

A Functional Haplotype of PADI4 Gene in Rheumatoid Arthritis: Positive Correlation in a French Population

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ABSTRACT. Objective. A functional haplotype of peptidyl arginine deiminase 4 (PADI4) was associated with susceptibility to rheumatoid arthritis (RA) in Asian populations, but the results are contradictory in Europeans. We investigated (1) the association of 2 single-nucleotide polymorphisms (SNP) located in exon 2 of PADI4 with RA in another Caucasian population; and (2) the association between PADI4 and anti-citrullinated protein (anti-CCP) antibodies.

Methods. DNA samples were obtained from 405 French RA patients and 275 controls. All RA patients met the revised criteria of the American College of Rheumatology. PADI4_89 163(G→A) and PADI4_90 245(T→C) SNP were genotyped using a PCR-RFLP method confirmed by direct sequencing. All patients and controls were genotyped for HLA-DRB1. The presence of anti-CCP antibodies was tested in 243 RA patients using an ELISA technique.

Results. We focused on PADI4_89 163(G→A) and PADI4_90 245(T→C) SNP that distinguished 2 main haplotypes: AC haplotype (PADI4_89*A PADI4_90*C) and GT haplotype (PADI4_89*G PADI4_90*T), described, respectively, as “nonsusceptible” and “susceptible.” A positive association between RA and presence of the GT haplotype was found in the heterozygous state ($p = 0.002$) and the homozygous state (RA patients 22%, controls 13%; $p = 0.005$). A correlation was observed between the presence but not the level of anti-CCP antibodies and the GT heterozygous ($p = 0.03$) and homozygous ($p = 0.05$) haplotypes. No correlation was found between the HLA-DRB1 shared epitope and any of the PADI4 haplotypes.

Conclusion. Our findings confirm those of Japanese, Korean, and Canadian studies and suggest that PADI4 may be a new susceptibility gene independent of HLA-DRB1 for RA in Caucasian populations. (First Release April 1 2009; J Rheumatol 2009;36:881–6; doi:10.3899/jrheum.080398)

Key Indexing Terms:

PADI4
ANTI-CITRULLINATED PROTEIN ANTIBODY

RHEUMATOID ARTHRITIS
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Rheumatoid arthritis (RA) is the most frequently occurring inflammatory rheumatic disease, affecting about 0.5% of the population. It is a chronic inflammatory disease of the synovial membrane, leading to progressive joint destruction with systemic manifestations. Although the pathogenic mechanisms of RA are not fully understood, it is clear that the concomitance of several factors (environmental, genetic, immunological, and hormonal¹⁻³) is necessary to initiate the disease. Twin and family-based studies have shown that the genetic component of RA may account for up to 60%⁴ of

disease susceptibility. To date, HLA-DRB1 remains the major genetic component in RA⁵⁻⁷. Strong susceptibility is conferred by certain HLA-DR alleles [*0101, *0102 (DR1), *0401, *0404, *0405, *0408 (DR4), *1402 (DR6), and *1001 (DR10)] that share a specific epitope (shared-epitope alleles). However, since the effect of this particular genetic component accounts for only one-third of the overall influence of genetic factors^{7,8}, studies have sought to identify other genes that could account for susceptibility to RA^{9,10}.

Genome screening in different ethnic groups has revealed a candidate region in the CHR1p36 D1S228 locus. This region contains clusters of enzymes — peptidyl arginine deiminases (PADI) — that are functionally associated with the production of RA-specific antibodies. PADI genes encode enzymes that induce the citrullination of peptides in a process involving the post-translational conversion of the latter (i.e., the replacement of an arginine residue by a citrulline residue^{11,12}). Anti-cyclic citrullinated peptide (anti-CCP) antibodies are very specific to RA and appear very early in the course of the disease¹³⁻¹⁵. This suggests that they may play a specific role in the pathogenesis of RA¹⁶⁻¹⁸.

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Recent studies have reported the simultaneous presence of citrullinated peptides, PADI2 or 4 enzymes, and anti-CCP antibodies in RA joints¹⁹⁻²¹. Japanese and Korean studies have reported the existence of an association between a functional haplotype of PADI4 and RA^{10,22}. This haplotype was also associated with the presence of antibodies against citrullinated filaggrin¹⁰. This association between PADI4 and risk of RA was confirmed in a genome-wide single-nucleotide polymorphism (SNP) analysis²³ and a recent metaanalysis in an East Asian population²⁴. Despite the strong associations reported in the Japanese and Korean studies, these results are still controversial in Caucasian populations. While this association between PADI4 and RA was confirmed in German and Canadian populations^{25,26}, British, Spanish, Hungarian, and French studies have not confirmed this link²⁷⁻³⁰. Moreover, genome-wide association studies in the USA³¹, Spain³², the UK^{33,34}, and other Caucasian populations³⁵ found no significant link between PADI4 and the risk of RA.

The aim of our study was to validate, in another Caucasian population, the previously observed associations between PADI4 and RA and PADI4 and anti-CCP antibodies.

MATERIALS AND METHODS

Patients. Patients who had been diagnosed with RA according to the American College of Rheumatology (American Rheumatism Association) criteria³⁶ were identified by record review. Demographic data included age, sex, rheumatoid factor (RF) status, anti-CCP antibodies detected by ELISA, and presence of erosions on radiographs. PADI4 genotyping was performed on 405 patients; 243 patients were tested for anti-CCP antibodies. Radiograph reports were available for 295 patients. The controls were drawn from a DNA bank created in our biological laboratory in 1993. All controls were Caucasian; none had any symptom of an inflammatory rheumatological disorder. Of the controls, 275 were genotyped for PADI, while 309 were genotyped for HLA. All subjects provided their informed consent.

PADI4 genotyping and selection of the SNP. Suzuki, *et al*¹⁰ have described 4 main SNP distributed in exon 2, exon 3, and exon 4 of the PADI4 gene: SNP 89 (A→G) rs11203366, SNP 90 (C→T) rs11203367, SNP 96 (C→T) rs874881, and SNP 102 (T→C) rs2240337. These SNP defined 4 haplotypes: haplotype 1 (PADI4_89*A 90*C 96*T 102*C); haplotype 2 (PADI4_89*G 90*T 96*C 102*C); haplotype 3 (PADI4_89*G 90*T 96*C 102*T); and haplotype 4 (PADI4_89*G 90*T 96*T 102*C). Haplotypes 1 and 2 were the main haplotypes in the general population (85%) and were associated with RA susceptibility (haplotype 2) and protection (haplotype 1).

Thus, we decided to restrict the genotyping to the 2 main PADI4 exon 2 SNP, PADI4_89 163(G→A) and PADI4_90 245(T→C), to enable us to define the protective haplotype 1 (PADI4_89*A PADI4_90*C) and susceptible haplotype 2 (PADI4_89*G PADI4_90*T). PADI4 exon 2 was amplified by polymerase chain reaction (PCR) assay using 2 primers, forward 5'-CAT CCT CTG CTT TCC CAT GT-3' and reverse 5'-CCA CAC ACA CTC TGT CCT GC-3'. Thermal cycling was performed at 59°C with 1 unit of TAQ polymerase (Invitrogen). SNP detection was performed using the restriction enzymes HaeIII and FnuHI (Biolab), which were selected by computer models to distinguish the haplotype 1 (PADI4_89*A PADI4_90*C), denoted AC, and haplotype 2/3/4 (PADI4_89*G PADI4_90*T), denoted GT. The nucleotide sequence of the allelic variants defined by PCR-restriction fragment-length polymorphism (RFLP) was confirmed by direct sequencing for a few samples. Fluorescence-based

automated cycle sequencing of the PCR products was performed using an ABI DNA sequencer (PE Applied Biosystems).

HLA-DRB1 genotyping was performed at the allelic level using PCR-RFLP as described and interpreted with the usual nomenclature^{37,38}.

Anti-CCP antibodies. Anti-CCP antibodies (Inova, San Diego, CA, USA) were measured using commercial ELISA tests (Diastat kit; Axis-Shield, Dundee, UK) and following manufacturers' instructions. A cutoff of 25 IU/ml and limit of linearity of 1600 IU/ml were selected.

Statistical analysis. Associations between RA and PADI4 haplotypes or genotype were assessed by chi-square test using Statview software. Statistical significance was set at 5%; p value was corrected by the number of comparisons. Patients were then subgrouped according to the presence of anti-CCP antibodies and HLA-DRB1 status. Comparisons of the serum anti-CCP levels and PADI4 genotypes were assessed by the Kruskal-Wallis test, a nonparametric method for testing equality of population medians among groups.

RESULTS

Patient characteristics. PADI4 genotyping was performed on 405 RA patients who had been seen in a university hospital rheumatology department. The characteristics of these patients are shown in Table 1. Mean age was 57.64 years (range 20–91). Most patients were female (308 women, 76%). Seventy-four percent of the patients tested positive for RF and 70% tested positive for anti-CCP. Information on radiological status was available for 295 patients, and 207 (70%) of them had erosions.

PADI4 genotyping was performed on 275 controls (mean age 35.4 yrs). Most controls were female (185 women, 67%) and all were Caucasian.

PADI4 haplotypes and genotypes in patients and controls. PADI4_89 163(G→A) and PADI4_90 245(T→C) were assessed using PCR-RFLP, defining 2 main haplotypes, the AC haplotype (PADI4_89*A PADI4_90*C) and the GT haplotype (PADI4_89*G PADI4_90*T). For both patients and controls, repartition of the genotypes from the most frequent to the least frequent were AC/GT (51% for both), AC/AC (27% vs 36%), and GT/GT (22% vs 13%); distribution of the 3 genotypes was significantly different in the 2 populations (chi-square 8.58, $p_{\text{corr}} = 0.000^{**}$; Table 2). This difference was explained by an increase of the GT/GT genotype in RA patients (OR 1.79, $p = 0.006$) and a decrease of the AC/AC genotype (OR 0.7, $p = 0.03$). An association between the GT haplotype and RA was also observed ($p = 0.02$). The effect of the homozygous state (GT/GT) accounted for a large part of this result. RA patients and controls

Table 1. Characteristics of the patients.

No. of Patients	Characteristics	
405	Mean age, yrs (range)	57.6 (20–91)
405	Female/male	308/97
345	Presence of RF, n (%)	256 (74)
243	Presence of anti-CCP, n (%)	172 (70)
295	Patients with radiographic erosions, n (%)	207 (70)

RF: rheumatoid factor.

Table 2. PADI4 haplotype and genotype frequencies in RA patients and controls.

	Patients, n = 405 (%)	Controls, n = 275 (%)	Chi-square	OR (95% CI)	p
Genotypes					
AC/AC	113 (27)	98 (36)	4.58	0.7 (0.5–0.97)	0.03
AC/GT	204 (51)	140 (51)	0.02	0.98 (0.72–1.33)	NS
GT/GT	88 (22)	37 (13)	7.47	1.79 (1.17–2.72)	0.006
Haplotype carriers					
GT	2 n = 810 (%) 380 (47)	2 n = 550 (%) 214 (39)	8.53	1.39 (1.11–1.73)	0.003

NS: nonsignificant.

were stratified into quartiles for age, and there was no statistically significant difference of distributions of AC/GT, GT/GT, and AC/AC among the 4 quartiles (< 48, 48–59, 59–68, > 68 years). We also analyzed the distribution of AC/GT, GT/GT, and AC/AC between women and men, in patients and controls, with no statistically significant difference (data not shown).

Association between PADI4 and presence of anti-CCP antibodies in RA patients. Among the 405 RA patients, 243 were tested for the presence of anti-CCP antibodies. PADI4 haplotypes and genotypes were distributed equally in this subgroup of 243 patients, as in the main group of 405 patients. Of these 243 patients, 172 tested positive for anti-CCP antibodies. An association between the presence of anti-CCP antibodies and PADI4 was observed (chi-square 5.7, $p = 0.05$). This association was due to a significant decrease of the AC/AC genotype in CCP-positive compared to CCP-negative patients (OR 0.48, $p = 0.02$). Patients carrying the GT haplotype were found significantly more frequently in the anti-CCP-positive subgroup (Table 3). Anti-CCP concentrations were then compared according to PADI4 genotype (Figure 1); median levels of anti-CCP were lower in the AC/AC group (median 146 AU/ml) compared to the AC/GT (median 185 AU/ml) and GT/GT groups (median 189 AU/ml), but this did not achieve significance (Kruskal-Wallis = 1.45, $p = 0.46$).

Association between HLA-DRB1 shared-epitope and presence of anti-CCP antibodies. We then compared HLA-DR

genotype distributions according to the anti-CCP status. We stratified HLA into 6 subgroups combining susceptible shared-epitope DR1, DR4 alleles, and nonsusceptible DRx alleles (those different from DR1 and DR4). A strong association was observed between HLA-DR and the presence of anti-CCP antibodies ($p = 0.0002$; Table 4). This was explained by a significant increase of DR4/DR4-susceptible genotype (OR 11.1, $p = 0.003$) and a significant decrease of DRx/DRx (OR 0.29, $p = 10^{-5}$) in CCP-positive patients. As with PADI4, no significant association was observed between the level of antibodies and the presence or absence of the shared-epitope (medians 161 and 193, respectively; data not shown).

DISCUSSION

In this study an association was found between the PADI4 susceptibility haplotype and RA. These findings are consistent with those of a Japanese study in which an association was found between a PADI4 haplotype and RA¹⁰. The results of that study were confirmed by another study involving 1230 Japanese patients with RA and 948 matched controls³⁹. The same association was also suggested in a Korean study involving a different Asian population²². However, in 4 European studies (England, Spain, France, and Hungary), no association was found between PADI4 and RA^{27–30}. In a German population and in a Canadian one (European descent) the PADI4 haplotype was found to be significantly associated with RA^{25,26}. A recent metaanaly-

Table 3. Frequencies of anti-CCP antibodies in the RA population. Association between presence of anti-CCP antibodies and different PADI4 genotypes and haplotypes.

	Anti-CCP- positive, n = 172 (%)	Anti-CCP- negative, n = 71 (%)	Chi-square	OR (95% CI)	p
Genotypes					
AC/AC	32 (18)	98 (32)	5.46	0.48 (0.25–0.59)	0.02
AC/GT	94 (55)	140 (48)	0.92	1.31 (0.75–2.28)	NS
GT/GT	46 (27)	37 (20)	7.47	1.49 (0.76–2.92)	NS
Haplotype carriers					
GT-positive	n = 172 (%) 140 (81)	n = 71 (%) 48 (67)	5.31	1.39 (1.12–3.93)	0.02

NS: nonsignificant.

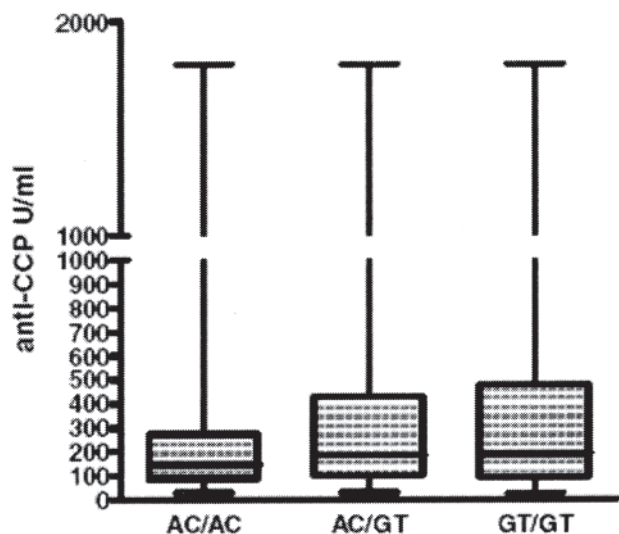


Figure 1. Associations among anti-CCP concentrations and PADI4 genotypes.

sis⁴⁰ concludes that PADI4 polymorphisms may play a larger role in susceptibility to RA in Asian than in European populations.

Our study further confirms the existence of an association between PADI4 and RA in a Caucasian population. To explain the discrepancy between Asian and European results, ethnic or environmental differences have been proposed. Kang and colleagues²² emphasize that the RA susceptibility conferred by the PADI4 functional haplotype might be modified by the presence of certain alleles. DRB1*0401, for example, occurs more frequently in European patients with RA than in Asian patients²². But in our study, DRB1*0401 was detected in 24% of RA patients, a much higher rate than in Asian patients (< 4%). Clinical characteristics, such as the percentages of female patients or average age at disease onset, have been suggested as factors that could influence the susceptibility to RA conferred by the PADI4 functional haplotype, but subanalysis did not confirm this hypothesis. The percentage of women involved in our study (76%) is similar to those in the UK (74%) and Spanish studies (76%) and therefore cannot explain the discrepancy in results. As PADI4 is involved in the generation

of anti-CCP antibodies, one could hypothesize that PADI4 haplotypes are associated with the production of anti-CCP antibodies that might contribute to the occurrence of RA. We could suggest that the association between PADI4 haplotypes and RA is preferentially observed in patients with anti-CCP antibodies (comparison between RA patients negative or positive for anti-CCP antibodies according to genotypes, $p = 0.05$, and to haplotypes, $p = 0.03$). In our study, anti-CCP antibodies were detected in 70% of patients with RA, whereas in the disease severity study by Barton, *et al*⁴¹, only 19.2% of patients had anti-CCP antibodies. Suzuki, *et al*¹⁰ found anti-CCP antibodies in 72% of patients with RA. But in the absence of an association between PADI4 and RA, the family-based study conducted by Caponi, *et al* showed anti-CCP antibodies in 71% of patients with RA, which does not confirm our hypothesis³⁰. Another suggestion to explain these discrepancies is that our RA population may have been more homogeneous or had more severe disease than those in the other European studies: only 26 (6.4%) of our patients tested negative for RF and had no radiological signs of erosion. Hoppe, *et al*⁴² observed that the PADI4 genotype in combination with anti-CCP antibodies and shared-epitope alleles modulates clinical and serological characteristics of RA.

HLA genotyping allowed us to validate our population of patients with RA. Indeed, our findings of an association between HLA-DRB1 shared-epitope and RA⁴³ were similar to those previously described in the literature. Moreover, we found an association between HLA susceptibility for RA and the presence of anti-CCP antibodies. In our study, this association was very strong for HLA-DR4/DR4, with a protection conferred by genotypes not containing DR1 and DR4 (DRx/DRx). Anti-CCP antibodies were detected in all patients carrying DR4/DR4 except one. This association has been reported in several studies^{19,25,44,45}. Sebbag, *et al*⁴⁴ have suggested that “susceptible” HLA-DR may have more affinity for citrullinated than for noncitrullinated peptides. Indeed, these investigators have shown that the affinity of susceptible HLA-DR for citrullinated vimentin is 10 to 100 times greater than for noncitrullinated vimentin^{44,46}. These findings were confirmed by Hill, *et al*⁴⁶. These studies tend to suggest that “shared-epitope” alleles may present a

Table 4. Association between subgroups of HLA-DR and anti-CCP antibodies.

	Anti-CCP-positive, n = 172 (%)	Anti-CCP-negative, n = 70 (%)	Chi-square	OR (95% CI)	p
DR4/DR4	24 (14)	1 (1.4)	8.43	11.1 (1.4–84.4)	< 0.003
DR1/DR4	18 (10.4)	3 (1.7)	2.4	2.61 (0.7–9.1)	NS
DR1/DR1	1 (0.5)	1 (1.4)			NS
DR4/DRx	61 (35)	21 (30)	0.6	1.28 (0.7–2.3)	NS
DR1/DRx	28 (16)	8 (11)	0.4	1.51 (0.6–3.5)	NS
DRx/DRx	40 (23)	36 (51)		0.29 (0.16–0.51)	< 2 × 10 ⁻⁵

greater affinity to citrullinated proteins and contribute to the autoimmune response and induction of production of anti-CCP antibodies. Data from these previous studies focus on the possible effect of citrullination in RA⁴⁷⁻⁵⁰. Indeed, anti-CCP antibodies present at the onset of disease, and even prior to onset, are very specific to RA and are associated with RA severity^{51,52}. Anatomopathological studies have revealed that the synovial tissue contains citrullinated peptides, anti-CCP antibodies, and PADI4 and PADI2 enzymes^{20,21,37}, reinforcing that citrullination is one mechanism involved in the development of RA³⁸. Our findings confirm the association between the GT haplotype and presence of anti-CCP antibodies. With 243 patients tested for anti-CCP antibodies and genotyped for PADI4, this is one of the largest studies addressing such an association. Farago, *et al*²⁹ found no association between the functional PADI4 haplotypes and patients with RA with anti-CCP, but Cha, *et al*⁵³ found that the PADI4 RA risk haplotype was associated with increased anti-CCP levels in RA patients with disease of short duration (less than 34 months), suggesting that PADI4 may play a role in early RA.

We found an association between PADI4 and RA as well as an association between PADI4 and the presence of anti-CCP antibodies. Our study is the third involving a Caucasian population to report findings similar to those reported in the Asian studies, thereby reopening this debate. The hypothesis suggesting that the discrepancies in results may be due to ethnic and/or environmental factors can no longer be retained.

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