).

The C1672T substitution in exon 9 of the OCTN1 was genotyped by a PCR amplification of specific allele assay, using two allele-specific reverse primers: octn1C, 5’ TCTGACTGTCTCCTGATTTGGAATCC 3’ for the wild type allele and octn1T: 5’ TCTGACTGTCTCCTGATTTGGAATCT 3’ for the mutant allele, in combination with a common forward primer octn1F: 5’ AGATGAGGTTTCACTATGGG 3’. The 3’-ends of the reverse primers were able to anneal to the regions that differed between the two alleles. The PCR profile included initial denaturation at 95 °C for 5 min, followed by 35 amplification cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 40 s and extension at 72 °C for 30 s and a final incubation at 72 °C for 10 min.

The mutation G207C in the OCTN2 promoter region resulted in the abolishment of a restriction site for NlaIV and was also genotyped by a combined PCR-RFLP method using primers 5’ TCACTTTCAGTTCTACCTGCTAC 3’ (forward) and 3’ f o r t h e w i l d t y p e a l l e l e a n d o c t n 1 T : 5’ TCTGACTGTCTCCTGATTTGGAATCT 3’ for the mutant allele, in combination with a common forward primer octn1F: 5’ AGATGAGGTTTCACTATGGG 3’. The 3’-ends of the reverse primers were able to anneal to the regions that differed between the two alleles. The PCR profile included initial denaturation at 95 °C for 5 min, followed by 35 amplification cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 40 s and extension at 72 °C for 30 s and a final incubation at 72 °C for 10 min.

The mutation G-207C in the OCTN2 promoter region resulted in the abolishment of a restriction site for NlaIV and was also genotyped by a combined PCR-RFLP method using primers 5’ TCACTTTCAGTTCTACCTGCTAC 3’ (forward) and 3’ for the wild type allele and octn1T: 5’ TCTGACTGTCTCCTGATTTGGAATCT 3’ (reverse). The presence of a wild-type allele resulted in a restriction site for NlaIV and was also genotyped by a combined PCR-RFLP method using primers 5’ TCACTTTCAGTTCTACCTGCTAC 3’ (forward) and 3’ for the wild type allele and octn1T: 5’ TCTGACTGTCTCCTGATTTGGAATCT 3’ (reverse). The presence of a wild-type allele resulted in a restriction site for NlaIV and was also genotyped by a combined PCR-RFLP method using primers 5’ TCACTTTCAGTTCTACCTGCTAC 3’ (forward) and 3’ for the wild type allele and octn1T: 5’ TCTGACTGTCTCCTGATTTGGAATCT 3’ (reverse).

### Table 1 Demographic characteristics and clinical features of 120 patients with Crohn’s disease and of 85 patients with ulcerative colitis

| Total number | Crohn’s disease | Ulcerative colitis |
|--------------|-----------------|-------------------|
| Sex (male/female) | 120 | 85 |
| Age of diagnosis (mean±SD yr) | 29.8±14.00 | 33.6±14.24 |
| Family history in first-degree relative (%) | 4 (3.3%) | 5 (5.9%) |
| Smoking habit (%) | | |
| Never | 50 (41.7%) | 48 (56.5%) |
| Ex-smoker | 11 (9.2%) | 14 (16.5%) |
| Current | 59 (49.2%) | 23 (27.1%) |
| Localization of disease | | |
| Ileal | 39 (32.5%) | 40 (47.1%) |
| Colonic | 11 (9.2%) | 12 (14.3%) |
| Ileocolitis | 67 (55.8%) | 24 (28.3%) |
| Upper gastroenteric | 3 (2.5%) | 2 (2.4%) |
| Disease features | | |
| Inflammatory | 78 (65%) | 67 (79.5%) |
| Fibrostenotic | 32 (26.7%) | 11 (12.9%) |
| Fistulizing | 10 (8.3%) | 6 (7.1%) |
| Extra-intestinal manifestations | | |
| Arthritis | 16 (13.3%) | 13 (15.3%) |
| Erythema nodosum | 5 (4.2%) | 2 (2.4%) |
Table 2 Allele and genotype frequencies of C1672T SNP in OCTN1 gene in CD and UC patients and in healthy controls

|        | C  | T  | T allele frequencies (%) | P [odds ratio (95%CI)] | CC | CT | TT | TT genotype frequencies (%) | P [odds ratio (95%CI)] |
|--------|----|----|-------------------------|------------------------|----|----|----|-------------------------------|------------------------|
| CD     | 25 | 75 | 0.01 [1.89 (1.16-3.07)]  |                        | 70 | 40 | 10 | 8.33                         | 0.095 [2.94 (0.78-10.99)] |
| UC     | 18 | 82 | 0.28 [0.71 (0.38-1.32)]  |                        | 67 | 17 | 1  | 1.17                         | 0.39 [0.38 (0.04-3.77)]  |
| Controls | 15 | 85 |                        |                        | 73 | 24 | 3  |                              |                        |

Table 3 Allele and genotype frequencies of G-207C SNP in OCTN2 gene in CD and UC patients and in healthy controls

|        | G  | C  | C allele frequencies (%) | P [odds ratio (95%CI)] | GG | GC | CC | CC genotype frequencies (%) | P [odds ratio (95%CI)] |
|--------|----|----|-------------------------|------------------------|----|----|----|-------------------------------|------------------------|
| CD     | 52 | 48 | 21.67                   | 0.038 [1.69 (1.02-2.81)]| 75 | 38 | 7  | 5.83                         | 0.53 [1.49 (0.42-5.25)] |
| UC     | 18 | 82 | 10.58                   | 0.32 [0.73 (0.39-1.37)] | 69 | 14 | 2  | 2.35                         | 0.53 [0.58 (0.10-3.24)] |
| Controls | 14 | 86 |                        |                        | 76 | 20 | 4  |                              |                        |

Table 4 Linkage disequilibrium (D' and r²) between 1672T and -207C are indicated), and TC haplotype frequencies in patients with CD, UC and in healthy individuals

|        | D' | r² | TC haplotype frequencies (%) | P [odds ratio (95%CI)] |
|--------|----|----|-----------------------------|------------------------|
| CD     | 0.51 | 0.22 | 13.3                        | 0.018 [2.21 (1.12-4.45)] |
| UC     | 0.5 | 0.23 | 5.9                         | 0.81 [0.89 (0.37-2.15)]   |
| Controls | 0.34 | 0.1 | 6.5                        |                        |

Table 5 Odds ratios for susceptibility to CD and UC of a NOD2/CARD15 mutation, and for the joint TC- NOD2/CARD15 effect

|        | TC     | NOD2/CARD15 | Joint TC- NOD2/CARD15 |
|--------|--------|-------------|------------------------|
| CD     | 2.21 (1.12-4.43) | 16.8 (8.6-32.7) | 9.22 (2.1-40.6) |
| UC     | 0.89 (0.37-2.15) | 3.34 (1.76-6.36) | 3.06 (0.58-16.21) |

RESULTS

We genotyped 120 patients with CD, 85 patients with UC and 100 healthy individuals in order to investigate a possible association of the genetic substitutions in the OCTN1, OCTN2 and DLG5 genes with a susceptibility to CD in the Greek population. These mutations were not tested using the 1 df χ² test. Allele frequency independent estimators of LD were used: the D' = D/Dmax, where Dmax is the maximum possible D (i.e., for border frequencies p1, p2, q1, q2, the lesser of p1q2 or p2q1 if D is positive or lesser of p1q2 or p2q1 if D is negative). Inference was aided by GraphPad InStat (version 3.00, GraphPad Software, Inc., San Diego, CA, USA).

Allele and genotype frequencies of the mutations G-207C of the OCTN2 gene are presented in Table 3. The presence of a wild-type allele resulted in five fragments of 40, 51, 65, 124, and 360 bp, whereas the RFLP profile of the 113A variant was characterized by four bands of 65, 91, 124, and 360 bp. The PCR conditions included initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 40 s and 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, 0.2 µmol/L of each primer, 200 ng of genomic DNA and 2.5 U of Taq DNA polymerase (Platinum Invitrogen). The specificity of PCR products was confirmed by sequencing analysis using a Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Darmstadt, Germany), and an ABI 377 automated sequencer.

Statistical analysis

Frequency and susceptibilities of mutations among the patients and controls were compared using the χ² test. Odds ratios (OR) were calculated with the corresponding χ² distribution test and 95% confidence intervals (95%CI). The two-tailed P<0.05 were considered statistically significant. Hardy-Weinberg equilibrium was verified by the calculation of expected frequencies and numbers, and significance testing was based on the 1 df χ². The hypothesis that there is no linkage disequilibrium (LD) was also tested using the 1 df χ². Allele frequency independent estimators of LD were used: the D' = D/Dmax, where Dmax is the maximum possible D (i.e., for border frequencies p1, p2, q1, q2, the lesser of p1q2 or p2q1 if D is positive or lesser of p1q2 or p2q1 if D is negative). Inference was aided by GraphPad InStat (version 3.00, GraphPad Software, Inc., San Diego, CA, USA).
distribution of genotypes was consistent with the Hardy-Weinberg equilibrium. C allele frequencies were markedly increased in only CD patients compared to the controls ($P<0.05$).

The G113A SNP of the DLG5 gene was completely absent in the Greek IBD cases as well as in the Greek healthy population.

The C1672T and G-207C were in strong linkage disequilibrium and created a two-allele risk haplotype (TC) (Table 3). The TC haplotype was significantly overrepresented in patients with CD (13.3%) as compared to the controls (6.5%) ($P<0.05$, Table 4). Odds ratios conferred by allele 1672T, allele -207C or the TC haplotype were similar. The risk for CD was much greater in the presence of both the TC haplotype and at least one of the three main alleles of NOD2/CARD15 gene (Table 5).

A significant association was found between ileocolitis and colitis and possession of TC haplotype. Twenty-three out of the thirty carriers of the TC haplotype had ileocolitis or colitis, whereas only seven TC carriers had exclusively ileal disease ($P<0.01$). Notably, when the presence of TC haplotype was evaluated jointly with the presence of one or more of the common NOD2/CARD15 variants, a significant association was observed with ileocolitis and ileitis. Seventeen of the nineteen carriers of both TC haplotype and at least one of the NOD2/CARD15 variants had ileocolitis or ileitis, whereas only two patients presented exclusively colitis ($P<0.05$).

In CD patients, disease behavior in 32 (26.7%) was defined as fibrostenotic, in 10 (8.3%) as fistulizing and in 78 (65%) as inflammatory. A significant association was observed between the presence of the TC haplotype and fibrostenotic $rs$ inflammatory phenotype of disease in our population. Twenty out of the thirty TC carriers presented a fibrostenotic phenotype since only 10 patients had inflammatory disease ($P<0.05$).

**DISCUSSION**

The precise etiology of CD and UC is uncertain, although it is widely accepted that IBD develops in a genetically predisposed individual following exposure to environmental stimuli$^{[10]}$. The genetic basis of IBD is adequately documented, since genetic factors that affect susceptibility to IBD have been disclosed through genetic linkage and population-based association studies$^{[12-14]}$. NOD2/CARD15 was the first gene which was found to be associated with IBD, specifically with CD$^{[9,10]}$. Through the candidate gene approach, various genes were identified as candidate genes to predispose to IBD in some populations$^{[10]}$. Very recently Peltekova et al$^{[21]}$, reported on two functional mutations in the OCN cluster on 5q31 (the IBD5 locus) that were associated with CD, while Stoll et al$^{[20]}$, reported the association of IB with mutations in the DLG5 gene.

Regarding the OCTN1 and OCTN2, it has been recently shown that mutations in these genes are associated with lower carnitine uptake rate and increased transport of xenobiotics$^{[14,17]}$. It is known that carnitine deficiency could be related with a disorder of fatty acid oxidation and consequently with insufficient fatty acid β-oxidation$^{[17]}$. On the other hand, there are some evidences that the inhibition of fatty acid oxidation in the epithelium of the colonic mucosa is associated with UC and inflammation$^{[18]}$. Taking all these into consideration, it seems reasonable that the OCTN cluster might have an active role in IBD pathogenesis.

Our case-control study for OCTN1 and OCTN2 genes showed that the frequency of the 1672T and -207C alleles was significantly higher in CD patients compared to UC patients and controls. Both mutations were, as expected from the previous studies on IBD5 haplotype, in strong linkage disequilibrium (LD) and created a two-allele risk haplotype, i.e. TC which in our cases had a frequency of 13.3% in CD patients compared to 6.5% in healthy individuals. Although the TC haplotype frequency that was observed was lower than that reported by Peltekova et al$^{[21]}$, our results confirmed an association between the OCTN cluster and CD. The TC haplotype was not increased in UC in our population, which was in agreement with Peltekova et al$^{[21]}$, but in contrast with several previous studies on IBD$^{[19,20]}$. It has to be pointed out that our results differed from those of a recent study in CD patients in a Japanese population, where other genetic variants have been associated with CD pathogenesis$^{[20]}$. Furthermore, it is known that variants in the IBD5 haplotype appear to be very rare in the Japanese population$^{[18]}$.

It has been hypothesized that the third member of the OCTN cluster, the OCTN3 gene, in the OCTN1-OCTN2 interval, is also associated with IBD$^{[24]}$. The OCTN3 gene might represent a homolog to the mouse gene Slc22a9 and several research groups were unable to identify a human counterpart or any other gene within this region$^{[12,24]}$.

Interestingly, the risk for CD was even greater in the presence of both TC haplotype and at least one of the NOD2/CARD15 variants, confirming the previously reported interaction between IBD5 haplotype and NOD2/CARD15$^{[25]}$. Notably, in agreement with our results, very recently, Torok et al$^{[30]}$, reported that TC haplotype was associated with an increased CD risk, which increases even more in the presence of NOD2/CARD15 mutations.

Patients with CD clinically present heterogeneous disease characteristics, including differences in disease behavior, localization and severity. Defining the relationship between OCTN-TC haplotype and disease phenotypic variation is not only crucial in probing the clinical diversity in disease presentation and behavior, but may also assist in defining rational treatment strategies. Concerning the disease location in the intestine, we found that the possession of the TC haplotype was associated mainly with colitis or ileocolitis, which was in agreement with previous findings that demonstrated the IBD5 association with colonic CD$^{[24,27]}$. However, when the TC haplotype was combined with the presence of at least one of the NOD2/CARD15 variants, a significant association with ileitis or ileocolitis was observed, which was in agreement with the results of a recent study by Newman et al$^{[28]}$. This observation suggests that these variants have a
biological involvement in CD pathogenesis. When disease behavior was examined, the presence of the TC haplotype was found to be associated with the fibrostenotic phenotype.

Concerning the DLG5 gene, which is important in maintaining the epithelial structure, the 113A variant was completely absent in our studied population. Our observations concerning the DLG5 gene were strongly in contrast with previous data reported by Stoll et al.,

Additionally, our results indicate that the carriage of the OCTN-TC haplotype is significantly associated with ileocolitis or colitis and the fibrostenotic phenotype, but the TC haplotype combined with the presence of NOD2/CARD15 variants, associates with ileocolitis or ileitis. However, further studies involving a larger number of cases and controls in a worldwide scale are needed to elucidate the complex biological mechanisms underlying IBD susceptibility.

Collectively, our study confirms recent findings suggesting that the mutations in OCTN1 and OCTN2 genes are associated with CD.

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