

Association of HLA-DRB1*07 and DRB1*04 to citrus red mite (*Panonychus citri*) and house dust mite sensitive asthma

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Summary

Background Specific IgE responses to allergens provide useful models for evaluating the genetic factors that control human immune responses. A recent survey demonstrated that the citrus red mite (*Panonychus citri*, CRM) is the most important allergen in the development of asthma in citrus farmers.

Objective The aim of this study was to evaluate whether susceptibility or resistance to CRM-induced asthma was associated with HLA-DRB1 gene.

Methods DNAs were extracted from two groups of unrelated Korean adults living around citrus farms: (1) Ninety-one adults with CRM-sensitive asthma; and (2) 98 exposed, healthy nonatopic controls. Genotypes of HLA-DRB1 alleles were carried out using PCR-based methods.

Results Allelic frequency of HLA-DRB1*07 was higher in the CRM-sensitive asthmatics compared to the controls (17.6% vs 4.1%, $P=0.01$). Conversely, the frequency of DRB1*04 was lower in the CRM-sensitive asthmatics compared to the controls (19.8% vs 40.8%, $P=0.01$). No significant difference was found in the distributions of the other HLA-DRB1 gene-encoded antigens between the two groups.

Conclusion HLA-DRB1 genes may be involved in the development of CRM-induced asthma. In addition, HLA-DR7 may increase, and DR4 decrease, the risk of developing the asthma in CRM-exposed adults.

Keywords: citrus red mite, asthma, HLA-DRB1, genetic, house dust mite, spider mite

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Introduction

House dust mites (HDMs) are major causes of asthma in subtropical areas including Korea [1]. We previously reported that the citrus red mite (*Panonychus citri*, CRM) was a very important allergen in citrus-cultivating farmers with asthma [2]. Asthma is associated with eosinophil-rich inflammation of the bronchial mucosa [3]. Specific IgE responses to allergens are believed to play a role in the pathogenesis of asthma, since exposure to allergens causes immediate bronchoconstriction and later allergic

inflammation [4], whereas removal of allergen exposure ameliorates the disease [5]. Numerous population and family studies have suggested that both genetic and environmental influences are involved in the pathogenesis of asthma [6,7]. Despite ample evidence for heritability of asthma, genetic analysis has been difficult and contradictory.

Human leucocyte antigen (HLA) class II genes encoding for HLA class II molecules are likely candidates for controlling the immune response to allergens. Many associations between class II alleles and specific IgE responses to specific allergens have been investigated. In general, significant associations are found only with highly purified simple allergens, and not with more complex ones [8–10].

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However, to the best of our knowledge, there has been no study linking CRM-induced asthma with HLA class II genes, although a few studies have linked HLA class II genes directly with allergic asthma [11–13]. Our study was designed to investigate whether susceptibility or resistance to CRM-induced asthma was associated with HLA-DRB1 alleles. To test this hypothesis, the distributions of HLA-DRB1 alleles were determined by polymerase chain reaction (PCR)-based methods in patients with CRM-sensitive asthma and in CRM-exposed, healthy controls.

Materials and methods

Subjects

Ninety-one CRM-sensitive asthmatic patients and 98 exposed, healthy control subjects were recruited for this study. They were unrelated subjects living around citrus farms on Cheju Island, Korea. The mean age of the CRM-sensitive asthmatic patients was 40.9 (range 21–68) years with 44 males and 47 females. The diagnosis of CRM-sensitive asthma was based on the following: (1) documented exposure to CRM; (2) typical asthmatic symptoms (paroxysmal cough, wheezing, nocturnal dyspnea, or dyspnea at rest) with aggravation after work on citrus farms; (3) bronchial hyperresponsiveness to methacholine challenge performed according to standard procedures (PC₂₀-methacholine less than 16 mg/mL) [14] or positive airway reversibility after antiasthmatic treatment (improvement of FEV₁ more than 15% of predictive value); (4) positive skin-prick test response to CRM (allergen/histamine ratio ≥ 1.0).

The mean age of the healthy control subjects was 36.8 (range 21–65) years with 47 males and 51 females. They had been exposed to the same environment as the CRM-sensitive asthmatic patients since childhood. They revealed no asthmatic symptoms on the questionnaire, which was based on the Respiratory Disease Questionnaires of the American Thoracic Society [15], and had negative skin prick test responses to common inhalant allergens including CRM. All the subjects gave informed consent, and the study protocol was approved by the Ethics Committee of Seoul National University Hospital.

Preparation of CRM extract

CRM extracts were prepared as previously described [16]. In brief, the mites were mixed with modified Coca's solution in 1:5 w/v ratio. After incubation at room temperature for 24 h, the mixture was centrifuged at 3000 g for 30 min. The supernatant was dialysed (the cut-off molecular weight was 6000 Da) against 4 L of distilled water for

48 h at 4°C, and then the same amount of glycerin was added with a final concentration of 1:20 w/v ratio. This was used for the skin prick tests.

Skin prick tests

All the subjects underwent skin prick tests using a panel of 11 inhalant allergens (Allergopharma Co., Germany) and CRM. None of the subjects had received antihistamines orally for three days preceding the study. The commercially available panel included *Dermatophagoides pteronyssinus*, *D. farinae*, cat fur, molds (*Alternaria* spp. and *Aspergillus* spp.), pollens (two mixtures of early blooming trees and late blooming trees, grass pollen mixture, mugwort, and ragweed), and cockroach. A positive control of histamine (1 mg/mL, Allergopharma Co., Germany), along with a negative control of diluent (Allergopharma Co., Germany), was included in all the tests. Skin prick tests were performed on the volar side of both forearms. Fifteen minutes after the skin prick, the mean diameter of a wheal formed by the allergen was compared with that formed by histamine. If the former was the same or larger than the latter (allergen/histamine ratio ≥ 1.0), the reaction was defined as positive.

DNA isolation

Genomic DNA from peripheral blood mononuclear cells of DP-sensitive subjects and the controls was extracted using the Wizard Genomic DNA Purification Kit (Promega Co., WI, USA) as directed by the supplier.

Low-resolution analysis of HLA-DRB1 alleles: PCR with sequence-specific oligonucleotide probe (SSOP)

Low-resolution analysis of HLA-DRB1 alleles was performed using the Dynal RELITM SSO HLA-DRB test (Dynal A.S., Oslo, Norway). PCR amplification of the second exon of the DRB1 gene was performed in a PCR reaction mixture, consisting of: 25 μ L DNA (0.5 μ g), 50 μ L master mix (Tris solution containing 10% glycerol, KCl, dATP, dCTP, dGTP, dUTP, biotinylated primers, AmpErase, AmpliTaq, and 0.05% sodium azide), and 25 μ g of MgCl₂. Amplification in a thermal cycler (Perkin Elmer, CA, USA) was performed, as follows: 35 cycles of 15 s at 95°C, 45 s at 60°C, and 15 s at 72°C after an initial holding process of 2 min at 50°C. The reactions were terminated at 72°C after a final elongation process of 5 min at 72°C.

Three ml of prewarmed hybridization buffer (50°C) was added to each well, and 70 μ L of denatured amplified sample or control added and hybridized at 30 g for 30 min using specific oligonucleotide probes as directed by the

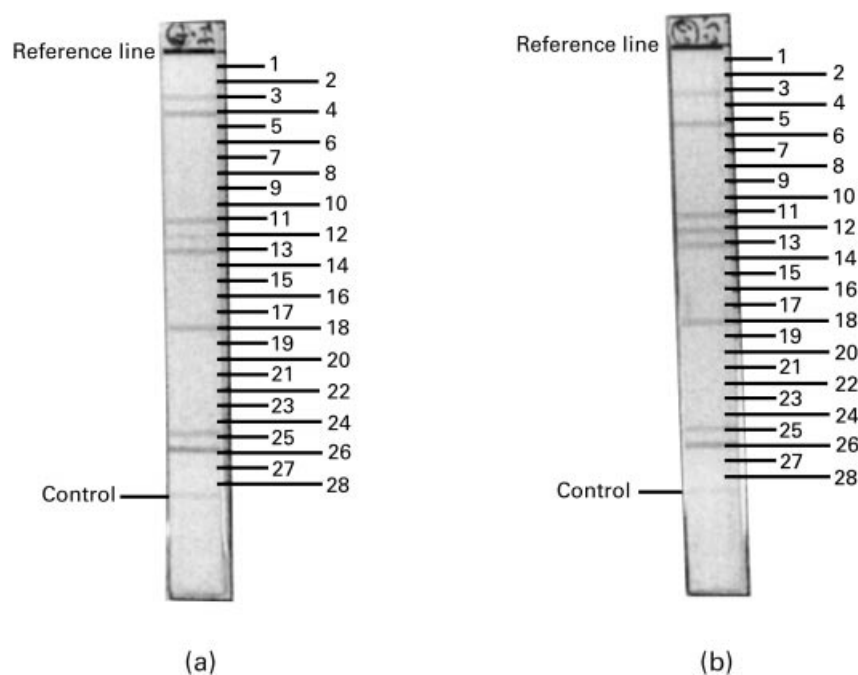


Fig. 1. PCR-SSOP low-resolution analysis of HLA-DRB1 alleles. (a) DRB1*04/DRB1*13, (b) DRB1*07/DRB1*13.

supplier. Genotypes of HLA-DRB1 alleles were interpreted using the Dynal FELI™ SSO Pattern Matching Computer Program (Fig. 1).

*High-resolution analysis of HLA-DRB1*04: PCR with single strand conformation polymorphism (SSCP)*

Samples typed as HLA-DRB1*04 using the PCR-SSOP, DRB1*04 subtypes (*0401–*0411) were discriminated using PCR-SSCP. PCR amplification of the second exon of the DRB1 gene was performed in 10 μ L of a PCR reaction mixture, consisting, 1 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.3), 10 mM ammonium sulphate, 20 mM of each of dATP, dCTP, dGTP, and dTTP, 0.5 mM of each primer (Table 1), 30 ng of template DNA, and 0.2 U of Taq DNA polymerase (Boehringer Mannheim, CA, USA). They were mixed gently, overlaid with 10 μ L of mineral oil, and then subjected to thermal cycling (Perkin Elmer, CA, USA). After preheating for 5 min at 95°C, 30 amplification cycles were performed, followed by incubation for 5 min at 72°C. Each amplification cycle consisted of two steps: 1 min at 95°C, for denaturing and 1 min at 58°C, to anneal and polymerase. Amplification of DNAs was verified using 5% polyacrylamide gel electrophoresis and ethidium bromide staining.

SSCP analysis was performed according to the method described by Bannai *et al.* [17] with a few modifications. Briefly, one ml of amplified solution was mixed with 7 μ L of denaturing solution (95% formaldehyde, 20 mM EDTA,

0.05% bromophenol blue, and 0.05% xylene cyanol FF). The mixtures were denatured for 5 min at 95°C, immediately cooled on ice, 2 μ L of the samples were then applied to a 10% polyacrylamide gel (acrylamide:bisacrylamide = 49:1, with 5% glycerol). A minigel electrophoresis apparatus with a constant temperature control system (90 $\times$ 80 $\times$ 1 mm, AE-6410, ATTO, Japan) was used. Electrophoresis was carried out in 45 mM Tris-borate (pH 8.0)/1 mM EDTA at 22°C. Single stranded DNA fragments in the gel were detected by silver staining (Daiichi Pure Chemicals, Tokyo, Japan). HLA-DRB1 subtypes (DRB1*0401–0411) were identified by comparison with reference DNA DR types (UCLA DNA Reference Panel, UCLA, LA, USA) (Fig. 2).

Table 1. Primers for DRB1*04 subtyping by PCR with single strand conformation polymorphism (SSCP)

Name	Primer sequence	Base position
<i>Sense primer</i>		
DRBAMP-4	GTTCTTGGAGCAGGTAA	17–37
<i>Antisense primer</i>		
DRBAMP-B	CCGCTGCACTGTGAAGCTCT	260–279

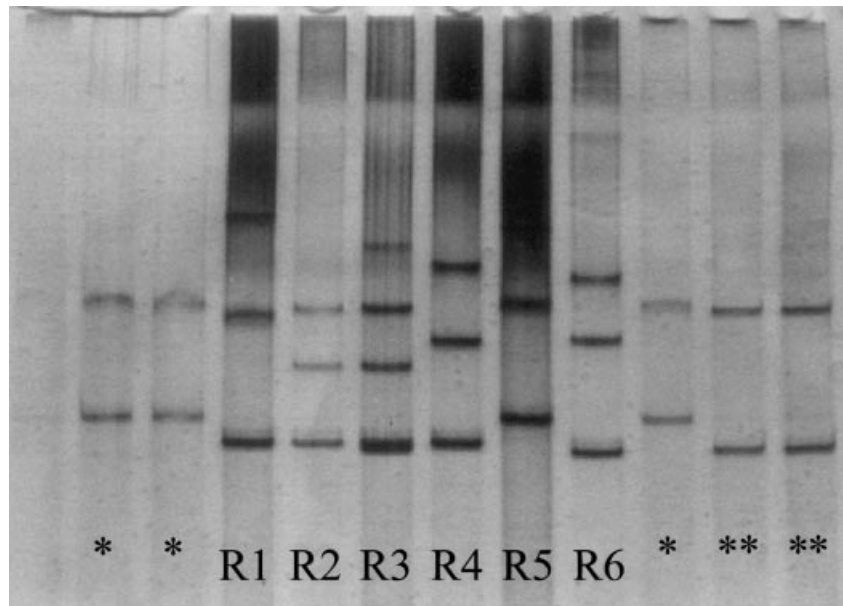


Fig. 2. PCR-SSCP high-resolution analysis of HLA-DRB1*04 subtypes. R1–6: reference DNA DR types. *HLA-DRB1*0405, **HLA-DRB1*0403.

Statistical analysis

HLA-DRB1 alleles were determined by direct counting. Allelic frequency (%) was calculated as follows:

$$GF = n/N \times 100$$

GF = genotype frequency

n = number of individuals positive for each genotype

N = total number of individuals tested

The allele frequencies were compared using the chi-square test, and odds ratio (OR) was calculated. When

either value was below 5, Fisher's exact test was used. P -values were corrected for the number of comparisons (P_c) which were considered significant when $P_c < 0.05$.

Results

The distribution of HLA-DRB1 genotypes among the patients with CRM-sensitive asthma and the control subjects is shown in Table 2. The frequency of subjects carrying HLA-DRB1*07 allele was significantly higher among

Table 2. Allelic frequency of HLA-DRB1 gene in citrus red mite sensitive asthmatics and controls

Alleles	Patients ($n = 91$)		Controls ($n = 98$)		OR (95% CI)	P_c^*
	No.	GF (%)	No.	GF (%)		
DRB1*01	20	22.0	21	21.4		NS
DRB1*02	20	22.0	21	21.4		NS
DRB1*03	8	8.8	4	4.1		NS
DRB1*04	18	19.8	40	40.8	0.36 (0.31–0.42)	0.01
DRB1*07	16	17.6	4	4.1	5.01 (3.65–6.87)	0.01
DRB1*08	18	19.8	19	19.4		NS
DRB1*09	17	18.7	18	18.4		NS
DRB1*10	3	3.3	1	1.0		NS
DRB1*11	2	2.2	3	3.1		NS
DRB1*12	11	12.1	14	14.3		NS
DRB1*13	24	26.4	17	17.3		NS
DRB1*14	18	19.8	23	23.5		NS

GF: genotype frequency; NS: not significant; OR: odds ratio * P corrected for the number of alleles (12) tested.

Table 3. Allelic frequency of HLA-DRB1 gene in asthmatics with positive skin responses both to citrus red mite and house dust mite

Alleles	Patients* (n = 51)		Controls (n = 98)		OR (95% CI)	Pc†
	No.	GF (%)	No.	GF (%)		
DRB1*01	9	17.6	21	21.4		NS
DRB1*02	10	19.6	21	21.4		NS
DRB1*03	5	9.8	4	4.1		NS
DRB1*04	7	13.7	40	40.8	0.23 (0.20–0.27)	0.008
DRB1*07	10	19.6	4	4.1	5.73 (4.18–7.86)	0.02
DRB1*08	13	25.5	19	19.4		NS
DRB1*09	11	21.6	18	18.4		NS
DRB1*10	2	3.9	1	1.0		NS
DRB1*11	2	3.9	3	3.1		NS
DRB1*12	7	13.7	14	14.3		NS
DRB1*13	12	23.5	17	17.3		NS
DRB1*14	8	15.7	23	23.5		NS

GF: genotype frequency; NS: not significant; OR: odds ratio. *Citrus red mite-sensitive asthmatics with positive skin response both to citrus red mite and house dust mites. †P corrected for the number of alleles (12) tested.

the patient group than among the controls [17.6% vs 4.1%, OR = 5.01 (95% CI 3.65–6.87), $P_c = 0.01$]. In addition, the HLA-DRB1*04 allele frequency was significantly lower among the patients than among the controls [19.8% vs 40.8%, OR = 0.36 (95% CI 0.31–0.42), $P_c = 0.01$]. There were no significant differences between the two groups in the distribution of the other alleles such as HLA-DRB1*01, DRB1*02, DRB1*03, DRB1*08, DRB1*09, DRB1*10, DRB1*11, DRB1*12, DRB1*13, or DRB1*14.

Among the 91 CRM-sensitive asthmatic patients, the distributions of HLA-DRB1 alleles in the patients who showed positive skin responses both to CRM and HDM were shown in Table 3. The HLA-DRB1*07 allele frequency was significantly higher among the patients than among the controls [19.6% vs. 4.1%, OR = 5.73 (95% CI 4.18–7.86), $P_c = 0.02$]. The HLA-DRB1*04 allele frequency was significantly lower among the patients than among the controls [13.7% vs 40.8%, OR = 0.23 (95% CI 0.20–0.27), $P_c = 0.008$]. There were no differences between the two groups in the distribution of the other HLA-DRB1 alleles.

Among the CRM-sensitive asthmatic patients, the distributions of HLA-DRB1 alleles in the patients who showed isolated positive skin responses to CRM is shown in Table 4. The HLA-DRB1*07 allele frequency was higher among the patients than among the controls (15.0% vs. 4.1%, OR = 4.15 (95% CI 3.03–5.69), $P = 0.02$, $P_c > 0.05$). However, the statistical significance was less

marked than those subjects with positive skin responses to both CRM and HDM. As for the HLA-DRB1*04 allele, the frequency was not significantly different between the two groups (27.5% vs 40.8%, $P > 0.05$). There were also no differences between the two groups in the distribution of the other HLA-DRB1 alleles.

Figure 3 shows the distributions of HLA-DRB1*04 subtypes by PCR-SSCP. HLA-DRB1*0405 showed a significant decrease amongst the CRM-sensitive asthmatics (4.4% vs 16.3%, $P = 0.008$). Among the CRM-sensitive asthmatics, the DRB1*0405 was higher in the patients with positive skin response both to CRM and HDM (2.0 vs 16.3%, $P = 0.01$), although the allele were not different between the patients with positive skin response only to CRM and the controls (7.5% vs 16.3%, $P > 0.05$). The allelic frequency of the other DRB1*04 subtypes were the same between the CRM-sensitive asthmatics and the controls.

Discussion

Our results showed that CRM-induced asthma not only had a strong positive association with HLA-DRB1*07, but also a strong negative association with HLA-DRB1*04. These associations were more remarkable in the patients who showed positive skin responses both to CRM and HDMs.

The HLA class II (HLA-DR, DQ, and DP) antigens are heterodimers (α and β chains) with a high proportion of

Table 4. Allelic frequency of HLA-DRB1 gene in asthmatics with a skin response only to citrus red mite

Alleles	Patients* (n = 40)		Controls (n = 98)		OR (95% CI)	P _c †
	No.	GF (%)	No.	GF (%)		
DRB1*01	11	27.5	21	21.4	4.15 (3.03–5.69)	NS
DRB1*02	10	25.0	21	21.4		NS
DRB1*03	3	7.5	4	4.1		NS
DRB1*04	11	27.5	40	40.8		NS
DRB1*07	6	15.0	4	4.1		NS‡
DRB1*08	5	12.5	19	19.4		NS
DRB1*09	6	15.0	18	18.4		NS
DRB1*10	1	2.5	1	1.0		NS
DRB1*11	0	0	3	3.1		NS
DRB1*12	4	10.0	14	14.3		NS
DRB1*13	12	30.0	17	17.3		NS
DRB1*14	10	25.0	23	23.5		NS

GF: genotype frequency; NS: not significant; OR: odds ratio. *Citrus red mite-sensitive asthmatics with positive skin response both to citrus red mite and house dust mites. †P corrected for the number of alleles (12) tested. ‡Uncorrected $P < 0.05$ in these cases.

polymorphisms. As with HLA-DQ and DP antigens, the α and β chains of HLA-DR antigens are encoded separately by DRA and DRB genes, respectively. The β chain of the HLA-DR antigen shows a remarkable polymorphism in the first domain, which is encoded by the second exon of HLA-DRB genes. Among the DRB genes, the DRB1 gene is

the most polymorphic of the human class II genes, in which over 120 distinct alleles have been identified, making it a powerful marker for individual identification. In this study, we targeted the HLA-DRB1 genes.

HLA genes play a major role in regulating immune responses. An HLA influence on the IgE response to an

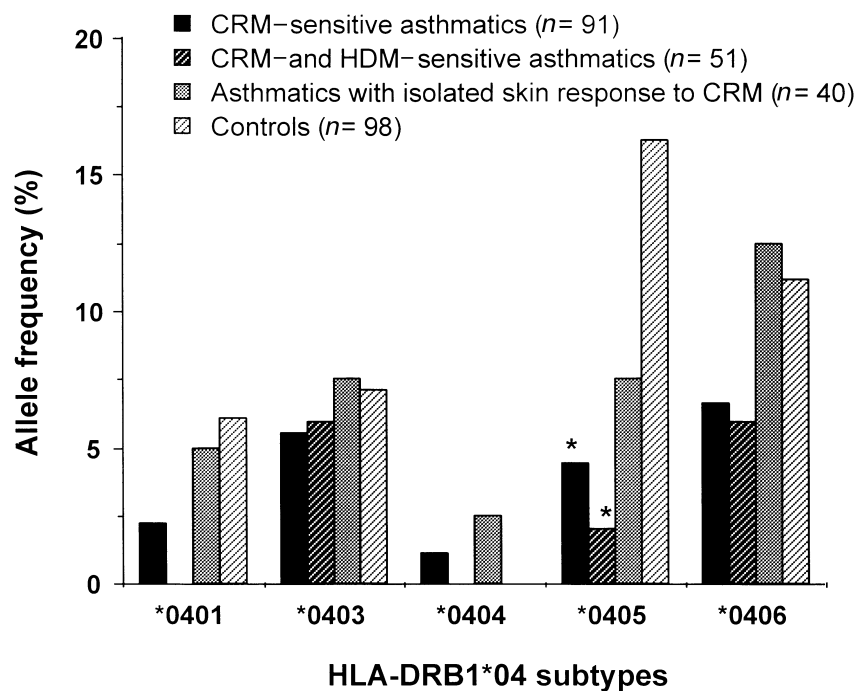


Fig. 3. Allele frequency of HLA-DRB1*04 subtypes by PCR-SSCP high-resolution analysis. *Significant difference compared to nonatopic controls ($P < 0.05$).

allergen was first noted by Levine *et al.* [8], who found an association between HLA class I haplotypes and IgE responses to antigen E derived from ragweed allergen (*Ambrosia artemisiifolia*). Marsh *et al.* [18] demonstrated that the response to a minor component of ragweed antigen (*Amb a V*) was restricted by HLA-DR2. However, many other possible positive [9,10,19,20] and negative [21,22] associations with allergen responses have been described.

Our recent studies revealed that CRM was the most important allergen in the development of asthma in citrus farmers [2]. To the best of our knowledge, this is the first association study between citrus red mite-sensitive asthma and HLA alleles, although a few studies have linked HLA antigens directly with asthma. Aron *et al.* [23] observed that HLA-DR4 and DR7 were significantly implicated in susceptibility to atopic asthma, while Caraballo *et al.* [12] have shown a negative association between HLA-DPB1*0401 and allergic asthma. Recently, Lara-Marquez *et al.* [13] have shown association of DRB1*1101 DQA1*0501 DQB1*0301 haplotype with *Dermatophagoides* spp.-sensitive asthma in a sample of the Venezuelan population. However, our study demonstrated that CRM-sensitive asthma had a strong positive association with DRB1*07, while DRB1*04, especially DRB1*0405, were more prevalent in exposed, healthy control subjects, suggesting a protective effect. These novel findings suggest that HLA-DRB1 gene-encoded molecules play an important role in the development of CRM-induced asthma.

Our previous studies showed that CRM-derived allergens contained species-specific allergens in addition to allergens commonly shared with HDMs [2,16]. Among the CRM-sensitive asthmatics, this study revealed that the allelic frequency of DRB1*07 was more marked among the patients with positive skin responses both to CRM and HDM compared to the patients with positive skin response only to CRM. A negative association with DRB1*04 (especially DRB1*0405) was more marked in the patients with positive skin responses both to CRM and HDM, but the association was not observed in the patients with an isolated skin response to CRM. These findings suggest that DRB1*07 is involved in the susceptibility to the development of CRM-sensitive asthma, and HLA-DRB1*04 plays a role in protecting against the development of the CRM- and HDM-sensitive asthma. However, we cannot preclude the possibility of association HLA-DQ alleles with CRM-sensitive asthma, because HLA-DR4 and DR7 have a linkage disequilibrium with HLA-DQ4 and DQ2, respectively, in Korean families [24].

In conclusion, we suggest that HLA-DRB1 gene may be involved in the development of CRM-induced asthma, and that HLA-DRB1*07 may increase, and DRB1*04 decrease, the risk of developing the asthma in exposed individuals.

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