Genetics, autoantibodies and clinical features in understanding and predicting rheumatoid arthritis

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Chapter 7

Refining the complex rheumatoid arthritis phenotype based on specificity of the HLA-DRB1 shared epitope for antibodies to citrullinated proteins

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ABSTRACT

Objective. The main genetic risk factor for rheumatoid arthritis (RA), the HLA region, has been known for 25 years. Previous research has demonstrated, within the RA population, an association between HLA-DRB1 alleles carrying the shared epitope (SE) and antibodies directed against cyclic citrullinated peptides (anti-CCP antibodies). We undertook this study to make the first comparison of SE allele frequencies in the healthy population with those in RA patients who do or do not harbor anti-CCP antibodies.

Methods. HLA-DRB1 typing was performed in 408 RA patients from the Leiden Early Arthritis Clinic (the Leiden EAC; a Dutch population-based inception cohort in which disease course was followed up over time), in 423 healthy Dutch controls, and in 720 affected members of 341 US multiplex (sibpair) families of Caucasian origin from the North American RA Consortium (NARAC) with well-established disease and fulfilling the American College of Rheumatology classification criteria for RA. The presence of anti-CCP antibodies was determined by enzyme-linked immunosorbent assay.

Results. For the Leiden EAC, the odds ratio (OR) describing the association of 2 copies of the SE allele with anti-CCP positivity (using no copies of the SE allele in the healthy control group as the referent) was 11.8 ($P < 0.0001$), while the OR for 1 SE allele was 4.4 ($P < 0.0001$). No association with the SE was observed in the Dutch anti-CCP-negative RA patients. For the NARAC families, linkage and association analysis revealed the SE to be associated only with anti-CCP-positive disease and not with anti-CCP-negative disease. Stratified analyses indicated that anti-CCP antibodies primarily mediated association of the SE with joint damage or disease persistence.

Conclusion. HLA-DRB1 alleles encoding the SE are specific for disease characterized by antibodies to citrullinated peptides, indicating that these alleles do not associate with RA as such, but rather with a particular phenotype.

INTRODUCTION

A definition of the rheumatoid arthritis (RA) phenotype has been developed through consensus procedures. The current RA classification criteria were developed by expert clinicians who examined the features of "classic" RA cases and analyzed their sensitivity and specificity using clinical judgment as the gold standard. As is the case with most complex disorders defined by consensus criteria, there is large variation in the phenotype. Despite the phenotypic heterogeneity encompassed by this disease definition, the genetic contribution is estimated to be 50-60% (1), with the HLA region having the largest impact on genetic risk. In particular, HLA-DRB1 alleles encoding a common amino acid sequence (the shared epitope SE) in the third hypervariable region of the DRB1 molecule have been identified as risk alleles for RA (2). The functional significance implied by the location of this SE sequence along the rim of the peptide-binding groove has stimulated efforts to search for the putative RA antigen.

A considerable proportion of RA patients have autoantibody responses, especially rheumatoid factor (RF). More recently, another autoantibody response has received much attention. These antibodies are directed against cyclic citrullinated peptides (CCPs) and are highly specific for RA (3,4). Intriguingly, these antibodies may be detected years before disease onset (5,6), are stable over time (7), and are associated with joint destruction, the hallmark of RA (8,9).

The immune response to citrullinated antigens may occur in the context of the SE, because conversion of arginine to citrulline at the peptide side-chain position interacting with the SE significantly increases peptide-major histocompatibility complex affinity and leads to the activation of CD4-positive T cells in mice transgenic for one of the SE alleles (10). Previous research has demonstrated, within the RA population, an association between HLA alleles and anti-CCP antibodies, but no comparison has been made between the HLA profile in the healthy population and that in RA patients who do or do not produce anti-CCP antibodies (8,9,11). Such a comparison may be important, since different genetic contributions associating with different phenotypes point to different etiopathologies.

PATIENTS AND METHODS

Patients

Four hundred eight Dutch RA patients were participants in the Leiden Early Arthritis Clinic (EAC), a population-based inception cohort described previously in detail (12). All RA patients fulfilled the 1987 revised criteria of the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) (13) and were of Caucasian

origin. The study protocol included collection of serologic, radiographic, and clinical data upon study entry and yearly thereafter. Informed patient consent was obtained, and the local medical ethics committee approved the study. Radiographs of the hands and feet were obtained for all EAC patients at baseline and at each subsequent annual visit and were scored for damage using the modified Sharp/van der Heijde method (14). Controls consisted of a random panel of 423 healthy unrelated Dutch individuals.

Patients with RA who had clinical remission of disease were defined as follows: at cohort entry they had 1) symmetric arthritis of the small joints, 2) morning stiffness of at least 1 hour duration, 3) a diagnosis of definite or probable RA according to the ACR criteria, and 4) they had achieved long-term and complete remission of disease (no signs of arthritis in the absence of disease-modifying drugs). Patients were not included in the remission group when their disease had been in remission for <1 year without the use of disease-modifying drugs. All other patients were classified as having persistent RA.

The US sample consisted of multiplex (sibpair) families of Caucasian origin from the North American RA Consortium (NARAC) with well-established disease and fulfilling the ACR classification criteria, as described previously (15). Radiographs of the hands and feet of all affected individuals were obtained at the time of study entry, unless films obtained within 2 years prior to entry were available for review. To document the presence of erosive disease, all radiographs were read by a single radiologist who was blinded to the patients' clinical and genetic information.

Serology and genotyping

Anti-CCP titers were determined based on a second-generation enzyme-linked immunosorbent assay (ELISA) (either the Immunoscan RA Mark 2 [Euro-Diagnostica, Arnhem, The Netherlands], in the case of the Leiden EAC, or an ELISA manufactured by Inova Diagnostics [San Diego, CA], in the case of the NARAC). HLA-DRB1 typing and subtyping were performed using polymerase chain reaction-based methods. The following alleles were classified as SE positive: DRB1*0101, *0102, *0104, *0401, *0404, *0405, *0408, *0413, *0416, *1001, and *1402 (2). Genotyping of NARAC families for 27 microsatellite markers on chromosome 6 was performed using markers from the Marshfield set8A combo list (available online at http://www.marshfieldclinic.org/research/genetics/sets/combo.html) with additional markers in the HLA complex, as described previously (15).

Statistical analysis

Nonparametric linkage analysis was performed using the Multipoint Engine for Rapid Likelihood INference (MERLIN) statistical package (16). Analysis of the anti-CCP-positive families (575 affected members in 271 families comprising 317 sibpairs) was restricted to anti-CCP-positive siblings (i.e., anti-CCP-negative siblings were excluded). Analysis of the anti-CCP-negative families (145 affected members in 70 families comprising 75 sibpairs)

was restricted to anti-CCP-negative patients and their siblings with negative or low titers of anti-CCP antibodies (<49), provided that at least 1 sibling in the family had anti-CCP-negative disease. Chi-square tests and odds ratios (ORs) were used to assess the statistical significance and magnitude of associations for categorical outcomes.

With regard to the issue of multiple testing, statistical significance levels accounted for the fact that we examined the following 4 hypotheses in this study: 1) whether the SE and anti-CCP antibodies are associated, 2) whether the SE and anti-CCP antibodies are associated differently in RA patients and in controls, 3) whether the SE and anti-CCP antibodies are associated with remission, and 4) whether the SE and anti-CCP antibodies are associated with joint destruction. Although one may argue that these 4 hypotheses are probably not completely independent based on previous data, we considered results to be statistically significant if the *P* values were less than or equal to 0.01.

RESULTS

As shown in Table 1, the presence of the SE allele was strongly associated with anti-CCP positivity. There was also evidence of a dose effect, with increasing copies of the SE allele being associated with increasing risk for RA. For the Leiden EAC, the OR describing the association of 2 copies of the SE allele with anti-CCP positivity (using no copies of the SE allele in the healthy control group as the referent) was 11.8 ($P < 0.0001$), while the OR for 1 SE allele was 4.4 ($P < 0.0001$). When no copies of the SE allele in the EAC was used as the referent, the OR describing the association of 2 copies of the SE allele with anti-CCP positivity was 8.6 ($P < 0.0001$), while the OR for 1 SE allele was 3.4 ($P < 0.0001$). Interestingly, however, no association with the SE was observed in the Dutch anti-CCP-negative RA patients (for 2 copies of the SE allele versus healthy Dutch controls, OR 1.4, $P = 0.3$; for 1 copy of the SE allele versus healthy Dutch controls, OR 1.3, $P = 0.2$), indicating that the SE does not associate with RA as such, but rather with a defined anti-CCP phenotype.

Table 1. Distribution of SE and anti-CCP positivity*

		Dutch EAC RA patients			
		Anti-CCP positive (n=195)		Anti-CCP negative (n=213)	
SE	Dutch controls (n=423), no (%)	No (%)	OR (95% CI)	No (%)	OR (95% CI)
+/+	26 (6)	49 (25)	11.79 (6.58-21.13)	16 (8)	1.38 (0.71-2.67)
+/–	153 (36)	107 (55)	4.37 (2.88-6.65)	88 (41)	1.29 (0.91-1.82)
–/–	244 (58)	39 (20)	1.0	109 (51)	1.0

***** The following alleles were classified as shared epitope (SE) positive: DRB1*0101, *0102, *0104, *0401, *0404, *0405, *0408, *0413, *0416, *1001, and *1402 (4). EAC = Early Arthritis Clinic; RA = rheumatoid arthritis; CCP = cyclic citrullinated peptide; OR = odds ratio; 95% CI = 95% confidence interval.

Other autoantibodies such as IgM RF have been reported to be associated with the SE as well. However, a comparison of the distribution of copies of the SE allele among RA patients who were RF negative and anti-CCP positive (10 SE+/+ patients, 21 SE+/- patients, 4 SE-/- patients) with the distribution among those who were RF positive and anti-CCP negative (4 SE+/+ patients, 11 SE+/- patients, 22 SE-/- patients) yielded a strikingly significant difference ($P < 0.0001$). Thus, the SE appears to be associated primarily with anti-CCP autoantibodies, but not with RF autoantibodies.

Analysis of NARAC families revealed a strong association between the SE and anti-CCP positivity. Specifically, the OR describing the association of 2 copies of the SE allele with anti-CCP positivity was 7.7 ($P < 0.0001$), and the OR for 1 SE allele was 3.5 ($P < 0.0001$), both relative to NARAC patients with no copies of the SE allele.

Results of nonparametric linkage analysis in NARAC families highlighted the strong relationship between the HLA region and anti-CCP positivity. Figure 1 shows the linkage analysis for 27 microsatellite markers on chromosome 6 among 720 affected members of 341 families. Among the 271 anti-CCP-positive families, the maximum logarithm of odds (LOD) score in the region was 10.63, in contrast to the maximum LOD score of 1.12 among the 70 anti-CCP-negative families. Anti-CCP-negative families were defined by anti-CCP-negative patients and their siblings with negative or low titers of anti-CCP antibodies (<49), provided that at least 1 sibling in the family had anti-CCP-negative disease. Therefore, sensitivity analyses employing stricter definitions of anti-CCP-negative families (e.g., all affected individuals within a family must be anti-CCP negative) were performed, and these revealed a similar pattern (data not shown). The level of 49 was chosen due to the fact that titers in the range of 25-49 are considered intermediate based on analyses of sensitivity versus specificity (3,17).

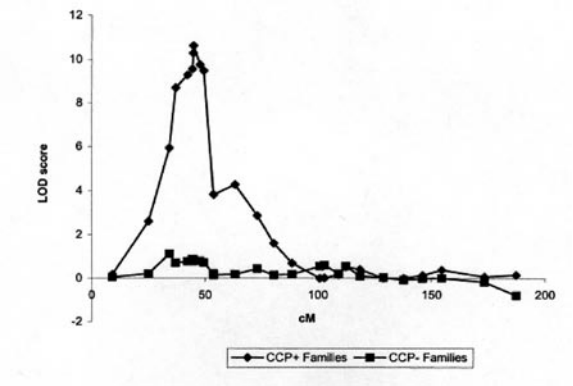

Figure 1. Results of nonparametric linkage analysis of chromosome 6 in anti-CCP-positive and anti-CCP-negative Caucasian multiplex families with well-established rheumatoid arthritis. The 2 curves correspond to anti-CCP-positive families (575 affected members of 271 families) and anti-CCP-negative families (145 affected members of 70 families). LOD = logarithm of odds.

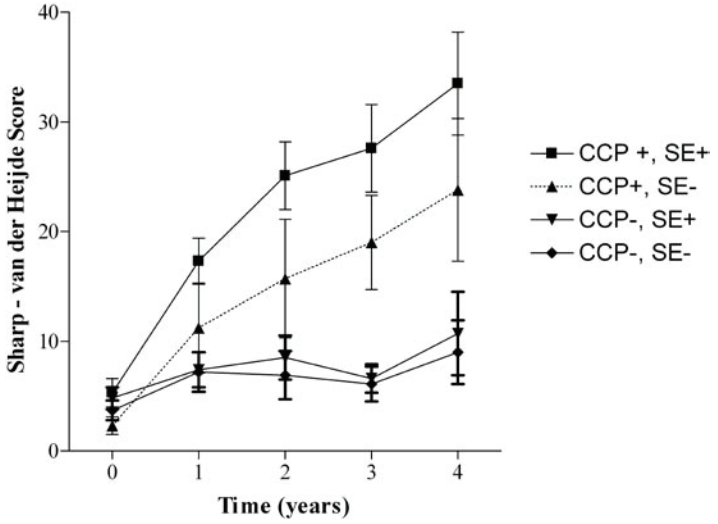

Figure 2. Progression of joint damage (in Sharp/van der Heijde units) according to the presence of the SE and anti-CCP antibodies. 74 patients were anti-CCP positive and SE positive, 18 were anti-CCP positive and SE negative, 30 were anti-CCP negative and SE positive, and 34 were anti-CCP negative and SE negative. Radiographs of the hands and feet were obtained at baseline and at each subsequent annual visit and were scored for damage using the modified Sharp/van der Heijde method. Shown are mean ± SEM.

The prospective design of the EAC cohort allowed us to examine the relative contributions of anti-CCP status and the SE to the rate of joint destruction (Figure 2). Large differences were observed between anti-CCP-positive and anti-CCP-negative patients. No apparent association was observed between SE positivity and progression of joint damage in anti-CCP-negative patients. In contrast, radiographic severity scores were higher among anti-CCP-positive patients who were SE positive than among those who were SE negative. Similar results were observed for the second hallmark of RA, chronic inflammation. The presence of anti-CCP antibodies yielded an OR of 9.5 ($P < 0.0001$) for persistent disease compared with RA patients who had clinical remission of their disease. In stratified analyses of the NARAC population, the number of copies of the SE allele was associated with the presence of erosive disease among patients with anti-CCP-positive disease ($P < 0.02$), but not among those with anti-CCP-negative disease.

DISCUSSION

Defining the phenotype for multifactorial diseases often occurs through consensus procedures. The current RA classification criteria were developed by expert clinicians who examined the features of "classic" RA cases and analyzed their sensitivity and specificity using clinical judgment as the gold standard. It is anticipated that phenotype definitions

based on specific biologic characteristics such as anti-CCP antibody production will facilitate the search for genetic risk factors, leading to greater understanding of underlying disease mechanisms.

It has been previously reported that the SE correlates strongly with another autoantibody, RF (18). Given the fact that RF is less specific for RA than anti-CCP, it is not surprising that the SE was more strongly associated with anti-CCP than with RF. Indeed, comparison of the distribution of copies of the SE allele among RF-negative, anti-CCP-positive patients with the distribution of copies of the SE allele among RF-positive, anti-CCP-negative patients revealed a much stronger association of the SE with anti-CCP antibodies. Although anti-CCP autoantibody titers were not available for the healthy controls in the current study, many previous studies have documented the absence of anti-CCP antibody production among healthy individuals (3-8,9). Further, previous research indicates that a double dose of the SE allele is relatively rare among controls. Finally, even among RA patients with a double dose of the SE allele, approximately one-third was anti-CCP negative. Thus, it is highly unlikely that anti-CCP antibodies are present in healthy controls.

Another intriguing observation is that the proportion of anti-CCP-positive RA patients is clearly less in inception cohorts with incident cases of arthritis than in groups of RA patients who have been selected for chronic, well-established disease (2,5,8), as was true for NARAC patients. Our analysis demonstrated differences in disease course for anti-CCP-positive disease compared with anti-CCP-negative disease. The strong tendency to achieve longstanding remission in anti-CCP-negative disease is compatible with the fact that cohorts of patients with longstanding disease are selected for anti-CCP positivity. The phenotypic data on joint destruction are compatible with the hypothesis that the presence of the SE is not only a risk factor for anti-CCP-positive disease but is also associated with more destructive disease. Although the current study had insufficient statistical power to examine associations of the SE with the rate of joint destruction among subgroups defined according to anti-CCP antibody production, this was due in part to the low rate of joint destruction in anti-CCP-negative patients. Thus, additional studies will be required to more definitively address this question.

Prior to this study, we defined 4 hypotheses to be tested and conservatively considered them as independent tests, and we therefore used a threshold for statistical significance of $P \leq 0.01$. Although one may argue that this threshold is too conservative, this decision did not alter the main findings in this study, since most of the results were associated with P values that were either much smaller than 0.01 or much larger than 0.05.

A strength of the current study was the demonstration of an association between the SE and anti-CCP-positive RA in 2 independent cohorts and using 2 analytic methods, association and linkage. Moreover, a recent study of juvenile RA demonstrated that anti-CCP-positive patients were 5 times more likely to be DR4 positive compared with anti-CCP-negative patients. Intriguingly, a strong association was observed between HLA-DR4

and the presence of anti-CCP antibodies in healthy children as well (4 of 688 children tested positive for anti-CCP antibodies, and all 4 children carried the DR4 allele) (19). Unfortunately, HLA typing in that study did not allow for determination of SE status, but those data also support the theory that a better fit in the SE of citrullinated antigens can give rise to the anti-CCP antibody response.

In summary, we propose that refinement of the RA phenotype (as defined by the ACR criteria) to "disease characterized by the presence of antibodies to citrullinated peptides" will facilitate the search for the mechanisms underlying the pathophysiology of the disease. Indeed, despite the fact that the most prominent genetic risk factor for RA has been known for 25 years, our current data illustrate that the SE is not associated with disease in a substantial proportion of RA patients, but rather that it is associated with disease in RA patients who have anti-CCP antibodies. Thus, our data suggest the presence of distinct pathways underlying disease induction/progression in anti-CCP-positive and anti-CCP-negative RA, since these 2 phenotypes exhibit different genetic associations.

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