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• *BRIEF REPORTS* •

IBD5 polymorphisms in inflammatory bowel disease: Association with response to infliximab

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Abstract

AIM: Inflammatory bowel diseases (IBD) are multifactorial pathologies of unknown etiology. One susceptibility locus, *IBD5*, has been mapped to chromosome 5q31. We analyzed our Spanish cohorts of Crohn's disease (CD) and ulcerative colitis (UC) patients to determine whether this locus is associated with IBD, and to ascertain the main clinical phenotype influenced by this risk factor. The kind of interaction, either genetic heterogeneity or epistasis, between this *IBD5* susceptibility region and the *NOD2/CARD15* gene mutations was studied as well. Finally, we assessed whether this locus can predict response to infliximab therapy.

METHODS: A case control study was performed with 274 CD and 211 UC patients recruited from a single center and 511 healthy ethnically matched controls. Two polymorphisms were genotyped in the *IBD5* locus and three in the *CARD15/NOD2* gene.

RESULTS: Our results evidence association only with CD especially with the fistulizing phenotype and in the absence of *NOD2/CARD15* variants (mutant allele frequency in patients *vs* controls: OR = 2.03, 95% CI = 1.35-3.06, $P<0.01$). The frequency of the *IBD5* homozygous mutant genotype significantly increased in CD patients lacking response to infliximab (RR = 3.88, 95% CI = 1.18-12.0, $P<0.05$). UC patients overall do not show association with 5q31 polymorphisms, although a similar trend to the one observed in CD is found within the worse prognosis group.

CONCLUSION: The *IBD5* variants may enhance an individual carrier's risk for CD, mainly in the absence of the *NOD2/CARD15* mutations and in fistulizing patients. The data presented suggest the potential role of the 5q31 polymorphisms as markers of response to infliximab.

Key words: Crohn's disease; Ulcerative colitis; 5q31 polymorphisms; Infliximab

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INTRODUCTION

Most patients with inflammatory bowel disease (IBD) are diagnosed as having Crohn's disease (CD) or ulcerative colitis (UC) according to clinical, endoscopic, radiological and pathological criteria[1]. Epidemiological studies claim significant contribution of genetics to the IBD etiology[2,3]. Even though differences are present between the two clinical forms of these autoimmune disorders of the gastrointestinal tract, relatives of patients with CD or UC are at increased risk of developing any form of IBD[4]. Therefore, it seems plausible that both CD and UC patients will share some susceptibility genes, while others will be phenotype-specific loci. A 250-kb haplotype in the 5q31 cytokine gene cluster was first described to confer CD risk in a Canadian population[5], and recently mapping to the OCTN cation transporter in the same population has been reported[6]. *IBD5* association with UC has also been found in a German cohort, in addition to the replication of the association of this region with CD[7]. The so-called *IBD5* locus might therefore be regarded as a general risk factor for IBD, at least in some populations. Simultaneously, a British study in a large European cohort of patients did not detect association with UC, and reported that the risk conferred by the 5q31 locus to CD patients was dependent on the presence of at least one of the *NOD2/CARD15* disease susceptibility alleles[8]. The *NOD2/CARD15* gene is an established CD risk locus, *IBD1*[9-11]. It encodes a protein with affinity for bacterial components that defectively activates the NF-κB pathway[12]. Therefore, whether the 5q31 region is a general risk factor for both intestinal diseases or for CD exclusively is open to debate, together with the putative epistatic interaction with the *NOD2/CARD15* predisposition gene. Further evidence from independent populations will aid in clarifying the importance of this locus in IBD.

Replication of the initial Canadian study associating the cytokine cluster region in 5q31 with CD has been obtained

in British and German populations, whereas the extremely low frequency of these polymorphisms in Japan precluded the analysis[13,14]. We aimed at replicating this finding in a Mediterranean population and we sought to determine the clinical forms showing the strongest impact of this risk factor.

Th1 cells are critical in the pathogenesis of CD and the release of Th1 cytokines increases during CD relapses. Tumor necrosis factor alpha (TNF-α) mediates mucosal inflammation and the efficiency of the TNF-α neutralizing agents has been proven. The infusion of chimeric anti-TNF-α antibodies (infliximab) has been shown to exert a pro-apoptotic effect on T-cells[15] and to inhibit the production of both Th1 type cytokines and granulocyte-macrophage colony stimulating factor (GM-CSF[16]). Given that the GM-CSF gene maps to the 5q31 cytokine cluster, we were interested in ascertaining whether this *IBD5* susceptibility locus had any influence on the response to infliximab treatment. Moreover, this 5q31 locus is a cluster of genes with relevance in the immune response, including several cytokine genes that map to this chromosomal region, and this alone may justify the approach.

MATERIALS AND METHODS

Patients and controls

The study group consisted of 274 unrelated adult white Spanish CD patients (53% women) with median follow-up 10.5 years (95% percentile values range from 3.4 to 26.9 years), recruited after informed consent from a single center. Diagnosis of CD was based on Lennard-Jones criteria[17]. Phenotypic details were obtained with the clinical history and personal interviews with patients. Disease phenotype was determined following the Vienna Classification[18]. Location: L_1 (Terminal ileum), L_2 (Colonic), L_3 (Ileocolonic) and L_4 (Upper Gastrointestinal). Behavior: B_1 (Inflammatory, Non-stricturing and non-fistulizing), B_2 (Stricturing) and B_3 (Fistulizing). Perianal disease was defined by the presence of perianal abscesses, fistulae and/or ulcers. In addition, 211 unrelated adult white Spanish UC patients (38% women) were recruited after informed consent from the same center. Their diagnosis was documented by conventional endoscopic, histologic, and clinical criteria. The median follow-up period was 8.5 years (95% percentile values range from 2.7 to 19.4 years). Disease was classified as extensive (inflammation proximal to the splenic flexure) or distal. Patients and data are regularly followed up in the Inflammatory Bowel Disease Unit at Hospital Clínico San Carlos, Madrid. A group of 511 healthy white, unrelated subjects (61% women) from the Madrid region (mainly hospital employees and blood donors) were used as controls.

Genotyping

***5q31* locus** Two variants, IGR2060a_1 and IGR3081a_1, were independently analyzed by using the SYBR Green Master Mix of Applied Biosystems, under conditions recommended by the manufacturer. Allelic genotyping was achieved in an ABI 7700 Sequence Detector (Applied Biosystems, Foster City, CA) with the following set of primers: IGR2060a_1: sense 5'-CTC ATT ACA TCC TTG CAA CCC T(G/C)-3' and antisense 5'-GAC ACA TGG TGT GAG CTC AGT CA-3'. IGR3081a_1: sense 5'-TCG CGT GAG TCC TAT TCT TTC T(T/G)-3' and antisense 5'-TTC ATA CTT CCA GCA GCG GG-3'.

***NOD2/CARD15* polymorphisms** Primers and probes used were previously described[19], in summary: Leu1007fsinsC was genotyped using a TaqMan assay (Applied Biosystems, Foster City, CA) and PCR products were analyzed in an ABI 7700 Sequence Detector (Applied Biosystems). Arg702Trp (sense, 5'-CAT CTG AGA AGG CCC TGC TC (C/T)-3'; antisense, 5'-CAG ACA CCA GCG GGC ACA-3') and 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. Wild-type/mutant genotype was assessed in an ABI 7700 Sequence Detector by SYBR Green assay. Previously sequenced samples were used as controls.

Treatments

Crohn's disease Forty patients received an intravenous infusion of infliximab at a dose of 5 mg/kg of body weight at wk 0, 2 and 6. The physicians assessing clinical response to infliximab were blinded for the genotype information. Clinical response was divided into two categories: response or non-response. Patients with active luminal (non-fistulizing) disease were considered responders if the CD activity index (CDAI) decreased below 150 or if a decrease of 70 points in CDAI after 4 wk was observed[20]. Patients with fistulizing disease were considered responders in case of complete fistulae healing or 50% decrease in the number of draining fistulae within two consecutive visits, i.e., if a response was observed at both weeks 10 and 14[21].

Ulcerative colitis Medical treatments (steroids, immunosuppressant agents and surgical intervention) were related to the severity of the disease. Immunosuppressant agents (azathioprine, 6-mercaptopurine and cyclosporine) were used in UC patients to induce remission in steroid refractory disease and as steroid-sparing agent in patients who were steroid-dependent. Procedures considered as surgical therapy were colonic resections.

Statistical analysis

Case-control analyses were performed with the χ^2 statistics or Fisher exact test. The association between genotypic and phenotypic characteristics of CD and UC was estimated by the odds ratio (OR) with 95% confidence interval (CI). The χ^2 test or Fisher exact test was used to compare responders and non-responders and association was expressed as relative risk (RR) with 95% CI. The statistical power considering a RR = 1.5 for the 5q31 polymorphisms is 84.38% for CD and 79.51% for UC. Logistic regression analysis was performed to assess whether 5q31 homozygous mutants were correlated with baseline characteristics of patients treated with infliximab. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 10.07 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Eleven SNPs significantly associated with CD initially defined

the 250-kb *IBD5* locus. Due to the strong linkage disequilibrium (LD) among them, any of those 11 polymorphisms would be equally informative once LD was confirmed in one specific population. We genotyped two of those 11 SNPs, IGR2060a_1 and IGR3081a_1 from the 5' and 3' ends of the *IBD5* haplotype, to verify whether the extent of linkage disequilibrium was similar in our population and in those previously described. The rate of LD observed between these two SNPs was confirmed to be almost complete, in keeping with literature (data not shown).

In a case-control study with 274 CD and 211 UC Spanish patients and 511 unrelated healthy controls, we first examined the CD cohort to determine whether there was any evidence of association with the two above-mentioned *IBD5* SNPs. The comparison of the frequencies of the mutant allele carriage in CD patients overall with healthy controls nearly reached significant levels (200/341 *vs* 74/170, OR [CI] = 1.35 [0.96-1.89], $P = 0.07$). Due to clinical heterogeneity of the disease, it seemed interesting to clarify in which group of patients *IBD5* had the strongest impact on susceptibility. According to the Vienna Classification, dealing with anatomical localization and behavior of the lesions, the analysis revealed that some unique clinical characteristics were correlated with the genotype. The extended follow-up of the patients allowed us to confidently describe the localization and behavior of their lesions. The patients were classified in three groups: terminal ileum (L_1, 48.6%), colonic (L_2, 16.5%) and ileocolonic (L_3, 34.9%). In terms of the behavior their distribution was inflammatory (B_1, 43.3%), stricturing (B_2, 16.1%) and fistulizing (B_3, 40.6%). The ileocolonic L_3 and fistulizing B_3 clinical forms were both significantly associated with *IBD5* when compared to control subjects in the Spanish sample (Table 1). Data supported a dominant model of inheritance. When allelic frequencies were studied, the fistulizing patients were also significantly different from the group formed by the inflammatory plus stricturing patients (B_3 (119/101) *vs* B_1+B_2 (141/179), OR [CI] = 1.5 [1.04-2.14], $P<0.05$). Moreover, the homozygous mutant genotype was significantly more abundant in fistulizing patients than that in the inflammatory and stricturing clinical groups (Table 1, B_3 (33/77) *vs* B_1+B_2 (31/132), OR [CI] = 1.82 [1-3.34], $P<0.05$).

Table 1 Genotype frequencies of the *IBD5* SNPs in Crohn's disease patients and controls

	TT (%)	GT (%)	GG (%)
Controls ($n = 511$)	170 (33.3)	236 (46.2)	105 (20.5)
CD patients ($n = 274$)	74 (27)	136 (49.6)	64 (23.4)
CD-L_1 ($n = 133$) terminal ileum + UG	41 (30.8)	66 (49.6)	26 (19.6)
CD-L_2 ($n = 46$) colon	12 (26.1)	23 (50)	11 (23.9)
CD-L_3 ($n = 95$) ileocolon	21 (22.1)	47 (49.5)	27 (28.4)[a]
CD-B_1 ($n = 119$) inflammatory	36 (30.2)	60 (50.4)	23 (19.3)
CD-B_2 ($n = 44$) stricturing	14 (31.8)	22 (50)	8 (18.2)
CD-B_3 ($n = 110$) fistulizing	24 (21.3)	53 (48.9)	33 (30)[c]
CD-perianal ($n = 47$)	10 (21.3)	23 (48.9)	14 (29.8)
CD-non perianal ($n = 227$)	64 (28.2)	113 (49.8)	50 (22)

(GG+ GT) *vs* TT: [a]$P<0.05$; OR (CI) = 1.76 (1.02-3.05) compared to controls. (GG+ GT) *vs* TT: [c]$P<0.05$; OR (CI) = 1.79 (1.07-3.00) compared to controls.

Since the *IBD1* locus in chromosome 16 is a well-known genetic factor conferring susceptibility to CD we searched for epistatic effects with the *IBD5* region. Three *NOD2/CARD15* variants (R702W, G908R and L1007fs) act cooperatively to increase risk in CD: in the presence of any one of them, risk rises 2-6-fold and when homozygotes or compound heterozygotes appear, risk reaches up to 40-fold. We found that 31.4% of the Spanish CD patients present at least one of those three polymorphisms compared to 9.3% controls. To assess whether there was a different association of the *IBD5* locus on stratification by the *NOD2/CARD15* genotype, we studied the allele distribution of both polymorphisms IGR2060a_1 and IGR3081a_1 in the *NOD2/CARD15*-negative and *NOD2/CARD15*-positive populations. In the *NOD2/CARD15*-positive group, no *IBD5*-association could be detected (data not shown). Interestingly, we could observe that the association with *IBD5* was present in the *NOD2/CARD15*-negative individuals (Table 2). In this case, the genotype distribution in *NOD2/CARD15*-negative CD patients was significantly different from the one displayed for the healthy controls (OR [CI] = 1.68 [1.07-2.66], $P<0.05$). Again, as seen in the overall CD cohort, in the *NOD2/CARD15*-negative population the L_3 and B_3 subtypes were associated with the 5q31 polymorphisms (Table 2).

Table 2 Genotype frequencies of the *IBD5* SNPs in *NOD2/CARD15*-negative Crohn's disease patients and ethnically-matched controls

	TT (%)	GT (%)	GG (%)
Controls ($n = 511$)	170 (33.3)	236 (46.2)	105 (20.5)
CD patients ($n = 140$)	32 (22.9)	69 (49.3)	39 (27.9)[a]
CD-L_1 ($n = 55$) terminal ileum + UG	16 (29.1)	25 (45.5)	14 (25.5)
CD-L_2 ($n = 31$) colon	8 (25.8)	15 (48.4)	8 (25.8)
CD-L_3 ($n = 54$) ileocolon	8 (14.8)	29 (53.7)	17 (31.5)[b]
CD-B_1 ($n = 65$) inflammatory	17 (26.1)	33 (50.8)	15 (23.1)
CD-B_2 ($n = 15$) stricturing	6 (40)	7 (46.7)	2 (13.3)
CD-B_3 ($n = 59$) fistulizing	9 (15.25)	28 (47.5)	22 (37.3)[d]
CD-perianal ($n = 34$)	6 (17.7)	19 (55.9)	9 (26.5)
CD-non perianal ($n = 106$)	26 (24.5)	50 (47.2)	30 (28.3)

(GG+GT) *vs* TT: [a]$P<0.05$; OR (CI) = 1.68 (1.07-2.66) compared to controls. (GG+GT) *vs* TT: [b]$P<0.01$; OR (CI) = 2.87 (1.27-6.73) compared to controls. (GG+GT) *vs* TT: [d]$P<0.01$; OR (CI) = 2.77 (1.28-6.2) compared to controls.

Forty CD patients were treated with infliximab, and only 25 of them responded to treatment (see Methods), while the remaining 37.5% were non-responders. This means that infliximab was effective in around two-third of the CD patients, concordant with the previously reported efficiency of this therapy[21]. This overall response rate did not differ among the patient groups, regardless of the fistulizing or luminal phenotype. We pursued checking whether the polymorphisms located in the 5q31 locus could act as markers of response to this monoclonal antibody. No distorted distribution of the 5q31 phenotype among treated and non-treated CD patients was observed (homozygous mutant: 24.4% non-treated *vs* 25% treated). However, when the response to the monoclonal antibody was studied in those 40 CD patients (Table 3), the homozygous mutant

genotype was significantly associated with lack of response to infliximab treatment (RR [CI] = 3.88 [1.18-12.8], $P<0.05$). Patients' demographics and characteristics did not differ significantly between responders and non-responders. We only found significant association between homozygous mutant carriers and lack of response to infliximab treatment, which was independent of demographics, disease phenotype and concomitant medications. The frequency of the homozygous mutant genotype in the non-responders group was 46.7%, more than twice the frequency found for the healthy control cohort (20.3%) (OR [CI] = 3.42 [1.09-10.68], $P<0.05$). Moreover, significant difference was maintained when responders and non-responders to infliximab were compared (Table 3).

Table 3 Patients' characteristics at the time of infliximab treatment: the differential response to infliximab therapy in CD patients depends on their 5q31 genotype

	Responder (n = 25)	Non-responder (n = 15)	P
Demographics			
Mean age, yr (range)	35 (21-66)	40 (17-68)	0.21
Mean duration of disease, yr (range)	10 (1-27)	12 (1-31)	0.37
Male:Female	13:12	6:9	0.46
Smoker, n (%)	13 (52)	8 (53.3)	0.93
Extraintestinal manifestations, n (%)	13 (52)	7 (46)	0.92
Previous surgery, n (%)	14 (56)	9 (60)	0.80
Disease distribution			
Small bowel only, n (%)	9 (36)	4 (26.7)	0.54
Colon only, n (%)	3 (12)	2 (13.3)	1
Colon and small bowel, n (%)	13 (52)	9 (66)	0.62
Indication for infliximab			
Inflammatory disease only, n (%)	7 (28)	6 (40)	0.62
Fistulizing disease only, n (%)	10 (40)	7 (46.7)	1
Inflammatory and fistulizing disease, n (%)	8 (32)	2 (13.3)	0.27
Concomitant medication			
6-mercaptopurine, azathioprine, methotrexate, n (%)	17 (68)	13 (86.7)	0.27
Mesalamine, n (%)	7 (28)	4 (26.7)	0.52
Corticosteroid, n (%)	6 (24)	5 (33.3)	1
5q31 genotype			
Homozygous wild type, n (%)	6 (24)	3 (20)	1
Heterozygous, n (%)	16 (64)	5 (33.3)	0.1
Homozygous mutant, n (%)	3 (12)	7 (46.7)	0.024[a]

[a]$P<0.05$, RR (CI) = 3.88 (1.18-12.8).

The UC cohort was then analyzed and no evidence of association with this pathology in the Spanish sample was found. No significant difference was observed when comparing the two clinical forms, extensive and distal, to healthy controls. The main genetic determinant of UC susceptibility and extension within the Major Histocompatibility Complex (MHC) in our population is DRB1*0103. Stratifying by this MHC factor did not yield any difference either. Although UC disease can be treated with steroids in order to control symptoms, some patients eventually become resistant to or dependent on this therapy. To treat disease exacerbation in those cases, immunosuppressive therapy or even surgery is performed. If immunosuppressive drugs and/or surgical therapy were used at least once during the disease, it was considered as severe colitis. When the distribution of the 5q31 alleles was compared between these severe colitis patients and the healthy controls, a trend for association was seen (56/48 *vs* 440/572, OR [CI] = 1.52 [0.99-2.32], $P = 0.05$). These results could point to clinical heterogeneity of the UC samples tested as the cause underlying the variability reported in the association with the 5q31 haplotype. Others[22] have already observed the different UC incidence rate between sexes present in our population. The gender distribution of the *IBD5* genotype was compared to discern the male predominance in UC as a factor altering our results and no difference was found between men and women (TT: 39/22; GT: 73/42 and GG: 26/22, respectively).

DISCUSSION

A haplotype spanning 250 kb in the cytokine gene cluster on chromosome 5q31 was originally reported to be associated with Crohn's disease in a Canadian population[5,23]. Moreover, functional variants of OCTN cation transporter genes have been recently found in association with CD in this population, probably affecting the uptake of physiologic compounds and toxins[6]. Replication trials have been performed in order to verify the association of the 5q31 locus in independent cohorts. There are important disagreements in the literature regarding the relevance of this chromosome 5-risk locus in IBD. The influence of this locus in UC has been reported only once[7]. The primary purpose of this work was testing the involvement of the *IBD5* risk alleles in IBD disease susceptibility in the Spanish population. We have been able to replicate the association previously found in CD, but not in our UC cohort, although both seem to share a similar trend. This difference could be due either to a lesser effect of this locus on UC susceptibility or to an association with a yet-unidentified specific subgroup of UC patients or to the combination of both.

The complex genetic background of IBD has been revealed by the identified locus to locus interactions. A previous study stated that the *IBD5* association was present only in individuals with at least one of the known *NOD2/CARD15* susceptibility alleles, but was not significantly elevated in CD patients who had no *NOD2/CARD15* mutation[8]. The present work shows that the association found with *IBD5* in the Spanish CD cohort was due to the predisposition conferred to the *NOD2/CARD15*-negative subpopulation, and no influence was detected in the *NOD2/CARD15*-positive group. Our results agree with others reporting a contribution of the 5q31 variants in CD independently of the presence of *NOD2/CARD15* susceptibility alleles[7,14]. Moreover, our group has already determined that *NOD2/CARD15* and the Major Histocompatibility Complex locus are also independent genetic risk factors for CD[24]. The data presented suggest that one CD susceptibility locus affect a definite patient subgroup. Therefore, IBD predisposition would not always

be conferred by the presence of the same polymorphisms in many susceptibility genes, each with a small effect. Most probably, few variants would be enough to increase risk to one specific clinical form, and a different combination of risk factors would lend susceptibility to another clinical form. Thus, the genetic background determines which pathways are preferentially activated during tissue damage.

To assess the repercussion at the clinical subtypes of the disease, a detailed analysis following the Vienna Classification was performed. The CD perianal phenotype has been reported essential in the association with *IBD5*[14]. In contrast, the fistulizing phenotype is more strongly associated in the Spanish cohort tested in the present study, and in our patients, perianal lesions do not seem to be so crucial. It is interesting that both the interactions with the *IBD1* locus and the relationship with the clinical phenotype are not unequivocally reproduced in all the populations studied, probably indicating that the underlying different clinical composition of the cohorts tested sometimes preclude from detecting an effect. One could postulate that something similar occurs within the UC cohort, i.e., the different clinical composition may explain the apparently contradictory results reported in terms of association with the 5q31 locus.

Infliximab is a chimerical monoclonal antibody that inhibits the action of TNF-α. Although both infliximab and etanercept show powerful TNF-α neutralization, only infliximab (the only one effective in CD) binds to peripheral blood lymphocytes and lamina propria T cells to induce apoptosis of activated lymphocytes[15]. This provides a biological basis for the difference in CD effect of both drugs. It has been recently demonstrated that T cells from CD patients produce increased amounts of GM-CSF and that this increase significantly correlates with CD activity[16]. Moreover, the same study shows that infliximab inhibits the production of GM-CSF by mucosal T cells. Therefore, given the location of the GM-CSF gene in the *IBD5* risk locus, we were interested in exploring these 5q31 variants as potential markers of infliximab response. In clinical trials in CD, infliximab significantly decreased the CD activity index compared to placebo in treatment-resistant disease. However, this agent is expensive and at least one-third of the eligible patients fail to show any useful response. Lack of response seems a stable trait even after repeated infusions, suggesting that it might be genetically determined. Identifying predictors of response to infliximab may lead to better selection of patients for this maintenance therapy for individuals with CD. Several genes have been studied for this purpose: the TNF-α gene polymorphisms show conflicting results as predictors of response. Polymorphisms in the TNF-α receptors and the three mutations in the *NOD2/CARD15* gene have been reported not to be associated with infliximab response[16]. Our results showed that chromosome 5 polymorphisms associated with susceptibility to CD could be considered as good markers for response to infliximab therapy. The homozygous mutant patients for these IBD5 variants present a significantly worse response to infliximab. However, as the number of patients recruited is small, this preliminary interesting finding should be further confirmed in a larger cohort of treated CD patients before making any definite conclusion.

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