Prevalence of Mutations of the NOD2/CARD15 Gene and Relation to Phenotype in Spanish Patients with Crohn Disease


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Background: We assessed the prevalence of R702W, G908R, and L1007fs coding mutations in the NOD2/CARD15 gene and the genotype–phenotype relation in Spanish patients with Crohn disease.

Methods: A cohort of 204 unrelated patients with Crohn disease and 140 healthy controls were studied. The phenotype was established before commencement of genotyping. Genotyping of the R702W, G908R, and L1007fs gene polymorphisms of NOD2/CARD15 was performed by two independent laboratories using different techniques. In the case of discordant results, specific sequencing of DNA strands was performed.

Results: At least one mutation was present in 32.8% of patients compared to 10.7% in controls (OR = 4.08, 95% CI 2.21 to 7.50). In patients with Crohn disease, the frequency of R702W, G908R, and L1007fs carriers was 13.7%, 8.3%, and 14.2%, respectively. Compound heterozygotes and homozygotes occurred in 3.4% and 2.9% of patients and in none of the controls. The correlation of genotype–Vienna classification showed a significant association with ileal disease (RR = 1.61, 95% CI 1.21–2.15, P = 0.001) and an inverse association with colonic localization (RR = 0.29, 95% CI 0.11–0.80, P = 0.007). There was a significant association between G908R carriership and previous appendectomy, surgical interventions, and stricturing behavior.

Conclusions: In a Spanish population from Madrid, mutations of the NOD2/CARD15 gene were a marker of susceptibility to Crohn disease and were associated with ileal disease. Carriers of the G908R mutation showed a stricturing disease behavior, history of appendectomy, and surgical interventions over the course of the disease.

Key words: CARD15/NOD2 gene; Crohn disease; Vienna classification

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Crohn disease is a chronic inflammatory disorder of the gastrointestinal tract. The inflammation may involve any segment of the digestive tract, from the mouth to the anus, and may affect the mucosa and the deeper layers of the digestive wall, with or without granulomas. The etiopathogenesis of the disease remains poorly understood. Experimental and observational data suggest that intestinal inflammation arises from abnormal immune reactivity to bacterial flora in the intestine of individuals who are genetically susceptible (1).

The combined strategies of positional cloning and candidate gene analysis of chromosome 16 have recently led three independent groups to identify NOD2—the nomenclature of NOD2 has been changed to CARD15—as a gene linked to Crohn disease (2–4). The NOD2/CARD15 gene is located at the Crohn disease susceptibility locus (IBD1) on chromosome 16q12. The respective gene product is expressed in monocytes and intestinal epithelial cells (5, 6), functions as an intracellular receptor for bacterial components, and is involved in apoptosis and nuclear factor κB (NFκB) activation, which is a key transcriptional factor involved in initiation of immunoinflammatory responses (7).

It has been demonstrated that three single-nucleotide polymorphisms (SNPs) R702W, G908R, L1007fs (also called SNP8, SNP12, and SNP13, respectively) within the NOD2/CARD15 gene are associated with Crohn disease. On the other hand, it has been suggested that various disease phenotypes including age at diagnosis, sex, family history, location of disease, response to treatment, and behavior of disease may be genetically determined (8). In fact, recent studies have provided a link between NOD2/CARD15 mutations and clinical characterization of Crohn disease (9–15). Marked racial differences are observed for common Crohn disease-associated variants. The frequency of mutant NOD2/CARD15...
Materials and Methods

Study population

We studied a cohort of 204 Caucasian unrelated consecutive patients with Crohn disease who were recruited in a Unit of Inflammatory Bowel Disease (IBD) from a single tertiary referral center in Madrid, Spain. Diagnosis of Crohn disease was based on standard clinical, radiologic, endoscopic, and histologic criteria (19). Phenotypic details were obtained by review of clinical charts and personal interview with the patients. The same clinical questionnaire was completed for each patient. This questionnaire included: date of birth, sex, familial IBD, age at diagnosis, duration of follow-up, smoking habits, history of surgery (t onsillectomy, appendectomy), definitions of the Vienna classification for age at diagnosis (A1, <40 years, A2, ≥40 years), disease location (L1, terminal ileum, L2, colon, L3, ileocolon, L4, upper gastrointestinal), and behavior (B1, non-strictureing non-penetrating, B2, stricturing, B3, penetrating), perianal disease defined as the presence of perianal abscess, fistulas and/or ulceration), extraintestinal clinical manifestations (articular, cutaneous, ocular, hepatic), and previous treatment as an indication of severity of disease (surgical intervention, corticosteroids, immunosuppressant agents, infliximab). All patient data were recorded by a gastroenterologist from the Unit of IBD (J.L.M.) who was blind from the genotype status of each patient.

A total of 104 normal healthy unrelated controls from the same geographic area were recruited from the blood-donor institution and all patients and controls included in the study gave their written informed consent.

Genotyping

Amplification and specific discrimination of alleles in all blood samples was performed by two independent laboratories using different techniques, gel electrophoresis at Vrije University Medical Centre, Amsterdam, and fluorimetric detection at Hospital Clinico San Carlos, Madrid. In the case of discordant results, specific sequencing of DNA strands was performed.

Briefly, genotyping at Vrije University Medical Centre was performed as follows: NOD2/CARD15 L1007fs. A frameshift mutation produced by a C-insertion in nucleotide 3020 of NOD2/CARD15 gene affecting the 10th leucine-rich-repeat region of the protein was amplified by multiple PCR as described previously (3).

NOD2/CARD15 G908R (G908R). The G→C substitution at position 2722 in the 8th exon of NOD2/CARD15 gene (NCBI SNP Id rs2066845) abolishes an HhaI site: 5'-AAGCTCTGTAATGTAAGGCCAC- 3' (sense) and 5'-CCCTGCTCCTCCCTCCTTC- 3' (antisense). The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturing at 95 °C for 5 s, annealing at 59 °C for 30 s and extension at 72 °C for 30 s, and final incubation at 72 °C for 7 min. HhaI digestions of the PCR product resulted in fragments that either remained intact (NOD2/CARD15 G908 allele) or were cut into two fragments (NOD2/CARD15 908R allele).

NOD2/CARD15 2104T gene polymorphism (R702W). A bi-allelic polymorphism produced for a C→T substitution at position 2104 in the 3rd intron of the NOD2/CARD15 gene (NCBI SNP Id rs2066844) produces the HpaII restriction site. This region was amplified by PCR with the following primers (Invitrogen Life Technologies, Breda, The Netherlands): 5'-CGCACAACCTTCAGATCACA- 3' (sense) and 5'-GGATGGAGTGGAAGTGCTTG- 3' (antisense). The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturing at 95 °C for 30 s, annealing at 59 °C for 30 s and extension at 72 °C for 1 min. HpaII digestion of the PCR product fragments was cut into either three fragments (OPCIONAL of 64-bp, 54-bp and 47-bp) (NOD2/CARD15 R702 allele) or two fragments (OPCIONAL of 114-bp and 47-bp, respectively) (NOD2/CARD15 702W allele).

Genotyping at Hospital Clinico San Carlos was performed as follows: The L1007fs was genotyped by use of the Taqman system (Applied Biosystems, Foster City, Calif., USA) with primers and probes that had been synthesized according to a previously described protocol (4). Taqman-PCR products were read directly into an ABI 7700 analyzer (Applied Biosystems).

The G908R was genotyped by specific amplification of the allele using the following primers: 5'-TGGCTTTTTCAGATTCTGGG- 3' (wild type, sense) and 5'-TGGCTTTTTCAGATTCTGGG- 3' (mutated, sense) and 5'-CAGCTGTCACCACCCTCTGTG- 3' as antisense primer in both cases. The PCR conditions were as follows: initial denaturation at 94 °C for 10 min, followed by 40 cycles of denaturing at 94 °C for 15 s and annealing at 65 °C for 30 s. Detection of the normal/mutant amplified product in an ABI 7700 analyzer was performed using the fluorescent stain SyberGreen.
according to the manufacturer’s instructions (Applied Biosystems).

Genotyping of the R702W was performed following the same method by substitution of C/T in exon 3/4 with primers 5'-CATCTGAGAGGGCCCCTGTC(C/T)-3' and 5'-CAGACACCAGGCGGCACA- 3' and amplification cycles at 94°C for 15 s and at 65°C for 30 s.

Statistical analysis

The frequencies for the NOD2/CARD15 mutations were estimated by counting gene and calculating sample proportions. Carrier status was considered if any subject inherited at least one copy of the variant allele. Compound heterozygous status was defined as the presence of two different variants, and no variant. Case-control analyses were performed with the chi-squared statistics or Fisher exact test. The association and no variant. Case-control analyses were performed with the chi-squared statistics or Fisher exact test. The association between NOD2/CARD15 mutations and phenotypic characteristics of Crohn disease was estimated by the odds ratio (OR) with the 95% confidence interval (CI). To assess whether NOD2/CARD15 variants influence synergically upon the course of Crohn disease, subjects were classified as carriers and non-carriers of variant allele at both polymorphic loci. The chi-squared test or Fisher exact test was used for the comparison of carriers and non-carriers. When the association between genotype and phenotypic variables was known, statistical correction was not applied. Logistic regression analysis was performed to assess whether NOD2/CARD15 mutations were correlated with a particular clinical phenotype. Association was expressed as relative risk (RR) with 95% CI. The multiple logistic regression analyses were adjusted for age (years). A two-tailed P value ≤0.05 was considered as significant. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 10.07 for Windows (SPSS Inc., Chicago, Ill., USA).

Results

The cohort of 204 patients with Crohn disease consisted of 108 men and 96 women. The median age at diagnosis was 27 years (mean 31.6, range 8–80) with an interquartile range of 22–26 years. The median duration of follow-up was 8 years (mean 9.9, range 0.6–44) with an interquartile range of 5–13 years.

At least one mutation was present in 32.8% of patients compared to 10.7% in controls (OR = 4.08, 95% CI 2.21 to 7.50, P < 0.05)). In patients with Crohn disease, the frequency of R702W, G908R, and L1007fs carriers was 13.7%, 8.3%, and 14.2%, respectively. Compound heterozygotes and homozygotes for a variant occurred in 3.4% and 2.9% of patients and in none of the controls (Table I).

The genotype–phenotype correlations are given in Table II. With regard to Vienna classification of the disease, strictureting behavior was significantly associated with G908R carriership (P = 0.002). Specifically, patients with strictureting disease had a 3.38 increased risk (95% CI, 1.86 to 6.28) of carrying the G908R allele variant. The presence of at least one mutant variant was positively associated with ileal disease (P = 0.001) and inversely associated with solely colonic involvement (P = 0.007), that is, patients with ileal disease had a 1.61 increased risk (95% CI, 1.21 to 2.15) of carrying at least one mutation. The risk ratio for solely colonic involvement was 0.29 (95% CI, 0.11 to 0.80). In the multivariate analysis, the L1007fs variant was mostly associated with ileal disease (P = 0.0061, RR = 3.10, 95% CI, 1.33 to 7.2). No other variables of the Vienna classification were associated with the presence or absence of NOD2/CARD15 variants. On the other hand, in relation to risk factors for Crohn disease, a significant association of the G908R allele carriership and history of appendectomy was observed (P = 0.02, RR = 2.54, 95% CI, 1.22 to 5.30). Surgery over the course of the disease was significantly more frequent in patients who were carriers of G908R allele (P = 0.005, RR = 2.4, 95% CI, 1.64 to 3.51), but in the logistic regression analysis this variable was dependent not only on the presence of G908R mutation (P = 0.028, RR = 5.13, 95% CI, 1.65 to 15.88) but also of ileal disease (P < 0.001, RR = 3.25, 95% CI, 1.73 to 6.10), and strictureting disease behavior (P < 0.001, RR = 6.15, 95% CI, 1.03 to 22.10). No other risk factors, clinical manifestations, and treatment modalities were associated with NOD2/CARD15 variants. A gene-dosage effect on phenotypic characteristics was not observed.

Discussion

We have performed a genotype-phenotype correlation in a cohort of 204 Caucasian patients with Crohn disease from the

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Table I. Distribution of the CARD15 L1007fs, G908R and R702W carriership. Frequencies in Spanish patients with Crohn disease and controls

<table>
<thead>
<tr>
<th>NOD2/CARD15 carriership</th>
<th>Patients (n = 204)</th>
<th>Controls (n = 104)</th>
<th>Odds ratio (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>R702W</td>
<td>28 (13.7)</td>
<td>6 (4.3)</td>
<td>3.55 (1.43–8.84)</td>
<td>0.0039</td>
</tr>
<tr>
<td>G908R</td>
<td>17 (8.3)</td>
<td>3 (2.1)</td>
<td>4.15 (1.19–14.45)</td>
<td>0.016</td>
</tr>
<tr>
<td>L1007fs</td>
<td>29 (14.2)</td>
<td>6 (4.3)</td>
<td>3.70 (1.49–9.17)</td>
<td>0.0028</td>
</tr>
<tr>
<td>At least one variant</td>
<td>67 (32.8)</td>
<td>15 (10.7)</td>
<td>4.08 (2.21–7.50)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Heterozygote</td>
<td>54 (26.5)</td>
<td>15 (10.7)</td>
<td>3.0 (1.61–5.57)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Compound heterozygote</td>
<td>7 (3.4)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homozygote</td>
<td>6 (2.9)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data as number and percentage in parentheses.
The prevalence of carriership for \textit{NOD2/CARD15} gene polymorphisms in the Spanish population with Crohn disease (R702W, 13.7%; G908R, 8.3%; L1007fs, 14.2%) is similar to that obtained in studies of Caucasian patients from central and northern Europe (9, 10, 12, 13, 17), Canada (14), and North America (3) and much higher than the prevalence of the three mutant variants found in healthy people from our same geographic area (R702W, 4.3%; G908R, 2.1%; L1007fs, 4.3%). Neither homozygosity nor compound heterozygosity for a variant was observed in controls, which confirms the strong genetic contribution of this gene to susceptibility for Crohn disease (2). However, a complex interplay of genetic and environmental factors is supported by patterns of geographic variation in the incidence of Crohn disease (e.g. three or more times higher in northern than in southern Europe) (22–24) and significant racial differences in the frequency of major mutations (R702W; G908R and L1007fs) in the \textit{NOD2/CARD15} gene (16–19). On the other hand, the possibility of characterizing phenotypic subtypes in a heterogeneous disorder such as Crohn disease according to location and behavior of the disease may convert genotyping of candidate genes, including the \textit{NOD2/CARD15} gene, to a promising tool for understanding disease development and progression (25).

Although in some studies mutations in the \textit{NOD2/CARD15} gene are not a marker of Crohn disease according to the Vienna classification (26, 27), most reports describe an association between \textit{NOD2/CARD15} polymorphisms and disease phenotype (10–15, 28). In our Spanish Crohn disease population and in agreement with findings in an Italian population (27), mutations of the \textit{NOD2/CARD15} gene were not associated with age at diagnosis, which is in contrast to data in northern and central Europe (2, 10, 12, 28) and in the Ashkenazi Jewish population (29) showing that patients with mutations in the \textit{NOD2/CARD15} gene presented earlier than patients with no \textit{NOD2/CARD15} variants. This is consistent with the epidemiologic observation in northern and central Europe that Crohn disease is diagnosed at a younger age and is associated with a higher rate of complications compared to cases from southern Europe, which in turn stresses the importance of studies designed to examine gene–gene and gene–environmental interactions (30).

Location of disease is the variable that remains more stable during the course of the disease (31) and that showing a stronger association with mutations of the \textit{NOD2/CARD15}...
gene in genotype–phenotype studies using the Vienna classification. In our study, like others (10, 15, 28), possession of a \textit{NOD2/CARD15} variant, particularly L1007fs, was significantly associated with ileal disease. On the other hand, mutations of the \textit{NOD2/CARD15} gene were exceptional in patients with solely colonic involvement. This subgroup of patients accounted for 16% of the patients and showed a prevalence of the variant allele similar to that in health controls, which may indicate that in this subgroup of patients other genes may be involved in the susceptibility to Crohn disease (28). In contrast to findings in the Norwegian and German populations (12) and similarly to findings in the British population (13, 28), we have not found an association with location of disease in the right colon and mutations of the \textit{NOD2/CARD15} gene.

In our population of patients with Crohn disease, an association between strictureing disease and carriers of the G908R polymorphism was observed, although strictureting behavior was mainly dependent on location of disease in the terminal ileum. In contrast to observations in other populations, especially from northern and central Europe (4, 12), no association of the L1007fs and R702W polymorphisms and a fibrostenotic behavior was found. In addition, and in contrast to other studies (10, 12, 14, 28), no other extraintestinal manifestations and perianal disease were associated with mutations of the \textit{NOD2/CARD15} gene.

With regard to risk factors for Crohn disease, we only found a significant association between carriership of the G908R allele and history of appendectomy, which was independent of location and behavior of the disease. Russel et al. (32) also noted a positive association of Crohn disease and previous appendectomy, suggesting that appendectomy in some cases was a result of still undiagnosed Crohn disease.

Surgery over the course of the disease was significantly more frequent in patients who were carriers of the G908R allele, but this variable was dependent not only on the carriership of G908R allele but also of ileal disease and a strictureing disease behavior. It therefore seems that mutations of the \textit{NOD2/CARD15} gene do not exert an isolated influence on the prognosis of Crohn disease. In other studies (33, 34), \textit{NOD2/CARD15} was not predictive of treatment outcome with infliximab in patients with Crohn disease. Finally, no gene-dosage effect (homozygotes, compound heterozygotes) on phenotypic characteristics was observed. This could indicate that a higher genetic load would increase susceptibility to Crohn disease but would not affect the clinical characteristics of the disease. In contrast, in the study of Lesage et al. (10) patients with double-dose mutations were characterized by a younger age at onset, a more frequent strictureting phenotype, and a less frequent colonic involvement than was seen in those patients who had no mutation.

This study has shown that in a Spanish population from Madrid, mutations of the \textit{NOD2/CARD15} gene were a marker of susceptibility for Crohn disease and were associated with ileal disease. Carriers of the G908R mutation were characterized by a strictureing disease behavior, history of appendectomy, and surgical interventions over the course of the disease. Identification of plausible factors that may interact with mutations in the \textit{NOD2/CARD15} gene is a promising step toward understanding how sequence variation influences disease susceptibility in Crohn disease.

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1240 J. L. Mendoza et al.


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