Polymorphisms in interleukin-10 gene according to mutations of NOD2/CARD15 gene and relation to phenotype in Spanish patients with Crohn’s disease

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INTRODUCTION

Crohn’s disease (CD) 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 mucosa and 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 arise from abnormal immune reactivity to bacterial flora in the intestine of individuals who are genetically susceptible [1]. Epidemiologic and linkage studies suggest that genetic factors play a significant role in determining CD susceptibility. CD has no simple Mendelian pattern of inheritance. As other immune diseases, CD is thought to be a heterogeneous, complex polygenic disease, where both genetic and environmental factors play an important role in the disease and, in which multiple interactions between susceptibility and resistance alleles are involved in disease pathogenesis [2,3].

Human genetic studies, notably the landmark identification in 2001 of NOD2/CARD15 within the linkage region IBD1, have confirmed a genetic influence on CD [4-6], and it is now clear that a genotype-phenotype relationship exists. In our population of Spanish CD patients carrying at least one NOD2/CARD15 mutation, the -1082G allele is associated with ileocolonic disease and the IL-10G14 microsatellite allele is associated with previous history of appendectomy and smoking habit at diagnosis. These data provide further molecular evidence for a genetic basis of the clinical heterogeneity of CD.

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Key words: Crohn’s disease; NOD2/CARD15 gene; Interleukin-10 gene

Abstract

AIM: To examine the contribution of interleukin-10 (IL-10) gene polymorphisms to Crohn’s disease (CD) phenotype, and the possible genetic epistasis between IL-10 gene polymorphisms and CARD15/NOD2 gene mutations.

METHODS: A cohort of 205 Spanish unrelated patients with Crohn's disease recruited from a single center was studied. All patients were rigorously phenotyped and followed-up for at least 3 years (mean time, 12.5 years). The clinical phenotype was established prior to genotyping.

RESULTS: The correlation of genotype-Vienna classification groups showed that the ileocolonic location was significantly associated with the -1082G allele in the NOD2/CARD15 mutation-positive patients (RR = 1.52, 95%CI, 1.21 to 1.91, P = 0.008). The multivariate analysis demonstrated that the IL-10 G14 microsatellite allele in the NOD2/CARD15 mutation positive patients was associated with two risk factors, history of appendectomy (RR = 2.15, 95%CI = 1.1-4.30, P = 0.001) and smoking habit at diagnosis (RR = 1.29, 95%CI = 1.04-4.3, P = 0.04).

CONCLUSION: In Spanish population from Madrid, in CD patients carrying at least one NOD2/CARD15 mutation, the -1082G allele is associated with ileocolonic disease and the IL-10G14 microsatellite allele is associated with previous history of appendectomy and smoking habit at diagnosis. These data provide further molecular evidence for a genetic basis of the clinical heterogeneity of CD.

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patients from Madrid, mutations in the NOD2/CARD15 gene were a marker of susceptibility to disease and were associated with ileal disease[7].

In CD, the mucosal inflammation is associated with an exaggerated and prolonged immune response because of a dysregulated production and interaction of pro-inflammatory and anti-inflammatory cytokines and their receptors[8]. A variety of genes encoding various proteins involved in the immune regulation have been postulated as possible candidates for disease susceptibility, including, among others, cytokines as interleukin-10 (IL-10). IL-10 is a regulatory cytokine that has several functions, but one important role is to act as an inhibitor of development of Th1 cells, activated macrophages and their products interleukin-12 (IL-12), tumor necrosis factor (TNF) and interferon-gamma (IFN-γ). Even though it is usually considered an inhibitory cytokine, it also has stimulatory effects (e.g. stimulating B cell proliferation)[9]. Recently, we have also shown that IL-10 polymorphisms contribute to susceptibility to CD in our Spanish population. IL-10G14 microsatellite allele as well as -1082G allele (guanine at position -1082) were significantly increased in Crohn’s disease patients. The combined presence of both alleles in one individual notably increased the risk to develop CD[10].

Although the pathogenetic mechanisms mediated by NOD2/CARD15 remain elusive, it has recently been shown that one of the mutations in the NOD2/CARD15 gene results in defective release of IL-10 from blood mononuclear cells after stimulation with Toll-like receptor (TLR) 2 ligands and this could contribute to the overwhelming inflammation seen in CD[11].

As IL-10 polymorphisms appear to confer a risk to develop CD in the Spanish population, the present study examined genotype-phenotype correlations in the disease process. Moreover, after stratifying the patients on the basis of the presence or absence of the well-established NOD2/CARD15 mutations, we looked for susceptibility factors being present in one specific phenotypic subpopulations.

**MATERIALS AND METHODS**

**Study population**

We studied a cohort of 205 Caucasian unrelated consecutive patients with CD who were recruited in a Unit of Inflammatory Bowel Disease (IBD) from a single tertiary referral center in Madrid, Spain. Diagnosis of Crohn’s disease was based on standard clinical, radiologic, endoscopic, and histologic criteria[12]. 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, follow-up interval, smoking habits, history of surgery (tonsillectomy, 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), behavior (B1, nonstricturing nonpenetrating; B2, stricturing; B3, penetrating), perianal disease (defined as the presence of perianal abscess, fistulas and/or ulceration), extraintestinal clinical manifestations (articular and cutaneous), 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 to the genotype status of each patient. The protocol was approved by the Ethics Committee of the Hospital Clínico San Carlos, Madrid, and all patients were included in the study after giving informed consent.

**Genotyping**

**IL-10 polymorphisms:** IL-10G and IL-10R microsatellites were amplified using primers and conditions as previously described[13]. Blood samples were subsequently denatured and run on an ABI Prism 3100 automatic sequencer (Applied Biosystems, Foster City, CA, USA). Each sample included an internal size standard (HD400 ROX, Applied Biosystems) in order to achieve a highly consistent measure. The results were analyzed using GeneMapper v3.0 (Applied Biosystems).

As previously described[14], a combined amplification of the IL-10G microsatellite and the -1082 and -819 SNPs was performed. Our typing method allowed us to construct haplotypes directly. SNPs at positions -1082, -819 and -592 only form three different haplotypes in our population[15,16], namely, ACC, ATA and GCC. Based on this previous finding, we only typed the samples for the two first SNPs, as the information provided by the third one is redundant.

**NOD2/CARD15 polymorphisms:** SNP13 (Leu1007fsinsC) was genotyped using a TaqMan assay (Applied Biosystems, Foster City, CA, USA). Primers and probes used were as previously described[15,17] and PCR products were analysed on an ABI 7700 Sequence Detector (Applied Biosystems). SNP8 (Arg702Trp) (sense, 5’-CAT CTG AGA AGG CCC TGC TC(C/T)-3’; antisense, 5´-CAG ACA CCA GCG GGC ACA-3´) and SNP12 (Gly908Arg) (sense, 5´-TTG GCC TTT TCA GAT TCT GG (G/C)-3´; antisense, 5´-CCC CTC GTC ACC CAC TCT G-3´) were typed by allele-specific PCR. Detection of wild-type/mutant variants was assessed in an ABI 7700 Sequence Detector by an SYBRGreen assay. Previously sequenced samples were used as controls. In cases of doubt, samples were sequenced to confirm the result.

**Statistical analysis**

The frequencies for the IL-10 polymorphisms and NOD2/CARD15 mutations were estimated by counting gene and calculating sample proportions. Subsequently, Hardy Weinberg equilibrium for each of the polymorphisms was tested to check for Mendelian inheritance using χ² test with one degree of freedom. Carrier status was considered if any subject inherited at least one copy of the mutant allele 2. The association between IL-10 polymorphisms and phenotypic characteristics of CD was estimated by the relative risk (RR) with the 95% confidence interval (CI). Logistic regression analysis was performed to assess whether IL-10 polymorphisms were correlated with a particular clinical phenotype. The multiple logistic regression analysis was
mutation positive patients (NOD2/CARD15 alleles) on phenotypic characteristics was not observed (data not shown). When we examined the associated IL-10G14 and -1082G alleles in the NOD2/CARD15 mutation positive and negative patients separately, three new positive associations were found. With regard to Vienna classifications of the disease, ileocolonic disease was significantly associated with -1082G allele in the CARD15/NOD2 mutation positive patients (P=0.002). On the other hand, relative to risk factors for Crohn disease, two significant associations of the IL-10G14 allele carriership and history of appendectomy (P=0.002) and smoking habit at diagnosis (P=0.02) in the NOD2/CARD15 mutation positive patients as compared with the negative patients were observed (Table 3). The multivariate analysis demonstrated that IL-10G14 allele was associated with history of appendectomy (P=0.001, RR=2.15, 95%CI=1.1-4.30) and with smoking habit at diagnosis (P=0.04, RR=1.29, 95%CI=1.04-4.3).

**DISCUSSION**

In this study, we performed a genotype-phenotype correlation study in a cohort of 205 Caucasian patients with Crohn’s disease from the community of Madrid...
(central Spain) who had been followed-up for a mean of 12.57 years. The clinical diagnosis of Crohn’s disease was confirmed by the criteria of Gasche et al[19]. Our results showed that a relation existed between disease location (ileocolon), risk factors for CD (appendectomy and smoking habit) and genetic heterogeneity in our population. This could suggest an epistatic interaction of both genes.

CD is an extensively heterogeneous disease. Epidemiologic and genetic data suggest that heterogeneity of CD may be genetically determined. Recently, Ahmad et al[20] have shown the importance of the NOD2/CARD15 gene and the HLA region in determining clinical subgroups of CD. Similarly, in our CD population, we confirmed the association between NOD2/CARD15 mutations and ileal disease and the strong association confirmed the association between ileocolonic location and mutations of the NOD2/CARD15 gene[7]. In contrast, this association has been found between the NOD2/CARD15 variants and IL-10 -1082G carriers. This suggests the importance of classifying the patients according to the different genes implicated in the etiopathogenesis of CD and, therefore, of performing the molecular characterization of CD patients. Tagore et al[21] have shown that IL-10 production is associated with three biallelic polymorphisms within the promoter region of the IL-10. The allele -1082G is associated with higher IL-10 production in peripheral blood leukocytes. This different levels of IL-10 expression has been found between the CARD15/NOD2 (-) and non-smokers (P = 0.02, RR = 2.47, 95% CI = 1.28-4.8). IL-10 G14 in NOD2/CARD2 mutation positive patients: smokers vs non-smokers (P = 0.002, RR = 3.29, 95% CI = 1.45-7.7)

### Table 3 Distribution of IL-10G14 allele among the different clinical subgroups of CD stratified by NOD2/CARD15 status

| Phenotypic characteristics | Phenotype frequency of IL-10.G14 (+) (n = 47) (%) | P | CARD15/NOD2 (+) IL-10.G14, (n = 22) (%) | P | CARD15/NOD2 (+) IL-10.G14, (n = 25) (%) | P |
|----------------------------|-------------------------------------------------|---|----------------------------------------|---|----------------------------------------|---|
| Sex                        |                                                 |   |                                        |   |                                        |   |
| Men                        | 23 (48.9)                                       | 0.74| 13 (59.1)                              | 0.08| 10 (40)                                | 0.32|
| Women                      | 24 (51.1)                                       | 1.00| 9 (40.9)                               | 0.60| 15 (60)                                | 0.32|
| Age at diagnosis (yr)      |                                                 |   |                                        |   |                                        |   |
| A1, < 40                   | 39 (83.0)                                       | 0.5| 21 (95.5)                              | 0.27| 18 (72)                                | 0.68|
| A2, ≥40                    | 8 (17.0)                                        | 0.93| 1 (4.5)                               | 0.56| 7 (28)                                 | 0.88|
| Family history             |                                                 |   |                                        |   |                                        |   |
| Smokers                    | 9 (19.1)                                        | 0.54| 4 (18.2)                              | 0.52| 5 (20)                                 | 0.88|
| Appendectomy               | 24 (51.1)                                       | 0.51| 16 (72.7)                             | 0.021| 8 (32)                                | 0.41|
| Nonstricturing, nonpenetrating (BI) | 5 (10.6) | 0.63| 3 (13.6)                              | 0.48| 2 (8)                                 | 0.36|
| Location of disease        |                                                 |   |                                        |   |                                        |   |
| Terminal ileum (L1)        | 23 (48.9)                                       | 0.26| 15 (68.2)                             | 0.54| 8 (32)                                | 0.24|
| Colon (L2)                 | 4 (8.5)                                         | 0.37| 1 (4.5)                               | 0.18| 3 (12)                                 | 0.12|
| Ileocolon (L3)             | 17 (36.2)                                       | 0.27| 5 (22.7)                             | 0.021| 12 (48)                               | 0.32|
| Upper gastrointestinal (L4) | 3 (6.4)                                         | 0.86| 1 (4.5)                               | 0.47| 2 (8)                                 | 0.24|
| Perianal                    | 14 (29.8)                                       | 0.08| 4 (18.2)                             | 0.47| 10 (40)                               | 0.28|
| Extraintestinal clinical manifestations | 8 (17.0) | 0.62| 3 (13.6)                              | 0.30| 5 (20)                                | 0.56|
| Cutaneous                  | 13 (27.7)                                       | 0.32| 6 (27.3)                             | 0.53| 7 (28)                                | 0.36|
| Articular                  |                                                 |   |                                        |   |                                        |   |
| Surgical intervention      | 20 (42.6)                                       | 0.98| 10 (45.5)                             | 0.81| 10 (40)                               | 0.22|
| Infliximab                 | 7 (14.9)                                        | 0.79| 5 (22.7)                             | 0.31| 2 (8)                                 | 0.21|
| Immunosuppressants         | 18 (38.3)                                       | 0.61| 10 (45.5)                             | 0.81| 8 (32)                                | 0.23|

1IL-10G14 in NOD2/CARD2 mutation positive patients: smokers vs non-smokers (P = 0.02, RR = 2.47, 95% CI = 1.28-4.8). 2IL-10 G14 in NOD2/CARD2 mutation positive patients: appendectomy vs non-appendectomy (P = 0.002, RR = 3.29, 95% CI = 1.45-7.7)

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The IL-10 gene knockout mouse spontaneously develops a chronic enterocolitis\(^{26}\) and gene therapy using an adenovirus IL-10 construct is successful in preventing experimental colitis in rats\(^{34}\). Moreover, there have been preliminary reports of amelioration of clinical symptoms of CD following administration of human recombinant IL-10\(^{27,23}\).

Regarding the risk factors for CD, we found two significant associations between carriage of the IL-10G14 microsatellite allele in CARD15-positive patients and previous history of appendectomy and smoking habit at diagnosis. The interaction between genetics and smoking has been demonstrated in siblings from mixed-disease families, where some individuals develop CD and others in the same family develop ulcerative colitis. There is a strong positive relationship between smoking and CD and an equally strong negative relationship between smoking and ulcerative colitis\(^{34}\). Appendectomy provides a spectrum of protection against ulcerative colitis development and progression, whereas its role in CD remains unclear. Russel et al\(^{35}\) have also noted a positive association of CD and previous appendectomy, suggesting that, in some cases, appendectomy is a result of still undiagnosed CD. Recently, other retrospective study concluded the risk of CD after appendectomy was associated with an increased risk of CD dependent on the patient’s sex, age, and the diagnosis at operation\(^{34}\). Future work should pursue to investigate the epidemiological relationships in CD, addressing a greater number of potentially important confounders, such as smoking, hygiene, and pathology of the appendix. And parallel with these clinical observations, new target could be defined by genetic and immunologic analysis to evaluate if appendicitis could be correlated with any particular genetic modification involved for patients with CD\(^{30}\).

In conclusion, our study has shown that in the Spanish population from Madrid, in CD patients carrying at least an NOD2/CARD15 mutant, the -1082G allele might be associated with ileocolonic disease, and the IL-10G14 microsatellite allele might be associated with previous history of appendectomy and smoking habit at diagnosis. Identification of plausible factors that may interact with genes is a promising step toward understanding how sequence variation influences disease susceptibility.

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