Interaction of HLA-DRB1*09:01 and *04:05 with Smoking Suggests Distinctive Mechanisms of Rheumatoid Arthritis Susceptibility Beyond the Shared Epitope

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ABSTRACT. ***Objective.*** Although HLA-DRB1 shared epitope (SE) alleles and HLA-DRB1*09:01 have repeatedly been shown to be associated with susceptibility to rheumatoid arthritis (RA), the effect of each allele on levels of anticyclic citrullinated peptide autoantibodies (anti-CCP) and interaction with cigarette smoking in RA remains to be fully defined. We investigated whether HLA-DRB1 risk alleles influence anti-CCP levels and whether each allele interacts with smoking in anti-CCP-positive or -negative RA.

Methods. All patients with RA (n = 1924) and controls (n = 1119) were Korean. The HLA-DRB1 4-digit genotyping was performed by standard PCR-sequencing based typing method. OR and biologic interactions as departures from additivity or multiplicity were analyzed by logistic regression.

Results. SE alleles were significantly associated with increased anti-CCP levels. Conversely, HLA-DRB1*09:01 was associated with reduced levels, in both SE-positive and SE-negative patients. Each of SE alleles interacted significantly with smoking, whereas HLA-DRB1*09:01 did not. Interactions between the 2 most significant risk alleles, HLA-DRB1*04:05 and HLA-DRB1*09:01, (attributable proportion = 0.68, 95% CI 0.46–0.89, multiplicity p = 0.012) significantly increased RA susceptibility regardless of anti-CCP and smoking status. Smoking increased the risk for RA by significant interaction with the heterozygote HLA-DRB1*04:05/*09:01.

Conclusion. HLA-DRB1*09:01 differs from SE alleles with regard to anti-CCP levels and interaction with smoking, suggesting a distinct mechanism of HLA-DRB1*09:01 in the pathogenesis of RA that may bypass anti-CCP formation. Also, a significant increase of the HLA-DRB1*04:05/*09:01 heterozygote in RA susceptibility may be attributable to the synergistic contribution of 2 different pathways in which 2 alleles participate independently. (J Rheumatol First Release May 1 2013; doi:10.3899/jrheum.121280)

Key Indexing Terms:
RHEUMATOID ARTHRITIS HLA-DRB1*09:01 SHARED EPITOPE
ANTICITRULLINATED PROTEIN AUTOANTIBODIES SMOKING

Rheumatoid arthritis (RA) is a chronic autoimmune arthritis to which both genetic and environmental factors contribute. The HLA-DRB1 shared epitope (SE) alleles that share conserved amino acid sequences (^{70}QRRAA^{74} *01:01, *01:02, *04:04, *04:05, *04:08, *04:10, *14:02, *14:06; ^{70}QKRAA^{74} *04:01; ^{70}RRRAA^{74} *10:01) in the third hypervariable region of the HLA-DRB1 molecule have consistently shown the strongest genetic association with RA[1]. The most significant of these SE alleles differ according to ethnic groups: the HLA-DRB1*04:01 and *04:04 alleles in whites, and the HLA–DRB1*04:05 allele in Asians, including Koreans, Japanese, and Chinese[2,3,4]. In addition to the SE alleles, HLA–DRB1*09:01 (^{70}RRRAE^{74}), which is relatively common in Asians but is rare in whites, consistently shows a significant association with RA in Asians[2,5] and whites[6,7]. In anticyclic citrullinated peptide autoantibodies (anti-CCP)-negative RA, while an association of HLA–DRB1*03:01 has been reported in whites[8], there was no significant association in our current study because of the lower frequency of *03:01 in Koreans (1.0%–1.7%)[2]. But the *09:01 allele was found to be significant in our study and in several previous studies in Asians[2,5,9,10], suggesting a non-SE contribution to RA susceptibility.

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Supported by a grant from the Korea Healthcare Technology R&D Project (A102065), Ministry for Health and Welfare, Republic of Korea.

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Accepted for publication February 20, 2013.

Cigarette smoking is the most consistent environmental risk factor for RA. It has been suggested that smoking may trigger the immune reaction to citrullinated protein with the SE-restricted form[11,12,13]. RA can be classified into 2 types, anti-CCP-positive and anti-CCP-negative, and the clinical course and prognosis are thought to be worse in anti-CCP-positive RA. It has also been suggested that the 2 subgroups have different genetic and environmental risk factors in whites[12,14], such that the SE alleles and smoking predispose to anti-CCP-positive RA, not to anti-CCP-negative RA[12,15,16]. However, we observed that SE alleles and smoking are associated with anti-CCP-negative RA as well as anti-CCP-positive RA in a Korean population[5], suggesting the existence of ethnic differences[4,17]. In addition, a recent European study suggested that SE alleles have a weaker effect on the risk of anti-CCP-negative RA[6]. Thus, further studies are needed to define the genetic and environmental risk factors for anti-CCP-negative RA.

Although many investigations have shown the effect of HLA-DRB1 and smoking on RA, several issues remain to be clarified. First, the effect of HLA-DRB1 risk alleles on anti-CCP levels is still uncertain. Some studies, but not others, have suggested a positive correlation between SE alleles and anti-CCP levels. In addition, a Japanese study reported that HLA–DRB1*09:01 lowered anti-CCP levels in patients with RA[18]. High anti-CCP levels may be relevant to a qualitatively broad anticitrullinated protein repertoire that predisposes to RA development[19,20,21]. In addition, high anti-CCP levels and SE alleles are associated with poor prognosis in RA. Moreover, new 2010 American College of Rheumatology (ACR)/European League Against Rheumatism classification criteria for RA emphasize that high anti-CCP levels (> 3 times the normal range) have greater diagnostic value than low levels[22]. Therefore, it would be worthwhile to investigate which genetic and environmental risk factors influence anti-CCP levels.

Second, although gene-environment interactions between SE alleles and smoking have been observed in anti-CCP-positive RA in both whites[12,23] and Asians[5,17,24], it is not clear whether each of the SE alleles (*01, *04, *10) and *09 interact with smoking in the same manner. Van der Helm-van Mil, *et al*[25] found a significant interaction between smoking and the HLA–DRB1*01 or *10 group, but there was no interaction between smoking and the HLA–DRB1*04 group in their case-only analysis. In contrast, interactions between smoking and the *04 group as well as the *01 or *10 group were observed in a case–control study of the Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA)[26].

Moreover, the interaction between genetic and environmental risk factors for anti-CCP-negative RA, which includes 20%–40% of individuals with RA, remains to be defined[14,27].

We investigated whether HLA–DRB1 susceptible alleles (SE and *09) influence anti-CCP levels and interact with smoking in the development of anti-CCP-positive or anti-CCP-negative RA in the Korean population.

MATERIALS AND METHODS

Patients and controls. A total of 1924 patients who satisfied the ACR 1987 classification criteria for RA[28] were enrolled from the BAE RA cohort at Hanyang University Hospital for Rheumatic Diseases. The BAE RA cohort consists of native Koreans, all recruited consecutively with a standard protocol since 2001 at Hanyang University Hospital for Rheumatic Diseases. A total of 1119 healthy Korean controls, excluding any with a personal or familial history of autoimmune disease, volunteered for our study. Informed consent was obtained from all the patients and controls through a questionnaire at the time of enrollment, when clinical information was also collected. Smoking information was recorded as never, past, or currently smoking, and was obtained from 1897 (98.6%) RA cases and 1101 (98.4%) controls. Past and current smokers were classified as ever-smokers, who began smoking before the onset of RA.

The study was approved by the local ethics committee of Hanyang University Hospital.

Laboratory procedures. Anti-CCP was measured using the ImmuLisa CCP ELISA test (Immco Diagnostics Inc.). Positive samples contained > 25 units/ml, according to the manufacturer.

HLA-DRB1 genotyping. Genomic DNA was extracted from peripheral blood mononuclear cells. Four-digit HLA–DRB1 typing was obtained by a conventional PCR-sequencing based typing method as described[5,29,30,31].

Statistical analysis. Frequencies and OR of the alleles and genotypes of HLA–DRB1 were calculated[32], and the chi-square test was used to compare RA cases and controls. We used the relative predispositional effects (RPE) method to examine the relative effects of HLA–DRB1 susceptible alleles on RA[33], and the Bonferroni method to control for errors inherent in multiple comparisons. For the RPE method, a chi-square value for RA group versus control group was calculated for each of the remaining markers (2 times all alleles table). The greatest-risk HLA–DRB1 allele for RA was *04:05. The comparison was repeated after *04:05 was removed. We continued removing the HLA–DRB1 allele that contributed most to the chi-square value until the serially reduced table no longer generated a significant value with Bonferroni correction. Differences in the median values of anti-CCP levels between groups were analyzed using the Mann-Whitney U test or Kruskal-Wallis test. All anti-CCP levels > 3200 units/ml were truncated and assigned as 3200 units/ml for analysis.

For the assessment of biologic interactions, we estimated the relative excess risk due to interaction, attributable proportion, and the synergy index as a departure from additivity as described elsewhere[5,12,16,20,24,31,34,35]. In addition, gene-gene interaction between the presence of HLA–DRB1 *04:05 and *09:01 alleles was evaluated using a multiplicative model[34,35]. All interaction variables were obtained from logistic regression analysis. We excluded the rare (< 0.5%) HLA–DRB1 alleles (*01:02, *14:02, and *14:06) in the analysis of combination effects of HLA–DRB1 susceptible alleles and smoking. All results were adjusted for age and sex to control for confounding. All statistical analyses were performed with PASW Statistics 18 for Windows (IBM SPSS).

RESULTS

The basic characteristics of RA cases and controls are shown in Table 1.

Effect of RA risk alleles on the development of RA. The RPE method revealed 5 SE-positive alleles (*01:01, *04:01, *04:04, *04:05, and *10:01) and 1 SE-negative allele (*09:01) as RA risk alleles in Koreans (Appendix 1). The 2 most significant risk alleles for RA were HLA–DRB1*04:05

Table 1. Basic characteristics of patients with rheumatoid arthritis (RA) and controls at enrollment to the study.

Characteristic	No. / Total No. (%) RA, n = 1924	Controls, n = 1119
Age, mean ± SD, yrs	51.2 ± 12.3	36.8 ± 12.3
Onset age, mean ± SD, yrs	41.2 ± 12.7	—
Disease duration, mean ± SD, yrs	9.9 ± 8.5	—
Female	1703/1924 (88.5)	976/1119 (87.2)
Ever-smokers	308/1897 (16.2)	139/1101 (12.6)
Anti-CCP	1420/1645 (86.3)	—
Rheumatoid factor	1673/1924 (87.0)	—
HLA–DRB1 shared-epitope†	1289/1924 (67.0)	388/1119 (34.7)
HLA–DRB1*09:01	482/1924 (25.1)	195/1119 (17.4)
ESR, mm/h, mean ± SD	37.1 ± 28.3	—
CRP, mg/dl, mean ± SD	1.3 ± 1.8	—
DAS28-CRP††, mean ± SD	3.8 ± 1.2	—
Radiologic severity#		
Stage 1	498/1918 (25.9)	—
Stage 2	597/1918 (31.1)	—
Stage 3	584/1918 (30.5)	—
Stage 4	239/1918 (12.5)	—
HAQ, mean ± SD	1.0 ± 0.7	—

† HLA-DRB1 shared epitope indicates *01:01, *04:01, *04:04, *04:05, *04:08, *04:10, and *10:01. †† 28-joint Disease Activity Score-CRP was obtained from 434 RA cases. # Stages according to the radiologic criteria of Steinbrocker, *et al*[41]. Stage 1 (early) RA defined as the absence of destructive changes on radiographs; stage 2 (moderate) RA was radiographic evidence of osteoporosis, with or without slight subchondral bone destruction or slight cartilage destruction; stage 3 (severe) RA was radiographic evidence of cartilage and bone destruction, subluxation, or ulnar deviation; stage 4 (terminal) RA was fibrous or bony ankylosis. Anti-CCP: anticyclic citrullinated peptide autoantibodies; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; HAQ: Health Assessment Questionnaire.

(OR 3.38, 95% CI 2.85–4.01, $p = 2.73 \times 10^{-48}$) and *09:01 (OR 1.92, 95% CI 1.61–2.28, $p = 6.33 \times 10^{-14}$), as reported[2,5]. The strongest associations of the 2 alleles with RA were maintained in both anti-CCP-positive and anti-CCP-negative RA groups (Appendix 1).

Effect of smoking on the development of RA. Smoking was independently related to the development of RA using a multiple logistic regression analysis, performed with adjustments for age, sex, and HLA–DRB1 risk alleles (OR 2.19, 95% CI 1.56–3.09). Smoking had significant effects on the risk of anti-CCP-positive RA (OR 1.99, 95% CI 1.37–2.88) and anti-CCP-negative RA (OR 3.39, 95% CI 1.96–5.88), as reported[5].

Effect of smoking on anti-CCP levels. The effects of SE alleles and smoking on anti-CCP levels were investigated in the 1225 anti-CCP-positive patients with RA whose levels were available. The median levels of anti-CCP were higher in the ever-smokers (552 units/ml) than in the never-smokers (427 units/ml), but this difference was not statistically significant (p = 0.16).

Effects of RA risk alleles on anti-CCP levels. It appeared that the presence of any SE allele was associated with markedly increased anti-CCP levels. As shown in Table 2, the anti-CCP levels in the group with SE were much higher than those in the group without SE (527 units/ml vs 316 units/ml, respectively; $p = 1.91 \times 10^{-9}$). A stronger effect on anti-CCP levels was found in the group carrying the double SE (614 units/ml) compared to the group carrying a single SE (493 units/ml). In addition, anti-CCP levels were, significantly, lowest in never-smokers without the SE and highest in smokers with the SE ($p = 2.05 \times 10^{-8}$).

In contrast, the presence of the HLA–DRB1*09:01 allele was strongly associated with reduced levels of anti-CCP: 268 units/ml in the group with HLA–DRB1*09:01 versus 515 units/ml in the group without ($p = 6.49 \times 10^{-10}$). The presence of double copies of the HLA–DRB1*09:01 allele (189 units/ml) was associated with lower anti-CCP levels than for the single copy (304 units/ml).

We then performed a conditional analysis, because this observation could have been due to the strong effect of SE alleles on anti-CCP levels, rather than an effect of the HLA–DRB1*09:01 allele. The HLA–DRB1*09:01 allele significantly decreased anti-CCP levels in both the SE-negative group and the SE-positive group. Interestingly, we found a hierarchy of anti-CCP levels depending upon the particular combination of RA risk alleles ($p = 5.55 \times 10^{-11}$). In the hierarchy, individuals carrying SE/SE had the highest (614 units/ml) and those carrying *09:01/*09:01 had the lowest levels (189 units/ml).

Interactions between RA risk alleles and smoking. We previously demonstrated that SE alleles and smoking were associated with RA susceptibility regardless of anti-CCP status, and that these gene-environmental risk factors interacted only in individuals with anti-CCP[5]. We further evaluated the interactions of each HLA–DRB1 risk allele with smoking for RA susceptibility. All HLA–DRB1 risk alleles increased the risk of RA in smokers (*01:01, OR 9.58, 95% CI 4.85–18.95; *04:05, OR 16.31, 95% CI 9.03–29.46; *10:01, OR 24.71, 95% CI 6.76–90.29; *09:01, OR 7.10, 95% CI 4.06–12.41) compared with nonsmokers without HLA–DRB1 risk alleles without subgrouping for anti-CCP (Appendix 2).

We measured the strength of gene-environment interactions by the method of attributable proportion (AP), relative excess risk due to interaction (RERI), and the synergy index (S). The AP, RERI, and S values for the interactions between all SE alleles and smoking were 0.48 (95% CI 0.26–0.70), 6.76 (95% CI 0.84–12.67), and 2.07 (95% CI 1.32–3.26), respectively, indicating statistical significance. In analyses for each DRB1 subtype, smoking interacted significantly with the *01:01 (AP = 0.45, 95% CI 0.09–0.80; *04:05 (AP = 0.37, 95% CI 0.02–0.73); and *10:01 (AP = 0.71, 95% CI 0.34–1.09), but no significant interaction was found between the *09:01 allele and smoking (AP = 0.15, 95% CI –0.28 to 0.59; Appendix 2). The HLA–DRB1*04

Table 2. Levels of anticyclic citrullinated peptide autoantibodies (anti-CCP) according to HLA–DRB1 risk alleles or smoking in anti-CCP-positive rheumatoid arthritis (RA).

	No. (n = 1225)	Median Level (IQR)	p†	
Shared epitope (SE)				
No	369	316 (135–717)		
Any	856	527 (197–1181)		1.91×10^{-9}
Single	666	493 (183–1112)		1.25×10^{-7}
Double	190	614 (254–1284)		6.82×10^{-8}
Non-SE/never-smoker	320	315 (136–728)		
Non-SE/ever-smoker	46	336 (122–699)		2.05×10^{-8}††
SE/never-smoker	712	493 (188–1133)		
SE/ever-smoker	139	601 (243–1265)		
HLA–DRB1*09:01				
No	937	515 (201–1103)		
Any	288	268 (121–717)		6.49×10^{-10}
Single	262	304 (123–742)		5.01×10^{-8}
Double	26	189 (77–401)		1.03×10^{-4}
SE/SE	190	614 (254–1284)		
SE/non-SE	555	535 (204–1141)	1.64×10^{-3}††	
Non-SE/non-SE	192	366 (157–867)		5.55×10^{-11}††
SE/*09:01	111	365 (122–927)		
Non-SE/*09:01	151	268 (125–618)	0.05††	
*09:01/*09:01	26	189 (77–401)		

† Comparing each group with the corresponding reference group [never-smokers group, no shared epitope (SE), or no *09:01 allele] by Mann-Whitney U test. (Anti-CCP levels were available for 1225 anti-CCP-positive patients with RA. Eight subjects whose smoking history was unavailable were excluded from the analysis. Non-SE indicates the absence of *09:01 allele and SE alleles. †† Kruskal-Wallis test. IQR: interquartile range.

risk group (*04:01, *04:04, *04:05, *04:08, *04:10) was found to have a significant interaction with smoking (AP = 0.45, 95% CI 0.16–0.73).

In further subgroup analysis according to anti-CCP status, the SE alleles interacted strongly with smoking in anti-CCP-positive RA (AP = 0.48, 95% CI 0.25–0.71) by all additive methods (AP, RERI, S) and slightly in anti-CCP-negative RA (AP = 0.43, 95% CI 0.02–0.81) only by the method of AP (Table 3). In addition, each of the SE alleles interacted significantly with smoking in anti-CCP-positive RA, as follows: *01:01, AP = 0.48, 95% CI 0.12–0.84; *04:05, AP = 0.37, 95% CI 0.01–0.74; and *10:01, AP = 0.76, 95% CI 0.42–1.08. In anti-CCP-negative RA, only *10:01 interacted with smoking (AP = 0.70, 95% CI 0.12–1.28). However, HLA–DRB1*09:01 did not interact with smoking in either anti-CCP-positive (AP = 0.16, 95% CI –0.32 to 0.65) or negative RA groups (AP = 0.28, 95% CI –0.32 to 0.87).

*Contribution of HLA-DRB1*04:05/*09:01 heterozygote and smoking to RA*. We previously showed that the heterozygote for HLA–DRB1*04:05/*09:01 dramatically increased the risk of RA in Koreans, and the risk tended to be higher than for either homozygote (HLA–DRB1 *04:05/*04:05 or *09:01/*09:01)[2]. In the present study, we tested whether the synergistic effect of *04:05/*09:01 heterozygote was sustained after stratification according to anti-CCP and smoking status.

Both alleles of heterozygote *04:05/*09:01 appear to be synergistically interactive for increasing RA susceptibility in anti-CCP-positive RA (AP = 0.66, 95% CI 0.43–0.89) and anti-CCP-negative RA (AP = 0.73, 95% CI 0.49–0.96; Table 4). Moreover, *04:05 and *09:01 were synergistically interactive regardless of smoking status: for the never-smokers group AP = 0.65 (95% CI 0.40–0.89), and for ever-smokers AP = 0.82 (95% CI 0.45–1.20; Appendix 3).

The *04:05/*09:01 heterozygote increased risk in smokers with RA who were anti-CCP-positive (OR 67.46, 95% CI 8.34–545.62) and anti-CCP-negative (OR 65.92, 95% CI 6.60–657.94) compared with nonsmokers without the HLA-DRB1 risk alleles; the heterozygote for the HLA–DRB1*04:05/*09:01 alleles also conferred a significantly higher risk than that of the double homozygote (HLA–DRB1*04:05/*04:05 or *09:01/*09:01) in both anti-CCP-positive (OR 10.43, 95% CI 2.55–42.65) and -negative (OR 13.15, 95% CI 2.45–70.63; Appendix 4).

In the analysis of interaction between the *04:05/*09:01 heterozygote and smoking, statistical significance was attained only in the anti-CCP-negative RA. In the anti-CCP-negative RA, all 3 genotypes consisting of HLA–DRB1 *04:05 and/or *09:01 (*04:05/*04:05, *09:01/*09:01, and *04:05/*09:01) showed significant interaction with smoking; that is, for all 3 genotypes (AP = 0.74, 95% CI 0.38–1.10), homozygote (*04:05/*04:05 and *09:01/*09:01, AP = 0.79, 95% CI 0.43–1.15), and heterozygote (*04:05/*09:01, AP = 0.80, 95% CI 0.32–1.28).

Table 3. Risk of having anticyclic citrullinated peptide autoantibody (anti-CCP)-positive and anti-CCP-negative RA according to HLA–DRB1 and smoking status.

HLA-DRB1/ Smoking	Anti-CCP-positive RA No. Cases/ Controls	Anti-CCP-positive RA OR† (95% CI)	Anti-CCP-negative No. Cases/ Controls	Anti-CCP-negative OR† (95% CI)
SE				
–/–	188/502	reference	58/502	reference
–/+	30/63	2.17 (1.25–3.77)	13/63	3.76 (1.74–8.15)
+/–	826/328	7.28 (5.85–9.06)	99/328	2.66 (1.87–3.79)
+/+	166/46	16.31 (9.96–26.74)	22/46	9.05 (4.43–20.37)
AP††		0.48 (0.25–0.71)		0.43 (0.02–0.81)
RERI††		7.87 (0.53–15.21)		4.08 (–2.34–10.50)
S††		2.06 (1.28–3.31)		1.92 (0.84–4.41)
HLA–DRB1 *01:01				
–/–	188/502	reference	58/502	reference
–/+	30/63	2.67 (1.43–4.97)	13/63	3.34 (1.43–7.78)
+/–	200/124	4.54 (3.39–6.08)	26/124	1.87 (1.15–3.22)
+/+	42/17	12.02 (5.69–25.39)	5/17	4.92 (1.43–16.91)
AP††		0.48 (0.12–0.84)		0.14 (–0.88–1.17)#
RERI††		5.81 (–2.56–14.17)		0.71 (–5.07–6.50)
S††		2.11 (0.99–4.54)		1.22 (0.27–5.62)
HLA–DRB1*04:05				
–/–	188/502	reference	58/502	reference
–/+	30/63	2.06 (1.15–3.67)	13/63	3.40 (1.53–7.57)
+/–	516/142	11.18 (8.60–14.54)	58/142	3.68 (2.44–5.57)
+/+	97/22	19.50 (10.43–36.45)	13/22	9.95 (4.03–24.54)
AP††		0.37 (0.01–0.74)		0.38 (–0.13–0.90)#
RERI††		7.26 (–4.24–18.75)		3.86 (–4.30–12.03)
S††		1.65 (0.88–3.07)		1.76 (0.68–4.53)
HLA–DRB1*10:01				
–/–	188/502	reference	58/502	reference
–/+	30/63	2.33 (1.20–4.50)	13/63	3.24 (1.34–7.87)
+/–	97/34	7.60 (4.91–11.76)	7/34	1.69 (0.72–4.01)#
+/+	22/3	36.39 (9.29–142.61)	2/3	13.06 (1.75–98.67)#
AP††		0.76 (0.42–1.08)		0.70 (0.12–1.28)
RERI††		27.46 (–21.69–76.62)		9.22 (–16.55–34.99)
S††		4.46 (1.10–18.08)		4.14 (0.48–35.65)
HLA–DRB1*09:01				
–/–	188/502	reference	58/502	reference
–/+	30/63	2.54 (1.41–4.60)	13/63	2.90 (1.30–6.45)
+/–	292/162	4.97 (3.83–6.44)	41/162	2.19 (1.41–3.40)
+/+	47/33	7.77 (4.12–14.63)	13/33	5.66 (2.38–13.45)
AP††		0.16 (–0.32–0.65)#		0.28 (–0.32–0.87)#
RERI††		1.25 (–3.21–5.72)		1.57 (–2.85–0.87)
S††		1.23 (0.63–2.38)		1.51 (0.54–4.20)

† OR were calculated by comparing each group with the corresponding reference group [individuals without HLA–DRB1 risk alleles (*01:01, *04:01, *04:04, *04:05, *04:08, *04:10, *09:01, *10:01)], adjusted for age and sex. HLA–DRB1 shared epitope (SE) indicates *01:01, *04:01, *04:04, *04:05, *04:08, *04:10, and *10:01. Subjects with no available smoking history or the rare alleles (*0102, *1402, and *1406) were excluded from the analysis. †† The attributable proportions (AP), relative excess risk due to interaction (RERI), and synergy index (S) were calculated for gene-environmental interaction. # Not statistically significant ($p > 0.05$). RA: rheumatoid arthritis.

DISCUSSION

In this study, the *04:05/*09:01 heterozygote strongly increased the risk of RA with multiplicative interaction between 2 risk alleles. We further demonstrated that the synergistic effect of this heterozygote persisted regardless of anti-CCP and smoking status. Interestingly, we observed that there was significant interaction between *04:05/*09:01 heterozygote and smoking in anti-CCP-negative RA (Table 4).

Even though *09:01 is the significant risk allele for anti-CCP-positive RA in North American Native populations[9] and for anti-CCP-negative RA in Japanese[10], the relative risk is modest compared to that for SE alleles, especially *04:05 in Asians.

It has been suggested that SE alleles bind citrullinated peptides with higher affinity[36] and make them more immunogenic, which leads to anti-CCP formation[23].

Table 4. Synergistic effect of having HLA–DRB1 *04:05 and *09:01 alleles in RA according to anticyclic citrullinated peptide autoantibodies (anti-CCP) status.

Genotype	RA		Anti-CCP-positive RA		Anti-CCP-negative RA	
	No. Cases/ Controls	OR † (95% CI)	No. Cases/ Controls	OR† (95% CI)	No. Cases/ Controls	OR† (95% CI)
Non-risk/non-risk	344/574	reference	220/574	reference	71/574	reference
*09:01/non-risk	251/151	2.72 (2.12–3.47)	173/151	2.94 (2.24–3.87)	31/151	1.63 (1.03–2.59)
*04:05/non-risk	465/135	6.08 (4.79–7.72)	359/135	7.54 (5.82–9.77)	51/135	3.11 (2.07–4.67)
*09:01/*09:01	41/11	6.36 (3.20–12.63)	29/11	6.92 (3.36–14.24)	2/11	1.44 (0.31–6.64)#
*04:05/*04:05	76/9	15.22 (7.48–30.94)	61/9	20.22 (9.77–41.82)	2/9	1.85 (0.39–8.74)#
*04:05/*09:01	127/10	24.09 (12.41–46.79)	90/10	28.03 (14.17–55.45)	16/10	13.66 (5.93–31.44)
AP††		0.68 (0.46–0.89)		0.66 (0.43–0.89)		0.73 (0.49–0.96)
RERI††		16.38 (0.48–32.27)		18.69 (–0.28–37.66)		9.98 (–1.23–21.20)
S††		3.40 (1.68–6.88)		3.19 (1.57–6.52)		4.63 (1.75–12.26)
Multiplicity††		p = 0.012		p = 0.025		p = 4.32×10^{-4}

† OR were calculated by comparing each group with the corresponding reference group [individuals without HLA-DRB1 risk alleles (*01:01, *04:01, *04:04, *04:05, *04:08, *04:10, *09:01, *10:01)] and were adjusted for age and sex. Non-risk alleles indicates the absence of risk alleles, SE, and *09:01. †† The attributable proportions (AP), relative excess risk due to interaction (RERI), synergy index (S) and multiplicity were calculated for interaction between *04:05 and *09:01 allele. # These values were not statistically significant (p > 0.05). RA: rheumatoid arthritis.

Smoking also promotes nonspecific citrullination of proteins that predispose to anti-CCP formation in individuals with the SE background[37]. Therefore, these 2 gene-environmental risk factors can interact synergistically through citrullination and subsequent autoantibody formation, as shown in previous and our current studies. However, SE, *09:01, and smoking are also risk factors in anti-CCP-negative RA, suggesting these risk factors also participate through unknown mechanisms unrelated to anti-CCP formation prior to development of RA.

We have shown that SE alleles were associated with markedly increased anti-CCP levels, with a gene-dosage effect in anti-CCP-positive RA (Table 2), confirming the previous observation of a positive correlation between SE alleles and anti-CCP levels. Conversely, HLA–DRB1 *09:01, the second most significant risk allele for RA in the Korean population, was significantly associated with reduced anti-CCP levels, in agreement with a Japanese study[18]. We demonstrated the existence of a clear hierarchy of anti-CCP levels according to SE alleles and the *09:01 allele (Table 2).

We previously observed that HLA–DRB1 SE alleles and smoking were associated with RA susceptibility in anti-CCP-positive as well as anti-CCP-negative RA[5], in contrast to many studies in whites, in which both SE and smoking contributed only to anti-CCP-positive RA[12,14,16]. In the present study, we confirmed that the 2 most significant HLA–DRB1 risk alleles, 04:05 and *09:01, increased susceptibility to RA in both anti-CCP-positive and anti-CCP-negative cases.

In addition, significant interactions between SE alleles and smoking were found in RA, but stronger effects were seen in anti-CCP-positive RA than anti-CCP-negative RA. Interestingly, the interaction between HLA–DRB1*10:01 and smoking was stronger than that for other SE alleles, as shown in a previous study[25] and also as observed in both anti-CCP-positive and negative RA (Table 3). It is currently unclear whether this observation might be due to different amino acids at positions 70 and 71 among SE alleles, which influence antigen-binding affinity. In contrast, the *09:01 allele itself did not interact significantly with smoking in either anti-CCP-positive or anti-CCP-negative RA.

As summarized above, several distinctive characteristics of *09:01 compared to SE have emerged from our study. First, *09:01 showed different effects on anti-CCP levels compared to SE alleles, as a replication of previous findings in other populations. Second, there was no interaction of *09 itself with smoking in either anti-CCP-positive or anti-CCP-negative RA. Third, the heterozygote *04:05/ *09:01 interacted with smoking only in anti-CCP-negative RA. All these findings suggest that *09:01 confers the risk for RA through different means from those of the SE alleles in anti-CCP-positive and -negative RA. The *09:01 allele may not be involved in anti-CCP formation even among anti-CCP-positive RA cases, but instead by some unknown mechanism. Although SE and smoking may have a pathway independent of the anti-CCP production as noted above, it may be minor compared to anti-CCP formation. However, the main mechanism by which the *09:01 allele contributes to RA might bypass anti-CCP formation. This hypothesis was supported by our observation that the *04:05/*09:01 heterozygote significantly increased RA susceptibility in both anti-CCP-positive and -negative RA, which may be attributable to the synergistic contribution of 2 different pathways in which 2 alleles participate independently.

The heritability of anti-CCP-positive RA (68%) and anti-CCP-negative RA (66%) were reported to be similar in

a twin study, suggesting that genetic predisposition plays an important role in the pathogenesis of each of them[38]. However, the extent of the contribution of SE alleles to genetic variance was shown to differ between the 2 types of RA: 18% in anti-CCP-positive RA but only 2.4% in anti-CCP-negative RA[38]. If *09:01 were analyzed together with SE in both anti-CCP-positive and -negative RA, the missing genetic heritability would be reduced, at least in the Korean population. In a recent study, amino acid residues at positions 11, 71, and 74 within peptide-binding grooves explained the effects of HLA-DRB1 on antigenic peptide presentation to T cells in RA[31]. While SE had the hydrophobic residues Val and Leu at position 11, HLA-DRB1*09:01 had Asp at position 11 and was found to be associated with RA (OR = 1.65)[31]. Fujisao, *et al*[39] reported that residues on HLA–DRB1*09:01 had more hydrophilic residues at the second pocket compared to HLA–DRB1*01:01, *04:01, and *04:05. Whether this difference may lead DR9 peptide motifs to bind specific autoantigens that are not related to citrullinated peptide and then to influence T cell responses, especially in anti-CCP-negative RA, remains to be defined.

A limitation of our study is that the number of individuals was large, but the numbers of smokers or those bearing the *04:05/*09:01 heterozygote among controls were very small. The prevalence of anti-CCP-positive RA in our cohort (86.3%; 1420/1645) was higher than that observed in other studies, but was similar to that seen in the Korean Observational Study Network for Arthritis (86.8%; 4098/4719)[40], a prospective multicenter cohort for RA.

We did not observe significant interaction of the *04:05/*09:01 heterozygote with smoking in anti-CCP-positive RA or interaction of each of the SE alleles (except *10:01) with smoking in anti-CCP-negative RA, probably because of the lack of power of our study. Additional studies are needed to confirm these findings in other populations.

Our results have shown that the *09:01 allele may not be involved in the production of anti-CCP and does not interact with smoking on its own. However, the *04:05/*09:01 heterozygote potentiates the risk of RA irrespective of anti-CCP status, and interacts strongly with smoking in anti-CCP-negative RA, suggesting a potential synergistic effect of this combination of risk factors that may bypass anti-CCP formation. Our findings may provide clues to identifying the distinctive mechanisms of anti-CCP-negative RA.

Appendix 1. Risks for HLA–DRB1 susceptible alleles in anticyclic citrullinated peptide autoantibody (anti-CCP) -positive and -negative rheumatoid arthritis (RA).

HLA–DRB1†	No. Cases	OR (95% CI)	p††
Anti-CCP-positive RA (2n = 2840)			
*04:05	675	3.61 (3.02–4.30)	1.23×10^{-50}
*09:01	369	1.85 (1.54–2.22)	2.48×10^{-12}
*10:01	120	3.33 (2.31–4.81)	1.25×10^{-11}
*01:01	252	1.99 (1.61–2.47)	1.79×10^{-10}
*04:04	61	4.10 (2.41–6.97)	1.84×10^{-8}
*04:01	53	4.41 (2.47–7.85)	4.30×10^{-8}
*04:10	45	2.87 (1.69–4.89)	4.96×10^{-5}
Anti-CCP-negative RA (2n = 450)			
*04:05	73	2.24 (1.67–3.01)	3.78×10^{-8}
*09:01	56	1.57 (1.14–2.16)	5.15×10^{-3}

† DRB1 alleles were sorted by the relative predispositional effects (RPE) method in RA patients and controls. †† Threshold values were calculated according to the procedure of Benjamini and Hochberg for controlling the Bonferroni method.

ACKNOWLEDGMENT

The authors are grateful to their colleagues for assistance with sample preparation, data collection, and technical studies.

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Appendix 2. Risk of developing RA according to HLA-DRB1 risk alleles and smoking status.

HLA-DRB1/ Smoking	No. Cases	OR (95% CI)
SE (*01:01, *04:01, *04:04, *04:05, *04:08, *04:10, *10:01)		
–/–	287	reference
–/+	52	2.38 (1.47–3.85)
+/–	1054	5.91 (4.86–7.19)
+/+	217	14.05 (8.85–22.30)
AP††		0.48 (0.26–0.70)
RERI††		6.76 (0.84–12.67)
S††		2.07 (1.32–3.26)
*01:01		
+/–	260	3.72 (2.85–4.86)
+/+	57	9.58 (4.85–18.95)
AP††		0.45 (0.09–0.80)
*04:05		
+/–	662	9.01 (7.09–11.46)
+/+	125	16.31 (9.03–29.46)
AP††		0.37 (0.02–0.73)
*10:01		
+/–	115	5.79 (3.82–8.77)
+/+	26	24.71 (6.76–90.29)
AP††		0.71 (0.34–1.09)
*09:01		
+/–	401	4.41 (3.48–5.59)
+/+	72	7.10 (4.06–12.41)
AP††		0.15 (–0.28–0.59)#
RERI††		1.09 (–2.52–4.71)
S††		1.22 (0.67–2.22)

† OR were calculated by comparing each group with the corresponding reference group [individuals without HLA–DRB1 risk alleles (*01:01, *04:01, *04:04, *04:05, *04:08, *04:10, *09:01, *10:01)], and were adjusted for age and sex. †† The attributable proportions (AP), relative excess risk due to interaction (RERI), and synergy index (S) were calculated for gene-environmental interaction. # Not statistically significant ($p > 0.05$). RA: rheumatoid arthritis.

Appendix 3. Synergistic effect of having *04:05 and *09:01 alleles in RA according to smoking status.

Genotype	RA		Never-smokers		Ever-smokers	
	No. Cases	OR † (95% CI)	No. Cases	OR † (95% CI)	No. Cases	OR † (95% CI)
Non-risk/non-risk	344	reference	287	reference	52	reference
*09:01/non-risk	251	2.72 (2.12–3.47)	211	2.93 (2.24–3.83)	35	1.59 (0.79–3.20)#
*04:05/non-risk	465	6.08 (4.79–7.72)	384	6.14 (4.74–7.94)	82	7.04 (3.44–14.43)
*09:01/*09:01	41	6.36 (3.20–12.63)	37	7.39 (3.50–15.59)	4	1.35 (0.20–8.84)#
*04:05/*04:05	76	15.22 (7.48–30.94)	68	18.49 (8.33–41.05)	7	12.27 (1.25–120.25)
*04:05/*09:01	127	24.09 (12.41–46.79)	106	22.71 (11.25–45.81)	18	42.92 (5.22–352.80)
AP††		0.68 (0.46–0.89)		0.65 (0.40–0.89)		0.82 (0.45–1.20)
SERI††		16.38 (0.48–32.27)		14.75 (1.11–30.62)		36.21 (–55.72–128.14)
S††		3.40 (1.68–6.88)		3.09 (1.46–6.51)		6.39 (0.72–56.90)

† OR were calculated by comparing each group with the corresponding reference group [individuals without HLA-DRB1 risk alleles (*01:01, *04:01, *04:04, *04:05, *04:08, *04:10, *09:01, *10:01)], and were adjusted for age and sex. Non-risk alleles indicates the absence of risk alleles, SE, and *09:01. †† The attributable proportions (AP), relative excess risk due to interaction (RERI), and synergy index (S) were calculated for interaction between *04:05 and *09:01 allele. # Not statistically significant ($p > 0.05$). RA: rheumatoid arthritis.

Appendix 4. Risk of developing RA in smokers exposed to different combinations of *04:05 and *09:01 alleles.

*04:05, *09:01/Smoking	Anti-CCP-positive RA		Anti-CCP-negative RA	
	No. Cases	OR† (95% CI)	No. Cases	OR† (95% CI)
No/–	188	reference	58	reference
No/+	30	2.20 (1.27–3.82)	13	2.99 (1.38–6.47)
Single/–	570	5.95 (4.75–7.47)	74	2.39 (1.64–3.47)
Single/+	112	10.33 (6.32–16.87)	15	4.47 (2.05–9.73)
Double/–	160	19.86 (12.49–31.56)	13	4.65 (2.25–9.62)
Double/+††	20	23.93 (7.54–75.96)	7	24.22 (6.33–92.68)
Heterozygote/–	78	27.85 (13.51–57.39)	12	12.72 (5.08–31.86)
Heterozygote/+	12	67.46 (8.34–545.62)	4	65.92 (6.60–657.94)

† OR were calculated by comparing each group with the corresponding reference group [individuals without HLA-DRB1 risk alleles (*01:01, *04:01, *04:04, *04:05, *04:08, *04:10, *09:01, *10:01)], and were adjusted for age and sex. †† Double indicates DRB1 *04:05/*04:05 or *09:01/*09:01 or *04:05/*09:01. Heterozygote indicates DRB1*04:05/*09:01. Anti-CCP: anticyclic citrullinated peptide autoantibodies; RA: rheumatoid arthritis.

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