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

REBECA DIEGUEZ-GONZALEZ, SERVET AKAR, MANUEL CALAZA, ISIDORO GONZALEZ-ALVARO, BENJAMIN FERNANDEZ-GUTIERREZ, JOSE RAMON LAMAS, ARTURO R. de la SERNA, RAFAEL CALIZ, FRANCISCO J. BLANCO, DORA PASCUAL-SALCEDO, MARIA LUISA VELLOSO, EVA PEREZ-PAMPIN, JOSE LUIS PABLOS, FEDERICO NAVARRO, JAVIER NARVAEZ, FRANCISCO JAVIER LOPEZ-LONGO, GABRIEL HERRERO-BEAUMONT, JUAN J. GOMEZ-REINO, and ANTONIO GONZALEZ

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.
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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[1]. 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[1]. 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[2]. 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[3,4], and of other genetic factors that seem largely specific for Asian ethnici-

From Laboratorio Investigacion 10 and Rheumatology Unit, Hospital Clinico Universitario de Santiago, Santiago de Compostela, Spain.

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R. Dieguez-Gonzalez, MSc; S. Akar, MD; M. Calaza, MSc, Laboratorio Investigacion 10 and Rheumatology Unit, Hospital Clinico Universitario de Santiago; I. Gonzalez-Alvaro, MD, Rheumatology Unit, Hospital Universitario de la Princesa, Madrid; B. Fernandez-Gutierrez, MD; J.R. Lamas, MD, Rheumatology Unit, Hospital Clinico San Carlos, Madrid; A.R. de la Serna, MD, Rheumatology Unit, Hospital Santa Creu e San Pau, Barcelona; R. Caliz, MD, Rheumatology Unit, Hospital Universitario Virgen de las Nieves, Granada; F.J. Blanco, MD, Laboratorio de Investigación Osteoarticular y del Envejecimiento, Complexo Hospitalario Universitario de A Coruña; D. Pascual-Salcedo, MD, Immunology, Hospital La Paz, Madrid; M.L. Velloso, MD, Rheumatology Unit, Hospital Universitario de Valme, Sevilla; E. Perez-Pampin, MD, Laboratorio Investigacion 10 and Rheumatology Unit, Hospital Clinico Universitario de Santiago; J.L. Pablos, MD, Rheumatology Unit, Hospital 12 de Octubre, Madrid; F. Navarro, MD, Rheumatology Unit, Hospital Universitario Virgen Macarena, Sevilla; J. Narvaez, MD, Rheumatology Unit, Hospital Universitario de Bellvitge, Barcelona; F.J. Lopez-Longo, MD, Rheumatology Unit, Hospital Universitario Gregorio Marañon, Madrid; G. Herrero-Beaumont, MD, Rheumatology Unit, Fundacion Jimenez Diaz, Madrid; J.J. Gomez-Reino, MD, Laboratorio Investigacion 10 and Rheumatology Unit, Hospital Clinico Universitario de Santiago, Department of Medicine, University of Santiago de Compostela; A. Gonzalez, MD, PhD, Laboratorio Investigacion 10 and Rheumatology Unit, Hospital Clinico Universitario de Santiago.

Address correspondence to Dr. A. Gonzalez, Laboratorio de Investigacion 10, Hospital Clinico Universitario de Santiago, Travesia de Choupana sn., 15706 Santiago de Compostela, Spain.
E-mail: Antonio.Gonzalez.Martinez.Pedrayo@sergas.es

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ty, like PADI4[5] and FCRL3[6]. 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[7] and STAT4[8] 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 RA[11]. 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 fever[12] and tuberculosis[13,14], 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 activation[15]. All of these processes are important for RA[16]. In addition, CD209 has shown a particular pattern of expression in RA[17]. 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 study[19]. 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 described[20]. The study included a total of 1587 patients and 1570 controls from tertiary-level Spanish hospitals. Patients with RA were classified according to the 1987 American College of Rheumatology criteria[21]. Clinical data for patients are provided in Table 1.

The Ethical Committee for Clinical Research of Galicia approved this study as well as the ethics committees from the recruiting centers, and all participants gave their written informed consent.

Table 1. Clinical characteristics of patients with RA. Data are percentages, except where indicated.

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

* 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 ExoSAP digestion with Exonuclease I (Epicentre, 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 3130xl Genetic Analyzer (Applied Biosystems). Primers and probes are shown in Table 2.

Statistical analysis. Analysis relied on 3 applications: Haploview[22], a customized version of Statistica 7.0 (StatSoft, Tulsa, OK, USA), and Phase2[23]. 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 software[24]. 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 meta-analysis 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 (ACPA), rheumatoid factor (RF), presence of joint erosions, and the R620W PTPN22 SNP.

Table 2. Primers and probes used for single-nucleotide polymorphism (SNP) genotyping.

Genes	SNP ID	Position	Minor Allele	Forward	Reverse	Minisequencing probe	Length
CFH	rs800292	193373890	A	TGC AAT GAA CTT CCT CCA AG	CCA TTC TCC CTT CCT GCA TA	GCA CCC AGG CTA TCT ATA AAT GCC GCC CTG GAT ATA GAT CTC TTG GAA AT	50
	rs3766404	193383489	C	TTG CAT CTC ATA GCT TTT GAC TTC	TCT TCA GTG TTT GAT GTT GAC ACT	GTA TAT GAA AAT ATG ATA GAG AAG AAT GAA GTG ACA GAA AAA AAG AAA AAA GGA ATA CAT TTA GGA CT	68
	rs1061170	194925860	C	TTG TTA TGG TCC TTA GGA AAA TGT	ACG GAT GCA TCT GGG AGT AG	GCA GGC AAC GTC TAT AGA TTT ACC CTG TAC AAA CTT TCT TCC AT	44
CD209	rs4804803	7718733	C	CAA AAA TGA GGA CAG CAG CA	CAG GGA GAA GGA CTC ATC CA	AAC TGG GGG TGC TAC CTG CC	20
Eotaxin-3	rs2302009	75043649	G	CCA GGA GGC AAA GAG TCT CA	GGG TCC ATG TAG CCT TCA GA	AAT GGG TGC AAA AAT ACA TTT CTT TAC TGA AAA CTC CGA AAC AAT TGT GAC TCA GCT GAA TT	62
	rs6965556	75237854	A	GCG GAG TTT GCA GGA GTA GA	GGG TTT CTC CAC GTT GGT	GCA GAT CAA TTA ATA CGA TAC CTG CGG GTT CTG TTC TTC GTT GAC ATG AGG TCT CCT GAG GTT GGG AGT TC	71
MHC2TA	rs3087456	10878403	G	GAA GGT TCC CCC AAC AGA CT	GCG GTC AGA TTT CTG TTT CTG	TCT GTC TTC ACC AAA TTC AGT CCA CAG TAA GGA AGT GAA ATT AAT TTC AGA GGT GT	56

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

Table 3. Allele and genotype frequencies of the 7 SNP in patients with RA and controls. None of the comparisons was significant at $p < 0.05$.

Gene	SNP	Minor Allele Frequencies*			Genotype Frequencies								
					Patients			Controls			OR (95% CI)		
		Patients	Controls	OR (95% CI)	11**	12	22	11	12	22	11 vs other	12 vs other	22 vs other
CFH	rs8002209	22.3 (692)	23.3 (722)	0.95 (0.8–1.1)	60.4 (937)	34.6 (536)	5.0 (78)	58.5 (908)	36.5 (566)	5.0 (78)	1.08 (0.9–1.25)	0.92 (0.8–1.1)	1.00 (0.7–1.4)
	rs3766404	16.2 (501)	16.1 (499)	1.01 (0.9–1.2)	70.7 (1096)	26.4 (409)	3.0 (46)	70.2 (1089)	27.5 (427)	2.3 (36)	1.02 (0.9–1.2)	0.94 (0.8–1.1)	1.29 (0.8–2.0)
	rs1061170	35.3 (1095)	35.8 (1110)	0.98 (0.9–1.1)	42.2 (655)	44.9 (697)	12.8 (199)	40.7 (631)	47.0 (728)	12.3 (191)	1.06 (0.9–1.2)	0.92 (0.8–1.1)	1.05 (0.85–1.3)
CD209	rs4804803	21.3 (660)	21.8 (677)	0.97 (0.9–1.1)	62.3 (967)	32.8 (508)	4.9 (76)	60.8 (944)	34.7 (538)	4.5 (70)	1.07 (0.9–1.2)	0.92 (0.8–1.1)	1.09 (0.8–1.5)
Eotaxin-3	rs2302009	23.1 (716)	22.1 (686)	1.06 (0.9–1.2)	59.4 (921)	35.1 (544)	5.5 (86)	60.7 (942)	34.3 (533)	5.0 (77)	0.95 (0.8–1.1)	1.03 (0.9–1.2)	1.12 (0.8–1.5)
	rs6965556	23.0 (715)	22.0 (682)	1.06 (0.9–1.2)	59.6 (924)	34.8 (539)	5.7 (88)	61.1 (947)	33.9 (526)	5.0 (78)	0.94 (0.8–1.1)	1.04 (0.9–1.2)	1.14 (0.8–1.55)
MHC2TA	rs3087456	25.6 (666)	25.4 (737)	1.01 (0.9–1.1)	56.2 (731)	36.3 (472)	7.5 (97)	56.4 (818)	36.5 (529)	7.2 (104)	0.99 (0.85–1.2)	0.99 (0.85–1.2)	1.04 (0.8–1.4)

* 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.

ICAM-3-positive T cells in RA synovium, provides more direct evidence of a significant involvement of this receptor in RA pathology[17]. 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 of the association with dengue fever and tuberculosis. 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[12-14].

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[18], with allergic rhinitis, asthma, IgE concentrations, and eosinophil counts also in Koreans[29,30]; and with eosinophilic esophagitis in Europeans[31]. 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[18,30]. No direct functional involvement of eotaxin-3 has been reported in RA, although a role in recruiting mast cells to the inflamed synovium can be hypothesized. These cells are present in limited numbers in normal human synovium, but they constitute 5% or more of all synovial cells in RA[32], and they are critical for joint inflammation in a murine arthritis model[33]. The rs3087456 SNP in the MHC2TA gene was associated with RA and with multiple sclerosis and myocardial infarction in a Swedish study[19]. This SNP was also associated with a lower level of expression of the gene[19]. However, subsequent case-control studies by different groups have been negative and a recent metaanalysis of 10 studies concluded that this SNP is not associated with RA[34]. 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*[35], 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[36] and multiple sclerosis[37,38].

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). 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)[18] 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[5] and FCLR3[6]. A false-positive in the original Korean study is also possible given its relatively small size, 292 cases and 281 controls[18]. 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[39]. 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 any of the 2 large genome-wide association studies for

which complete data were available to us, and which included 5,000[40] and 3,300[41] subjects. This lack of association is consistent with our results and of important significance given the size of the studies, but it cannot be taken as definitive because only 2 of the SNP from *CFH*, rs800292 and rs3766404, are included in the SNP panels used in one of the studies[40]. Therefore, for *CFH* and *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[1]. It is still possible that other polymorphisms in *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[9].

In summary, our investigation of selected SNP in 4 candidate RA genes, *CFH*, *CD209*, *eotaxin-3*, and *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 *CFH* relevant for AMD and of the SNP in *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.

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