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Analysis of Crohn's disease-related CARD15 polymorphisms in Spanish patients with idiopathic uveitis

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Abstract. We wished to analyse the frequency of Crohn's disease-linked CARD15 polymorphisms (P268S, R702W, G908R and 1007fs) in a group of Spanish patients with idiopathic uveitis. To this aim, DNA samples were obtained from 111 unrelated patients. P268S, R702W and G908R polymorphisms were detected using TaqMan Genotyping kits (Applied Biosystems), and the 1007fs variation by direct DNA sequencing. Control group consisted of 105 healthy subjects.
None of the polymorphisms studied revealed a significant increase in the groups of patients, when compared to the control group. Thus, P268S is found in 50% of patients (gene frequency 0.284) *vs* 44% of control individuals (gene frequency 0.245); R702W in 7% of patients (0.036) *vs* 7% (0.033); G908R in 2% of patients (0.009) *vs* 4% (0.019) and, finally, 1007*fs* in 2% of uveitis patients (0.008) *vs* 4% (0.021). Moreover, DNA sequencing has allowed us to define two new intronic polymorphisms in phase, in the 5' and 3' boundaries of the exon 11 (GenBank accession number #DQ 869189).
Altogether, our results suggest that the Crohn's disease-linked CARD15 polymorphisms do not seem to predispose to idiopathic uveitis in the Spanish population.

Keywords: Uveitis, CARD15, SNPs

1. Introduction

Idiopathic uveitis (uveitides) encompasses a group of inflammatory conditions (immune-mediated) affecting mainly the uveal tract of the eye, which is the middle layer of the eye, between the sclera, conjunctiva and the anterior chamber on the outside, and the retina on the inside [15]. Depending on the anatomic part of the eye affected, idiopathic uveitis can be classified in anterior, intermediate or posterior uveitis, and panuveitis (affecting the anterior chamber, vitreous and retina or choroids). Annual incidence in Western countries is approximately 17/100,000, and the prevalence is estimated to be 38/100,000 [20]. Although its etiology is not well understood both, genetic and environmental factors, seem to play key roles in the disease appearance. The genetics of idiopathic uveitis remains unknown and several genes have been involved: major histocompatibility complex (HLA-B27), cytokine gene polymorphism or chemokine receptors genes [12].

Many uveitic entities are associated with systemic auto-immune or inflammatory diseases, such as inflammatory bowel disease (IBD). Ocular manifestations are not uncommon in IBD patients, with an incidence spanning between 4 and 12%. Amongst them the most frequent are uveitis, episcleritis and scleritis [6]. Interestingly, altered intestinal permeability, a feature of IBD, has also been reported in uveitis, suggesting that either the intestinal mucosa or its lymphoid tissue may be implicated in the pathogenesis of uveitis [2].

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Susceptibility to Crohn's disease (CD), one of the forms of IBD, has been linked to variations in the caspase recruitment domain (CARD15) gene [7,18]. This gene encodes a cytosolic protein, termed nucleotide oligomerization domain 2 (Nod2), involved in intracellular detection of bacterial components (muramyldipeptide, MDP). It is expressed mainly on monocytes, granulocytes and dendritic cells, and consists of several functional domains: two amino-terminal CARD domains, a central NOD domain and 10 carboxi-terminal leucine rich repeat (LRR) domains. The LRR domains are involved in the interaction with the infecting pathogen, whereas the CARD domains enable the protein to induce apoptosis and the NF-κB signalling pathways.

Three major CARD15 variants have been associated with susceptibility to CD [10]. These are a 2104 C $\rightarrow$ T transition responsible for the R702W polymorphism where an arginine is replaced by a tryptophan (SNP accession number rs17860491, according to the SNP database at http://www.ncbi.nlm.nih.gov/SNP/), a trasversion 2722 G $\rightarrow$ C, that causes the G908R polymorphism, where a glycine is replaced by an arginine (rs2066845), and a C insertion at position 3020 (3020*insC*) of the coding sequence, which causes a frameshift leading to a premature stop codon in aminoacid 1007 (1007fs, rs5743293). These mutations lay at exons 4, 8 and 11, respectively, the two latter in the LRR domains of the molecule. An additional mutation, a 802 C $\rightarrow$ T transition, yielding a proline to serine change at position 268 (P268S, rs2066842), is also associated with CD, although this variant was shown to occur in phase with the R702W, G908R and 1007fs mutations [7].

These protein variants are impaired in their ability to sense MPD, and, therefore are defective in mounting the adequate NF-κB-mediated response [8]. As a result, defects in the initial innate response to bacterial exposure result in increased susceptibility to inflammation [11].

Recognizing the association between CARD15 and an inflammatory condition (CD), we sought to analyse the four previously mentioned CD-linked CARD15 mutations in a large group of Spanish patients with idiopathic uveitis, another inflammatory condition.

2. Materials and methods

2.1. Experimental subjects

Written consent was obtained from 111 unrelated Spanish patients with idiopathic uveitis, diagnosed according to standardized criteria [9], and disclosed as follows: 70 were anterior uveitis (AU) patients, 19 intermediate uveitis (IU) and 22 posterior uveitis (PU). Table 1 shows the main features of the patients. The inclusion of patients in the group of idiopathic uveitis is irrespective of the anatomic part of the eye inflamed or of whether they present any concomitant inflammatory condition. Thus, in this regard, the group of patients is fairly homogeneous and can be confidently pooled. As a control group, 105 healthy volunteers (race-matched) were used. This research was approved by the Ethic Committee of the Institution.

2.2. DNA extraction

Blood samples were obtained by venopunction in EDTA-containing tubes. DNA was extracted by means of the DNAzol technique, according to the manufacturer's protocol (DNAzol reagent, Carlsbad, CA, USA).

2.3. Analysis of P268S, R702W and G908R polymorphisms

TaqMan SNP Genotyping Assay kits (Applied Biosystems, Foster City, CA, USA) were used for each mutation (references c_11717468-20, c_11717470-20 and c_11717466-20, respectively), according to manufacturer's protocols, using an Applied Biosystems Real-Time PCR 7900 HT Fast System.

2.4. Analysis of the 1007fs polymorphism

This polymorphism was analysed by direct DNA sequencing. Exon 11 was amplified by PCR using previously published primers and conditions [10].PCR amplification products were resolved in a 2% agarose gel, and the band of interest sliced, eluted from the gel (MiniElute Gel Extraction Kit, QIAGEN, Valencia, CA, USA) and submitted to the DNA sequencing facilities of the Universidad Complutense de Madrid (CAI de Genómica), where it was sequenced (direct sequencing) in an automated DNA sequencer. Both, forward and reverse amplifications from each sample were analysed to ensure the quality of the sequences.

Sequences from patients were compared to that of control group, as well as with previously published sequences of the CARD15 gene (GenBank #AJ303140).

Table 2 summarizes the methodology used.

Table 1
Clinical features of the patients with idiopathic uveitis studied

Patients	$n = 111$
Age (mean; range)	42.6 yrs; 16–78 yrs
Sex M/F	53/58
Uveitis subtypes:	
Anterior uveitis	70
Intermediate uveitis	19
Posterior uveitis	22
Concomitant systemic inflammatory conditions:	
Crohn's disease	4
Spondyloarthropathy	15
Behcet's Disease	3
Other (SLE, RA, fibromyalgia, etc. . . .)	10
Control individuals	$n = 105$

SLE, Systemic lupus erythematosus; RA, Rheumatoid arthritis.

Table 2
Polymorphisms studied, localization and method of detection

EXON	DOMAIN	SNP	AMINOACID CHANGE	METHOD ANALYSIS
4	5' NOD boundary	802 C → T	P268S	TaqMan SNP
4	5' LRR1 boundary	2104 C → T	R702W	TaqMan SNP
8	LRR6	2722 G → C	G908R	TaqMan SNP
11	LRR10	3020*ins*C	1007fs	DIRECT DNA SEQUENCING

Table 3
Results obtained for the polymorphisms studied in the total group of patients with uveitis, and its subtypes

POLYMORPHISM	PATIENTS (N = 111; 70 AU, 19 IU, 22 PU) %/ allele frequency	CONTROL POPULATION (N = 105) %/ allele frequency	p VALUE
P268S	Total group 50%/ 0.284 AU 57%; IU 47%; PU 32%	44%/ 0.245	n.s.
R702W	Total group 7%/ 0.036 AU 10%; IU 0%; PU 5%	7%/ 0.033	n.s.
G908R	Total group 2%/ 0.009 AU 1%; IU 0%; PU 5%	4%/ 0.019	n.s.
1007fs*	Total group 2%/ 0.008 AU 0%; IU 6%; PU 0%	4%/ 0.021	n.s.
Positive for at least one of the above markers	Total group 51% AU 57%; IU 47%; PU 32%	43%	n.s.

Gene frequency was assessed by direct gene counting.
AU Anterior uveitis; IU Intermediate uveitis; PU Posterior uveitis
*Only 62 patients (32 AU, 16 IU and 14 PU) and 70 healthy subjects were sequenced.

2.5. Allele frequency

Allele frequency of the polymorphisms considered was calculated by direct gene counting.

2.6. Statistical analysis

The frequency of the different polymorphisms in the group of patients was compared to that of the group of healthy subjects, using the Chi-squared with Yates' correction test, or Fisher exact probability test when needed. A p value less than 0.05 was considered significant.

3. Results

Table 3 shows the results obtained. The frequencies found for the markers tested in the healthy group matches that previously reported in the Spanish [4,17] or European [10] population.

P268S polymorphism is found in 50% of patients (gene frequency 0.284) and in 44% of control subjects (gene frequency 0.245), a difference that is not statistically different. When the various subsets of uveitis are considered, once again no statistical differences are found when compared to the control group, although a higher frequency of the P268S change is found in AU

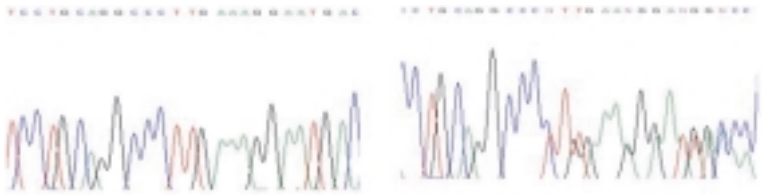

Fig. 1. Exon 11 sequence. Chromatogram depicts a homozygous individual lacking the 3020*ins*C insertion (left) and a heterozygous individual with the insertion (right).

patients (57%, $n = 70$), than in the IU group (47%, $n = 19$) or the PU group (32%, $n = 22$).

Regarding the R702W change, it is found in 7% of patients (0.036) and in 7% (0.033) of healthy individuals (n.s). If the group of patients is disclosed, it is found in 10% of AU patients ($n = 70$), 0% of IU patients ($n = 19$) and 5% of PU patients ($n = 22$).

As for the G908R SNP, it is found in roughly 2% of patients (0.009) and 4% (0.019) of control individuals (n.s). If patients are analysed depending on the type of uveitis, this polymorphism is found in 1% of AU ($n = 70$), 0% of IU ($n = 19$) and 5% of PU patients ($n = 22$).

The sequence of the exon 11 obtained in all subjects was identical to the consensus sequence previously published, except one patient ($n = 62$, 2%; 0.008) and three control individuals ($n = 70$, 4%; 0.021), in whom a cytosine insertion at position 3020 (3020*ins*C) was found (Fig. 1, left). This insertion has been reported in 11% of patients with CD [10], in marked contrast to our group of uveitis patients, and in 2% of the control population [10], similar to our data. With regard to this polymorphism, our results resemble previously published data on ankylosing spondylitis [22].

Finally, postivity for any of the markers previously mentioned (i.e.: positive for at least one marker) was also considered and, as before, no difference was found between patients and control subjects (50% *vs* 43%, p n.s,).

Analysis of the 5' and 3' exon 11 flanking regions.

DNA sequencing has allowed us to study the 5' and 3' flanking regions of exon 11, which revealed an intronic polymorphism not previously described, at positions 128723 (5' end) and 128943 (3' end) (see Fig. 2A). Whereas most individuals present an A in homozygosis at position 128723, and a C at position 128943, 9 patients (15%) and 4 control subjects (6%, p n.s.), bear an A/T polymorphism in heterozygosis in the first position (see Fig. 2B) and, simultaneously, a C/A polymorphism, also in heterozygosis, in the second position (see Fig. 2C). No homozygous individuals for these changes were found. This new intronic polymorphism has been submitted to the GenBank, and the accession number #DQ869189 assigned.

4. Discussion

IBD is a chronic inflammatory disorder of the digestive tract, and includes two entities: CD and ulcerative colitis (UC) [19], and several mutations at the CARD15 gene have been involved in the susceptibility to suffer CD. Given the fact that some IBD patients may present uveitis and that altered intestinal permeability, a feature present in CD, has been reported in uveitis [2], we wished to analyse the status of the CD-linked CARD15 gene mutations in a group of patients with uveitis.

No differences were found in the allele frequency of the markers tested (P268S, R702W, G908R, 1007fs) between patients and control subjects (Table 3), nor did it in the number of individuals carrying more than one mutation: eight patients and twelve control individuals presented two mutations, and no individual presented more than two mutations (data not shown).

Since uveitis may, in some instances, manifest with concomitant chronic diseases (spondyloarthropathy, Behcet's disease, etc. . . .), we next tested whether any difference with regard to the polymorphisms herein analysed existed between the group of patients with systemic manifestations ($n = 27$) and those lacking them ($n = 84$). Once again, we found no differences in the frequency of the markers between both groups (data not shown).

Given that 57 of the 111 patients with uveitis presented at least one CD-linked mutation, we were prompted to analyse what the frequency of CD was in our cohort. Only four patients suffer also from CD and, out of these, only two carried a CD-predisposing mutation (P268S, in heterozygosis). This result fits with our current knowledge that mutated CARD15 alleles are neither sufficient not necessary for the development of CD, since mutations are found in 0.5%–2% of the general population, and 60%–70% of CD patients show no CARD15 mutations [5].

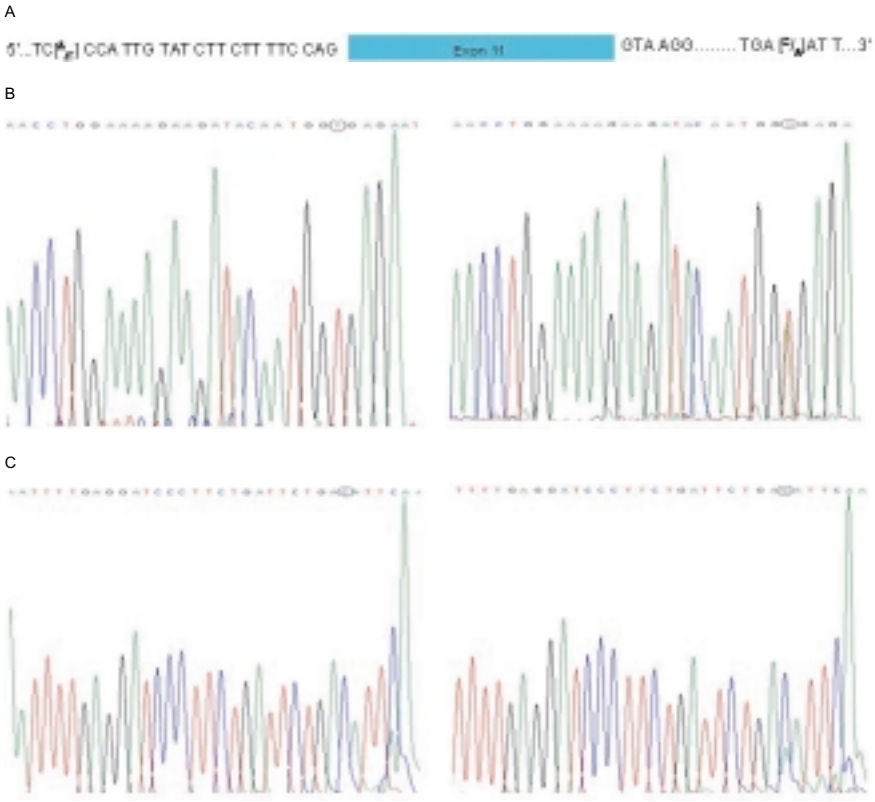

Fig. 2. Exon 11 5' and 3' flanking regions. a) Localization of the intronic polymorphisms described, with regard to exon 11. b) Sequence showing a homozygous (left, see a clear T base) and a heterozygous individual (right, note T and A bases) at position 128723. c) Sequence showing a homozygous (left, see a clear C base) and a heterozygous individual (right, note C and A bases) at position 128943.

Altogether our results suggest that the polymorphisms in the CARD15 gene predisposing to CD may not be linked to uveitis. Similar findings have been reported for ankylosing spondylitis [22], aggressive periodontitis [16], or Behçet's disease [1,21], where no differences were found for these CARD15 mutations between patients and controls. In addition, a study carried out in 52 patients with sarcoidosis, it was concluded that uveitis, a feature that may appear in some, but not all, patients with sarcoidosis, was not linked to CARD15 mutations [13].

The fact that the frequency of the polymorphisms studied in uveitis patients do not differ from that of the control group, does not discard this gene be responsible for the susceptibility to present uveitis. Other regions of the CARD15 gene may be involved. In fact, this is the situation in other inflammatory diseases.

Blau syndrome (BS, MIM 186580) is a rare Mendelian trait with autosomal-dominant inheritance, characterized by multiorgan granulomatous inflammation of the skin, eyes and joints [3]. In this disease, CARD15 is mutated in the NOD domain of the molecule, a region different to that of CD [14,23]. Interestingly, uveitis is also present in this syndrome, and it is thus conceivable that our patients may present mutations in this region. Ongoing work in our laboratory will focus on this issue.

Analysis of the 5' and 3' exon 11 flanking regions.

The fact that all the individuals bearing the A → T transition in the 5' flanking region, bore also the C → A mutation in the 3' region is intriguing. This prompted us to analyze the DNA sequences, to determine whether these changes had any effect on the exon/intron structure of the gene, or on the generation of new splicing sites. Analysis carried out with GENSCAN (http://genes.mit.edu/GENSCAN.html), FCGNESH (http://www.softberry.com/berry.phtml) and GRAIL (http://grail.lsd.ornl.gov/grailexp/) softwares, suggested that these polymorphisms apparently induced no change. The A → T transition may affect the so called branch point, an adenosine located 20–50 bases from the splice site, and important for the lariat formation during the correct mRNA splicing. Further

sequencing and biochemical analyses are required to confirm this hypothesis.

In summary our data suggest that none of the CARD15 mutations linked to CD seem to be involved in the pathogenesis of idiopathic uveitis. However, to reach firmly based statistical conclusions, increasing number of patients are required. This involves multicentric cooperation, which would then allow gathering the adequate number of carefully selected patients with idiopathic uveitis. Moreover, we describe a new intronic polymorphism affecting simultaneously two positions, one at the 5' boundaries and the other at the 3' boundaries of exon 11. To our knowledge, this is the first report on the CARD15 gene in idiopathic uveitis.

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