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RAPID COMMUNICATION

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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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.

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

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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 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/CAR15* 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[5, 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 χ^2 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 estimatied by the relative risk (*RR*) with the 95% confidence interval (*CI*). Logistic regression analysis was perfomed to assess whether IL-10 polymorphisms were correlated with a particular clinical phenotype. The multiple logistic regression analysis was

Table 1 Distribution of IL-10G14 microsatellite allele and -1082G allele stratified by NOD2/CARD15 status

Allele frequencies	At least one CARD15/NOD2 mutation positive *n* = 76 (%)	CARD15/NOD2 mutation negative *n* = 129 (%)	*P*
IL-10G14 (*n* = 47)	22 (28.9)	25 (19.4)	0.115
-1082G (*n* = 146)	54 (71.1)	92 (71.3)	0.96
IL-10G14+-1082G (*n* = 29)	13 (17.1)	16 (12.4)	0.35

Table 2 Distribution of -1082G allele among different clinical subgroups of CD stratified by NOD2/CARD15 status

Phenotypic characteristics	Phenotype frequency of -1082 IL-10G(+) (*n* = 146) (%)	*P*	CARD15/NOD2(+) -1082 IL-10 G(+) (*n* = 54) (%)	*P*	CARD15/NOD2(-) -1082 IL-10 G(+) (*n* = 92) (%)	*P*
Sex						
Men	67 (45.9)		24 (44.4)	0.77	43 (46.7)	0.45
Women	79 (54.1)	0.67	30 (55.5)		49 (53.3)	
Age at diagnosis (yr)						
A1, < 40	117 (80.1)		46 (85.2)	0.5	71 (77.2)	0.41
A2, ⩾40	29 (19.9)	0.72	8 (14.8)		21 (22.8)	
Family history	27 (18.5)	0.43	10 (18.5)	0.43	17 (18.5)	0.76
Smokers	64 (43.8)	0.55	24 (44.4)	0.86	40 (43.5)	0.65
Appendectomy	20 (13.7)	0.55	7 (12.9)	0.93	13 (14.1)	0.49
Tonsillectomy	22 (15.1)	0.11	5 (9.3) 0.66		17 (18.5)	0.19
Disease behavior						
Nonstricturing, nonpenetrating (B1)	63 (43.2)	0.87	24 (44.4)	0.58	39 (42.4)	0.86
Stricturing (B2)	22 (15.1)		9 (16.7)		13 (14.1)	
Penetrating (B3)	61 (41.8)		21 (38.9)		40 (43.5)	
Location of disease						
Terminal ileum (L1)	70 (47.9)	0.63	29 (53.7)	0.021	41 (44.6)	0.43
Colon (L2)	24 (16.4)		5 (9.3)		19 (20.7)	
Ileocolon (L3)	47 (32.2)		19 (35.2)		28 (30.4)	
Upper gastrointestinal (L4)	5 (3.4)		1 (1.9)		4 (4.3)	
Perianal	39 (26.7)	0.30	11 (20.4)	1	28 (30.4)	0.3
Extraintestinal clinical manifestations						
Cutaneous	27 (18.5)	0.56	9 (16.7)	0.29	18 (19.6)	0.93
Articular	49 (33.6)	0.69	16 (29.6)	0.83	33 (35.9)	0.83
Treatment						
Surgical intervention	61 (41.8)	0.76	22 (40.7)	0.71	39 (42.4)	0.92
Infliximab	19 (13.0)	0.06	5 (9.3) 0.032		14 (15.2)	0.61
Immunosuppressants	63 (43.2)	0.81	25 (46.3)	0.72	38 (41.3)	0.63

[1] -1082G allele in NOD2/CARD15 mutation positive patients: ileocolonic location ($P = 0.008$, $RR = 1.52$, 95% $CI = 1.21$-1.91). [2] -1082G allele in NOD2/CARD15 mutation positive patients: infliximab ($P = 0.03$, $RR = 0.54$, 95%$CI = 0.27$-1)

adjusted for age (years). A two-tailed *P* value equal to or less than 0.05 was considered statistically 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 205 patients with Crohn's disease consisted of 109 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 11 years (mean 12.57, range 3-47) with an interquartile range of 7-16 years.

No statistically significant difference in IL-10G14 and -1082G or both allele frequencies was found between *NOD2/CARD15* mutation positive and negative patients (Table 1).

The genotype-phenotype correlations are shown in Tables 2 and 3. No Vienna classifications of the disease, neither risk factors, clinical manifestations, or treatment modalites were associated with either IL-10G14 or -1082G alleles. A gene-dosage effect (IL-10G14 and -1082G 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 *NOD2/CARD15* mutation positive patients ($P = 0.008$, Table 2). 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

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)		9 (40.9)		15 (60)	
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)		1 (4.5)		7 (28)	
Family history	9 (19.1)	0.54	4 (18.2)	0.52	5 (20)	0.88
Smokers	24 (51.1)	0.51	16 (72.7)	0.02[1]	8 (32)	0.41
Appendectomy	9 (19.1)	0.32	6 (27.3)	0.002[2]	3 (12)	0.59
Tonsillectomy	5 (10.6)	0.63	3 (13.6)	0.48	2 (8)	0.36
Disease behavior						
Nonstricturing, nonpenetrating (B1)	19 (40.4)	0.61	6 (27.3)	0.19	13 (52)	0.62
Stricturing (B2)	9 (19.1)		6 (27.3)		3 (12)	
Penetrating (B3)	19 (40.4)		10 (45.5)		9 (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)		1 (4.5)		3 (12)	
Ileocolon (L3)	17 (36.2)		5 (22.7)		12 (48)	
Upper gastrointestinal (L4)	3 (6.4)		1 (4.5)		2 (8)	
Perianal	14 (29.8)	0.86	4 (18.2)	0.47	10 (40)	0.28
Extraintestinal clinical manifestations						
Cutaneous	8 (17.0)	0.62	3 (13.6)	0.30	5 (20)	0.56
Articular	13 (27.7)	0.32	6 (27.3)	0.53	7 (28)	0.36
Treatment						
Surgical intervention	20 (42.6)	0.98	10 (45.5)	0.8	10 (40)	0.82
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

[1]IL-10G14 in NOD2/CARD2 mutation positive patients: smokers *vs* non-smokers ($P = 0.02$, $RR = 2.47$, 95%$CI = 1.28$-4.8). [2]IL-10 G14 in NOD2/CARD2 mutation positive patients: appendectomy *vs* non-appendectomy ($P = 0.002$, $RR = 3.29$, 95%$CI = 1.45$-7)

(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*[18]. Our results showed that a relation existed between disease location (ileocolon), risk factors for CD (appendicectomy 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*[19] 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 between DRB1*0103 allele and colonic disease[17]. These studies may provide an initial basis for the construction of a molecular classification of CD[20].

Location of disease is the variable that remains more stable during the course of the disease[21] and it showed a stronger association with mutations of the *NOD2/CARD15* gene in genotype-phenotype studies using the Vienna classification. In our population[7], like others[19,22,23], possession of an *NOD2/CARD15* variant was significantly associated with ileal disease. On the contrary, mutations of the *NOD2/CARD15* gene were exceptional in patients with solely colonic involvement[7]. In contrast to findings in the Norwegian and German populations[24] and similar to findings in the British population[19,25], we could not find an 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*[26] have shown that IL-10 production is associted with three biallelic polymorphisms within the promotor 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 could explain the diverse phenotypic clinical behaviour of CD in specific genetic susceptibility background, like *NOD2/CARD15*.

No other factor is likely to contribute in isolation to the pathogenesis of CD. The modulation of the immune system either locally or systemically has an important role to play in the etiology and pathogenesis of this disease. The IL-10 gene has special interest for a candidate gene approach in CD. The biology of IL-10 is highly complex: a potent down-regulator of CD peripheral monocyte and intestinal lamina propria mononuclear cells activator *in vitro* and *in vivo*[27]. Both human IL-10 and murine IL-10 exert immunostimulatory effects by up-regulating MHC class II expression in B lymphocytes and inducing cytotoxic T-cell differentiation[28]. Due to the dual regulatory function of IL-10 itself, its gene is indeed an interesting susceptibility candidate and it would be worthwhile to know whether environment factors could modulate the IL-10 function.

The IL-10 gene knockout mouse spontaneously develops a chronic enterocolitis[29] and gene therapy using an adenovirus IL-10 contruct is successful in preventing experimental colitis in rats[30]. Moreover, there have been preliminary reports of amelioration of clinical symptoms of CD following administration of human recombinant IL-10[27,31].

Regarding the risk factors for CD, we found two significant associations between carriership 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[32]. Appendectomy provides a spectrum of protection against ulcerative colitis development and progression, whereas its role in CD remains unclear. Russel *et al*[33] 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[35].

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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