The three most common CARD15 mutations associated with Crohn’s disease and the chromosome 16 susceptibility locus for systemic lupus erythematosus

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Objective. To test if the three most common mutations contributing to Crohn’s disease on the CARD15/NOD2 gene could contribute also to genetic susceptibility to systemic lupus erythematosus (SLE), which has been found to be linked to the region of chromosome 16q13 where the CARD15 gene is located.

Methods. We obtained DNA samples from the blood of 189 SLE patients (according to the American College of Rheumatology classification criteria) and 194 controls of Spanish ancestry. Genotypes for the three CARD15 mutations (3020insC, 2722G > C and 2104C > T) were determined by hybridization with fluorescence resonance energy transfer probes on a LightCycler real-time polymerase chain reaction system.

Results. CARD15 genotypes were similar in SLE patients and in controls from the general population (allelic frequencies for 3020insC 0.013 in SLE patients vs 0.013 in controls; for 2722G > C 0.011 vs 0.008; and for 2104C > T 0.032 vs 0.051).

Conclusion. We did not find evidence that the Crohn’s disease-associated mutations on CARD15 contributed to SLE susceptibility.

KEY WORDS: Systemic lupus erythematosus, Crohn’s disease, Genetic predisposition to disease, Autoimmune diseases.

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by immune dysregulation resulting in the production of antinuclear antibodies, generation of circulating immune complexes and activation of the complement system, which is most frequent in women of childbearing age. SLE is notable for its protean clinical manifestations with unpredictable exacerbations and remissions. There is a predilection for clinical involvement of the joints, skin, kidney, brain, serosa and haematological abnormalities. Given SLE clinical heterogeneity, classification criteria have been developed and their generalized use allows for uniformity between the patient cohorts studied by different groups. However, there is still phenotypic heterogeneity between the patients as any combination of four among 11 criteria is enough for its classification [1]. The aetiology remains unknown, but a combination of genetic predisposition, sex hormones and environmental triggers probably results in the disease.

A role for genetics in SLE aetiology is firmly established. Familial aggregation of cases is reflected in a recurrence risk for siblings 10–20 times larger than the population risk [2]. There is also an increased concordance rate among monozygotic (MZ) twins, 24–69%, compared with dizygotic (DZ) twin pairs, 2–3% [3]. This marked difference between MZ and DZ concordance rates (more than a 10-fold drop from MZ to DZ twins) is evidence of the polygenic nature of the hereditary component. Already, several genes that are important for immune reactions have been found to play a role in SLE susceptibility. Among them the histocompatibility antigens, which have been associated with most...
autoimmune diseases. Two alleles are over-represented in SLE in European-Caucasian populations: HLA-DR2 and HLA-DR3. Many other immune genes have been associated with SLE over the years, but clear evidence is available only for members of the complement cascade, and the low affinity receptors for IgG [2].

Four different groups have performed genome-wide linkage analyses on SLE families obtaining a complex and to some extent heterogeneous picture [4–9]. All agree on the lack of any single gene with a major effect and on the likely presence of many loci with small effects. The different groups have found significant linkage with a total of four loci, but they have not been replicated in the different studies. This lack of reproducibility probably reflects several factors: the small effect of each of the susceptibility genes; the number of families studied by each group was not very large; there is also some evidence suggesting that SLE could have genetic heterogeneity, with different genes playing a role in particular groups of patients; and another possible factor is the intrinsic lack of certainty in the diagnosis, which makes misclassification possible. Unfortunately, these problems will not be easy to tackle and finding larger sets of families will be very difficult given the relative rarity of SLE with sporadic cases outnumbering by far familial cases. Therefore, it seems that a purely genetic approach to SLE will be a daunting challenge and it will be desirable to find shortcuts and leads that will contribute to the search for susceptibility genes. Two possible paths will be to follow biological hypothesis and to explore genes found in other, more manageable, autoimmune diseases.

In the genome-wide linkage studies, at least some degree of reproducibility was found: some loci that were significantly linked to SLE in one of the studies received some support in a second one. One of these SLE-linked loci was found in chromosome 16q12-13 in a study of 187 families [4, 5] (maximum LOD score of 3.85), and was supported by some linkage in a different cohort of 80 SLE families [6]. The 16q region has been linked also to susceptibility to other autoimmune diseases: Crohn’s disease, psoriasis, type 1 diabetes, rheumatoid arthritis, ankylosing spondylitis and asthma. Recently, several mutations in the leucine-rich repeat regulatory region of CARD15 (previously known as NOD2), a gene located in chromosome 16q13, has been identified as a major susceptibility factor for one of them, Crohn’s disease [10–13]. Three of these CARD15 mutations account for 81% of the Crohn’s disease mutations—3020insC, 2722G > C and 2104C > T—and are independently associated with the disease [10, 13].

The CARD15 gene, a member of a growing family of proteins containing a nucleotide-binding oligomerization domain (NOD), which regulate apoptosis and host defence against pathogens, is expressed almost exclusively in monocytes [14, 15]. Its known function is to mediate the activation of NFκB in response to bacterial products. Disruption of the regulatory region, the leucine-rich repeat, of CARD15 modifies the magnitude of NFκB activation, a key transcription factor in the induction of many inflammatory genes, leading to an uncontrolled inflammatory response to enteric bacteria that through an unknown path culminates in intestinal pathology in Crohn’s disease [11, 14]. The discovery of CARD15 as the underlying gene for the chromosome 16q susceptibility locus for Crohn’s disease offers an interesting lead to investigate the susceptibility locus for SLE on this chromosome: both map to the same place and among the myriad immune abnormalities associated with SLE some are related with macrophages [16–19], though most interest has focused on the study of abnormalities of B and T cells. For these reasons CARD15 has been singled out in a recent review as the best candidate for the chromosome 16q SLE susceptibility locus [2].

Patients and methods

Patients

We studied 189 SLE patients, according to American College of Rheumatology classification criteria [1]. 116 followed in the Hospital 12 de Octubre in Madrid and 73 in the Hospital Clínico Universitario in Santiago de Compostela: and 194 controls recruited in Santiago de Compostela. They were questioned to ensure that they have a homogeneous Spanish ancestry. The regional Ethics Committee (Comite Etico de Investigacion Clinica de Galicia) approved the study, and written informed consent was obtained from all participants.

Genotyping

DNA was extracted from peripheral blood with the DNA Isolation kit for Mammalian Blood (Roche Diagnostics, Mannheim, Germany) following the manufacturer’s protocol. Three alleles of the CARD15 gene (3020insC, 2722G > C, 2104C > T) were typed by analysis of the melting temperature of the hybrids formed between the polymerase chain reaction (PCR) products and specific fluorochrome-labelled oligonucleotides. Detection of the hybridization signal was based on fluorescence resonance energy transfer (FRET) on a LightCycler (Roche) real-time PCR system. Primers and fluorescently labelled probes for FRET were synthesized by TIB MOLBIOL (Berlin, Germany) and are listed in Table 1. We performed multiplex PCR with primers and probes for the 2722G > C and 2104C > T alleles in the same reaction capillary; the 3020insC allele was typed separately. Both reactions were carried out in a total volume of 15 μl in the LightCycler glass capillaries. The PCR mixture contained 50–100 ng of genomic DNA, 0.5 μM of each primer, 0.1 μM of each probe and 1.5 μl of the DNA-Master Hybridization Probes (Roche Molecular Biochemicals) reagent, which includes Taq DNA polymerase, reaction buffer and dNTPs. Additionally, the multiplex PCR contained 9 mM MgCl2, and the PCR for 3020insC 4 mM MgCl2. PCR conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 45 cycles of denaturation at 95 °C for 0 s, annealing at 55 °C for 10 s, and extension at 72°C for 5 s. After amplification, the melting analysis was performed by denaturation at 95°C for 5 s, annealing at 50°C for 10 s, and increasing the temperature to 90°C at a rate of 0.5°C/s.

Sequence

To confirm the genotypes some samples were sequenced from the products of the PCR for genotyping. We used the ABI
**Table 1. Oligonucleotides**<sup>a</sup> used for genotyping CARD15 mutations<sup>b</sup>

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Type of oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>3020insC</td>
<td>Forward</td>
<td>TCTCTTTTCCAGTGTTCCAA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TAGAGTTGGAGGACTAAACAG</td>
</tr>
<tr>
<td></td>
<td>Anchor</td>
<td>L-CCATCTGGAGATCTGGTAAGGCp</td>
</tr>
<tr>
<td></td>
<td>Sensor</td>
<td>AGGCCCTTGGAAAAAGAACG-X</td>
</tr>
<tr>
<td>2722G &gt; C</td>
<td>Forward</td>
<td>GACATATCAGTTACTACTGACT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TTACCTGAGCCACCTTCAGC</td>
</tr>
<tr>
<td></td>
<td>Anchor</td>
<td>R-CTGAAAAGGCCCAAGAGTCAACAGAp</td>
</tr>
<tr>
<td></td>
<td>Sensor</td>
<td>CCACTCTGTGCAGCCAGAAX</td>
</tr>
<tr>
<td>2104C &gt; T</td>
<td>Forward</td>
<td>AGCCGCACACCTTCAGATCAC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCGGCCACAGGCCCTAGC</td>
</tr>
<tr>
<td></td>
<td>Anchor</td>
<td>L-GTCCTGGCCTCAGGCAGGAGGAGGCCcp</td>
</tr>
<tr>
<td></td>
<td>Sensor</td>
<td>GGCAGGAGCCAGGGCTTCTCA-X</td>
</tr>
</tbody>
</table>

<sup>a</sup>Oligonucleotide sequences are written from 5’ to 3’.

<sup>b</sup>Genotyping was done by analysis of the melting temperature of the hybrids formed between the PCR products (PCR primers were Forward and Reverse) and specific fluorochrome-labelled oligonucleotides (Anchor and Sensor). Detection of the hybridization signal was based on fluorescence resonance energy transfer (FRET) on a LightCycler (Roche) real-time PCR system. Anchor probes were labelled at the 5’ end and specific fluorochrome-labelled oligonucleotides (Anchor and Sensor). Detection of the hybridization signal was based on fluorescence resonance energy transfer (FRET) on a LightCycler (Roche) real-time PCR system. Anchor probes were labelled at the 5’ end by phosphorylation (p). Sensor probes were labelled at the 3’ end with fluorescein (X).

**Statistical analysis**

Allelic frequencies, odds ratios (OR), their confidence intervals (CI) and Fisher’s exact test were calculated (done on-line at: http://home.clara.net/sisa/index.htm). The sample size and the post hoc power of the study were determined with the Gpower software (available at: http://www.psycho.uni-duesseldorf.de/aap/projects/gpower/index.html).

**Results**

Frequencies of the three most common CARD15 alleles (3020insC, 2722G > C and 2104C > T) predisposing to Crohn’s disease were similar in SLE patients and controls (Table 2) with an overall OR of 0.8 (95% CI 0.4–1.4). The size of the study, 189 SLE patients and 194 controls, should be enough to test association with alleles having a sizeable effect. To quantify how large this effect should be, we calculated the post hoc power to detect a doubling effect on the SLE risk due to the three disease alleles taken together, and it was larger than 0.99 and larger than 0.8 for attributable risks over 1.5. This should be a valid approximation because the three mutations are known to be inherited in separate chromosomes and contribute independently to the disease (we did not find homozygotes or compound heterozygotes among our patients or controls). Other conditions of validity were also fulfilled: both groups were drawn from the same homogeneous Spanish population and the SLE patients were selected in agreement with the American College of Rheumatology classification criteria. Genotyping was done unambiguously by analysis of the melting curves after hybridization with FRET probes on a real-time PCR system (Table 1) and a selected fraction of the samples were confirmed by sequencing. Owing to the low frequency of the mutant alleles, it was not possible to do other analyses, such as haplotype analysis or stratification on clinical features.

**Discussion**

The genetic basis for susceptibility to SLE has been the subject of intense research for the last 20 yr, but progress has been hampered by its daunting complexity [2]. However, several new insights have been obtained both via the analysis of the association with candidate genes and through genome-wide linkage analysis. One of the lessons learned is that pure genetic investigation will progress very slowly given the unfavourable characteristics of SLE: its low incidence, lack of certainty in the diagnosis, a high phenotypic and genetic heterogeneity and a large degree of genetic complexity with the likely involvement of many low penetrance genes. In some
regards, biological hypothesis or information gathered on other autoimmune diseases may help to make the investigation of SLE susceptibility more tractable. One of these possible shortcuts is the investigation of genes influencing predisposition to other autoimmune diseases and having coincident mapping with susceptibility loci for SLE.

Coincident mapping of susceptibility loci for different autoimmune diseases happens much more often than randomly expected or than among non-autoimmune diseases [20, 21]. Up to 18 clusters, where linkage-positive results for human autoimmune diseases overlap, have been reported. One of the clearest of them, with a larger range of diseases linked and showing the strongest linkages, is the cluster on chromosome 16q, which includes susceptibility loci for Crohn’s disease and SLE [2, 20, 22]. The clustering of autoimmune susceptibility loci is a common finding in both human diseases and experimental models that needs to be explained. A possibility is that clinically distinct autoimmune diseases may be controlled by a common set of susceptibility loci. Other analogies between different autoimmune diseases reinforce this hypothesis of common genetic factors. For example, it is often found that members of the same family suffer from different autoimmune diseases, and it is easy to point to common features in the pathogenesis, epidemiology or clinical manifestations of many autoimmune diseases. Following these ideas, we surmised that CARD15 mutations predisposing to Crohn’s disease could explain the previously reported SLE linkage on chromosome 16q13 [4–6], though, in this case, there is no clinical overlap or common occurrence of both diseases in members of the same family. But, at least there is some role for macrophages on the pathogenesis of both diseases. In SLE, macrophages participate in the clearance of the nuclear elements freed from cells during apoptosis and in the inflammatory reaction induced by immune complexes deposited on vascular walls [16–19] and it is conceivable that CARD15 could be involved in some of these functions as it is almost exclusively expressed on this type of cell.

Our results on SLE showing no association between any of the three most common mutations, which account for 81% of the mutations found in Crohn’s disease patients [13], indicate that CARD15 is not a common genetic factor for Crohn’s disease and SLE. The recently reported lack of association between one of the three mutations, 3020insC, which accounts for 31% of the Crohn’s disease CARD15 mutations, and psoriasis [23], and the lack of association of the same three mutations studied here and ankylosing spondylitis (I. Ferreiros-Vidal et al., submitted) indicate that Crohn’s disease and these three autoimmune diseases have a different basis for the susceptibility loci on chromosome 16q.

An attractive alternative to explain the cluster of susceptibility loci is to hypothesize that they are due to genes with related function that have appeared by duplication and divergence from an ancestral single gene and that persist in neighbouring sites on the same chromosome, each playing a role in different diseases [20–22]. Several examples of these clusters of evolutionary and functionally related genes have been identified in the immune system, with the best known example being the MHC genes on chromosome 6. However, no such group of CARD15-related genes has been found on chromosome 16q by homology searches using BLAST (I. Ferreiros-Vidal et al., unpublished).

Further studies will be required to determine whether the other autoimmune diseases in this chromosome 16q cluster (type 1 diabetes, rheumatoid arthritis and asthma) are linked to Crohn’s disease CARD15 mutations and to find the underlying causes of the different loci. Unfortunately, what seemed a useful lead to accelerate the search for genes involved in genetic predisposition to SLE, the use of genes identified in other autoimmune diseases happens much more often than randomly expected or than among non-autoimmune diseases.

Acknowledgements

We thank the patients and the staff of the rheumatology units for their contribution. We thank also L. Fernandez Blanco for her help in processing samples and O. Landt (TIB MOLBIOL, Berlin, Germany) for designing the LightCycler hybridization probes. Supported by grant 01/3138 of the Fondo de Investigaciones Sanitarias, MSC-ISCHII (Spain) and Schering-Plough SA, Spain.

References


