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## **Genetic studies in rheumatoid arthritis**

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## **Chapter 14**

### **Association of a Haplotype in the Promoter Region of the Interferon regulatory Factor 5 Gene With Rheumatoid Arthritis**

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## **Abstract**

### ***Objective***

To determine whether genetic variants of the interferon regulatory factor 5 (IRF-5) and Tyk-2 genes are associated with rheumatoid arthritis (RA).

### ***Methods***

Five single-nucleotide polymorphisms (SNPs) in *IRF5* and 3 SNPs in *Tyk2* were analyzed in a Swedish cohort of 1,530 patients with RA and 881 controls. A replication study was performed in a Dutch cohort of 387 patients with RA and 181 controls. All patient sera were tested for the presence of autoantibodies against cyclic citrullinated peptides (anti-CCP).

### ***Results***

Four of the 5 SNPs located in the 5' region of *IRF5* were associated with RA, while no association was observed with the *Tyk2* SNPs. The minor alleles of 3 of the *IRF5* SNPs, which were in linkage disequilibrium and formed a relatively common haplotype with a frequency of ~0.33, appeared to confer protection against RA. Although these disease associations were seen in the entire patient group, they were mainly found in RA patients who were negative for anti-CCP. A suggestive association of *IRF5* SNPs with anti-CCP–negative RA was also observed in the Dutch cohort.

### ***Conclusion***

Given the fact that anti-CCP–negative RA differs from anti-CCP–positive RA with respect to genetic and environmental risk factor profiles, our results indicate that genetic variants of *IRF5* contribute to a unique disease etiology and pathogenesis in anti-CCP–negative RA.

## Introduction

The type I interferons (IFNs) comprise a large family of cytokines that are typically produced during viral infections, mainly by plasmacytoid dendritic cells (1). In addition to direct antiviral effects, type I IFNs have many important immunomodulatory functions (1). For instance, they cause maturation of dendritic cells, promote T cell activation, and stimulate B cell development and production of antibodies. Data from previous studies indicate that the type I IFN system plays a pivotal role when self tolerance is broken and autoimmune reactions develop (2). Accordingly, the type I IFN system is activated in many autoimmune diseases, including systemic lupus erythematosus (SLE) (2), primary Sjogren's syndrome (3), psoriasis (4), polymyositis (5), and type 1 diabetes mellitus (6).

In a previous report we described a strong association between SLE and 2 key genes from the type I IFN signaling system, namely the Tyk-2 and interferon regulatory factor (IRF-5) genes (7). The association of the single-nucleotide polymorphisms (SNPs) in the IRF-5 gene with SLE has recently been convincingly replicated in 4 independent case-control studies (8). A causative role of type I IFN in the initiation and maintenance of autoimmunity is suggested by the finding that up to 19% of IFN- $\alpha$ -treated patients with a malignant disease ultimately developed an autoimmune disorder (2). Development of rheumatoid arthritis (RA [MIM no. 180300]) during IFN $\alpha$  treatment has been reported (9), providing evidence that the type I IFN system may be involved in the pathogenesis of this disease.

RA is a common autoimmune disease characterized by chronic arthritis with progressive joint destruction, and if left untreated RA can lead to severe disability. RA affects women 2–3 times more frequently than it affects men, and both genetic and environmental components are involved in the etiopathogenesis of the disease (10). A majority of RA patients are positive for autoantibodies against the Fc portion of the IgG molecule (rheumatoid factor [RF]). The presence of RF in patients correlates with a severe form of RA (11), but RF can occasionally be detected in normal healthy individuals and in patients with conditions other than RA (12).

It has been shown that RA patients also produce antibodies against cyclic citrullinated peptides (anti-CCP), and that anti-CCP antibodies are rarely detectable in healthy individuals (13). They are thus a more specific disease marker for RA than is RF. Anti-CCP antibodies can be detected years before the appearance of any clinical RA symptoms (14), and they are prognostic indicators of a greater degree of inflammation and more destructive disease (15). Recent data on gene-environment interactions also show that the major environmental risk factor, smoking, is associated with anti-CCP-positive RA, but not with anti-CCP-negative RA (10,16). Furthermore, anti-CCP antibodies display a strong association with specific alleles at the HLA-DRB1 gene (10), which are collectively known as shared epitope alleles. In contrast, the HLA-DR3 allele is associated with anti-CCP-negative RA, which suggests that the syndrome called RA may consist of at least 2 distinct diseases (anti-CCP positive and anti-CCP negative), each with different risk factors and pathogenetic pathways (17). Genetic variants of several non-major histocompatibility complex (MHC) genes have also shown an association with RA (18), and among them the most convincing evidence for association has been observed for variants of the *PTPN22* (MIM no. 600716) (19), *PADI4* (MIM no. 605347) (20), and *CTLA4* (MIM no. 123890) (21,22) genes.

The *PTPN22* allele, which has shown the most consistent association with RA in previously published studies, is associated with anti-CCP–positive RA (18). So far, no genetic risk factor outside the HLA locus has been identified for anti-CCP–negative disease.

The role of the type I IFN system in RA has not been extensively investigated, but expression of IFN $\alpha$  (23) and the type I IFN–inducible protein myxovirus resistance protein A (24) has been detected in RA synovial tissue, along with increased levels of IFN $\alpha$  in synovial fluid (25). Furthermore, plasmacytoid dendritic cells are recruited to inflamed synovium (26), perhaps due to increased levels of production of chemoattractants for these cells in the synovial fluid (24). Moreover, the cytokines interleukin-6 (IL-6), IL-10, IL-12, and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), which are connected to the type I IFN pathway, have all been suggested to be involved in the disease process in RA (27).

The JAK Tyk-2 is crucial for signaling via the type I IFN receptor. Tyk-2 also interacts with the receptors for several other cytokines, such as IL-6, IL-10, and IL-12. A possible role of Tyk-2 in RA is more directly indicated by the finding that Tyk-2–deficient mice are resistant to experimental arthritis (28). IRF-5 is a transcription factor that is activated by Toll-like receptor 7 (TLR-7), TLR-8, and TLR-9, which are involved in induction of IFN $\alpha$  genes in human cells (29,30). IRF-5 is constitutively expressed in cells of the immune system, including plasmacytoid dendritic cells (31), and its expression is regulated by type I IFN (32).

Given the potential role of the type I IFN system in RA, we hypothesized that genetic variants of *Tyk2* and *IRF5* could be associated with RA, by analogy with our previous findings in SLE (7). To test this hypothesis, we genotyped 5 SNPs in the *IRF5* gene and 3 SNPs in the *Tyk2* gene in a large cohort of RA patients and controls from Sweden. We found that 4 of the 5 *IRF5* SNPs and a commonly observed haplotype formed by the rare alleles of these SNPs were strongly associated with RA, and that the evidence of association was mainly found in anti-CCP–negative RA. We replicated the association between *IRF5* and anti-CCP–negative RA in an unrelated cohort of Dutch patients.

## Patients & Methods

### *Samples*

The Swedish RA patients and controls were from the Epidemiological Investigation of Rheumatoid Arthritis (EIRA), a population-based case–control study of incident cases of RA. The EIRA included subjects ages 18–70 years who were living in the central and southern parts of Sweden during 1996–2003. Briefly, all potential cases were examined and diagnosed by a rheumatologist at a study unit. All rheumatology units linked to the public health care system, as well as almost all of the few existing privately run rheumatology units, in the relevant geographic area participated in the study. In total there were 19 reporting clinics, 15 of which were Early Arthritis Clinics. Initially, some centers reported cases that did not satisfy the criteria for RA, in order to enable investigations of undifferentiated arthritis, but these subjects were eventually excluded from the study.

For each potential case, a control subject was randomly selected from the study base, with consideration of age, sex, and area of residence. Controls were selected using the Swedish national population register, which is continuously updated. If a proposed control declined to participate, was not traceable, or was reported as having RA, a new control was selected using the same criteria. Controls selected to match cases who were eventually excluded because they did not fulfill study criteria remained in the study.

In the present study we used part of the EIRA cohort consisting of 1,530 newly diagnosed patients who fulfilled the 1987 American College of Rheumatology (formerly, the American Rheumatism Association) criteria for RA (33), and 881 controls who answered a questionnaire and provided a blood sample. Among the cases, the mean duration between estimated disease onset and study entry was 10 months. Of all participants in the EIRA study, 97% were Caucasian. Seventeen controls who were found to be positive for anti-CCP antibodies and 3 controls with unknown anti-CCP status were excluded from the analysis.

The replication cohort consisted of RA patients and controls from the Leiden Early Arthritis Clinic, as previously described in detail (34). Briefly, in 1993, an Early Arthritis Clinic was established at the Department of Rheumatology of the Leiden University Medical Center. General practitioners in an area with ~300,000 inhabitants referred patients directly to the clinic when arthritis was suspected. All patients were evaluated at the Early Arthritis Clinic within 2 weeks of referral. Patients were included in the study only if the duration of symptoms was <2 years, and the mean duration between estimated disease onset and study entry was 7 months. Diagnoses were made according to international classification criteria, as previously described (35).

Patients from the Early Arthritis Clinic who were diagnosed as having definite RA, and from whom DNA was available, were included in the present study ( $n = 387$ ). Other anti-CCP–negative and RF–negative patients from the Early Arthritis Clinic with diagnoses such as gout, pseudogout, viral or bacterial reactive arthritis, posttraumatic osteoarthritis, Lyme arthritis, or paraneoplastic arthritis made up the control group ( $n = 181$ ). Patients with undifferentiated arthritis, psoriatic arthritis, or SLE were excluded from the control group. All subjects in the case and control groups were Caucasian.

All subjects in both cohorts gave informed consent, and the local ethics committee approved the study. DNA was isolated from EDTA-treated blood by a standard desalting method.

### **Autoantibody Analysis**

Patient sera obtained from the EIRA cohort at the time of diagnosis were examined for RF by nephelometry and for anti-CCP antibodies by enzyme-linked immunosorbent assay (ELISA) (second-generation test; Euro-Diagnostica, Arnhem, The Netherlands). Antinuclear antibodies (ANAs) were measured in 194 anti-CCP–positive and 191 anti-CCP–negative patients with RA using indirect immunofluorescence on HEp-2 cells (Bio-Rad, Stockholm, Sweden) in a 1:200 screening dilution with a secondary antibody directed against the  $\gamma$ -chain of IgG (Dako, Glostrup, Denmark). All sera were interpreted blindly in parallel, by the same investigator (JR). Using this procedure, ANAs were detected in sera from 5 (5%) of 100 healthy controls. In the Early Arthritis Clinic study, serum anti-CCP antibodies were assessed with a commercial ELISA (Immunoscan RA [Mark 2]; Euro-Diagnostica). Anti-CCP antibodies were measured in serum collected within 4 months after the first visit (in 94% of the patients) or, when serum was not available within this period, in the first stored serum sample available thereafter.

### **Genotyping**

In the Swedish EIRA cohort we genotyped 4 SNPs in the promoter and first intron of the IRF-5 gene (no. rs729302, no. rs2004640, no. rs752637, and no. rs3807306) and 2 SNPs in protein-coding exons of the Tyk-2 gene (no. rs12720356 and no. rs2304256) for which we had observed strong or suggestive signals for joint linkage and association with SLE in a previous study (7). We also included in the analysis an additional SNP in the promoter region of *IRF5* (no. rs3757385) and a *Tyk2* SNP (no. rs91755) located in intron 9 between the SNPs rs2304256 in exon 8 and rs12720356 in exon 15 of *Tyk2*.

Six of the SNPs were genotyped by multiplex minisequencing (fluorescence single-base extension) with the SNPstream Genotyping System (Beckman Coulter, Fullerton, CA) (36), and 2 SNPs (rs752637 and rs2304256) were genotyped using a homogeneous single-base extension assay with fluorescence polarization detection (FP-TDI; PerkinElmer, Emeryville, CA) (37). Four SNPs in the IRF-5 gene (rs729302, rs3757385, rs2004640, and rs3807306) were genotyped for replication in samples from the Dutch Early Arthritis Clinic study, using the SNPstream System as described above. The SNPs were genotyped at the SNP technology platform in Uppsala ([www.genotyping.se](http://www.genotyping.se)). The sequences of the polymerase chain reaction and minisequencing primers used in the genotyping assays are available from the corresponding author upon request.

The mean SNP genotype call rate in the EIRA samples was 94% (range 92–97%), and the accuracy estimated from 3,700 genotype comparisons between repeated assays (18% of the genotypes) was 99.9%. In the Early Arthritis Clinic study samples, the mean call rate was 98% (range 95–99%), and the accuracy based on repeated determination of 10% of the genotypes was 100%. All SNPs exhibited Hardy-Weinberg equilibrium ( $P > 0.01$  by Fisher's exact test) in both sample sets.

### Statistical Analysis

Unadjusted 2-tailed  $P$  values calculated by Fisher's exact test were used to compare the SNP allele frequencies in the RA patient groups versus the controls and for assessing Hardy-Weinberg equilibrium of the SNP genotypes. Haploview, version 3.2. (38) was used to construct the haplotypes, to calculate pairwise  $D'$  and  $r^2$  values for linkage disequilibrium, to perform haplotype association analysis using a chi-square test, and to perform permutation tests. Odds ratios (ORs) were calculated using the formula  $[i(G1)/i(G2)]/[j(G1)/j(G2)]$ , where  $i$  and  $j$  are the allele counts in patients with RA and in controls, respectively.

Prior to performing a pooled analysis of the 2 independent cohorts, lack of heterogeneity in the populations was confirmed using the Breslow-Day test of homogeneity (39). The EasyMA 2001 free software package (39) was used for the pooled analysis, using the Mantel-Haenszel test for calculating ORs. The EIRA and Early Arthritis Clinic cohorts demonstrated  $P$  values for homogeneity  $> 0.01$  for all 4 loci tested. The frequencies of ANAs in the EIRA patient and control sera were compared using a chi-square test.

### Results

Association analysis using the genotype data for the individual SNPs from all Swedish patients and controls revealed that 4 of the 5 analyzed SNPs in the *IRF-5* gene were associated with RA ( $P < 0.05$ ), with SNP rs3807306 exhibiting the strongest association ( $P = 0.00063$ ) (Table 1). In contrast, we did not find evidence of an association between RA and any of the SNPs in the *Tyk-2* gene ( $P > 0.05$ ). We also analyzed the genotype data from RA patients stratified into subgroups according to presence or absence of RF and stratified into subgroups according to presence or absence of anti-CCP autoantibodies, compared with the data from all controls. In the RF-positive patients, only SNP rs3807306 showed evidence of association with RA, whereas in the RF-negative patients 4 of the *IRF5* SNPs showed evidence of association.

No significant differences in the minor allele frequencies of any of the SNPs were observed when RF-positive and RF-negative patients were compared (data not shown). There was a slight difference between anti-CCP-positive and anti-CCP-negative patients in the minor allele frequency of SNP rs3807306 ( $P = 0.036$ ). The most striking result of this analysis was the strong evidence of association observed for the 4 *IRF5* SNPs in the group of anti-CCP-negative patients. Despite the fact that this subgroup of patients ( $n = 590$ ) constituted only approximately one-third of all patients with RA, evidence of association for SNP rs3807306 was stronger in this subgroup ( $P = 0.000091$ ) than it was in the original analysis of all patients and controls (Table 1). This association was also evident in a codominant model ( $P = 0.0003$ ; data not shown).



When the RA patients were stratified into subgroups according to a combination of presence or absence of RF and anti-CCP antibodies, most of the evidence of association was found in the group of patients who were negative for both anti-CCP and RF. In this group, which included only approximately one fifth of all patients ( $n = 366$ ), the observed  $P$  values were 0.0035, 0.0082, and 0.0026 for SNPs rs3757385, rs2004640, and rs3807306, respectively. Stratification of the patients according to anti-CCP and RF status did not reveal any evidence of association of the 3 *Tyk2* SNPs with RA.

We did not detect any significant sex-specific differences in *Tyk2* or *IRF5* SNP allele frequencies in the RA patient or control groups (data not shown). The minor allele frequencies of the *IRF5* SNPs were, however, lower in the male patients who were negative for both anti-CCP and RF ( $n = 100$ ) than in the male controls ( $n = 250$ ), and a significantly lower minor allele frequency was noted for SNP no. rs2004640 in this subgroup compared with that in male controls (0.35 versus 0.49;  $P = 0.00091$ ). Table 1 shows the ORs for the 5 *IRF5* SNPs in the subgroups of anti-CCP-positive and anti-CCP-negative patients. The frequency of the minor, variant alleles of the associated SNPs was lower in the RA patient groups than in the controls, reducing the risk of RA to 0.7–0.8 compared with carriers of the major alleles. Thus, these alleles appeared to be protective against RA.

**Table 1.** Analysis of the association of individual SNPs in the *IRF-5* and *Tyk-2* genes with RA in all Swedish patients and controls, and in Swedish patients after stratification based on the presence or absence of RF or anti-CCP antibodies\*

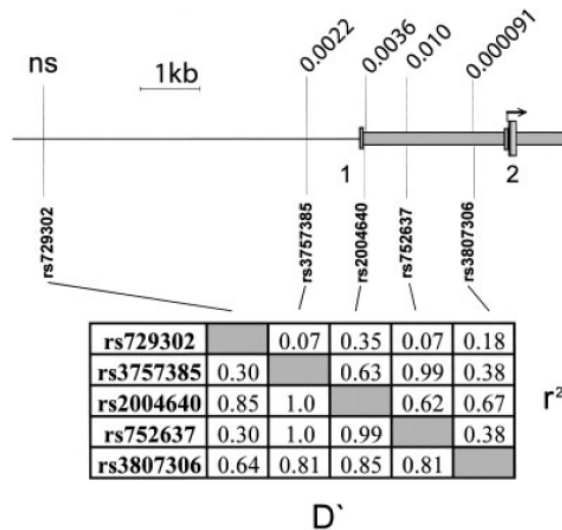
	RefSNP no., major/minor SNP allele							
	rs729302, A/C	rs3757385, C/A	rs2004640, T/G	rs752637, G/A	rs3807306, A/C	rs12720356, T/G	rs91755, G/T	rs2304256, C/A
Minor allele frequency								
Controls ( $n = 861$ )	0.31	0.36	0.48	0.37	0.50	0.09	0.48	0.30
All patients ( $n = 1,530$ )	0.32	0.31	0.44	0.33	0.45	0.09	0.47	0.29
RF+ patients ( $n = 956$ )	0.33	0.33	0.45	0.34	0.46	0.08	0.47	0.28
RF- patients ( $n = 489$ )	0.31	0.30	0.42	0.32	0.44	0.11	0.46	0.29
Anti-CCP+ patients ( $n = 919$ )	0.33	0.32	0.45	0.34	0.47	0.08	0.47	0.29
Anti-CCP- patients ( $n = 590$ )	0.31	0.30	0.43	0.32	0.43	0.11	0.47	0.28
$P$ versus controls†								
All patients ( $n = 1,530$ )	NS	0.0012	0.0067	0.007	0.00063	NS	NS	NS
RF+ patients ( $n = 956$ )	NS	NS	NS	NS	0.008	NS	NS	NS
RF- patients ( $n = 489$ )	NS	0.0032	0.0035	0.012	0.0023	NS	NS	NS
Anti-CCP+ patients ( $n = 919$ )	NS	0.013	NS	0.043	0.035	NS	NS	NS
Anti-CCP- patients ( $n = 590$ )	NS	0.0022	0.0036	0.01	0.000091	NS	NS	NS
OR (95% CI)‡								
Anti-CCP+ patients ( $n = 919$ )	1.09 (0.94–1.26)	0.83 (0.72–0.96)	0.89 (0.77–1.01)	0.86 (0.75–0.99)	0.86 (0.75–0.98)	ND	ND	ND
Anti-CCP- patients ( $n = 590$ )	1.02 (0.86–1.19)	0.77 (0.65–0.91)	0.80 (0.68–0.92)	0.81 (0.69–0.95)	0.73 (0.62–0.85)	ND	ND	ND

\* Single-nucleotide polymorphisms (SNPs) rs729302, rs3757385, rs2004640, rs752637, and rs3807306 are in the interferon regulatory factor 5 (*IRF-5*) gene; SNPs rs12720356, rs91755, and rs2304256 are in the *Tyk-2* gene. Rheumatoid factor (RF) status could not be determined in 85 patients, and anti-cyclic citrullinated peptide antibody (anti-CCP) status could not be determined in 21 patients. 95% CI = 95% confidence interval; NS = not significant; ND = not determined.

† Unadjusted 2-tailed  $P$  values comparing each patient group with controls were calculated using Fisher's exact test. The empirical  $P$  values for association with anti-CCP-negative rheumatoid arthritis (RA) (100,000 permutations obtained using Haploview software, version 3.2) were  $P = 0.024$  for SNP rs3757385,  $P = 0.042$  for SNP rs2004640, and  $P = 0.0008$  for SNP rs3807306. The  $P$  value for SNP rs752637 was not significant.

‡ Odds ratios (ORs) were calculated using the formula  $[i(G_1)/i(G_2)] / [j(G_1)/j(G_2)]$ , where  $i$  and  $j$  are allele counts in patients with RA and in controls, respectively.

Figure 1 shows the positions of the SNPs in the IRF-5 gene and the pairwise linkage disequilibrium values between the SNPs rs3757385, rs2004640, rs752637, and rs3807306 in the control samples from Sweden. Table 2 shows the frequencies of the haplotypes formed by these 4 IRF-5 SNPs in anti-CCP–negative RA patients, anti-CCP–positive RA patients, and controls. The 3 most common haplotypes, H1–H3, accounted for almost 90% of the genetic variation conferred by these SNPs in the sample set. The most common haplotype, H1, which is formed by the more frequent alleles of the SNPs, appeared to be a risk haplotype for anti-CCP–negative RA, with an OR of 1.23 (95% confidence interval [95% CI] 1.06–1.43). The frequency of the second most common haplotype, H2, which is formed by the minor alleles of the 4 SNPs, displayed the strongest evidence of association with RA ( $P = 0.00080$ ), and was associated with protection against anti-CCP–negative RA, with an OR of 0.71 (95% CI 0.60–0.84). For anti-CCP–positive RA, the OR for haplotype H2 was 0.85 (95% CI 0.74–0.98).



**Figure 1.** Location and pairwise linkage disequilibrium values of the single-nucleotide polymorphisms (SNPs) in the interferon regulatory factor 5 (IRF-5) gene. Top, Location of the SNPs in the IRF-5 promoter and first intron. P values for association with rheumatoid arthritis in Swedish patients who were negative for anti-cyclic citrullinated peptide antibodies are shown at the top. Exons 1 and 2 are indicated. NS, not significant. Bottom, Pairwise linkage disequilibrium values for the SNPs in the Swedish control samples.

**Table 2.** Analysis of the association of IRF-5 haplotypes with RA in Swedish patients\*

	Haplotype				
	H1 (CTGA)	H2 (AGAC)	H3 (CGGC)	H4 (CTGC)	H5 (AGAA)
Haplotype frequency					
Controls	0.46	0.33	0.11	0.06	0.03
Anti-CCP+ patients	0.49	0.30	0.12	0.05	0.03
Anti-CCP- patients	0.51	0.26	0.11	0.05	0.05
<i>P</i> for association					
Anti-CCP+ patients	0.088	0.026	0.51	0.65	0.33
Anti-CCP- patients	0.006	0.00069	0.73	0.99	0.049
OR (95% CI)					
Anti-CCP+ patients	1.12 (0.98–1.283)	0.85 (0.74–0.98)	1.07 (0.87–1.32)	0.93 (0.69–1.25)	0.82 (0.56–1.21)
Anti-CCP- patients	1.23 (1.06–1.43)	0.71 (0.60–0.84)	0.96 (0.75–1.21)	1.00 (0.71–1.38)	1.45 (0.99–2.09)

\* Haplotypes were formed by the 4 IRF-5 SNPs rs3757385, rs2004640, rs752637, and rs3807306. The empirical *P* value for association between the AGAC haplotype and RA in anti-CCP–negative patients (100,000 permutations obtained using Haploview software, version 3.2) was  $\leq 0.001$ . See Table 1 for definitions.

To attempt to replicate the association between the IRF-5 gene and anti-CCP–negative RA, we genotyped 4 of the IRF-5 SNPs in an unrelated cohort of Dutch RA patients (Table 3). The SNP allele frequencies were similar in the Swedish and Dutch patient and control groups, and evidence of association was observed for SNP rs2004640 in the group of anti-CCP–negative patients ( $P = 0.024$ ). Also, *P* values for SNPs rs729302 and rs3757385 ( $P = 0.072$  and  $P = 0.091$ , respectively) suggested an association in the anti-CCP–negative patients, but, possibly due to the small number of individuals in this group, the evidence of this association did not reach the formal threshold for statistical significance.

In a combined analysis of all 741 anti-CCP–negative patients in the cohorts from both populations, which were genetically homogeneous ( $P > 0.01$  by heterogeneity test), strong evidence of association of SNPs rs2004640 and rs3807306 with anti-CCP–negative RA was exhibited ( $P < 0.001$ ). Table 3 shows the ORs for the 4 IRF-5 SNPs analyzed in the Dutch cohort and the combined ORs for all anti-CCP–negative patients in both cohorts. In the Dutch samples the haplotypes formed by SNPs rs3757385, rs2004640, and rs3807306 occurred with similar frequencies as in the Swedish samples (data not shown).

**Table 3.** Analysis of association of SNPs in the IRF-5 gene with RA in all Dutch patients and controls, and in Dutch and Swedish patients after stratification based on the presence or absence of anti-CCP antibodies\*

	RefSNP no., major/minor SNP allele			
	rs729302, A/C	rs3757385, C/A	rs2004640, T/G	rs3807306, A/C
Minor allele frequency				
Dutch controls (n = 181)	0.35	0.40	0.51	0.49
All Dutch patients (n = 387)	0.30	0.36	0.45	0.48
Anti-CCP– Dutch patients (n = 151)	0.28	0.33	0.42	0.46
<i>P</i> versus controls†				
All Dutch patients (n = 387)	0.17	0.27	0.08	0.41
Anti-CCP– Dutch patients (n = 151)	0.072	0.091	0.024	0.279
Anti-CCP– Dutch and Swedish patients (n = 741)‡	0.23	0.0019	0.00089	0.00029
OR (95% CI)				
All Dutch patients (n = 387)	0.83 (0.62–1.09)	0.80 (0.66–1.13)	0.80 (0.61–1.04)	0.90 (0.69–1.17)
Anti-CCP– Dutch patients (n = 151)	0.73 (0.51–1.04)	0.76 (0.54–1.06)	0.70 (0.50–0.96)	0.84 (0.61–1.16)
Anti-CCP– Dutch and Swedish patients (n = 741)§	0.98 (0.85–1.13)	0.81 (0.70–0.92)	0.75 (0.65–0.86)	0.78 (0.68–0.89)

\* SNPs rs729302, rs3757385, rs2004640, and rs3807306 are in the IRF-5 gene. See Table 1 for definitions.

† Unadjusted 2-tailed *P* values comparing patient groups with controls were calculated using Fisher's exact test.

‡ Fisher's procedure was used for combining the *P* values for anti-CCP–negative RA in the Dutch and Swedish data sets. *P* values for the Swedish anti-CCP–negative patients are shown in Table 1.

§ EasyMA 2001 software was used for the pooled analysis, using the Mantel-Haenszel test to calculate ORs.

The strongest evidence of association with RA in the Swedish cohort was observed for the *IRF5* SNP rs3807306. This SNP is in relatively high linkage disequilibrium with SNP rs2004640 (Figure 1), which is associated with SLE, and the minor alleles of these 2 SNPs are on the same protective haplotype in both SLE and RA. To test for phenotypic similarities between SLE and anti-CCP–negative RA, we measured ANAs, which are characteristic of SLE, in a subgroup of 385 RA patients and 100 age- and sex-matched controls from the EIRA cohort. We noted an increased frequency of ANAs in the RA patient samples (78 of 385) compared with the healthy control sera (5 of 100) ( $P = 0.0005$ ), but there was no difference between anti-CCP–positive and anti-CCP–negative RA patients with respect to the frequency or staining intensity of ANAs (data not shown).

## Discussion

The data presented here provide strong evidence of an association between IRF-5 gene variants and RA, and show that the IFN pathway can be involved in this disease. Interestingly, in both the Swedish and Dutch cohorts we detected the most significant association with the *IRF5* SNPs in the subgroup of anti-CCP–negative RA patients, which constituted approximately one-third of the RA patient population. Previously, no gene, with the exception of the HLA–DR3 allele (17,40), has been found to be exclusively associated with anti-CCP–negative RA. This is particularly important because the pathogenesis of anti-CCP–negative disease is poorly understood, and specific drugs for this subset of patients with RA are lacking.

Other genes that have displayed an association with RA, such as the shared epitope alleles (17) and the *PTPN22* gene (18), are associated with anti-CCP–positive RA. Anti-CCP–positive and anti-CCP–negative RA patients have clinically similar phenotypes at diagnosis, but can differ with respect to the course of the disease, and anti-CCP–negative RA patients usually develop a milder form of the disease (41). The results of the present study further support the notion that genetic background is important in the differences observed between anti-CCP–positive and anti-CCP–negative RA.

The relevant SNPs are located in or close to the first intron of the IRF-5 gene. This region contains a CpG island, which is an indication of regulatory activity, and several promoters, including one with an IFNstimulated response element site that is activated by type I IFN and one with an IRF element site that is activated by IRF-5 and IRF-1 (42). SNP rs2004640 is located in a 5' splice donor site of an alternate exon 1, and recent data indicate that there is an allele-specific effect of this SNP on the expression pattern of different isoforms of the IRF-5 gene (8).

Because IRF-5 is an important mediator of signals from TLR-7–TLR-9 (29,43), we speculate that certain expression patterns of the IRF-5 gene can modify IRF-5–dependent induction of type I IFN, proinflammatory cytokines (e.g., IL-6, TNF $\alpha$ , IL-12, and IL-1 $\beta$ ), and several chemokines (44,45). Furthermore, the level of TLR-7 expression is increased in RA synovium and can contribute to synergistic cytokine production by dendritic cells (46). These cytokines can obviously influence the development and expression of inflammatory diseases, both at the level of the underlying autoimmune process and by promoting the inflammatory process. However, IRF-5 can also increase the expression levels of several genes coding for proteins that mediate cell growth arrest and apoptosis (45). Consequently, polymorphisms within the IRF-5 gene may affect several cellular functions of importance for RA susceptibility, besides the type I IFNs. However, understanding the possible functions of the allele variants of the IRF-5 gene on the molecular level requires further experimental studies.

The clear association of SNPs in the IRF-5 gene with anti-CCP–negative RA that we observed in the present study leads us to believe that the pathogenesis of this variant form of RA may be similar to the proposed pathogenesis of SLE that we described earlier (2). The findings of the present study suggest that future investigations of anti-CCP–negative RA should focus on the role of IFN $\alpha$ , as well as on the role of environmental agents, such as infections, that may trigger IFN $\alpha$ -related pathways. In a more general sense, the present clear demonstration of an association of an important non-MHC gene with anti-CCP–negative RA, but not with anti-CCP–positive RA, provides further evidence that appropriate phenotyping of RA patients and inclusion of sufficiently large study cohorts are fundamental prerequisites for future genetic and functional studies.

In summary, we now have evidence that in RA one set of genes and environmental triggers related to T cell activation and immune reactions against specific types of autoantigens (citrullinated proteins) is related to one subset of the disease, the anti-CCP–positive variant, whereas other sets of genes appear to be related to the other major subset of the disease, the anti-CCP–negative one. Thus, anti-CCP–positive RA and anti-CCP–negative RA should be considered as phenotypically distinct subsets of RA with respect to their genetic and environmental risk factors, as well as with respect to their molecular pathogenesis. In fact, we suggest that these subsets of RA should be regarded as 2 distinct diseases and treated as such in future studies.

**Addendum.**

After submission of this manuscript, reports of 2 studies showing no association between RA and 2 SNPs in the IRF-5 gene were published (47,48). The reasons for this seeming discrepancy could be related to the fact that the sizes of the cohorts used in the present study were larger and, perhaps more importantly, that patients in the present study were stratified according to anti-CCP antibody status.

## References

- (1) Theofilopoulos AN, Baccala R, Beutler B, Kono DH. Type I interferons ( $\alpha/\beta$ ) in immunity and autoimmunity. *Annu Rev Immunol* 2005;23:307–36.
- (2) Ronnblom L, Eloranta ML, Alm GV. The type I interferon system in systemic lupus erythematosus [review]. *Arthritis Rheum* 2006; 54:408–20.
- (3) Bave U, Nordmark G, Lovgren T, Ronnelid J, Cajander S, Eloranta ML, et al. Activation of the type I interferon system in primary Sjögren's syndrome: a possible etiopathogenic mechanism. *Arthritis Rheum* 2005;52:1185–95.
- (4) Nestle FO, Conrad C, Tun-Kyi A, Homey B, Gombert M, Boyman O, et al. Plasmacytoid predendritic cells initiate psoriasis through interferon- $\alpha$  production. *J Exp Med* 2005;202:135–43.
- (5) Greenberg SA, Pinkus JL, Pinkus GS, Bursleson T, Sanoudou D, Tawil R, et al. Interferon- $\alpha/\beta$ -mediated innate immune mechanisms in dermatomyositis. *Ann Neurol* 2005;57:664–78.
- (6) Stewart TA. Neutralizing interferon = as a therapeutic approach to autoimmune diseases. *Cytokine Growth Factor Rev* 2003;14: 139–54.
- (7) Sigurdsson S, Nordmark G, Goring HH, Lindroos K, Wiman AC, Sturfelt G, et al. Polymorphisms in the tyrosine kinase 2 and interferon regulatory factor 5 genes are associated with systemic lupus erythematosus. *Am J Hum Genet* 2005;76:528–37.
- (8) Graham RR, Kozyrev SV, Baechler EC, Reddy MV, Plenge RM, Bauer JW, et al. A common haplotype of interferon regulatory factor 5 (IRF5) regulates splicing and expression and is associated with increased risk of systemic lupus erythematosus. *Nat Genet* 2006;38:550–5.
- (9) Gota C, Calabrese L. Induction of clinical autoimmune disease by therapeutic interferon- $\gamma$ . *Autoimmunity* 2003;36:511–8.
- (10) Klareskog L, Stolt P, Lundberg K, Kallberg H, Bengtsson C, Grunewald J, et al. A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA–DR (shared epitope)–restricted immune reactions to autoantigens modified by citrullination. *Arthritis Rheum* 2006;54:38–46.
- (11) Van Zeben D, Hazes JM, Zwinderman AH, Cats A, van der Voort EA, Breedveld FC. Clinical significance of rheumatoid factors in early rheumatoid arthritis: results of a follow up study. *Ann Rheum Dis* 1992;51:1029–35.
- (12) Renaudineau Y, Jamin C, Saraux A, Youinou P. Rheumatoid factor on a daily basis. *Autoimmunity* 2005;38:11–6.
- (13) Schellekens GA, Visser H, de Jong BA, van den Hoogen FH, Hazes JM, Breedveld FC, et al. The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis Rheum* 2000;43:155–63.
- (14) Rantapaa-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, et al. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis Rheum* 2003;48:2741–9.
- (15) Ronnelid J, Wick MC, Lampa J, Lindblad S, Nordmark B, Klareskog L, et al. Longitudinal analysis of citrullinated protein/peptide antibodies (anti-CP) during 5 year follow up in early rheumatoid arthritis: anti-CP status predicts worse disease activity and greater radiological progression. *Ann Rheum Dis* 2005;64: 1744–9.

- (16) Padyukov L, Silva C, Stolt P, Alfredsson L, Klareskog L, for the Epidemiological Investigation of Rheumatoid Arthritis Study Group. A gene–environment interaction between smoking and shared epitope genes in HLA–DR provides a high risk of seropositive rheumatoid arthritis. *Arthritis Rheum* 2004;50:3085–92.
- (17) Irigoyen P, Lee AT, Wener MH, Li W, Kern M, Batliwalla F, et al. Regulation of anti–cyclic citrullinated peptide antibodies in rheumatoid arthritis: contrasting effects of HLA–DR3 and the shared epitope alleles. *Arthritis Rheum* 2005;52:3813–8.
- (18) Plenge RM, Padyukov L, Remmers EF, Purcell S, Lee AT, Karlson EW, et al. Replication of putative candidate-gene associations with rheumatoid arthritis in >4,000 samples from North America and Sweden: association of susceptibility with PTPN22, CTLA4, and PADI4. *Am J Hum Genet* 2005;77:1044–60.
- (19) Begovich AB, Carlton VE, Honigberg LA, Schrodi SJ, Chokkalingam AP, Alexander HC, et al. A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis. *Am J Hum Genet* 2004;75:330–7.
- (20) Suzuki A, Yamada R, Chang X, Tokuhiko S, Sawada T, Suzuki M, et al. Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. *Nat Genet* 2003;34:395–402.
- (21) Rodriguez MR, Nunez-Roldan A, Aguilar F, Valenzuela A, Garcia A, Gonzalez-Escribano MF. Association of the CTLA4 3' untranslated region polymorphism with the susceptibility to rheumatoid arthritis. *Hum Immunol* 2002;63:76–81.
- (22) Wesoly J, van der Helm-van Mil AH, Toes RE, Chokkalingam AP, Carlton VE, Begovich AB, et al. Association of the PTPN22 C1858T single-nucleotide polymorphism with rheumatoid arthritis phenotypes in an inception cohort. *Arthritis Rheum* 2005;52: 2948–50.
- (23) Van Holten J, Smeets TJ, Blankert P, Tak PP. Expression of interferon  $\beta$  in synovial tissue from patients with rheumatoid arthritis: comparison with patients with osteoarthritis and reactive arthritis. *Ann Rheum Dis* 2005;64:1780–2.
- (24) Lande R, Giacomini E, Serafini B, Rosicarelli B, Sebastiani GD, Minisola G, et al. Characterization and recruitment of plasmacytoid dendritic cells in synovial fluid and tissue of patients with chronic inflammatory arthritis. *J Immunol* 2004;173:2815–24.
- (25) Jongbloed SL, Lebre MC, Fraser AR, Gracie JA, Sturrock RD, Tak PP, et al. Enumeration and phenotypical analysis of distinct dendritic cell subsets in psoriatic arthritis and rheumatoid arthritis. *Arthritis Res Ther* 2006;8:R15.
- (26) Cavanagh LL, Boyce A, Smith L, Padmanabha J, Filgueira L, Pietschmann P, et al. Rheumatoid arthritis synovium contains plasmacytoid dendritic cells. *Arthritis Res Ther* 2005;7:R230–40.
- (27) Firestein GS. Immunologic mechanisms in the pathogenesis of rheumatoid arthritis. *J Clin Rheumatol* 2005;11 Suppl 3:S39–44. THE IRF-5 GENE IN RA 2209
- (28) Shaw MH, Boyartchuk V, Wong S, Karaghiosoff M, Ragimbeau J, Pellegrini S, et al. A natural mutation in the Tyk2 pseudokinase domain underlies altered susceptibility of B10.Q/J mice to infection and autoimmunity. *Proc Natl Acad Sci U S A* 2003;100: 11594–9.
- (29) Schoenemeyer A, Barnes BJ, Mancl ME, Latz E, Goutagny N, Pitha PM, et al. The interferon regulatory factor, IRF5, is a central mediator of Toll-like receptor 7 signaling. *J Biol Chem* 2005;280: 17005–12.
- (30) Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, Mizutani T, et al. Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature* 2005;434:243–9.



- (31) Izaguirre A, Barnes BJ, Amrute S, Yeow WS, Megjugorac N, Dai J, et al. Comparative analysis of IRF and IFN- $\alpha$  expression in human plasmacytoid and monocyte-derived dendritic cells. *J Leukoc Biol* 2003;74:1125–38.
- (32) Barnes BJ, Moore PA, Pitha PM. Virus-specific activation of a novel interferon regulatory factor, IRF-5, results in the induction of distinct interferon  $\alpha$  genes. *J Biol Chem* 2001;276:23382–90.
- (33) Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;31:315–24.
- (34) Van Aken J, van Bilsen JH, Allaart CF, Huizinga TW, Breedveld FC. The Leiden Early Arthritis Clinic. *Clin Exp Rheumatol* 2003;21(5 Suppl 31):S100–5.
- (35) Van der Horst-Bruinsma IE, Speyer I, Visser H, Breedveld FC, Hazes JM. Diagnosis and course of early-onset arthritis: results of a special early arthritis clinic compared to routine patient care. *Br J Rheumatol* 1998;37:1084–8.
- (36) Bell PA, Chaturvedi S, Gelfand CA, Huang CY, Kochersperger M, Kopla R, et al. SNPstream UHT: ultra-high throughput SNP genotyping for pharmacogenomics and drug discovery [published erratum appears in *Biotechniques* 2003;34:496]. *Biotechniques* 2002;Suppl:70–2, 74, 76–7.
- (37) Hsu TM, Chen X, Duan S, Miller RD, Kwok PY. Universal SNP genotyping assay with fluorescence polarization detection. *Biotechniques* 2001;31:560–8.
- (38) Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005;21: 263–5.
- (39) Cucherat M, Boissel JP, Leizorovicz A, Haugh MC. EasyMA: a program for the meta-analysis of clinical trials. *Comput Methods Programs Biomed* 1997;53:187–90.
- (40) Verpoort KN, van Gaalen FA, van der Helm-van Mil AH, Schreuder GM, Breedveld FC, Huizinga TW, et al. Association of HLA-DR3 with anti-cyclic citrullinated peptide antibody-negative rheumatoid arthritis. *Arthritis Rheum* 2005;52:3058–62.
- (41) Van der Helm-van Mil AH, Verpoort KN, Breedveld FC, Toes RE, Huizinga TW. Antibodies to citrullinated proteins and differences in clinical progression of rheumatoid arthritis. *Arthritis Res Ther* 2005;7:R949–58.
- (42) Mancl ME, Hu G, Sangster-Guity N, Olshalsky SL, Hoops K, Fitzgerald-Bocarsly P, et al. Two discrete promoters regulate the alternatively spliced human interferon regulatory factor-5 isoforms: multiple isoforms with distinct cell type-specific expression, localization, regulation, and function. *J Biol Chem* 2005;280:21078–90.
- (43) Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006;124:783–801.
- (44) Barnes BJ, Kellum MJ, Field AE, Pitha PM. Multiple regulatory domains of IRF-5 control activation, cellular localization, and induction of chemokines that mediate recruitment of T lymphocytes. *Mol Cell Biol* 2002;22:5721–40.
- (45) Barnes BJ, Richards J, Mancl M, Hanash S, Beretta L, Pitha PM. Global and distinct targets of IRF-5 and IRF-7 during innate response to viral infection. *J Biol Chem* 2004;279:45194–207.
- (46) Roelofs MF, Joosten LA, Abdollahi-Roodsaz S, van Lieshout AW, Sprong T, van den Hoogen FH, et al. The expression of Toll-like receptors 3 and 7 in rheumatoid arthritis synovium is increased and costimulation of Toll-like receptors 3, 4, and 7/8 results in synergistic cytokine production by dendritic cells. *Arthritis Rheum* 2005;52:2313–22. 47. Rueda B, Reddy MV, Gonzalez-Gay MA, Balsa A, Pascual-Salcedo D, Petersson IF, et al. Analysis of IRF5 gene functional polymorphisms in rheumatoid arthritis. *Arthritis Rheum* 2006;54: 3815–9.

- (47) Garnier S, Dieude P, Michou L, Barbet S, Tan A, Lasbleiz S, et al. The systemic lupus erythematosus new genetic factor IRF5 rs2004640-T allele is not linked to, nor associated with rheumatoid arthritis, in a family-based study from the French Caucasian population. *Ann Rheum Dis* 2006. E-pub ahead of print.

