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Association of Anti–Cyclic Citrullinated Peptide Antibody Levels With *PADI4* Haplotypes in Early Rheumatoid Arthritis and With Shared Epitope Alleles in Very Late Rheumatoid Arthritis

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Objective. **Anti–cyclic citrullinated peptide (anti-CCP) antibodies are rheumatoid arthritis (RA)–specific serologic markers. RA susceptibility has been associated with HLA–DRB1 shared epitope (SE) alleles and single-nucleotide polymorphism (SNP) haplotypes in the peptidyl arginine deiminase 4 gene (*PADI4*). This study was undertaken to determine whether anti-CCP levels are associated with *PADI4* haplotypes and/or SE alleles in Korean patients with RA.**

Methods. **Three nonsynonymous SNPs in *PADI4* (padi4_89, padi4_90, and padi4_92) and SE alleles were genotyped, and serum anti-CCP levels were measured, in 311 patients with nonerosive or erosive RA. The relationships between anti-CCP levels and *PADI4* haplotypes and/or SE alleles were analyzed statistically.**

Results. **Anti-CCP levels were significantly higher in patients carrying the *PADI4* RA risk haplotype than in patients who did not have the risk haplotype, among anti-CCP–positive patients with RA with a disease duration of ≤34 months ($P = 0.041$), but not among patients with a longer disease duration or among those who had erosive RA versus nonerosive RA. In contrast, the levels were significantly higher in SE carriers than in noncarriers among patients with RA with a disease duration of ≥141 months ($P = 0.0037$) and among those who had erosive RA ($P = 0.000098$), but not among patients who had a shorter disease duration or those who had nonerosive RA.**

Conclusion. **The *PADI4* RA risk haplotype is associated with increased anti-CCP levels in RA patients with disease of short duration, and *PADI4* may play a role in early RA. In contrast, SE alleles are associated with increased anti-CCP levels in RA patients with very longstanding disease and in patients with erosive RA, suggesting that SE alleles play a role in very late RA.**

Rheumatoid arthritis (RA), a chronic systemic autoimmune disease characterized by destructive inflammation of the synovial joints (1), affects ~1% of the world population and is more common in women than in men. RA is a multifactorial disease involving a variety of environmental and genetic components, with genetics determining 50–60% of the RA phenotype (2). Early and accurate diagnosis of RA is critical for treatment efforts. Rheumatoid factor has been used as a serologic marker for diagnosis of RA (3), but it has relatively low specificity, being sometimes detected in patients with other autoimmune diseases as well as in unaffected people (4). In contrast, antibodies against cyclic citrullinated peptides (anti-CCP) are good serologic markers for RA (5,6), with a specificity of >95% and a sensitivity comparable with that of rheumatoid factor (~80%) (4).

Members of the peptidyl arginine deiminase

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(PAD) gene family (*PADI1–PADI4* and *PADI6*), which generate the citrullinated peptides recognized by anti-CCP (6) via posttranslational modification of arginine residues to citrullines (7,8), appear to be good candidates for involvement in the pathogenesis of RA. Indeed, several single-nucleotide polymorphisms (SNPs) and related haplotypes in *PADI4* have been associated with RA susceptibility in 5 independent studies of Japanese (9,10), Korean (11), North American Caucasian (12), and German (13) cohorts. Although other studies of UK (14,15), Spanish (16), and Swedish (12) cohorts and French families (17) failed to replicate the association, a recent meta-analysis of the 6 previous replication study results using the Japanese (10), UK (14,15), Spanish (16), Swedish (12), and North American (12) cohorts confirmed the association of a SNP in *PADI4* with RA susceptibility across these populations (18). Thus, there appears to be good evidence linking SNPs in *PADI4* to RA susceptibility.

Notably, an RA susceptibility haplotype composed of 4 exonic SNPs (including 3 nonsynonymous SNPs) in *PADI4* was associated with increased stability of *PADI4* messenger RNA (mRNA) (9), which could lead to accumulation of PADI4 protein, with subsequent increases in citrullinated proteins and enhanced production of autoantibodies against these citrullinated peptides. Human type I collagen and translation initiation factor 4G1 have been identified as the substrates of PADI4, suggesting that their citrullinated forms might be autoantigens in RA (19,20). However, not all studies have demonstrated associations between *PADI4* haplotypes and anti-CCP across various ethnic groups. In the initial Japanese study, antibodies against citrullinated filaggrin were detected in individuals homozygous for the susceptibility haplotype more often than in heterozygotes and individuals homozygous for the nonrisk haplotypes (9). In contrast, subsequent studies of UK (15,21), French (17), Belgian (22), and German (13) cohorts showed no evidence of *PADI4* haplotypes being associated with anti-CCP detection or serum levels.

A Japanese study demonstrated increased rates of anti-PADI4 detection in RA patients (50% in patients versus 2.5% in controls), suggesting that the natural immunologic tolerance to PADI4 is decreased in RA (23). The levels of anti–rabbit muscle PAD antibodies in RA patient serum samples decreased as the disease duration increased (24), and anti-CCP antibodies were detected up to 14 years before disease onset (25), suggesting that protein citrullination and PADI4 may be involved in the initiation and/or very early stages of RA pathogenesis (23). However, it remains unclear whether *PADI4* functional haplotypes are strictly associated with levels of anti-CCP, especially in populations where the *PADI4* haplotype is associated with RA susceptibility.

Another genetic component of RA is the HLA–DRB1 genotype, which has long been known to be a strong RA susceptibility locus. The shared epitope (SE), a common amino acid motif in the HLA–DRB1 molecule, is thought to contribute to formation of a binding pocket for citrullinated proteins (26). Various SE alleles have been significantly associated with increased susceptibility to RA (11,27–29), but the results of a recent study have indicated that these alleles are associated only with anti-CCP–positive RA (30). More recently, SE alleles have been found to be associated with increased anti-CCP levels in anti-CCP–positive RA patients, and the presence of anti-CCP has been associated with RA development, but SE alleles have not been found to contribute to RA development in patients with undifferentiated arthritis (31).

We recently reported that a haplotype in *PADI4* consisting of minor alleles at 3 nonsynonymous SNPs was associated with RA susceptibility in Koreans (11). In the present study, we recruited a new cohort of Korean patients with RA and measured their serum anti-CCP levels. Then, we examined possible relationships between anti-CCP levels and *PADI4* haplotype and/or SE alleles.

PATIENTS AND METHODS

Patients. The study population consisted of 311 Korean patients with RA (86.5% women) recruited from the Hospital for Rheumatic Diseases (Hanyang University). Their characteristics are shown in Table 1. All subjects provided written informed consent, and the study was approved by the Institutional Review Board of Hanyang University Medical Center. All patients satisfied the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 revised criteria for RA (3). The mean ± SD age of the patients was 51.1 ± 12.6 years, and the mean ± SD age at onset of RA was 40.7 ± 12.3 years. The patients were classified into 4 groups based on radiographic stages of severity, according to the Steinbrocker criteria (32). Since the 4-stage classification may be ambiguous (33,34) but the difference between stage I and stages II–IV is distinct (i.e., the absence and presence, respectively, of destructive changes), for the purposes of this study the 4 groups were collapsed into 2, nonerosive RA (Steinbrocker stage I) and erosive RA (Steinbrocker stages II–IV). Blood samples were obtained from all subjects, and genomic DNA and serum were collected using standard protocols and stored at −70°C until use.

Genotyping of *PADI4* SNPs and HLA–DRB1 alleles. All RA patients were successfully genotyped for 3 nonsynonymous SNPs in *PADI4*, namely padi4_89 (no. rs11203366), padi4_90 (no. rs11203367), and padi4_92 (no. rs874881). The

Table 1. Characteristics of the RA patients and control subjects*

Characteristic	Patients			Controls (n = 392)†
	Total (n = 311)	Nonerosive RA (n = 62)	Erosive RA (n = 249)	
Age, mean ± SD years	51.1 ± 12.6	49.2 ± 12.3	51.6 ± 12.7	42.4 ± 14.2
Female, %	86.5	80.7	88.0	87.8
Disease duration, mean ± SD years	10.4 ± 7.8	3.9 ± 4.3	12.1 ± 7.6	–
SE positivity, %	70.1	59.7	72.7	34.7
RF positivity, %	92.8	86.9	94.2	–
Anti-CCP positivity, %	82.3	79.0	83.1	–
ESR, mean ± SD mm/hour	61.3 ± 29.0	50.7 ± 28.8	64.0 ± 28.4	–
CRP, mean ± SD mg/dl	3.9 ± 4.4	3.4 ± 6.0	4.0 ± 3.9	–

* RA = rheumatoid arthritis; SE = shared epitope; RF = rheumatoid factor; anti-CCP = anti–cyclic citrullinated peptide; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein.
† The control group in this study was the same as that in a previous study by Kang et al (11).

padi4_89 SNP refers to 163G>A or 55Gly>Ser, padi4_90 refers to 245T>C or 82Val>Ala, and padi4_92 refers to 335G>C or 112Gly>Ala, where the numbers indicate the nucleotide or amino acid positions. Genotyping was performed using the MassArray system (Sequenom, San Diego, CA), as previously described (11). Briefly, the genomic templates were amplified by polymerase chain reaction (PCR), the DNA primers were extended on the PCR products, and the extended primers were detected by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry. SpectroTyper software (Sequenom) was used for allele identification. The alleles were found to be in Hardy-Weinberg equilibrium in all 3 SNPs ($P = 0.714$, $P = 0.213$, and $P = 0.522$ for padi4_89, padi4_90, and padi4_92, respectively).

For determination of the HLA–DRB1 alleles in each patient, samples were subjected to PCR, followed by hybridization with sequence-specific oligonucleotide probes, according to the reference protocol of the Twelfth International Histocompatibility Workshop (35). The results were analyzed by subsequent direct DNA sequencing analysis (36). HLA–DRB1*0101, *0401, *0404, *0405, *0408, *0410, *1001, *1402, and *1406 were regarded as SE alleles (11,22).

Measurement of serum anti-CCP levels. Serum anti-CCP levels in all RA patients were quantitatively measured in duplicate by enzyme-linked immunosorbent assay using the Diastat Anti-CCP kit FCCP 200, according to the recommendations of the manufacturer (Axis-Shield, Dundee, UK). The results were measured as absorbance at 550 nm, determined using the Model 550 microplate reader (Bio-Rad, Richmond, CA). Of the 311 patients, 256 were positive for anti-CCP (defined as a serum level of >5 units/ml). Thus, the sensitivity of the anti-CCP assay was 82.3%, which is slightly higher than the 78% reported by the manufacturer of the kit (http://www.axis-shield.com/product/104/productdetailpage).

Statistical analysis. Haplotypes of the 3 nonsynonymous SNPs in *PADI4* were constructed for each study subject using the Bayesian algorithm–based program Phase, version 2.1 (37,38). The RA patients were classified based on RA risk haplotype status, SE allele status, and/or presence of erosive disease, and anti-CCP levels were compared between groups using Wilcoxon's rank sum test, since the levels were nonnormally distributed in most groups. Differences in the percentage of female patients and in age between subgroups in all comparisons were not significant enough to necessitate adjustment for these features ($P > 0.05$).

The associations of *PADI4* haplotypes with RA susceptibility were assessed by logistic regression analyses, which included all of the control subjects in our previous study (11), whose characteristics are shown in Table 1. Although the patient and control samples were genotyped at different time points, the methods of genotyping and haplotype construction and the association tests were the same. Multiple logistic regression analyses were performed with adjustments for age, sex, and presence of the SE in the susceptibility association tests of haplotype carriage, and with adjustments for age, sex, and presence of the haplotype in the association tests of SE carriage. All statistical tests were performed using SPSS, version 11.5 (SPSS, Chicago, IL).

RESULTS

Association of a functional *PADI4* haplotype with RA susceptibility in the Korean cohort. When haplotypes were constructed from the individual genotyping results on the 3 nonsynonymous SNPs in *PADI4* in all 311 RA patients in this study, we identified 5 different haplotypes (Table 2). Two common haplotypes, ACC and GTG (with letters representing the nucleotides found at padi4_89, padi4_90, and padi4_92, respectively), constituted almost all of the patient haplotypes (99.2%). The most frequent haplotype, ACC (52.7% of all patient haplotypes), carried the major RA nonrisk alleles, whereas the other common haplotype, GTG (46.5% of all patient haplotypes), carried the minor RA risk alleles. Three other very rare haplotypes were found in only 5 heterozygous patients (Table 2).

Table 2. Association of *PADI4* single-nucleotide polymorphism (SNP) haplotypes with susceptibility to rheumatoid arthritis*

Haplotype†	Present study				Kang et al (11) (1,090 patient haplotypes)‡
	Patients (n = 622)‡	Controls (n = 784)‡	OR (95% CI)§	*P*§	
ACC	328 (52.7)	484 (61.7)	1		563 (51.7)
GTG	289 (46.5)	299 (38.1)	1.4 (1.2–1.8)	1.1×10^{-3}	525 (48.2)
ACG	3 (0.5)	1 (0.1)	–	–	2 (0.2)
GCC	1 (0.2)	0	–	–	0
ATG	1 (0.2)	0	–	–	0

* Values are the number (%).
† SNPs are listed in the order padi4_89, padi4_90, and padi4_92.
‡ The n values are the numbers of haplotypes. The control group in this study was the same as that in a previous study by Kang et al (11).
§ The odds ratio (OR), 95% confidence interval (95% CI), and *P* value for the GTG haplotype were calculated by logistic regression comparisons with the reference haplotype, ACC.

When this patient group was compared with the control group (n = 392) from our previous study (11), we found that the GTG haplotype was significantly associated with RA versus the ACC haplotype (Table 2), with an odds ratio (OR) of 1.4 and a 95% confidence interval (95% CI) of 1.2–1.8 ($P = 0.0011$), which was similar to our previous results from the same type of analysis using a different patient group but the same control group (OR 1.5 [95% CI 1.3–1.8], $P = 0.000012$) (11).

Since the anti-CCP levels were to be compared among patient genotypes rather than haplotypes, genotype associations with RA susceptibility were tested (Table 3). Increased susceptibility to RA was significantly associated with the homozygous GTG genotype (adjusted OR 2.2 [95% CI 1.3–3.6], $P = 0.0036$) and the heterozygous GTG genotype (adjusted OR 1.5 [95% CI 1.0–2.2], $P = 0.035$) versus the homozygous non-GTG genotype (GTG−). Not only did these 95% CIs overlap, but the homozygous GTG genotypes were not significantly different from the heterozygous GTG genotypes in affecting RA susceptibility in a direct comparison (adjusted OR 1.4 [95% CI 0.86–2.2], $P = 0.19$).

Because the dose effect of the GTG haplotype was not significant, we then combined the 2 RA risk genotypes. The GTG genotypes (GTG+) were significantly associated with RA versus the GTG− genotypes (adjusted OR 1.6 [95% CI 1.1–2.3], $P = 0.0075$). Therefore, in the subsequent analyses of *PADI4* haplotype associations with anti-CCP levels, the heterozygous genotypes were grouped together with the homozygous GTG genotypes to be compared with the GTG− genotypes. These results confirmed our previous finding that the GTG haplotype is associated with increased susceptibility to RA in Koreans (11), indicating that the enrolled study population was suitable for analyzing the associations of *PADI4* haplotypes with anti-CCP levels. The findings also indicated that the associations could be

Table 3. Association of *PADI4* genotypes or HLA–DRB1 SE carriage with increased RA susceptibility*

Subject group	Patients (n = 311)	Controls (n = 392)†	OR (95% CI)‡	*P*‡
PADI4 genotype				
GTG−	85 (27)	152 (39)	1	–
GTG+	226 (73)	240 (61)	1.6 (1.1–2.3)	7.5×10^{-3}
GTG/non-GTG	163 (52)	181 (46)	1.5 (1.0–2.2)	3.5×10^{-2}
GTG/GTG	63 (20)	59 (15)	2.2 (1.3–3.6)	3.6×10^{-3}
SE carriage				
SE−	93 (30)	256 (65)	1	–
SE+	218 (70)	136 (35)	6.1 (3.7–10.0)	1.6×10^{-12}

* Values are the number (%).
† The control group in this study was the same as that in a previous study by Kang et al (11).
‡ The odds ratio (OR), 95% confidence interval (95% CI), and *P* values were calculated by multiple logistic regression with adjustment for age, sex, and SE carriage in the analysis of haplotype carriage effects, and with adjustment for age, sex, and GTG carriage in the analysis of SE carriage effects. See Table 1 for other definitions.

Table 4. Anti-CCP levels in anti-CCP–positive RA patients, grouped by presence or absence of erosions, *PADI4* risk haplotype carriage, and SE allele carriage*

Subgroup (n)	Anti-CCP, median (range) units/ml	*P* for association†
GTG carriage		
GTG− (71)	78 (6–883)	0.96
GTG+ (183)	93 (5–1,280)	
GTG− nonerosive (13)	66 (13–265)	0.24
GTG+ nonerosive (35)	95 (7–631)	
GTG− erosive (58)	91 (6–883)	0.61
GTG+ erosive (148)	92 (5–1,280)	
SE carriage		
SE− (70)	68 (5–756)	3.9×10^{-3}
SE+ (186)	104 (6–1,280)	
SE− nonerosive (19)	100 (17–549)	0.15
SE+ nonerosive (30)	65 (7–631)	
SE− erosive (51)	59 (5–756)	9.8×10^{-5}
SE+ erosive (156)	114 (7–1,280)	

* For GTG carriage, 254 patients were examined; for SE carriage, 256 patients were examined. See Table 1 for definitions.
† Versus the comparison subgroup, determined by Wilcoxon's rank sum test.

tested using the dichotomy of the presence or absence of the RA risk haplotype rather than the 3-genotype variable.

Associations between *PADI4* haplotypes and anti-CCP levels. To analyze possible associations between anti-CCP levels and *PADI4* haplotypes, we compared anti-CCP levels in patients who had GTG genotypes with those in patients who did not have GTG genotypes, excluding the 3 patients (1 anti-CCP negative and 2 anti-CCP positive) carrying ACG, GCC, or ATG but not GTG, because we did not know the definite RA risk effects of these rare haplotypes. We also compared the anti-CCP levels in patients who had erosive RA with those in patients who had nonerosive RA. The anti-CCP level was not significantly associated with the presence of GTG ($P = 0.43$) or with erosive RA ($P = 0.22$).

When the presence of anti-CCP was treated as a dichotomous variable, there was no significant association between anti-CCP and GTG ($P = 0.25$) or between anti-CCP and erosive RA ($P = 0.39$). Since the RA disease phenotype would be unrelated to cyclic peptide citrullination in the 54 anti-CCP–negative patients (anti-CCP $\leq$5 units/ml), we excluded these individuals and performed further statistical analyses on the remaining data set. Among the anti-CCP–positive patients (n = 254), we did not observe any significant difference in anti-CCP levels between the patients who had GTG genotypes and those who did not ($P = 0.96$) (Table 4).

We then examined whether there was an association between the *PADI4* GTG haplotype and anti-CCP levels dependent on whether a patient had erosive or nonerosive RA, because a previous finding of the progressive decrease in the anti-PAD antibody level as RA disease duration increased (24) raised the possibility that PADI4 levels might also decrease as disease duration increases and/or disease severity progresses. We divided the anti-CCP–positive patients into 4 groups, based on GTG status and presence of erosive or nonerosive RA (GTG+ nonerosive, GTG− nonerosive, GTG+ erosive, and GTG− erosive).

The anti-CCP levels in the GTG− nonerosive group were not significantly different from those in the GTG+ nonerosive group ($P = 0.24$) (Figure 1A), although the GTG+ nonerosive group had a 2.0-fold higher mean level and a 1.4-fold higher median level than the GTG− nonerosive group (Table 4). The GTG− erosive and GTG+ erosive groups (Figure 1A) showed no significant difference in anti-CCP levels ($P = 0.61$) (Table 4). These results indicated that the RA risk haplotype in *PADI4* was not significantly associated with anti-CCP levels in either nonerosive or erosive RA.

We also found that among the anti-CCP–positive patients, the levels in patients with nonerosive RA and in patients with erosive RA did not significantly differ from one another ($P = 0.41$). Anti-CCP levels were not associated with erosive RA either in the presence ($P = 0.96$) or in the absence ($P = 0.13$) of the GTG haplotype (Figure 1A), although the mean and median levels in the GTG− erosive group were 2.4-fold and 1.4-fold higher, respectively, than those in the GTG− nonerosive group.

Associations between SE alleles and anti-CCP levels. Because SE alleles are thought to interact with citrullinated proteins (26) and contribute to development of anti-CCP (31), we examined the possible associations of SE alleles with anti-CCP levels. In this cohort, the presence of an SE allele or SE alleles was significantly associated with RA compared with the absence of any SE alleles (OR 6.1 [95% CI 3.7–10.0], $P = 1.6 \times 10^{-12}$) (Table 3), indicating that the study population was suitable for analyzing associations between the presence of SE alleles and anti-CCP levels. Since SE alleles were previously found to be associated with anti-CCP–positive RA but not anti-CCP–negative RA (30), however, we primarily focused on the anti-CCP–positive patients, including those with rare haplotypes who were excluded from the tests of association between GTG and anti-CCP levels, rather than the entire group of patients. Thus, 256 patients were included in this analysis. Our results revealed that the SE alleles were significantly associated with increased levels of anti-CCP

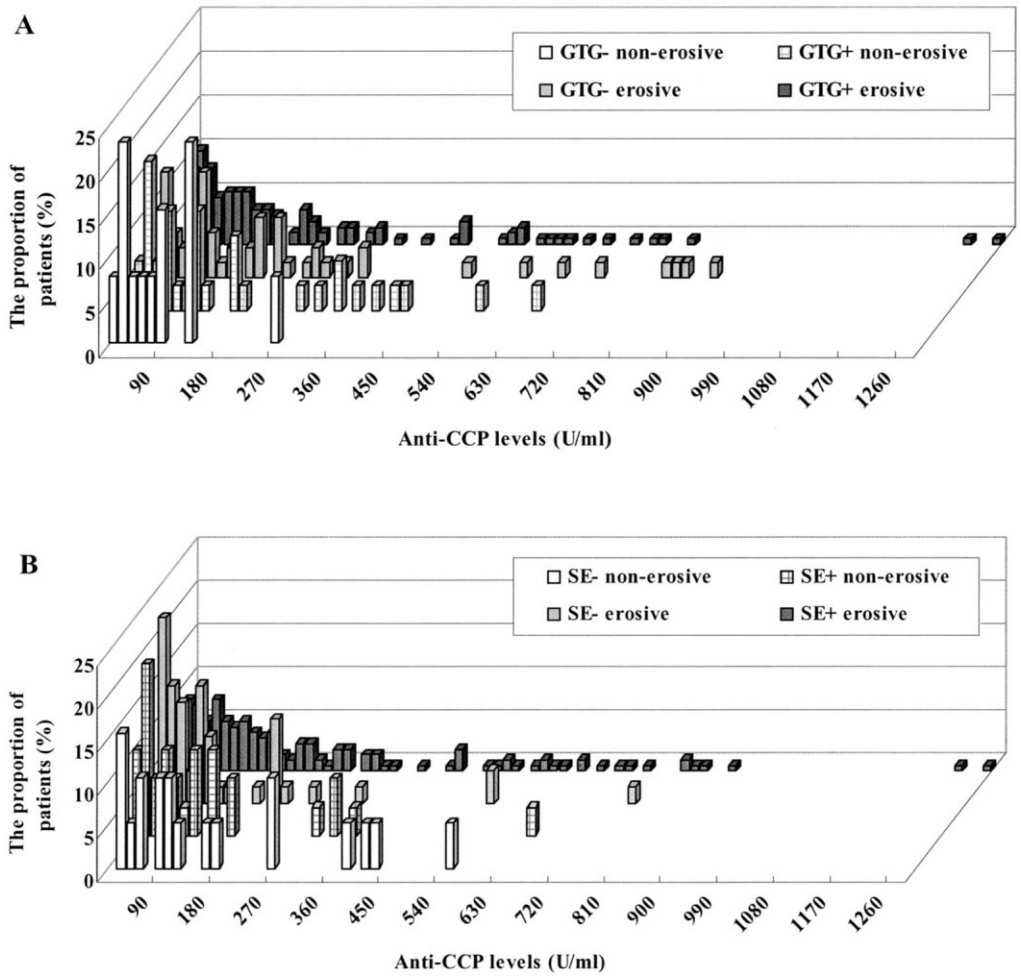

Figure 1. A, Distribution of anti–cyclic citrullinated peptide (anti-CCP) antibody levels in rheumatoid arthritis (RA) patients grouped by *PADI4* haplotype status and presence of erosive or nonerosive RA. The anti-CCP–positive RA patients, excluding 2 with rare *PADI4* haplotypes (n = 254), were divided into 4 groups, as follows: nonerosive RA without the GTG haplotype, nonerosive RA with the GTG haplotype, erosive RA without the GTG haplotype, and erosive RA with the GTG haplotype. **B,** Distribution of anti-CCP levels in RA patients grouped by shared epitope (SE) status and presence of erosive or nonerosive RA. The anti-CCP–positive RA patients (n = 256) were divided into 4 groups, as follows: nonerosive RA without SE alleles, nonerosive RA with SE alleles, erosive RA without SE alleles, and erosive RA with SE alleles. Serum anti-CCP levels were measured twice in each patient, and the mean value was recorded. Samples with mean values ≤5 units/ml were considered anti-CCP negative and were excluded from the histograms, which are shown in intervals of 15 units/ml.

in the anti-CCP–positive RA patients ($P = 0.0039$) (Table 4).

We then examined whether the SE alleles demonstrated a specific association with erosive disease, because they have recently been shown not to contribute to very early development of RA from undifferentiated arthritis (31). We divided the anti-CCP–positive patients into 4 groups, based on presence of the SE allele and presence of erosive or nonerosive RA (SE− nonerosive, SE+ nonerosive, SE− erosive, and SE+ erosive). Anti-CCP levels were significantly higher in the SE+ erosive group than in the SE− erosive group ($P = 0.000098$) (Figure 1B), with 2.0-fold and 1.9-fold differences in the mean and median values, respectively (Table 4). In contrast, we did not observe a significant association between SE alleles and anti-CCP levels in the patients with nonerosive RA ($P = 0.15$) (Figure 1B and Table 4). These findings indicated that SE alleles were significantly associated with increased anti-CCP levels in anti-

CCP–positive patients with erosive RA, but not in those with nonerosive RA.

We also found that erosive RA was associated with increased anti-CCP levels in the presence of SE alleles but with decreased anti-CCP levels in the absence of SE alleles. Among SE carriers, anti-CCP levels were significantly higher in anti-CCP–positive patients with erosive RA than in those with nonerosive RA ($P = 0.027$) (Figure 1B), with 1.8-fold differences in both the mean and median values. In contrast, the levels were significantly lower in the SE− erosive group versus the SE− nonerosive group ($P = 0.024$) (Figure 1B), with 1.7-fold differences in both the mean and median values.

Anti-CCP level association tests with stratification for RA duration. In the entire patient group (n = 311), the disease duration was substantially shorter among those with nonerosive RA (median 26 months) versus those with erosive RA (137 months) ($P = 3.2 \times 10^{-16}$). Thus, anti-CCP association tests for GTG and SE carriage were corrected for disease duration, although anti-CCP levels were not correlated with disease duration among the anti-CCP–positive patients ($r = -0.058$, $P = 0.36$; n = 254). The anti-CCP–positive patients were divided into 5 quintiles, according to RA duration: quintile 1 (0–34 months; n = 54), quintile 2 (35–93 months; n = 50), quintile 3 (95–140 months; n = 51), quintile 4 (141–196 months; n = 50), and quintile 5 (197–496 months; n = 49). The association tests were performed within each quintile (Table 5).

In quintile 1, anti-CCP levels were significantly higher in patients with GTG genotypes than in those with non-GTG genotypes ($P = 0.041$), with 2.9-fold and 2.8-fold differences in the mean and median levels, respectively. Anti-CCP levels were not significantly different with respect to the GTG genotype in quintile 2 ($P = 0.85$), 3 ($P = 0.73$), 4 ($P = 0.17$), or 5 ($P = 0.70$). In contrast, the association of the SE with increased anti-CCP levels was significant in quintiles 4 ($P = 0.027$) and 5 ($P = 0.026$) and nearly significant in quintile 3 ($P = 0.060$), but was not significant in quintile 1 ($P = 0.98$) or 2 ($P = 0.88$). When quintiles 4 and 5 were pooled together, anti-CCP levels were also positively associated with SE carriage ($P = 0.0037$), with 2.0-fold and 1.8-fold differences in the mean and median levels, respectively.

Taken together, these results suggested that increased anti-CCP levels were associated with the GTG haplotype in early RA (in patients with disease duration of ≤34 months) but with SE alleles in very late phases of RA (in patients with disease duration of ≥141 months). Anti-CCP levels were not associated with the GTG haplotype or SE alleles in patients with disease durations of 35–140 months.

Table 5. Anti-CCP levels in anti-CCP–positive RA patients, grouped by disease duration, *PADI4* risk haplotype carriage, and SE allele carriage*

Subgroup (n)	Anti-CCP, median (range) units/ml	*P* for association†
GTG carriage		
GTG− quintile 1 (17)	57 (11–265)	4.1×10^{-2}
GTG+ quintile 1 (37)	159 (5–1,280)	
GTG− quintile 2 (14)	77 (14–801)	0.85
GTG+ quintile 2 (36)	88 (7–516)	
GTG− quintile 3 (14)	93 (9–883)	0.73
GTG+ quintile 3 (37)	77 (8–1,239)	
GTG− quintile 4 (12)	134 (30–643)	0.17
GTG+ quintile 4 (38)	90 (8–808)	
GTG− quintile 5 (14)	90 (6–831)	0.70
GTG+ quintile 5 (35)	78 (5–670)	
SE carriage		
SE− quintile 1 (13)	111 (5–549)	0.98
SE+ quintile 1 (41)	122 (7–1,280)	
SE− quintile 2 (9)	82 (17–420)	0.88
SE+ quintile 2 (41)	85 (7–801)	
SE− quintile 3 (16)	55 (9–534)	0.060
SE+ quintile 3 (35)	113 (8–1,239)	
SE− quintile 4 (20)	74 (8–756)	2.7×10^{-2}
SE+ quintile 4 (30)	130 (10–808)	
SE− quintile 5 (12)	49 (5–201)	2.6×10^{-2}
SE+ quintile 5 (37)	98 (6–831)	

* Quintiles 1, 2, 3, 4, and 5 correspond to disease durations of 0–34 months, 35–93 months, 95–140 months, 141–196 months, and 197–496 months, respectively. See Table 1 for definitions.
† Versus the comparison subgroup, determined by Wilcoxon's rank sum test.

DISCUSSION

Since anti-CCP antibodies are gaining popularity as RA-specific serologic markers, it is important to investigate the possible genetic mechanisms underlying these markers. This is the first study to show that in a population of Korean patients with RA, increased serum levels of anti-CCP are associated with an RA risk haplotype of *PADI4* in early phases of RA (within 34 months of disease onset) but not later phases of RA, and with SE alleles in very late phases (≥141 months after disease onset) but not earlier phases. Furthermore, increased anti-CCP levels are associated with HLA–DRB1 SE alleles in erosive RA but not in nonerosive RA.

Our results revealed that a haplotype corresponding to the minor alleles of 3 nonsynonymous *PADI4* SNPs (padi4_89G, padi4_90T, and padi4_92G) was significantly associated with increased serum levels of anti-CCP in the quintile of RA patients who had a

disease duration of ≤34 months. Among these patients with early disease, anti-CCP levels were significantly higher in those with the GTG haplotype than in those without GTG ($P = 0.041$). A significant difference in anti-CCP levels in patients with the GTG haplotype versus patients without the GTG haplotype was also observed among patients with a disease duration of ≤18 months ($P = 0.026$), but disappeared in quintiles 2–5, which included patients with longer disease durations.

Our findings are consistent with the hypothesis that the anti-CCP level–associated RA risk haplotype in *PADI4* affects the onset or early phases of RA, but has little effect on the later phases. It has been suggested that PADI4-mediated citrullination is linked to the onset of RA and/or represents an RA-triggering event (9). This notion is supported by the observation that anti-CCP is detectable several years before the onset of RA (25).

A significant association between the presence of SE alleles and anti-CCP levels was observed in the overall patient set, but there were differences specific to erosive disease. SE alleles were significantly associated with increased anti-CCP levels in patients with erosive RA, showing 2.0-fold and 1.9-fold increases in the mean and median levels, respectively, versus patients without SE alleles ($P = 0.000098$). In contrast, this association was not significant in patients with nonerosive RA ($P = 0.15$). The apparent association between SE alleles and anti-CCP levels observed in the patient group as a whole was probably due to the relatively high rate of erosive RA (81%) in our patient cohort.

Previous studies have shown that levels of antibody against rabbit muscle PAD decreased during the progression of RA (24), suggesting that PADI4 levels might also decrease as RA progresses and/or disease duration increases. If SE alleles were not responsible for the increased anti-CCP levels in patients with erosive RA, the anti-CCP levels should decrease upon progression from nonerosive to erosive RA, due to the decrease in PADI4 level. In fact, within the SE− group, the anti-CCP levels were significantly lower in the erosive subgroup versus the nonerosive subgroup ($P = 0.024$), with a 1.7-fold decrease in both mean and median levels. Within the SE+ group, however, the anti-CCP levels were significantly higher in the erosive subgroup than in the nonerosive subgroup ($P = 0.027$), with a 1.8-fold increase in both mean and median levels. These observations further support the hypothesis that the presence of SE alleles increases anti-CCP levels in erosive RA.

It has been suggested that there is a functional difference between the RA risk and nonrisk haplotypes in *PADI4* with regard to the stability of *PADI4* mRNA (9), implying a possible gene-dose effect of RA risk haplotype on anti-CCP levels. In the present study, however, anti-CCP levels were not significantly different in patients homozygous for GTG from those in patients heterozygous for GTG, among patients in the quintile with the shortest disease duration ($P = 0.41$). Also, susceptibility to RA in GTG homozygotes was not significantly different from that in heterozygotes ($P = 0.19$). The absence of a significant haplotype-dose effect raises the possibility that there is another functional difference between risk and nonrisk haplotypes in PADI4 enzyme functions. Although the nonsynonymous SNP amino acid changes occur far from the active site and do not affect dimer contacts, based on the crystal structure of dimeric PADI4 (39), it remains unclear whether they affect protein interactions or the stability of dimeric PADI4.

In summary, increased serum levels of an RA-specific serologic marker antibody, anti-CCP, are associated with an RA risk *PADI4* haplotype in RA patients who have short disease duration, but with SE alleles in those with very longstanding disease. Additionally, the increased anti-CCP levels are associated with SE alleles in erosive RA but not in nonerosive RA. Taken together, these results suggest that PADI4 and SE alleles play roles in the early and very late phases of RA pathogenesis, respectively. Additional work is needed to validate these findings in Koreans and other populations, since the subgroups of patients with nonerosive disease in this study were small, and no adjustments were made for multiple comparisons.

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AUTHOR CONTRIBUTIONS

Dr. Changwon Kang had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Changwon Kang, Bae.
Acquisition of data. Cha, Choi, Han, Changsoo Paul Kang.
Analysis and interpretation of data. Cha, Choi, Han, Changwon Kang.
Manuscript preparation. Cha, Changwon Kang, Bae.
Statistical analysis. Cha, Han.

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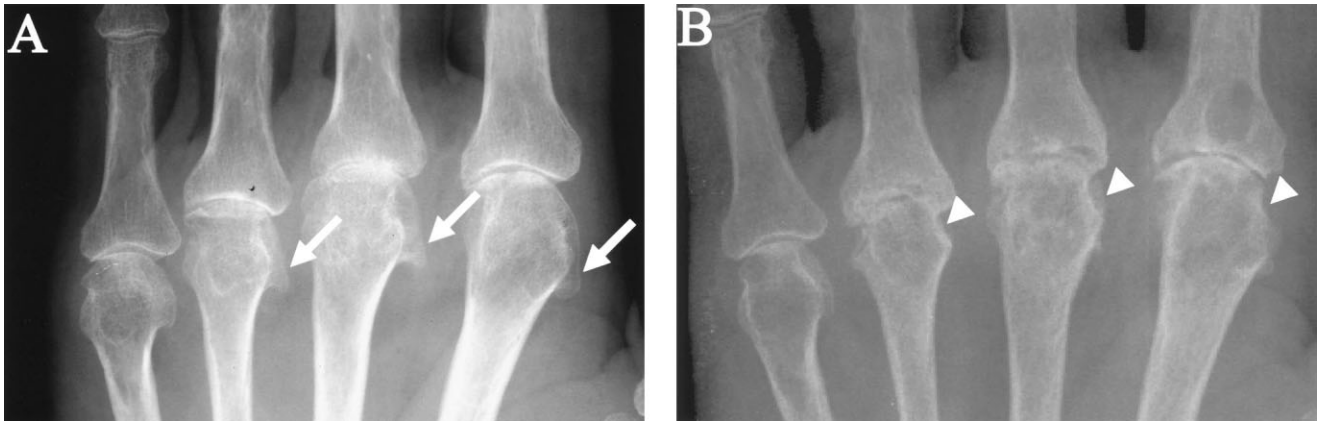
Clinical Images: Rheumatologic irony



The patient, a 57-year-old man, first presented with mild arthralgia in the metacarpophalangeal (MCP) joints. Radiographs obtained at that time (**A**) clearly showed degenerative changes in the MCP joints, with hook-like osteophytes (**arrows**). Laboratory evaluation revealed an elevated ferritin level as well as transferrin saturation, and genetic analysis confirmed hereditary hemochromatosis (C282Y homozygous). The patient subsequently underwent regular phlebotomy, but reported worsening of joint pain with new-onset symmetric synovitis. Three years later, he presented to our outpatient clinic with recent radiographs (**B**), which revealed complete resorption of the osteophytes seen 3 years earlier, and additional bone erosions in the MCP and carpal joints (**arrowheads**). Magnetic resonance imaging of the right hand showed massive synovitis, and seronegative rheumatoid arthritis was diagnosed. Coincident occurrence of hereditary hemochromatosis and rheumatoid arthritis has been described. However, to our knowledge, this is the first documented case in which the 2 diseases sequentially involved the same joints, with a complete transition from a proliferative phenotype with osteophyte formation to an erosive disease with complete resorption of the osteophytes that had previously grown over a long period of time.

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