Lack of Association of Ankylosing Spondylitis with the Most Common *NOD2* Susceptibility Alleles to Crohn's Disease

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ABSTRACT. ***Objective.*** To investigate whether the 3 most common mutations in the *NOD2* gene that confer susceptibility to Crohn's disease (CD) are also associated with ankylosing spondylitis (AS).

Methods. DNA from 112 patients with AS and 168 controls of homogenous Spanish ancestry were studied. The frequencies of the pathogenic alleles of *NOD2* (*3020insC*, *2722G>C*, and *2104C>T*) were determined by analysis of the melting curves after hybridization with FRET probes on a Light Cycler real-time polymerase chain reaction (PCR) system.

Results. *NOD2* allelic frequencies in controls (*3020insC*, 0.009; *2722G>C*, 0.009; *2104C>T*, 0.042) did not significantly differ from patients with AS (*3020insC*, 0.009; *2722G>C*, 0.004; *2104C>T*, 0.031).

Conclusion. The 3 most common CD *NOD2* mutations do not contribute to disease susceptibility to AS, and therefore do not explain the susceptibility locus for AS in chromosome 16q. (J Rheumatol 2003;30:102–4)

Key Indexing Terms:
ANKYLOSING SPONDYLITIS
CROHN'S DISEASE
GENETIC PREDISPOSITION TO DISEASE
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Different autoimmune and inflammatory diseases share common features: clinical manifestations, epidemiological distribution, and characteristics of pathogenesis. Some coincide in the same individual or, more often, in different members of the same family, leading to the hypothesis that these diseases have common genetic susceptibility factors[1]. Genetic studies on a multiplicity of autoimmune and inflammatory diseases have lent support to this hypothesis: many of the susceptibility loci overlap. The overlap is much more frequent than would be expected by random distribution or than is observed with susceptibility loci for nonautoimmune diseases[1-3]. The existence of these clusters of susceptibility loci is indicative of common disease genes, and the gene predisposing to a disease of this group may also be a susceptibility gene for the other diseases in the same cluster.

A particular cluster has been found in chromosome 16q[2,3]. The diseases showing linkage with this cluster include Crohn's disease (CD), ankylosing spondylitis (AS), systemic lupus erythematosus, psoriasis, rheumatoid arthritis, insulin dependent diabetes mellitus, asthma, and a murine model of multiple sclerosis. Recently, it has been found that some independent mutations on the regulatory region of the *NOD2* gene cause the susceptibility locus for CD[4-6]. These mutations account for a large fraction of CD heritability [at least one of the most common variants, *3020insC*, is also strongly associated with CD in Spanish patients (C. Lopez-Larrea, Hospital General de Asturias, personal communication)]. The *NOD2* gene, expressed almost exclusively in monocytes, mediates the activation of NF-κB, a key transcription factor in the induction of many inflammatory genes, in response to bacterial products. Disruption of the regulatory region of *NOD2* alters the magnitude of NF-κB activation, leading to an uncontrolled inflammatory response to enteric bacteria[5].

This observation prompted investigations of the susceptibility factors for the autoimmune and inflammatory diseases clustering in chromosome 16q. AS has the highest heritability among them, with a recurrence risk for first-degree relatives 82 times higher than the risk in the general population[7]. The genetic component in AS is not limited to the HLA-B27 allele: B27 positive brothers of patients with AS have a recurrence risk 5.6 to 16 times higher than unrelated B27 positive individuals. Genome-wide linkage studies have confirmed this with the identification of up to 7 additional loci causing susceptibility to AS[8,9]. The stronger non-MHC locus was found in the cluster of chromosome 16q, in the vicinity of *NOD2*, with a LOD score of 4.7. The likelihood that this locus is caused by *NOD2* mutations is reinforced by the special relationship

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between AS and CD. Some CD patients have symptoms or signs characteristic of AS and *vice versa*[10-13]. AS and CD also overlap in pathogenic mechanisms: in both diseases, gastrointestinal bacteria seem to trigger an inappropriate immune response mediated by macrophages and T cells[14,15]. Tumor necrosis factor-α (TNF-α) plays a central role, and treatment with TNF-α blocking agents causes a marked improvement in patients with CD[16] or AS[17]. Also, there is an increased incidence of inflammatory bowel diseases in first-degree relatives of patients with AS[13], and the HLA-B27 positive first-degree relatives of patients with CD have an increased incidence of developing AS[10]. Collectively, these observations suggest that the AS susceptibility locus on chromosome 16q could pertain to the same *NOD2* mutations predisposing to CD.

MATERIALS AND METHODS

Patients. We studied 112 patients with AS, according to the New York criteria[18], and 168 controls of homogenous Spanish ancestry. The study was approved by the regional Ethical Committee, and written informed consent was obtained from all patients.

Genotyping. DNA was extracted from peripheral blood with the Puregen kit (Gentra Systems, Minneapolis, MN, USA) following the manufacturer's protocol. Three alleles of the *NOD2* gene (*3020insC*, *2722G>C*, and *2104C>T*) were typed by analysis of the melting temperature of the hybrids formed between the polymerase chain reaction (PCR) products and specific fluorochrome labeled oligonucleotides. Detection of the hybridization signal was based on fluorescence resonance energy transfer (FRET) on a Light Cycler (Roche) real-time PCR system. Primers and fluorescent labeled probes for FRET were synthesized by TIB Molbiol (Berlin, Germany). The *3020insC* allele was amplified with forward primer 5′-TCTTCTTTTCCAGGTTGTCCAA-3′ and reverse primer 5′-TGAGGTTCGGAGAGCTAAAACAG-3′. In addition, an anchor probe, 5′-CCATCCTGGAAGTCTGGTAAGGCCp-3′, labeled at the 5′ end with Light Cycler Red 640 (LC-Red 640) and modified at the 3′ end by phosphorylation, and a sensor probe, 5′-AGGCCCCTTGAAAGGAATGACX-3′, labeled at the 3′ end with fluorescein (X), were used. The melting temperature of this probe is 64°C for the wild-type allele and 60°C for the mutated allele. The *2722G>C* allele was amplified with forward primer 5′-GCACATATCAGGTACTCACTGACACT-3′ and reverse primer 5′-TTACCTGAGCCACCTCAAGC-3′. The anchor probe 5′-CTGAAAAGGCCAAAAGAGTCAACAGACp-3′ was labeled at the 5′ end with LC-Red 705, and phosphorylated at the 3′ end. The sensor probe 5′-CCACTCTGTTGCGCCAGAAX-3′ was labeled with fluorescein. The melting temperature for the wild-type allele is 67°C and 61°C for the pathogenic allele. The primers to amplify the *2104C>T* allele were 5′-AGCCGCACAACCTTCAGATCAC-3′ forward and 5′-GCGGGCACAGGCCTAGC-3′ reverse. We used the anchor probe 5′-GTCTGGCACTCAGCCAGCAGGCCCCp-3′ labeled with LC-Red 640 and the 3′ fluorescein labeled sensor probe 5′-GCGCCAGAGCAGGGCCTTCTCAX-3′. The transition C>T produces a 6°C shift in melting temperature to 62°C.

We performed multiplex PCR with primers and probes for the 2722G>C and 2104C>T alleles in the same reaction capillary; the *3020inC* allele was typed separately. Both reactions were carried out in a total volume of 15 µl in the Light Cycler 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, Barcelona, Spain) reagent, which includes Taq DNA polymerase, reaction buffer, and dNTPs. Additionally, the multiplex PCR contained 9 mM $MgCl_2$, and the PCR for *3020insC* contained 4 mM $MgCl_2$. 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.

Sequencing. To confirm the genotypes, some samples were sequenced from the products of the PCR used for genotyping. We used the ABI Prism® dRhodamine terminator cycle sequencing ready reaction kit (Applied Biosystems, Madrid, Spain). Cycling conditions were as follows: initial denaturation at 96°C for 4 min, followed by 36 cycles of denaturation at 96°C for 15 s, annealing at 50°C for 10 s, and extension at 60°C for 2 min, and final elongation at 60°C for 10 min. The ABI Prism 377 DNA sequencer (Applied Biosystems) was used to read the sequences.

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

RESULTS

The allelic frequencies of the 3 most common *NOD2* alleles predisposing to CD were determined in DNA samples from 112 patients with AS fulfilling the New York diagnostic criteria and attending the Rheumatology Unit at the Hospital Clinico Universitario de Santiago and from 168 controls. All the genotypes were determined unambiguously and the samples with any of the 3 disease susceptibility alleles were confirmed by sequencing. The allele frequencies in the control population were different from those previously reported[4], with more *2104C>T* mutations and fewer of the other 2 mutations, but the overall frequency we found was very similar to the previous report (0.06 vs 0.07, respectively). There were no differences (Table 1) between the allele frequencies in AS patients and controls for any of the 3 alleles measured individually or collectively (OR 0.74; 95% CI 0.34–1.61). This lack of association most likely was not due to lack of power of the study as the *post-hoc* power to detect a doubling effect on the AS risk due to the 3 disease alleles together was 0.96. As in other European populations, most patients with AS were HLA-B27 positive (84.4%), although the percentage in Spain is somewhat lower than in other countries[19], and there was no difference in the frequency of *NOD2* mutations between HLA-B27 negative and positive patients (0.088 vs 0.037; OR 1.7, 95% CI 0.4–6.7). There were 5 AS patients who referred intestinal disorders, but none of the 5 had any of the 3 *NOD2* mutations we studied.

DISCUSSION

We investigated whether *NOD2*, recently described as a causative susceptibility gene for CD, also has a role in AS. The 3 mutations we investigated account for 81% of the

Table 1. Allelic frequencies of the 3 mutations of *NOD2* in patients with AS and controls.

	3020insC	*2722G>C*	*2104C>T*	Total*
Controls (n = 336)**	0.009	0.009	0.042	0.059
Cases (n = 224)	0.009	0.004	0.031	0.045
OR (95% CI)	1 (0.2–6.0)	0.5 (0.1–4.9)	0.7 (0.3–1.9)	0.7 (0.3–1.6)

* Joint frequency of the 3 mutations. No homozygous or compound heterozygous mutations were found.
** Number of studied chromosomes.

NOD2 mutations associated with CD, while the remaining 19% are due to 27 rare mutations[20]. Given the *post-hoc* power of our study and the strength (LOD 4.7) of the chromosome 16q AS susceptibility locus[8,9], our results show that the most common CD-associated *NOD2* mutations are not the basis for the susceptibility locus for AS. This is significant because the *NOD2* mutations we investigated, unlike many other gene variants analyzed in association studies, have functional consequences affecting monocyte-dependent responses through NF-κB activation in response to Gram negative bacteria[5]. Similarly to CD, a link of immune response to enteric bacteria and AS has been proposed[21]. Lack of influence of the *NOD2* mutations on AS indicates that enteric bacteria do not act in the same way in both diseases. Differences might include the bacteria involved, the type of response elicited, or the absence of this kind of trigger for AS.

Coincident mapping of susceptibility loci as a guide for selecting a candidate gene for the chromosome 16q AS locus has not been successful. *A priori*, a specific cluster of susceptibility loci could be due to a single gene underlying all the loci, a cluster of functionally or evolutionarily related genes, each playing a role in different diseases, or to poor precision mapping[1-3]. Either of the latter 2 alternatives might explain our results.

Finally, our results point to the weakness of reasoning from clinical or pathogenic similarities, or even from coinheritance in families, as guides to genetic investigation. Although these features are still appealing and indicate a genetic link, our current understanding of disease mechanisms does not allow sound inferences about specific genetic factors. The similarities between AS and CD may be due to other pathogenic mechanisms related to common susceptibility genes different from *NOD2* or to other unexplored *NOD2* mutations. A similar interpretation could be given to the recent description of the lack of association of one of the *NOD2* variants, *3020insC*, with susceptibility to psoriasis, another of the diseases with a susceptibility locus in the same region of chromosome 16 sharing pathogenic and clinical features with CD[22].

Note added in proof: Since submission of this manuscript 2 studies have been published reporting similar results on the same 3 *NOD2* mutations in AS: Crane AM, Bradbury L, van Heel DA, et al. Role of *NOD2* variants in spondylarthritis. Arthritis Rheum 2002;46:1629-33; and Miceli-Richard C, Zouali H, Lesage S, et al. *CARD15*/*NOD2* analyses in spondylarthropathy. Arthritis Rheum 2002;46:1405-6.

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