C.802C>T NOD2/CARD15 SNP is Associated to Crohn’s Disease in Italian Patients

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Abstract

The incidence of Crohn’s Disease (CD), a complex inflammatory bowel disease, is rapidly increasing. NOD2/CARD15 gene variants have been associated with early CD onset, terminal ileal involvement, and structuring disease. We comparatively analyzed, by PCR and direct sequencing, the exons 4, 8 and 11 of NOD2/CARD15 gene in CD Italian patients (n=42) and in healthy controls (n=66). Our results show that the frequency of the allele T of the c.802C>T SNP (rs2066842) results in linkage disequilibrium with allele T of the c.1377 C>T (p. R459R) SNP. Moreover, the frequency of the allele T of the c.802C>T (p.P268S) SNP (rs2066842) is significantly higher in CD’s patients than in control subjects (p=0.018; OR=2.02). Similarly, the frequency of the insertion c.3020insC (p.L1007fs) is significantly higher (p=0.0347; OR=14.59) in CD patients. Our results suggest that molecular analysis of the NOD2/CARD15 gene could represent a contributory tool for the identification of subjects genetically predisposed to CD.

Keywords: NOD2/CARD15 gene; Variants; Molecular analysis; Crohn disease

Introduction

Crohn’s Disease (CD) is a chronic relapsing inflammatory disorder of the gastrointestinal tract carrying a high morbidity and a poor quality of life [1,2]. In the last two decades, the incidence of CD is rapidly increasing [3]. Crohn’s disease diagnosis is often established following considerable diagnostic delay. An European study reported that the median diagnostic delay in CD was 9 months with 75% of all study subjects receiving a final diagnosis within 24 months [4]. Delay in CD diagnosis not only reduces the quality of life of the patients, but also has important clinical implications, such as significantly reduced response to medical therapy. It has been demonstrated that the length of diagnostic delay correlates with an increased risk of bowel stenosis and CD-related intestinal surgery. For these reasons, efforts should be undertaken to shorten the diagnostic delay [5]. The pathogenesis of CD is still largely unclear, it meanly derives from interaction of environmental factors and genetic predisposition. Genetically predisposed individuals have a dysregulated mucosal immune response to commensal gut microbiota which determines chronic bowel inflammation. Moreover genome wide association studies indicated several genetic factors associated with CD susceptibility [6-9]. Nucleotide-binding Oligomerization domain (NOD2)/Caspase-Recruitment Domain (CARD15) was the first gene identified as susceptibility gene for CD. It is located on chromosome 16q12-21 and constituted by 12 exons encoding a protein involved both in defence against microbial infections and in regulation of inflammation and apoptosis [10,11]. NOD2 protein contains 4 functional domains: 2 regions called CARD, involved in apoptosis, located at the N-terminus; a central domain NBD (nucleotide-binding domain), which possesses ATPase activity and is important for the oligomerization of the protein; a region of 10 leucine-rich repeat sequences (LRR), located at the C-terminal, involved in the interaction with the muramyl dipeptide [12]. A number of polymorphisms has been described in the NOD2 gene with a wide heterogeneity between different ethnic CD groups [10,13-15]. However, the variants associated with CD are c.2104 C>T (p.R702W), c.2722G>C (p.G908R), and 3020insC (p.L1007fs) localized in exons 4, 8 and 11, corresponding to LRR protein domain or adjacent region [16,17]. The impact of these mutations, two amino acid substitutions and one single base insertion, are still unclear [6,11,16,17].

In order to investigate the possible association between polymorphisms in NOD2/CARD15 gene and CD Italian patients, we comparatively analyzed by direct sequencing of the 4, 8 and 11 exons a cohort of CD patients and healthy controls.

Materials and Methods

Subjects

Consecutive forty-two subjects (11–69 years, mean 26.6 years) with a well-established CD diagnosis, according to standardized criteria [4,5], observed at Department of Translational Medical Science at the University of Naples Federico II were invited to participate into the study. All patients accepted to participate and a blood sampling was performed during routine visit for the follow up of CD (6 ml of whole blood from venipuncture). The control group consists of 65 healthy unrelated adults consecutively observed at the same Department without first and second degree family members of these subjects had an history of suspected or defined diagnosis of inflammatory bowel disease. A blood sampling was performed in these healthy subjects during a screening program. All study subjects were Caucasians. All subjects enrolled in the study, provided written informed consent. The study was approved by the Ethics Committee of our Institution.

DNA extraction and PCR

Genomic DNA was extracted from whole blood samples using a commercial kit (Nucleon BACC 2; Amersham Biosciences). The exons

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4, 8, 11 of NOD2/CARD15 gene were amplified using the following primers, designed by Primer3 software:

- Exon 4F 5'AGTGCACAGCTTGTGAATGG 3',
- Exon 4R 5'GCTCCCACACTTAGCCTTGA3',
- Exon 8F 5'CCACTCTGGATGGATGGTG3',
- Exon 8R 5'TCCATTGCCTAACATTTG3',
- Exon 11F 5'GGACAGGTGGGCTTCAGTAG3',
- Exon 11R 5'CCTCAAAATTCTGCCATTCC 3'

Protocol was performed as previously described [18,19]. For the amplification reaction was used a touchdown PCR protocol, consisting in 1 cycle of 3 min of denaturation at 94°C, after which the DNA was amplified during 39 cycles, of which 14 cycles consisted of 20s of denaturation at 94°C, decreasing 0.5°C each cycle, and 45s of extension at 72°C; then 25 cycles of denaturation at 94°C for 20s, 40s of annealing at 55°C and 45s of extension at 72°C. After amplification, the reaction mixture was subjected to a final cycle of 7 min of extension at 72°C. PCR products were subjected to sequence analysis performed on both strands with an automated procedure using the 3100 Genetic Analyzer (Applied Biosystems). PCR fragments were sequenced using the same primers used for PCR amplification.

### Results

We amplified by PCR and direct sequenced exons 4, 8, 11 of NOD2/CARD15 gene to analyze the allele genotype in control subjects and CD patients. Molecular analysis revealed the following variants: c.802C>T (p.P268S), c.1377 C>T (p.R459R), c.1761 T>G (p.R587R), c.2104 C>T (p.R702W), c.2722G>C (p.G908R), and 3020insC (p.L1007fs).

The frequency of the allele T of the c.802C>T (p.P268S) SNP results in linkage disequilibrium with allele T of the c.1377 C>T (p.R459R) SNP. Moreover, the frequency of the allele T of the c.802C>T (p.P268S) SNP is significantly higher in CD's patients than in control subjects ($p = 0.018; OR=2.02$). In addition, the C insertion of the c.3020insC (p.L1007fs) is significantly higher ($p = 0.0347; OR=14.59$) in CD patients.

No significant differences of the allelic frequency were observed for the variants c.1761 T>G (p.R587R), c.2104 C>T (p.R702W), c.2722G>C (p.G908R) (Table 1). The genotype frequencies of the c.802C>T (p.P268S) SNP and variant 3020insC (p.L1007fs) were differently distributed between cases and controls ($p = 0.03$ and $p = 0.01$).

### Discussion

Epidemiological and linkage studies suggest that genetic factors play a significant role in determining CD susceptibility [6-9], among these the most associated is NOD2/CARD15 gene [10,11,13-17]. Polymorphisms in NOD2 gene reduce NOD2/CARD15 protein function impairing a balanced inflammatory response to external stimuli [14,20]. Several variants were identified as genetic determinants of CD susceptibility, even if with a remarkable heterogeneity among racial and geographical groups [11,16,17,20]. The most frequent variants in CD are c.2104 C>T (p.R702W), c.2722G>C (p.G908R), and

| SNP/ genotypes Location | Control frequencies, % | CD frequencies, % | Control MAF | CD MAF | Armitage's trend test | p value | OR (C.I.) |
|-------------------------|------------------------|-------------------|-------------|-------|-----------------------|---------|-----------|
| c.802 C>T (p.P268S)     |                        |                   |             |       |                       |         |           |
| CC                      | 60.6 (40)              | 42.8 (18)         | 0.24        | 0.39  | 0.03                  | 0.018   | 2.022 (1.119-3.654) |
| CT                      | 30.3 (20)              | 35.7 (15)         |             |       |                       |         |           |
| TT                      | 9.1 (6)                | 21.4 (8)          |             |       |                       |         |           |
| c.1377 C>T (p.R459R)    |                        |                   |             |       |                       |         |           |
| CC                      | 60.6 (40)              | 45.2 (19)         | 0.24        | 0.37  | 0.07                  | 0.045   | 1.828 (1.007-3.316) |
| CT                      | 30.3 (20)              | 35.7 (15)         |             |       |                       |         |           |
| TT                      | 9.1 (6)                | 19.0 (8)          |             |       |                       |         |           |
| c.1761 T>G (p.R587R)    |                        |                   |             |       |                       |         |           |
| TT                      | 50 (33)                | 52.4 (22)         | 0.33        | 0.30  | 0.62                  | 0.580   | 0.847 (0.469-1.531) |
| TG                      | 33.3 (22)              | 35.7 (15)         |             |       |                       |         |           |
| GG                      | 16.6 (11)              | 12 (5)            |             |       |                       |         |           |
| c.2104 C>T (p.R702W)    |                        |                   |             |       |                       |         |           |
| CC                      | 92.4 (61)              | 83.3 (35)         | 0.04        | 0.09  | 0.10                  | 0.083   | 2.674 (0.844-8.469) |
| CT                      | 7.6 (5)                | 14.3 (6)          |             |       |                       |         |           |
| TT                      | 0 (0)                  | 2.4 (1)           |             |       |                       |         |           |
| c.2722 G>C (p.G908R)    |                        |                   |             |       |                       |         |           |
| GG                      | 95.4 (63)              | 90.5 (38)         | 0.02        | 0.06  | 0.21                  | 0.269   | 2.722 (0.633-11.701) |
| GC                      | 4.5 (3)                | 7.1 (3)           |             |       |                       |         |           |
| CC                      | 0 (0)                  | 2.4 (1)           |             |       |                       |         |           |
| c.3020insC (p.L1007fs)  |                        |                   |             |       |                       |         |           |
| WT                      | 66 (100)               | 90.5 (38)         | 0.01        | 0.05  | 0.01                  | 0.0347  | 14.590 (0.775-274.584) |
| WT/INS                  | 0 (0)                  | 9.5 (4)           |             |       |                       |         |           |
| INS/INS                 | 0 (0)                  | 0 (0)             |             |       |                       |         |           |

INS= insertion; WT= wild type; MAF= Minor allele frequency; OR= Odds Ratio; CI= confidence interval. The statistical significance was established at $p < 0.05$.
3020insC (p.L1007fs) [16,17]. These polymorphisms alter the structure of the protein at the level of the LRR domain or the adjacent regions, interfering with bacteria recognition and increasing the production of IL-12, IL13, IL23 and IFN- pro-inflammatory cytokines leading to a state of chronic inflammation [12]. In presence of one NOD2/CARD15 allele mutation, the risk of developing CD increases to 2-4 times, and even up to 20-40 in the case of a double mutation (heterozygous or homozygous) [14,15]. The present study confirms that molecular analysis of NOD2/CARD15 gene could represent an effective diagnostic tool for the identification of subject genetically predisposed to CD. In fact, our findings revealed a significant association between the allele T of c.802C>T (p.P268S) SNP and CD. Moreover, our data show linkage disequilibrium of c.802C>T (p.P268S) with c.1377 C>T (p.R459R). In addition, we found that the variant 3020insC (p.L1007fs) is significantly associated with CD susceptibility. Our results are in agreement with recent data reporting a genetic association between allele T of c.802C>T (p.P268S) and CD in Chinese patients [6,10].

In conclusion, our results suggest that molecular analysis of the NOD2/CARD15 gene could represent an indicative diagnostic tool for the identification of subject genetically predisposed to CD.

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