

Acute intestinal obstruction and NOD2/CARD15 mutations among Italian Crohn's disease patients

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Abstract. – Three CARD15 mutations (SNP8, SNP12, SNP13) were significantly associated with CD, however ethnic variations and genotype-phenotype relationships are still to be defined.

Aims: To evaluate the prevalence of three CARD15 mutations in 91 in-out consecutive CD, 109 Ulcerative Colitis (UC), 101 healthy controls; to examine the genotype-phenotype relationships among Italian pts with CD.

Material and Methods: The three mutations were determined by direct sequencing analysis. In CD were evaluated several features of disease phenotype. Data analysis was performed by using χ^2 or Fisher Test applying Bonferroni's correction.

Results: The allelic and genotype frequencies of CARD15 mutations were significantly associated with CD. None of controls or UC were homozygotes (OM) or compound heterozygotes (CET). In CD the carriers of at least one mutation were 26/91 (28.6%). The frequencies of simple heterozygotes (ET), CET and OM were: 19/26, 4/26, 3/26 respectively. A significant positive association was found between small bowel location and an acute intestinal obstruction at diagnosis and the carriers of at least one mutation ($p = 0.036$, OR:0.33 [0.12-0.9] and $p = 0.0025$, OR:0.125 [0.03-0.5], respectively), particularly with OM and CET genotype ($p = 0.005$, OR:0.07 [0.01-0.6]). A positive trend between the number of surgery and the carriers of at least one mutation was found, but it didn't reach statistical significance ($p = 0.0469$, OR:0.3 [0.1-0.96]). No relationship between CARD15 mutations and the other phenotype characteristics was found.

Conclusions: Our data confirms that CARD15 mutations are significantly associated with CD also in Italian population and with small bowel location (OM and CET genotype). A new positive association was also found between the carriers of at least one mutation and the acute intestinal obstruction at diagnosis.

Key Words:

CARD15, Crohn's disease (CD), Phenotype, Intestinal obstruction.

Introduction

The aetiology of Crohn's disease (CD) remains unknown. The disease results from a dysregulated intestinal immune response to intestinal microbes or antigens, in genetically susceptible individuals¹. Epidemiological studies have shown the consistent role of genetic factors in CD susceptibility and it is now clear that gene-environment interactions are central to understanding disease pathogenesis.

Many studies have identified putative loci on several chromosomes to localize Inflammatory Bowel Disease (IBD) susceptibility genes through genome-wide linkage studies². In 1996, Jean-Pierre Hugot's group identified a susceptibility locus for CD adjacent to the centromere on chromosome 16 (IBD1), confirmed by a number of centres³. In 2001, three independent mutations within NOD2 gene, mapping to chromosome 16, have been found to be associated with CD⁴⁻⁶. The identified mutations were one frameshift mutation (3020insC: SNP13) and two missense mutations (C2104T: SNP8 and C2722C: SNP12).

10-30% of CD patients are heterozygotes for at least 1 of the 3 mutations and 3-15% are homozygotes or compound heterozygotes. In comparison, in healthy control sub-

jects and ulcerative colitis (UC) patients, 8-15% are heterozygotes and 0-1% are homozygotes, respectively. Heterozygotes have a slightly increased risk (1.5-3-fold) of developing CD, whereas homozygotes and compound heterozygotes have a 10-40-fold risk. In conclusion, less than 30% of the overall susceptibility to CD may result from the effect of CARD15 mutations⁷. In clinical practice, the screening of healthy high risk individuals (relatives of CD patients) is not recommended on account of the limited penetrance of the CARD15 genotypes. However, there is considerable potential for genetic screening of already diagnosed CD patients, but this could only be proposed when the results of such testing can be used for improving the management of disease.

The contribution of CARD15 mutations to disease susceptibility varies in different racial and ethnic populations. The presence of CARD15 mutations confer an increased risk of CD primarily in Caucasian population. There are no such associations within Japanese, Korean and African-American populations and there are now clear differences between Ashkenazi Jewish and non-Jewish population⁸⁻¹². This data indicates that the mechanisms of disease pathogenesis varies between different populations, suggesting that CARD15 mutations may be sufficient, but not necessary to develop CD and other specific gene-gene and gene-environment interactions are needed.

A number of controversial areas remain unresolved in defining genotype-phenotype relationships, because this data is limited by the quality of phenotype data and by the limitations of the currently adopted clinical classifications. The most frequently reported relationship between variant CARD15 alleles is associated with ileal disease, particularly with double-dose carriers¹³⁻¹⁵. CD patients with ileal disease has 2.5 fold risk of being a carrier of at least one of three CARD15 mutations. This pattern of disease may be related to the distribution of Paneth cells in the gastrointestinal tract, because the Paneth cells that express CARD15 gene, are concentrated in the terminal ileum and have a key role in the regulation of the innate immune response (defensins). An inverse association with colonic disease has been identified.

The association of CARD15 risk alleles with disease behaviour are less well established, since different studies have used different definitions to classify CD patients and moreover disease behaviour may change with the increase in fistulous and stricturing complications over time¹⁶. The CARD15 mutations are associated with a stricturing disease, however it is clear that ileal disease is strongly associated with stricturing disease. It is the same for the variably reported association between CARD15 mutations and the need for more frequent surgery¹⁷⁻¹⁸. Finally, age at disease onset is approximately 2 years earlier in double-dose carriers of CARD15 risk alleles compared with that of all CD patients. Recently, it has been demonstrated that the presence of Crohn's-associated CARD15 alleles does not affect therapeutic responses to Infliximab and a considerable amount of work is still needed to indicate if this genotype evaluation helps to predict response to the drugs currently in use.

The aims of this study were to evaluate the prevalence of three CARD15 mutations in 91 in-out consecutive CD, 109 Ulcerative Colitis (UC), 101 healthy controls and to examine the genotype-phenotype relationships among Italian CD patients.

Materials and Methods

Patients

The study group consisted of 91 Italian patients with a diagnosed CD including clinical, radiologic, endoscopic and histological findings according to standardized criteria. The two control groups consisted of 109 Italian patients with a diagnosed UC and healthy subjects. All patients from the disease and control groups were Caucasians. Informed consent was obtained from each participant and disease phenotype data was obtained through retrospective collection from the patients clinical charts.

The following data of patients with CD were obtained: age, age at diagnosis, gender, familial or sporadic disease (familial disease was considered if one first or second degree relative had IBD), smoking habits (current smoking/history of smoking/never smoked), appendectomy, symptoms at diagnosis, dis-

ease location (ileal, right ileocolon, left ileocolon, total ileocolon, colon and upper), disease behaviour at diagnosis and at present (stricturing, inflammatory and fistulizing), extraintestinal manifestations (type I peripheral arthralgia, affections of eyes or skin, primary sclerosing cholangitis), type and date of surgery, number of stenosis and number and type of fistules, perianal disease and pharmacological and surgical therapeutic management.

The following definitions were taken into account: disease localization was defined as the maximum extent of digestive involvement at the latest follow-up. Information was obtained through endoscopic (including upper endoscopy or colonoscopy), radiological (small bowel X-ray) or histological examination. Stenotic CD was considered if persistent intestinal obstruction was found either in small bowel X-ray, ultrasonography, RM or colonoscopy. Perforating disease was recorded if patients had enterocutaneous, enteroenteric, enterovesical or enterovaginal fistula, intrabdominal abscess or small bowel perforation. Perianal disease was diagnosed if perianal fistula, ulcers or abscesses were present. Extraintestinal manifestations were defined as follows: Type I peripheral arthralgia¹⁹, presence of primary sclerosing cholangitis diagnosed by endoscopic cholangiography, affections of skin (presence of erythema nodosum or pyoderma gangrenosum) or eye (presence of episcleritis or anterior uveitis).

Polymerase Chain Reaction and Sequencing

Genomic DNA from all the patients' blood were prepared using commercially available extraction columns purification kit according to the manufacturer's protocol (NucleoSpin Blood Quick Pure). Genotyping of the three major CARD15 mutations (Arg702Trp: SNP8, Gly908Arg: SNP12, Leu1007fsinsC: SNP13) was performed using allele-specific PCR amplification with the following primers: 5'-ACCTTCAGATCACAGCAGCC-3' e 5'-GCTCCCCATACCTGAAC-3' (SNP8 422 bp), 5'-AAGTCTGTAATGTAAAGCCAC-3' e 5'-CCCAGCTCCTCCCTCTTC-3' (SNP12 380 bp), 5'-CTCACCATTGTATCTTCTTTTC-3' e 5'-GAATGTCAGAATCAGAAGGG-3' (SNP13 228 bp). The PCR was performed using the following conditions: 92°C for 2 min, 35

cycles (95° C for 50 s, specific annealing temperature for 1 min, 72° C for 1 min and 50 s) and an additional extension at 72° C for 7 min. PCR products were electrophoresed on 2% agarose and visualized with ethidium bromide.

DNA sequencing of the amplified products was performed by cycle sequencing with fluorescent dye terminators. Analysis was performed using an ABI 377 automatic sequencer (Applied Biosystems, Weiterstadt, Germany).

Statistical Analysis

Comparison of the frequency of the CARD15 mutations between patients and control groups and the association to the phenotype was performed by Chi-Square test or Fisher's exact test where appropriate, applying Bonferroni's correction. Allele and genotype frequencies were performed.

Results

Distribution of CARD15 Mutations

We investigated the frequencies of the three CARD15 mutations in 91 CD, 109 UC and 101 healthy controls. Genotypes and allele frequencies are shown in Figure 1. In total 28.6% (26/91) of CD patients carried at least one mutant allele within CARD15 compared with UC and healthy control. In the two controls groups differences between patients with UC and healthy controls were not significant. The frequencies of simple heterozygotes, compound heterozygotes and homozygotes were: 19/26, 4/26, 3/26 respectively. 7.7% of CD patients whereas none of the UC patients or healthy controls were homozygous for any of the three CARD15 mutations.

Genotype-Phenotype Analysis

In the next step a detailed genotype-phenotype analysis was performed in the 91 patients with CD. The percentages of CD patients carrying at least one CARD15 mutation were correlated to following data: age, age at diagnosis, gender, familial or spontaneous disease, smoking habits, appendectomy, symptoms at diagnosis, disease location, disease behaviour at diagnosis and at present, extraintestinal manifestations, type and date

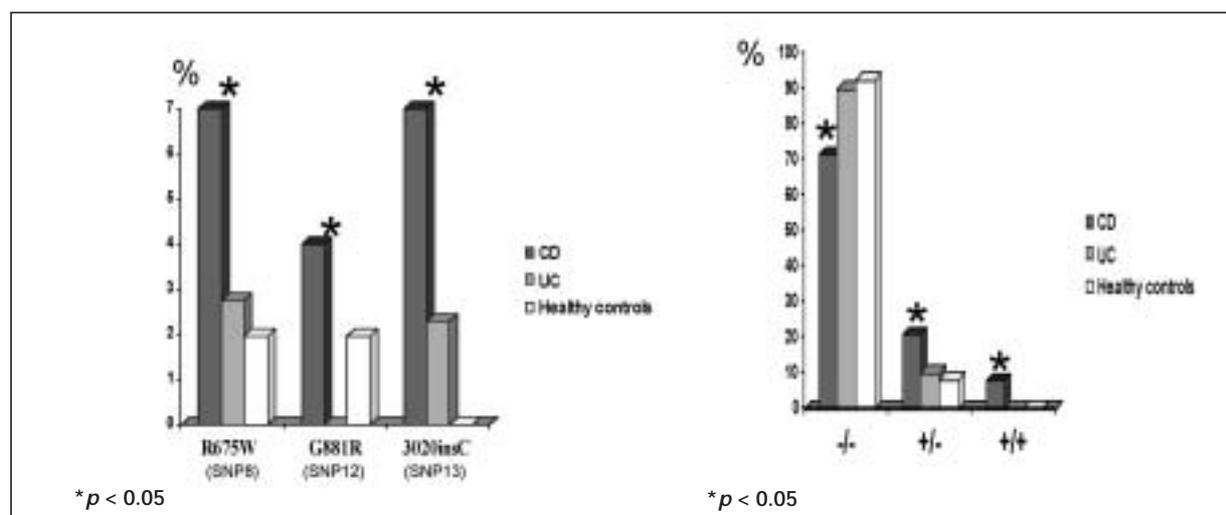


Figure 1. Genotypes and allele frequencies of three CARD15 mutations.

of surgery, number of stenosis and number and type of fistules, perianal disease and pharmacological and surgical therapeutic management.

A significant positive association was found between the small bowel location and the carriers of at least one mutation ($p = 0.036$, OR:0.33 [0.12-0.9]), particularly with omozygous and compound heterozygotes patients. The small bowel location was present in 16 of 57 (28.1%) wild type patients and in 12 of 22 (54.5%) carriers of at least one mutations. This association was higher in homozygous patients, presenting in 6 of 7 patients carried homozygous genotype ($p = 0.0053$, OR: 0.07 [0.01-0.6]). No relationship between CARD15 mutations and the other disease location was found.

The CD patients were subclassified on the basis of the symptoms at diagnosis and a significant association was found between acute onset and the carriers of at least one mutations ($p = 0.017$, OR: 0.24 [0.08-0.7]). The CD patients with acute onset were subclassified in: appendectomy, intestinal acute obstruction and other type of acute abdomen. A new significant association was found between an acute intestinal obstruction at diagnosis and the carriers of at least one mutation ($p = 0.0025$, OR:0.125 [0.03-0.5]), particularly with homozygous and compound heterozygous genotype ($p = 0.005$, OR:0.07 [0.01-0.6]) (Figure 2).

A positive trend between the number of surgery and the carriers of at least one mutation was found, but it didn't reach statistical significance ($p = 0.0469$, OR:0.3 [0.1-0.96]). No relationship between CARD15 mutations and the other phenotype characteristics was found (age at diagnosis, familial or spontaneous disease, smoking habits, appendectomy, disease behaviour at diagnosis and at present, extraintestinal manifestations, number of stenosis, number and type of fistules, perianal disease, pharmacological and surgical therapeutic management).

Discussion

In this research 91 Italian CD patients were genotyped for the most common CD-associated CARD15 mutations. In agreement with previous findings, we confirm that CARD15 mutations were significantly associated with CD also in Italian population. The prevalence of CARD15 mutations in this CD patients cohort was 28.6%, a figure similar to that reported in other studies wherein CARD15 genotyping was restricted to analysis of the three major CD-associated CARD15 alleles.

In addition, among the genotype-phenotype relationship, we are analyzed for the

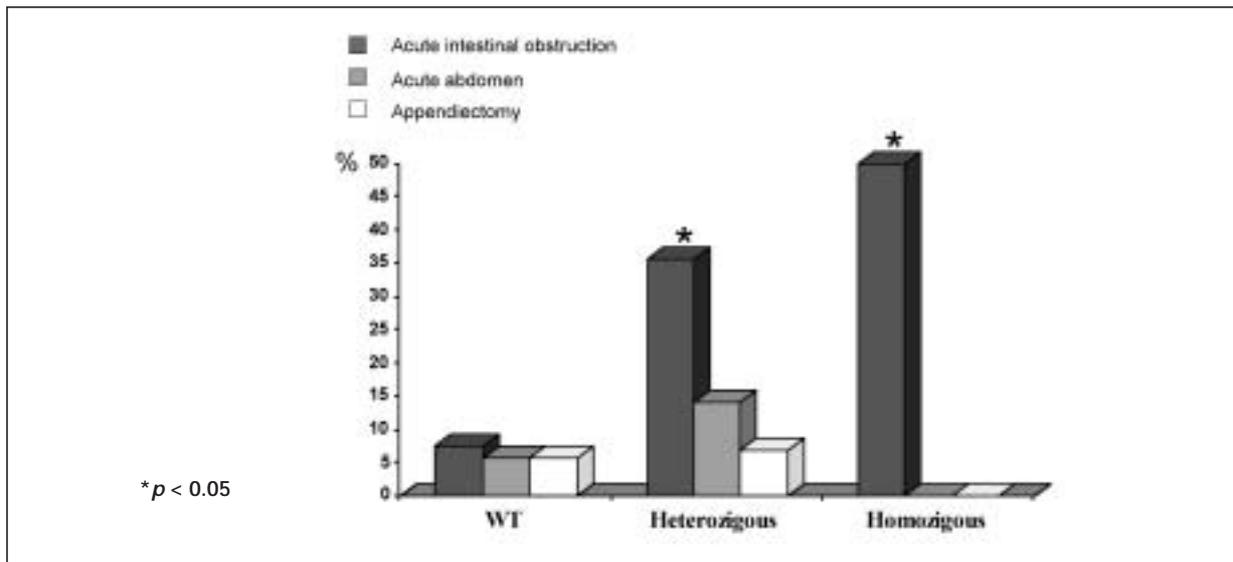


Figure 2. A significant association was found between an acute intestinal obstruction at diagnosis and the carriers of at least one mutation ($p = 0.0025$, OR:0.125 [0.03-0.5]), particularly with homozygous and compound heterozygous genotype ($p = 0.005$, OR:0.07 [0.01-0.6])

first time the association between CARD15 mutations and the feature of symptoms at diagnosis of CD patients, showing that these mutations are associated with acute onset and acute intestinal obstruction at diagnosis. It will be important to analyze the development of disease among these patients to show if there is an association with more aggressive disease phenotype.

Finally, we confirm the previous showed association between CARD15 mutations and small bowel location. There is now emerging evidence that the association between CARD15 mutations and small bowel location may be related to the distribution of Paneth cells within gastrointestinal tract that express CARD15 gene²⁰. These cells are concentrated in the terminal ileum and absent from the colon, and have a role in innate immunity via secretion of defensins. Recent provocative data suggest that carriage of CARD15 variants may be associated with reduced α -defensin release from Paneth cells in response to bacterial cell wall components. One may suggest that defective defensin release by the Paneth cell provide the missing link whereby reduced CARD15 activity impair host defences against bacteria and underlie persistent inflammation²¹.

In particular, CARD15 is an intracellular receptor protein involved in the recognition of intracellular pathogen-associated molecular patterns such as muramyl dipeptide (MDP), a component of both gram negative and positive bacterial cell walls. This receptor plays a key role in detecting the bacterial antigen and in transmitting several signalling cascades that induce a complex innate immune response. Its structural domains are related to the well-describes R proteins in plants that mediate host resistance to microbial pathogens. CARD15 protein has three structural domains: the N-terminus portion contains 2 caspase associated recruitent domains (CARD) which induce the nuclear factor- κ B (NF κ B) signalling cascade and active apoptosis; the central nucleotide-binding domain which induces self-oligomerization required for activation; and the C-terminus leucine-rich repeat (LRR) domain which is a pattern-recognition receptor for several types of microbial components²². The mutations in CARD15 gene result in reduced NF κ B activation, but conversely the uncontrolled mucosal inflammation of CD is characterised by upregulation of NF κ B activation²³. Recent study proposed that CARD15 is a negative regulator of the

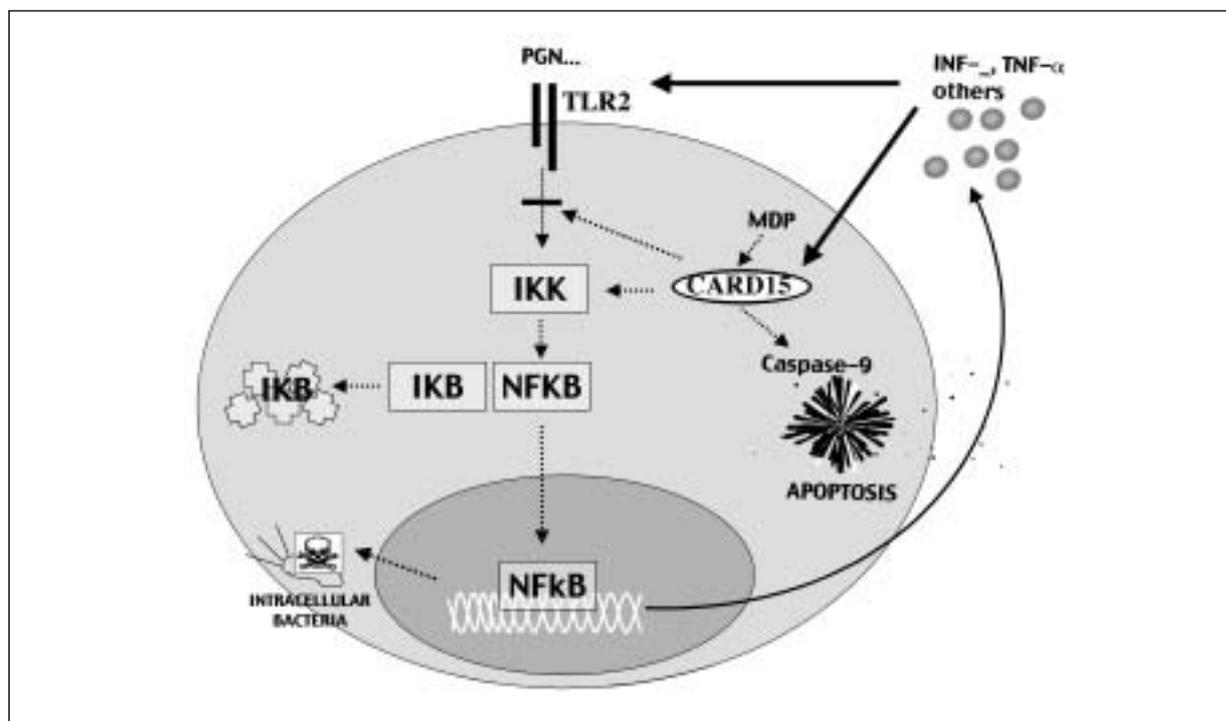


Figure 3. CARD15 is an intracellular protein that induces apoptosis via caspase-9 and induces nuclear factor- κ B (NF κ B) activation in epithelial cells (IECs). Activation of NF κ B, via I κ B kinase complex (IKK) responsible for degradation of its inhibitor I κ B, induces the expression of proinflammatory cytokines such as tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ). In turn, the expression of CARD15 protein is up-regulated by activity of TNF- α and IFN- γ . Activated NF κ B plays also an important role in the eradication of intracellular bacteria (*Salmonella typhimurium*) in IECs. The CARD15 receptor protein is the functionally intracellular counterpart of the cell surface Toll-like receptor 2 (TLR2) and acts as negative regulator of TLR2 signalling. Activation of TLR2 with specific antigen such as peptidoglycan (PGN) leads to activation of NF κ B via IKK producing proinflammatory cytokines.

Toll-like receptor2-(TLR2)-mediated cytokine response²⁴. The intact CARD15 signaling inhibits TLR2-driven activation of NF κ B that induce Th1 immune response (Figure 3). Moreover, CARD15 deficiency increase TLR2-mediated activation of NF κ B and Th1 immune response. The results reported in this study suggest that activation of CARD15 signalling by gram-positive but not gram-negative bacteria decrease the inflammation of CD, down-regulating Th1 immune response and the patients with intact CARD15 protein could be treated by the introduction of gram-positive bacteria into their intestinal flora. Although the precise role played by CARD15 mutations in the pathogenesis of CD remains to be clarified and the association of these mutations with CD support the hypothesis of the close relationship be-

tween genetic, environmental and innate immune system.

In conclusion, the identification of CARD15 mutations as the first susceptibility gene for CD opens up challenging questions for the future research towards our understanding of the etiology of chronic inflammatory disorders and to classify the several subtype of CD patients. Research on NOD molecules and particularly on CARD15 has provided an interesting example of scientific synergy between disciplines as diverse as clinical medicine, biochemistry, microbiology, immunology and genetics. From these discoveries, the challenge for the next years will be to understand how defects in intracellular bacterial sensing can lead to chronic inflammatory disorders and to high heterogeneity of disease phenotype.

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