Lack of Association with Rheumatoid Arthritis of Selected Polymorphisms in 4 Candidate Genes: CFH, CD209, Eotaxin-3, and MHC2TA

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ABSTRACT. Objective. To investigate associations with rheumatoid arthritis (RA) of single-nucleotide polymorphisms (SNP) in 4 candidate genes, complement factor H (CFH), CD209 or DC-SIGN, eotaxin-3, and the MHC class II Transactivator (MHC2TA) genes. These SNP have been reported as important for RA (eotaxin-3 and MHC2TA) or for other immune-mediated diseases (CFH and CD209).

Methods. Genotypes for the 7 selected SNP were obtained from 1587 patients with RA and 1570 controls of Spanish ancestry. Analyses were carried out after stratification for sex, erosions, rheumatoid factor, shared epitope, anti-cyclic citrullinated peptide antibodies, and the R620W PTPN22 SNP.

Results. None of the comparisons between patients with RA and controls or between the different strata of patients according to disease features was significant.

Conclusion. None of the SNP in CFH and CD209 showed evidence of association with RA. We did not replicate the association of eotaxin-3 with RA described in Koreans, or that of the MHC2T SNP.

Key Indexing Terms:
RHEUMATOID ARTHRITIS 
CANDIDATE GENES 
GENETICS 
CASE-CONTROL STUDY 
REPLICATION

Rheumatoid arthritis (RA) has a complex genetic component that accounts for about 50% of disease liability and is made up of multiple low penetrance polymorphisms. Great efforts have been made in recent years to identify these polymorphisms, with some notable successes, but a large fraction of the RA genetic component still awaits explanation. The most comprehensive results have been obtained with genome-wide association studies. Each of these has addressed several hundred thousand single-nucleotide polymorphisms (SNP) along the genome in large sample collections. Recently, a metaanalysis of some of these studies was performed. As a result of these different studies, there is definitive evidence supporting a series of RA genetic factors that include the classical HLA and PTPN22 factors and the newly discovered factors in the STAT4, C5-TRAF1, CD40, and 6q23 loci. Also very compelling, although not supported in the genome-wide association studies, is the evidence of the involvement of IRF5 in a subset of patients with RA that is still incompletely defined, and of other genetic factors that seem largely specific for Asian ethnicity.

From Laboratorio Investigacion 10 and Rheumatology Unit, Hospital Clinico Universitario de Santiago, Santiago de Compostela, Spain. Supported by grants PI04/1513 and PI06/0620 from the Instituto de Salud Carlos III (Spain) with participation of funds from FEDER (European Union). Dr. Akar was the recipient of an ARTICULUM Fellowship.

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Accepted for publication March 5, 2009.

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ty, like PADI4 and FCRL3. Much more research is needed because all the known loci together account for less than half the genetic component, and because no causal polymorphism has been identified for most of the loci. At present it is unwarranted to rely only on genome-wide association studies to identify the remaining factors because they do not yet cover all the variation in the genome. In addition, other less comprehensive approaches have recently been successful in RA, leading to identification of PTPN22 and STAT4 among others. With this in mind, we selected 7 SNP in 4 genes, complement factor H (CFH), CD209, eotaxin-3, and MHC class II transactivator (MHC2TA), for study in RA. They have been found to be associated with RA or with related diseases in previous studies.

Three SNP are from the CFH gene, which shows a definitive association with age-related macular degeneration (AMD)9,10, the most common form of blindness in adults. The at-risk alleles of CFH determine deficient complement inhibition, and we hypothesized they could be involved in RA given the importance of the complement system in the pathogenesis of RA11. We also considered that a regulatory SNP in the promoter of CD209 (rs4804803), coding for dendritic cell-specific intercellular adhesion molecule (ICAM-3) grabbing nonintegrin (DC-SIGN), whose minor allele is quite convincingly associated with protection from dengue fever12 and tuberculosis13,14, deserved to be analyzed in RA. CD209 is a dendritic cell (DC)-specific C-type lectin superfamily receptor that has functions of pattern recognition receptor in the innate response to infection, DC migration, and the initial steps of T cell activation15. All of these processes are important for RA16. In addition, CD209 has shown a particular pattern of expression in RA17. The other 3 SNP have been associated with RA in previous studies. Two of the SNP are in eotaxin-3 or CC chemokine ligand 26 (CCL26). They showed a remarkable association with RA in Koreans (odds ratio 2.99)18. The last SNP we tried to replicate is rs3087456 in the MHC2TA gene, which is a good candidate for a role in RA because this gene is the master regulator of MHC class II gene expression. The SNP we selected was associated with RA and with other immune-mediated diseases in a large Swedish study19. In spite of these suggestive hypotheses and previous associations, none of the 7 SNP was associated with RA in the 1600 Spanish patients that we have studied.

MATERIALS AND METHODS

**DNA samples.** DNA samples were obtained from peripheral blood of Spanish patients with RA and healthy controls. Recruiting of study subjects was as described20. The study included a total of 1587 patients and 1570 participants gave their written informed consent.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>75.2</td>
</tr>
<tr>
<td>Age of disease onset, yrs, median (IQR)</td>
<td>48 (37–57)</td>
</tr>
<tr>
<td>Morning stiffness</td>
<td>96.1</td>
</tr>
<tr>
<td>Arthritis of 3 or more joint areas</td>
<td>99.7</td>
</tr>
<tr>
<td>Arthritis of hand joints</td>
<td>99.0</td>
</tr>
<tr>
<td>Symmetric arthritis</td>
<td>99.3</td>
</tr>
<tr>
<td>Rheumatoid nodules</td>
<td>19.4</td>
</tr>
<tr>
<td>Rheumatoid factor</td>
<td>72.5</td>
</tr>
<tr>
<td>Erosions</td>
<td>71.9</td>
</tr>
<tr>
<td>Sicca syndrome</td>
<td>9.0</td>
</tr>
<tr>
<td>Intestinal pneumonitis</td>
<td>3.0</td>
</tr>
<tr>
<td>Shared epitope (carrier %)*</td>
<td>54.2</td>
</tr>
<tr>
<td>ACPA**</td>
<td>66.5</td>
</tr>
</tbody>
</table>

* Data available for 578 patients. ** Data available for 639 patients. IQR: interquartile range; ACPA: anticitrullinated protein antibodies.

**SNP genotyping.** We studied 7 polymorphisms from 4 genes, CFH (rs1061170, rs800292, rs3766404), CD209 (rs4804803), eotaxin-3 (rs6965556, rs2302009), and MHC2TA (rs3087456), in a single multiplex reaction. They were amplified in a single polymerase chain reaction (PCR) with the Qiagen Multiplex PCR kit (Qiagen, Valencia, CA, USA) on 30 ng of genomic DNA. PCR conditions were as follows: initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 90 s, and extension at 72°C for 90 s. Final extension was performed for 10 min at 72°C. PCR products were purified by Exo-SAP digestion with Exonuclease I (Epipcentred, Madison, WI, USA) and shrimp alkaline phosphatase (GE Healthcare, Barcelona, Spain) for 1 h at 37°C, and 15 min at 75°C to inactivate the enzymes. Next, single-base extension reactions with the SNaPshot Multiplex Kit (Applied Biosystems, Foster City, CA, USA) were done. Reaction conditions were: 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and single-base extension at 60°C for 30 s. Post-extension treatment with SAP was done for 1 h at 37°C. Genotypes were obtained in the ABI Prism 3130x Genetic Analyzer (Applied Biosystems). Primers and probes are shown in Table 2.

**Statistical analysis.** Analysis relied on 3 applications: Haploviet22, a customized version of Statistica 7.0 (StatSoft, Tulsa, OK, USA), and Phase23. Hardy-Weinberg equilibrium was tested in control samples with a threshold of 0.05 without correction for multiple testing. Chi-square association tests were performed to compare allele frequencies in 2 × 2 contingency tables. Effects on clinical subgroups of patients were explored by sample stratification. Power estimates were obtained with the power and sample size software24. We excluded from analysis of the MHC2TA SNP the samples from 2 sites (Hospital Clinico San Carlos and Hospital Universitario La Paz, total 251 patients and 101 controls) because they had been analyzed in previous reports and their inclusion here would lead to overlapping results, which are a handicap for overall assessment of results by metaanalysis or similar approaches.

RESULTS

Genotypes of the 7 SNP were obtained in 99.9% of the samples and they were in Hardy-Weinberg equilibrium. None of the 7 SNP was associated with RA (p < 0.05; Table 3). Allele frequency comparisons were also done after stratification of the samples by sex, shared epitope (SE), anti-cyclic citrullinated peptide antibodies (ACP), rheumatoid factor (RF), presence of joint erosions, and the R620W
PTPN22 SNP. Most of these analyses did not show any significant difference between the 2 RA patient groups or between each of these groups and the controls (data not shown). There was only a nominal difference in a specific comparison: the rare allele of the rs1061170 SNP in the CFH gene was less common in SE-negative patients than in the SE-positive patients (31.5% vs 37.9%, respectively; p = 0.02), but it was not different from controls (35.8%; p = 0.06) and the difference did not persist after correction for multiple testing. The corresponding genotype frequency comparisons gave similar results (Table 3). Haplotype analysis of the CFH gene, where 3 SNP were studied, showed no difference between cases and controls (data not shown). The 2 eotaxin-3 SNP were in a linkage disequilibrium so close (r^2 = 0.98) as to be almost completely redundant and the haplotypes were not more informative than the individual SNP.

**DISCUSSION**

We took a candidate gene approach to confirm or to identify RA genetic factors. The selected SNP have already been described as influential in susceptibility to RA or to other diseases that share pathological mechanisms with RA. However, none of them was associated with RA in our samples. Considering these negative results, it is important to critically assess the strength of the previous evidence suggesting a role of these SNP in RA and the power of our study.

We chose 3 SNP in CFH because they represent independent association signals to AMD^10^ However, most studies have focused on one of them, rs1061170, a nonsynonymous SNP in exon 9 of the CFH gene, resulting in the Y402H amino-acid change. CFH is necessary to prevent spontaneous activation of the alternative complement pathway in fluids or in tissue surfaces bearing polyanions^25^. We hypothesized that this deficiency could also influence RA due to multiple pieces of evidence, including complement consumption in RA synovial fluid together with increased levels of C5a and C5b-9, and a positive correlation between complement activation in synovial fluid and RA disease activity^11^ In addition, CFH is expressed and secreted by synovial fibroblasts and is present at increased levels in the synovium and synovial fluid of patients with RA^26^; the alternative activation pathway is critical in some experimental models of arthritis^27^, and CFH is encoded in a locus that has been linked to RA susceptibility^28^.

The known roles of CD209 in DC migration and function and more specifically its importance in the triggering of T cell activation suggest its possible implication in activation of RA^15,16^ Interestingly, CD209 is expressed by most RA synovial macrophages, in addition to DC, but not by osteoarthritis or control synovial macrophages or by macrophages in other tissues or in the blood of patients with RA^17^ This particular expression pattern, together with the
specific colocalization of the CD209-positive cells with ICAM-3-positive T cells in RA synovium, provides more direct evidence of a significant involvement of this receptor in RA pathology. However, CD209 involvement has not yet been investigated in in vivo or in vitro models of RA. The SNP examined here, rs4804803, is probably the causal SNP that has shown association with RA in Koreans, with allergic rhinitis, asthma, IgE concentrations, and eosinophil counts also in Koreans; it is a promoter SNP (in position –336) that affects the level of expression of CD209 and that is associated with these diseases in several ethnic groups.

The remaining SNP were associated with RA in previous studies, and our aim was to replicate these results. The rs2302009 SNP in eotaxin-3 has been found to be associated with RA in Koreans, with allergic rhinitis, asthma, IgE concentrations, and eosinophil counts also in Koreans, and with eosinophilic esophagitis in Europeans. It is located in the 3′ untranslated region of the gene, but does not have an identified functional effect. The rs6965556 SNP in the same gene is an intronic SNP that has shown association in some of the same studies, including the one addressing RA, given its relatively small size, 292 cases and 281 controls. No signal of association (p < 0.05) was found in any of the comparisons. None of the comparisons was significant at p < 0.05.

Table 3. Allele and genotype frequencies of the 7 SNP in patients with RA and controls.

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Minor Allele Frequencies*</th>
<th>Genotype Frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Patients</td>
<td>Controls</td>
</tr>
<tr>
<td>CFH</td>
<td>rs8002209</td>
<td>22.3</td>
<td>23.3</td>
</tr>
<tr>
<td></td>
<td>rs3766404</td>
<td>16.2</td>
<td>16.1</td>
</tr>
<tr>
<td></td>
<td>rs1061170</td>
<td>35.3</td>
<td>35.8</td>
</tr>
<tr>
<td>CD209</td>
<td>rs4804803</td>
<td>21.3</td>
<td>21.8</td>
</tr>
<tr>
<td>Eotaxin-3</td>
<td>rs2302009</td>
<td>23.1</td>
<td>22.1</td>
</tr>
<tr>
<td></td>
<td>rs6965556</td>
<td>23.0</td>
<td>22.0</td>
</tr>
<tr>
<td>MHC2TA</td>
<td>rs3087456</td>
<td>25.6</td>
<td>25.4</td>
</tr>
</tbody>
</table>

* Allele frequencies as percentages (number of minor alleles); genotype frequencies as percentages (number of genotypes). ** Genotype codes: 11 = homozygote for the major allele; 12 = heterozygote; 22 = homozygote for the minor allele.

Regarding the statistical power of our study, it was enough to detect an effect size with an odds ratio (OR) between 1.21 and 1.16 for SNP rs3766404 and rs1061170, respectively (for alpha = 0.05 and 1 – beta = 0.8 and allele frequencies 16.1% and 35.8%, the allele frequencies of rs3766404 and rs1061170, respectively (for alpha = 0.05 and 1 – beta = 0.8 and allele frequencies 16.1% and 35.8%, the allele frequencies of rs3766404 and rs1061170, respectively). These 2 SNP showed the lowest and the highest power. Therefore, lack of replication of the eotaxin-3 results (OR 2.99 and 2.13) was not due to insufficient power. More likely factors are ethnic differences in RA genetics, false-positive associations in the Korean study, or differences in linkage disequilibrium between the 2 populations. The first possibility is suggested by the differences between Europeans and Asians in other RA genetic factors including PADI4 and FCLR3. A false-positive in the original Korean study is also possible given its relatively small size, 292 cases and 281 controls. We consider it less likely that lack of replication could be due to differences between Koreans and Caucasians in the linkage disequilibrium and haplotype patterns because these patterns are very similar in the Chinese (the most similar population) and European HapMap samples. However, only a complete analysis covering genetic variability in the gene will exclude this possibility.

No signal of association (p < 0.05) was found in any of the blocks of linkage disequilibrium including the 7 SNP in RA. Our results reinforce this conclusion and we will not discuss them further. However, it is possible that a different RA genetic factor is present in this gene or its neighbors. This has been suggested by a haplotype study of MHC2TA, or can be hypothesized based on the association of the C-type lectin domain family 16, member A (CLEC16A) gene, which is 19.6 kb from MHC2TA, with type 1 diabetes and multiple sclerosis.

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No signal of association (p < 0.05) was found in any of the blocks of linkage disequilibrium including the 7 SNP in RA.
any of the 2 large genome-wide association studies for which complete data were available to us, and which included 5,000\textsuperscript{40} and 3,300\textsuperscript{41} subjects. This lack of association is consistent with our results and of significant importance given the size of the studies, but it cannot be taken as definitive because only 2 of the SNP from \textit{CFH}, rs800292 and rs3766404, are included in the SNP panels used in one of the studies\textsuperscript{40}. Therefore, for \textit{CFH} and \textit{CD209} no other RA association analysis is available and we cannot definitively exclude effects below OR 1.18, which corresponds to moderate-weak effects in the current context of RA genetics\textsuperscript{1}. It is still possible that other polymorphisms in \textit{CFH} or nearby sequences could have an effect on RA because the genetics of this locus is very complex, and new associations to AMD have been discovered in the same gene and in neighboring CFH-related genes\textsuperscript{9}.

In summary, our investigation of selected SNP in 4 candidate RA genes, \textit{CFH}, \textit{CD209}, \textit{eotaxin-3}, and \textit{MHC2TA}, did not show association with any of them in a large collection of Spanish samples. Our results therefore exclude a large effect in RA of the SNP in \textit{CFH} relevant for AMD and of the SNP in \textit{CD209} associated with dengue fever and tuberculosis. They confirm the lack of association with RA of the rs3087456 SNP in the MHC2TA gene, and indicate that the eotaxin-3 SNP described in Korean patients with RA are not associated with this disease in European Caucasians. However, our results do not exclude that other polymorphisms in the same genes or in neighboring sequences could be associated with RA susceptibility.

**ACKNOWLEDGMENT**

We thank Cristina Fernandez-Lopez for her excellent technical assistance.

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