

Genetic effect of *CCR3* and *IL5RA* gene polymorphisms on eosinophilia in asthmatic patients

June-Hyuk Lee, MD,^{a*} Hun Soo Chang, PhD,^{a*} Ji Hyun Kim, MS,^a Se-Min Park, MS,^a Yong Mok Lee, MD,^a Soo Taek Uh, MD,^a Taiyoun Rhim, PhD,^a Il Yup Chung, PhD,^b Yong-Hoon Kim, MD,^a Byung Lae Park, PhD,^c Choon-Sik Park, MD, PhD,^a and Hyoung Doo Shin, PhD^c Gyeonggi Do and Seoul, Korea

Background: Eosinophilic infiltration and peripheral blood eosinophilia in asthma require the cooperation of eosinophil-specific cytokines and chemokines and their receptors. **Objective:** We investigated the association of polymorphisms in *CCR3* and *IL5RA* with asthma susceptibility or peripheral blood eosinophilia and the effects of the polymorphisms on receptor expression. **Methods:** Polymorphisms in *CCR3* and *IL5RA* were identified and genotyped in 576 asthmatic patients and 180 healthy control subjects. *CCR3* and IL-5 receptor α (IL-5R α) protein expression on eosinophils was measured by means of flow cytometry. **Results:** Although polymorphisms in *CCR3* were not associated with asthma susceptibility, the *CCR3* haplotype *ht2* showed a negative gene dose effect on the eosinophil count ($P = .003-.009$). *IL5RA* c.-5091G>A was weakly associated with eosinophil count. The effects of *ht2* were greater when paired with *IL5RA* c.-5091A ($P = .001-.002$). *CCR3* protein expression was higher on eosinophils of asthmatic patients without *ht2* than in those with *ht2*. Asthmatic patients with the *IL5RA* c.-5091A allele showed higher IL-5R α expression than those who were homozygous for the G allele. **Conclusion:** The genetic association between *CCR3* polymorphisms and the number of circulating eosinophils was revealed as a novel finding. These associations were more pronounced when the *CCR3* polymorphisms were paired with polymorphisms in *IL5RA*. The protein expression levels of *CCR3* and IL-5R α on peripheral blood eosinophils are associated with the polymorphisms on their own genes.

Clinical implications: The identification of single nucleotide polymorphisms and haplotypes of *CCR3* and *IL5RA* might be useful in developing markers for intermediate phenotypes of eosinophil number and in designing strategies to control diseases related to hypereosinophilia. (J Allergy Clin Immunol 2007;120:1110-7.)

Key words: Asthma, eosinophilia, single nucleotide polymorphism, CC chemokine receptor 3, IL-5 receptor α

Asthma is a common respiratory disease characterized by intermittent airway obstruction and respiratory symptoms caused by chronic airway inflammation and remodeling.^{1,2} Excessive infiltration of eosinophils has been identified within the target tissues of allergic disorders.^{3,4} The extent of eosinophilic inflammation is a determinant of the severity of asthma symptoms,⁵ and the number of peripheral blood eosinophils is correlated with restricted airflow.^{6,7} The processes of eosinophilic infiltration and peripheral blood eosinophilia are dependent on the cooperative action of eosinophil-specific cytokines and chemokines, such as IL-5 and members of the eotaxin family.^{8,9}

CCR3 is a G protein-coupled receptor¹⁰ for chemokines, which provoke an eosinophilic response in the peripheral blood and airways.^{11,12} *CCR3* mRNA and protein levels are increased in the bronchial mucosa of asthmatic patients in association with airway hyperresponsiveness.¹³ The human *CCR3* gene (MIM no. 601268) is located on chromosome 3p21.3,^{14,15} which is linked to atopic dermatitis^{16,17} and asthma.¹⁸

IL-5 is important for the terminal differentiation, activation, and survival of committed eosinophil precursors.^{19,20} It stimulates the release of eosinophils into the circulation and prolongs their survival through the IL-5 receptor (IL-5R).^{21,22} The human IL-5R complex is a heterodimer consisting of a unique α subunit (IL-5R α) and a β subunit.²³ The α subunit is required for ligand-specific binding.²⁴ The *IL5RA* gene (MIM no. 147850) is located on chromosome 3p26-p25, which is linked to asthma development (D3S3564).¹⁸ *IL5RA* mRNA-positive cells are increased in the airways of asthmatic patients.²⁵

Given the biologic effects of *CCR3* and IL-5R α and their linkage to the chromosomal loci of asthma susceptibility, *CCR3* and *IL5RA* polymorphisms might be

From ^athe Genome Research Center for Allergy and Respiratory Diseases, Soonchunhyang University Bucheon Hospital, Gyeonggi Do; ^bthe Division of Molecular and Life Science, College of Science and Technology, Hanyang University, Gyeonggi Do; and ^cthe Department of Genetic Epidemiology, SNP Genetics, Inc, Seoul.

*These authors contributed equally to this work.

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Reprint requests: Choon-Sik Park, MD, PhD, Division of Allergy and Respiratory Medicine, Department of Internal Medicine, Soonchunhyang University Bucheon Hospital, 1174, Jung Dong, Wonmi Ku, Bucheon, Gyeonggi Do, 420-021, Korea. E-mail: mdcspark@unitel.co.kr.

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Abbreviations used

CCR3: CC chemokine receptor 3
IL-5R α : IL-5 receptor α
SNP: Single nucleotide polymorphism
UTR: Untranslated region

related to asthma development or peripheral blood eosinophilia in asthma. In this study we screened and genotyped single nucleotide polymorphisms (SNPs) in *CCR3* and *IL5RA* and examined the genetic effects of the SNPs on the risk for asthma and eosinophilia in asthmatic patients. Furthermore, we tested the interactive effects between the polymorphisms in *CCR3* and *IL5RA* on eosinophilia. We also validated the genetic effects of the SNPs on the expression of CCR3 and IL-5R α protein on eosinophils.

METHODS

Subjects

We enrolled 576 Korean asthmatic patients and 180 healthy control subjects from 3 tertiary hospitals in Korea (Soonchunhyang University Seoul, Bucheon, and ChunAn hospitals). Among the 180 healthy subjects, 166 were spouses of asthmatic patients, and 14 were healthy volunteers who performed Heath Screening Check Up for Job Application. The institutional review boards of the hospitals approved the study, and all patients gave informed consent. All the patients with asthma met the American Thoracic Society definition of asthma.²⁶ Additional details are provided in the **Methods** section in this article's Online Repository at www.jacionline.org. The clinical characteristics of all the subjects studied are summarized in Table I.

Sequencing analysis of the human *CCR3* gene

We sequenced all 4 exons, 3 introns, and the promoter region (~1.5 kb) of the *CCR3* gene in the DNA samples from 24 Korean subjects using an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, Calif). Four primer sets (*CCR3*) for the amplification and sequencing analysis were designed based on GenBank sequences (reference genome sequence, NT_022517). Information on the primers is available in Table E1 in this article's Online Repository at www.jacionline.org.

Genotyping by means of single-base extension and electrophoresis

Amplifying and extension primers were designed for single-base extension to genotype the SNPs. Reactions were performed with a SNaShot ddNTP primer extension kit (Applied Biosystems), according to the manufacturer's recommendations. Information on the primers is available in Table E2 in this article's Online Repository at www.jacionline.org. Data were analyzed with ABI Prism GeneScan 3.5.1 and Genotyper 3.6 software (Applied Biosystems).

Measurement of CCR3 and IL-5R α expression on peripheral blood eosinophils of asthmatic subjects

Eosinophil-rich polymorphonuclear leukocytes were purified from the peripheral blood of the asthmatic patients or subjects having

TABLE I. Clinical characteristics of study subjects

	Healthy control subjects	Asthmatic patients	P value
No. of subjects	180	576	—
Age, y [median (range)]	36.0 (7-81)	38.0 (8-80)	.791
Male sex (%)	50.3	45.2	.259
Current smoker (%)	23.1	12.3	.001
FVC (% predicted)*	90.88 $\pm$ 0.97	87.97 $\pm$ 0.74	.017
FEV ₁ (% predicted)*	96.71 $\pm$ 0.99	80.80 $\pm$ 0.89	<.001
PC ₂₀ (mg/mL)*	24.63 $\pm$ 0.20	2.78 $\pm$ 0.21	<.001
Total IgE (IU/mL)*	191.04 $\pm$ 27.95	414.58 $\pm$ 26.11	<.001
Atopy (%)	43.9	62.8	<.001
Geometric mean blood eosinophil (% [95% CI])	1.74 (1.42-2.15)	4.64 (4.33-4.98)	<.001
Geometric mean blood eosinophil count ($\times 10^9$ /L [95% CI])	105.5 (85.5-130.1)	336.9 (313.3-362.2)	<.001

FVC, Forced vital capacity.

*The data were presented as means $\pm$ SEMs.

peripheral blood eosinophil counts higher than 10% because of other diseases as described previously.²⁷ Purified cells were stained by using immunofluorescence with anti-human CCR3 (R&D Systems, Minneapolis, Minn) or IL-5R α antibody (BD Biosciences, San Jose, Calif). Eosinophils were distinguished from neutrophils by using a gated autofluorescence/forward-scatter method.^{27,28} Additional details are provided in the **Methods** section of the Online Repository at www.jacionline.org.

Statistical analysis

To examine Hardy-Weinberg equilibria, the Fisher exact test was performed with a modified version of the Markov-chain random walk algorithm.²⁹ Linkage disequilibria between loci were quantified as $|D'|$ and r^2 values^{30,31} by using the program Arlequin (<http://anthro.unige.ch/arlequin>) and Microsoft Excel. The haplotypes and their frequencies were inferred by using the algorithm implanted in PHASE.³² The allele and haplotype frequencies between phenotypes were compared by using logistic regression analysis, controlling for age and sex as covariates. The number of peripheral blood eosinophils was log transformed. The differences in the log-transformed values among genotypes or haplotypes were examined by using a generalized linear model type III SS. Data were analyzed with SAS (version 8.1; SAS Institute, Cary, NC). The effective number of independent marker loci in *CCR3* and *IL5RA* was calculated by using the software SNPSpD (<http://www.genepi.qimr.edu.au/general/daleN/SNPSpD/>) to correct for multiple testing.³³ Statistical significance was defined at the standard 5% level. The ratios of fluorescence intensity for CCR3 or IL-5R α were analyzed with the Mann-Whitney *U* test.

RESULTS

Association between *CCR3* gene polymorphisms and development of asthma

Direct sequencing of the *CCR3* gene, including the -1500 bp of the 5'-flanking region, identified 4 SNPs. Of 10 SNPs (-22557G>A, -520T>G, -174C>T,

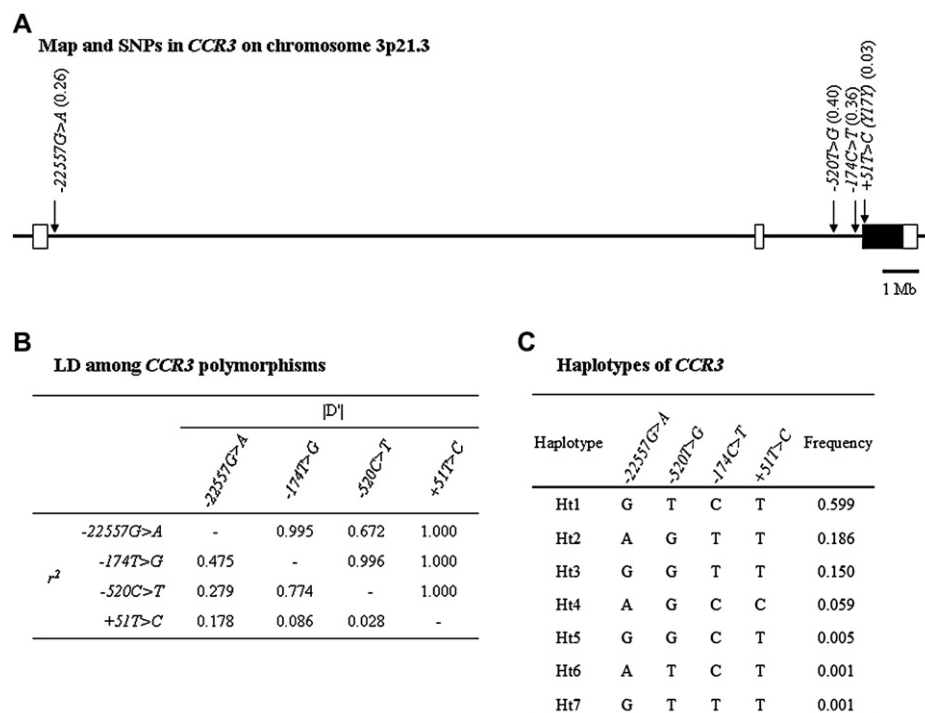


FIG 1. Gene map (A), linkage disequilibrium (LD) coefficients (B), and haplotypes (C) in *CCR3*. In the map (Fig 1, A), coding exons are marked by solid blocks and UTR by open blocks. The frequencies of SNPs were based on sequencing data ($n = 24$). The first base of the translation site was denoted as nucleotide +1.

+51T>C, +240C>T, +652T>A, +824G>A, +827G>C, +828C>G, and +1052T>C) reported in white and Japanese subjects,³⁴⁻³⁶ the last 6 listed were not identified in the 24 Korean subjects. Therefore the other 4 SNPs were selected for larger-scale genotyping. The genotype distributions of the 4 SNPs were in Hardy-Weinberg equilibrium within all subjects ($P > 0.05$, see Table E3 in this article's Online Repository at www.jacionline.org). Strong linkage disequilibria ($|D'| > 0.9$) were noted between the other alleles, except between -22557G>A and -174C>T (Fig 1, B), and 7 haplotypes were inferred from unphased genotype data (Fig 1, C). Among them, one haplotype (ht4: AGCC) was equivalent to +51T>C. Three haplotypes (ht1: GTCT, ht2: AGTT, and ht3: GGTT) were analyzed for the association study, and the other 3 haplotypes were excluded because of low frequency (<1%). The haplotype distributions were in Hardy-Weinberg equilibrium within all subjects ($P > 0.05$, see Table E3 in this article's Online Repository at www.jacionline.org). None of the SNPs or haplotypes of *CCR3* showed a significant association with asthma development on logistic regression analysis (see Table E4 in this article's Online Repository at www.jacionline.org).

Association between *CCR3* and *IL5RA* gene polymorphisms and peripheral blood eosinophil count in asthmatic patients

The asthmatic patients with rare alleles for SNPs of the *CCR3* gene (-22557G>A and -174C>T) showed a trend

toward having a smaller number of peripheral blood eosinophils than those with the common alleles among asthmatic patients ($P = .014-.043$, $P_{corr} = .042-.129$; Table II). The asthmatic patients with ht1 (GTCT) showed a trend toward having a greater number of eosinophils than those without ht1 ($P = .049$, $P_{corr} = .125$, recessive model). The asthmatic patients with ht2 (AGTT) showed a significantly smaller number of eosinophils than those without ht2 ($P = .003$, $P_{corr} = .008$, codominant model; $P = .009$, $P_{corr} = .023$, dominant model; $P = .005$, $P_{corr} = .013$, recessive model). ht2 (AGTT) showed a clear gene dose-dependent effect on the peripheral blood eosinophil count in all 3 models (Table II). The results for diplo-type analysis were similar to the results obtained from haplotype analysis (see Table E5 in this article's Online Repository at www.jacionline.org). The genetic effect of ht2 on the peripheral blood eosinophil count showed the same trends in atopic asthmatic patients and in nonatopic asthmatic patients (see Table E6 in this article's Online Repository at www.jacionline.org). The asthmatic patients who were ht1 homozygous had the highest number of eosinophils, and ht2 homozygotes had the lowest number of eosinophils. The asthmatic patients with ht1/ht2 had an intermediate number of eosinophils ($P = .004$, codominant model; Fig 2).

Among the polymorphisms identified in our previous publication,³⁷ 10 polymorphisms in *IL5RA* were selected for larger-scale genotyping based on location, linkage disequilibrium, frequency, and haplotype-tagging status. *IL5RA* can be parsed into 2 haplotype blocks. There are 6

TABLE II. Association of SNPs and haplotypes of the *CCR3* gene with the number of blood eosinophils of asthmatic patients

Locus	Reference SNP ID	Genotype	N	GM* (95% CI)	P (P_{corr})†		
					Cod	Dom	Rec
<i>CCR3</i> -22557 G>A	rs9853223	GG	294	340.1 (306.1-377.9)	.056	.144	.023
		GA	177	311.2 (273.5-354.1)	(.168)	(.432)	(.069)
		AA	34	229.2 (174.2-301.8)			
<i>CCR3</i> -520 T>G	rs3091250	TT	183	352.0 (309.0-401.1)	.114	.072	.110
		TG	233	312.2 (277.3-351.7)	(.342)	(.216)	(.330)
		GG	80	281.4 (236.3-335.0)			
<i>CCR3</i> -174 C>T	rs3091311	CC	224	358.7 (319.3-402.9)	.043	.014	.189
		CT	218	298.3 (263.6-337.5)	(.129)	(.042)	(.567)
		TT	56	283.1 (231.3-346.6)			
<i>CCR3</i> +51 T>C	rs4987053	TT	456	318.0 (292.8-345.2)	.217	.217	—
		TC	54	365.8 (289.2-462.5)	(.651)	(.651)	—
		CC	0	—			
<i>CCR3</i> <i>ht1</i>	—	<i>ht1/ht1</i>	197	356.5 (314.4-404.2)	.109	.167	.049
		<i>ht1/—</i>	237	309.2 (274.5-348.3)	(.278)	(.426)	(.125)
		<i>—/—</i>	80	288.2 (242.9-341.9)			
<i>CCR3</i> <i>ht2</i>	—	<i>ht2/ht2</i>	23	200.9 (146.0-276.3)	.003	.009	.005
		<i>ht2/—</i>	149	290.3 (252.6-333.6)	(.008)	(.023)	(.013)
		<i>—/—</i>	342	349.4 (317.2-384.8)			
<i>CCR3</i> <i>ht3</i>	—	<i>ht3/ht3</i>	10	563.8 (338.6-938.9)	.098	.562	.060
		<i>ht3/—</i>	123	302.2 (258.1-353.8)	(.250)	(1.000)	(.153)
		<i>—/—</i>	381	325.2 (297.0-356.0)			

Cod, Codominant model; Dom, dominant model; Rec, recessive model.

*Values are presented as geometric means.

†P values were obtained by using general linear model type III SS, controlling age and sex as covariables, and were corrected with the software SNPSpD (<http://www.genepi.qimr.edu.au/general/daledN/SNPSpD/>).

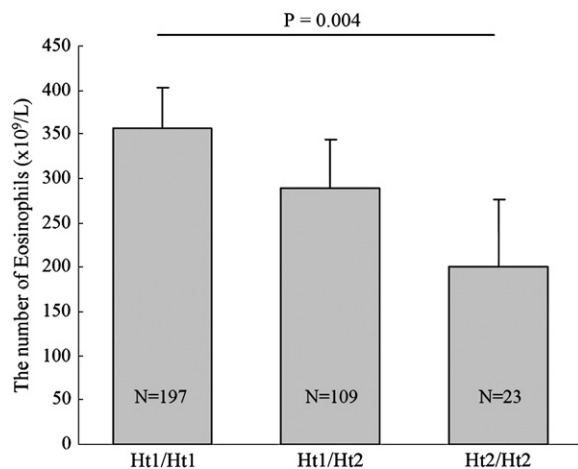


FIG 2. The comparison of the number of eosinophils in peripheral blood between the subjects possessing *ht1*, *ht2*, or both of *CCR3*. The bars and error bars represent geometric means and 95% CIs, respectively. P values were obtained by means of linear regression analysis, controlling age and sex as covariates in the codominant model.

and 5 common haplotypes in block 1 and block 2, respectively.³⁷ Block 1-*ht1*, Block 1-*ht2*, Block 1-*ht5*, Block 2-*ht2*, Block 2-*ht3*, and Block 2-*ht5* were not analyzed because they were equivalent to *c.*-6173T>C, *c.*-5091G>A, *c.*-6783T>C, *c.*-3643G>A, *c.*-480_482insdelGTT, and *c.*82+2068T>C, respectively. Block 1-*ht6* was not

analyzed because of its low frequency. Among the 10 SNPs on the *IL5RA* gene, *c.*-5091G>A showed a gene dose-dependent trend in association with the number of peripheral blood eosinophils (Table III). The asthmatic patients with rare alleles at *c.*-5091G>A of *IL5RA* showed a greater number of peripheral blood eosinophils than those with the allele common among asthmatic patients ($P = .031$, $P_{corr} = .287$, codominant model; $P = .010$, $P_{corr} = .091$, dominant model; Table III). The genetic effect of *IL5RA* *c.*-5091G>A on the peripheral blood eosinophil count showed the same trends in atopic asthmatic patients and in nonatopic asthmatic patients (see Table E6 in this article's Online Repository at www.jacionline.org). *IL5RA* *c.*-480_482insdelGTT showed an association with the number of eosinophils ($P = 0.005$, $P_{corr} = 0.045$, codominant model) but did not show an apparent gene dose effect.

Gene-gene interactions between *CCR3* *ht2* and *IL5RA* *c.*-5091 polymorphism on peripheral blood eosinophil number in asthmatic patients

The combined genetic effect of *CCR3* and *IL5RA* on the number of peripheral blood eosinophils was analyzed. The genetic effect of *CCR3* *ht2* became more pronounced when the asthmatic subjects were stratified according to *IL5RA* *c.*-5091G>A (Table IV). The asthmatic patients

TABLE III. The association of SNPs and haplotypes of the *IL5RA* gene with the values of the number of blood eosinophils in asthmatic patients

Locus	Reference SNP ID	Position	Genotype	N	GM* (95% CI)	P (P_{corr})†		
						Cod	Dom	Rec
<i>c.-6783T>C</i>	rs17878989	5' Flanking	<i>TT</i>	326	338.5 (306.8-373.3)	.456	.487	.342
			<i>TC</i>	52	307.8 (242.5-390.7)	(1.000)	(1.000)	(1.000)
			<i>CC</i>	1	712			
<i>c.-6475G>A</i>	rs17881995	5' Flanking	<i>GG</i>	173	306.7 (266.7-352.8)	.228	.165	.165
			<i>GA</i>	169	353.7 (310.5-402.9)	(1.000)	(1.000)	(1.000)
			<i>AA</i>	37	391.4 (294.6-519.9)			
<i>c.-6173T>C</i>	rs17887110	5' Flanking	<i>TT</i>	181	348.2 (308.5-393.0)	.716	.413	.795
			<i>TC</i>	161	325.0 (280.9-376.0)	(1.000)	(1.000)	(1.000)
			<i>CC</i>	36	300.0 (213.6-421.4)			
<i>c.-5993G>A</i>	rs17886800	5' Flanking	<i>GG</i>	91	305.4 (250.7-371.9)	.797	.659	.528
			<i>GA</i>	185	339.6 (297.6-387.7)	(1.000)	(1.000)	(1.000)
			<i>AA</i>	103	353.5 (301.8-414.2)			
<i>c.-5567C>G</i>	rs3806681	5' Flanking	<i>CC</i>	107	373.9 (318.6-438.8)	.168	.101	.139
			<i>CG</i>	171	331.5 (290.1-378.8)	(1.000)	(.916)	(1.000)
			<i>GG</i>	100	297.6 (246.3-359.7)			
<i>c.-5091G>A</i>	rs2290608	Exon 1 (5'-UTR)	<i>GG</i>	214	301.2 (267.1-339.8)	.031	.010	.142
			<i>GA</i>	132	372.7 (320.0-434.1)	(.281)	(.091)	(1.000)
			<i>AA</i>	33	431.1 (319.5-581.8)			
<i>c.-3643G>A</i>	Novel	Exon 2 (5'-UTR)	<i>GG</i>	205	355.4 (316.7-398.7)	.337	.220	.257
			<i>GA</i>	151	320.6 (275.4-373.2)	(1.000)	(1.000)	(1.000)
			<i>AA</i>	24	264.5 (173.8-402.6)			
<i>c.-480_482insdelGTT</i>	rs3217672	Intron 3	<i>TGT/TGT</i>	237	347.5 (310.6-388.7)	.005	.143	.025
			<i>TGT/-</i>	119	291.1 (245.2-345.6)	(.045)	(1.000)	(.227)
			<i>-/-</i>	22	508.6 (390.0-663.4)			
<i>c.82+2068T>C</i>	rs13097407	Intron 4	<i>TT</i>	305	322.1 (291.0-356.5)	.472	.264	.509
			<i>TC</i>	71	374.0 (307.0-455.7)	(1.000)	(1.000)	(1.000)
			<i>CC</i>	2	688.2 (0.2-2409905)			
<i>c.385A>G (p.I129V)</i>	rs17882248	Exon 7	<i>AA</i>	163	366.2 (323.7-414.3)	.119	.157	.058
			<i>AG</i>	162	328.2 (283.9-379.3)	(1.000)	(1.000)	(.526)
			<i>GG</i>	52	260.2 (198.0-342.0)			
<i>Block 1-ht3</i>	—	—	<i>+/+</i>	20	361.2 (274.4-475.6)	.180	.108	.756
			<i>-/+</i>	100	299.3 (247.1-362.6)	(.355)	(.213)	(1.000)
			<i>-/-</i>	260	347.5 (312.1-387.0)			
<i>Block 1-ht4</i>	—	—	<i>+/+</i>	6	203.9 (122.5-339.4)	.692	.678	.408
			<i>-/+</i>	79	332.2 (277.8-397.2)	(1.000)	(1.000)	(.805)
			<i>-/-</i>	295	338.9 (304.9-376.6)			
<i>Block 2-ht1</i>	—	—	<i>+/+</i>	40	366.3 (285.2-470.3)	.243	.140	.218
			<i>-/+</i>	170	355.8 (312.5-405.0)	(.471)	(.271)	(.423)
			<i>-/-</i>	170	308.5 (267.2-356.1)			
<i>Block 2-ht4</i>	—	—	<i>+/+</i>	6	268.2 (82.5-871.4)	.443	.206	.622
			<i>-/+</i>	66	289.7 (230.5-364.3)	(.859)	(.399)	(1.000)
			<i>-/-</i>	308	346.8 (314.3-382.7)			

Cod, Codominant model; Dom, dominant model; Rec, recessive model.

*Values are presented as geometric means.

†P values were obtained by using general linear model type III SS, controlling age and sex as covariables, and were corrected by using the software SNPSpD (<http://www.genepi.qimr.edu.au/general/daledN/SNPSpD/>).

possessing the rare allele at *c.-5091G>A* exhibited a stronger gene dose effect of *CCR3 ht2* on the number of peripheral blood eosinophils; those having *ht2/ht2* at *CCR3* showed the lowest number of peripheral blood eosinophils, whereas those without *ht2* had the highest number of peripheral blood eosinophils ($P = .001-.002$). The same trends of the genetic interactions were observed in atopic and nonatopic asthmatic populations (see Table E7 in this article's Online Repository at www.jacionline.org). The genetic effect of *CCR3 ht2* on the peripheral

blood eosinophil number did not change when the asthmatic subjects were stratified according to *IL5RA c.-480_482insdelGTT* (data not shown).

Flow cytometric analysis for CCR3 and IL-5R α protein expression on peripheral blood eosinophils from asthmatic patients

To investigate whether the sequence variants of *CCR3* or *IL5RA* could affect the expression of CCR3 or IL-

TABLE IV. The association of the combination between *IL5RA* c.-5091G>A genotypes and *CCR3* *ht2* with the number of blood eosinophils in asthmatic patients

		<i>IL5RA</i> c.-5091G>A				
		<i>GG</i>		<i>GA</i> or <i>AA</i>		
		N	GM* (95% CI)	N	GM* (95% CI)	<i>P</i> value†
<i>CCR3 ht2</i>	+/+	6	213.7 (82.1-556.6)	10	181.7 (113.5-290.7)	.643
	-/+	58	270.3 (223.1-327.6)	30	314.6 (240.8-411.1)	.426
	-/-	150	318.4 (273.3-371.0)	92	444.9 (378.8-522.6)	.003
<i>P</i> value‡	Cod		.444		.001	
	Dom		.344		.002	
	Rec		.283		.002	

Cod, Codominant model; *Dom*, dominant model; *Rec*, recessive model.
*Values are presented as geometric means.
†*P* values of the association of *IL5RA* c.-5091G>A were obtained by using general linear model type III SS, controlling age and sex as covariables.
‡*P* values of the association of *CCR3 ht2* were obtained by using general linear model type III SS, controlling age and sex as covariables.

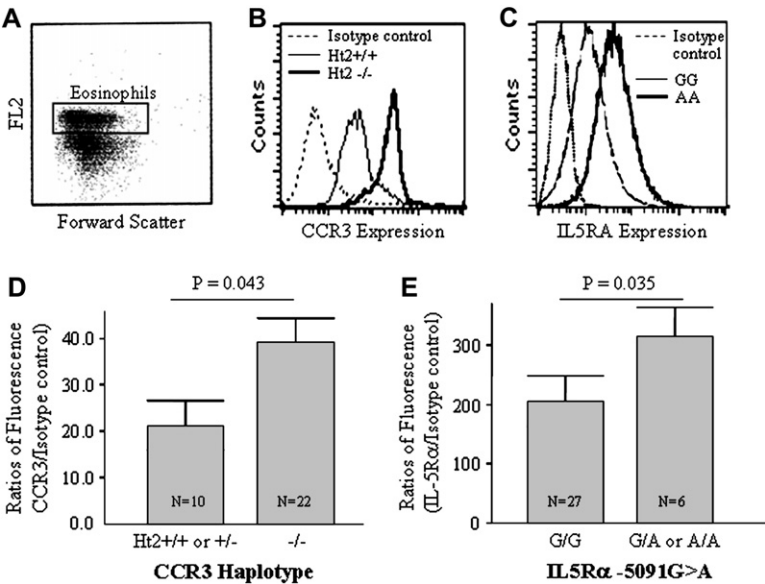


FIG 3. The CCR3 and IL-5R α expression on the peripheral blood eosinophils from asthmatic patients. **A**, Gating for eosinophils among peripheral blood granulocytes by using autofluorescence is shown. **B** and **D**, Levels of CCR3 expression were compared between eosinophils from asthmatic patients having *ht2* and having other haplotypes. **C** and **E**, Levels of IL-5R α expression were compared between eosinophils from asthmatic patients who were G or A allele homozygotes on *IL5RA*-5091G>A. Each datum was presented by the ratio of fluorescence intensities for each molecule to the values of isotype-matched control.

5R α protein, we measured CCR3 and IL-5R α expression on eosinophils from asthmatic patients (Fig 3, A). The level of CCR3 expression was significantly lower on eosinophils from asthmatic patients with *ht2* (*n* = 10) than in those without *ht2* (*n* = 22; *P* = .043; Fig 3, B and D). In addition, the eosinophils from asthmatic patients with the A allele at *IL5RA* c.-5091G>A (*n* = 27) showed greater IL-5R α expression than those with the G allele (*n* = 6; *P* = .035; Fig 3, C and E). The similar trends were observed in subjects with peripheral blood eosinophilia of more than 10% caused by diseases other than asthma (see Fig E1 in the Online Repository at www.jacionline.org).

DISCUSSION

Eotaxin plays an important role in the infiltration of eosinophils into target tissues in allergic disorders in a cooperative mode with IL-5.^{38,39} Whole-genome scans have suggested that the chromosome region 3p21-24, which contains a gene cluster of CC chemokine receptors, including *CCR3*, is linked to asthma.¹⁸ *D3S3567* on chromosome 3p was closely linked to the asthma phenotype in a founder population.¹⁸ *CCR3* is located very close to *D3S3567* (distance apart, 4.1 Mb), and *IL5RA* is close to *D3S3564* (distance apart, 39 Mb). These results suggest that *CCR3* and *IL5RA* gene polymorphisms might have

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an effect on genetic susceptibility to asthma and eosinophilia as a subphenotype.

In our study we examined the 10 *CCR3* gene polymorphisms previously reported in white and Japanese subjects.³⁴⁻³⁶ Only 4 of these ($-22557G>A$, $-520T>G$, $-174C>T$, and $+51T>C$) were found to be present in the Korean subjects. This might be attributable to racial differences, as well as to the extremely low frequency (0.01–0.005) of the variations ($+240C>T$, $+824G>A$, and $+1052T>C$) in white subjects.³⁶ The allele frequencies of $-520T>G$, $-174C>T$, and $+51T>C$ in our study were comparable with those in the Japanese population.^{35,36} The absence of an association between $-520T>G$, $-174C>T$, and $+51T>C$ and asthma development in our study concurs with 2 studies in Japanese populations,^{34,35} despite the report of a significant association in a British population.³⁴ The $+51T>C$ C allele is more frequent in the British population (12.8%) than in Korean (5.5%) or Japanese (4.2%) populations. This indicates the presence of a racial difference in *CCR3* gene polymorphisms between white and Asian subjects.

The *CCR3* gene contains 4 exons with 4 mRNA species by means of alternative splicing.⁴⁰ Intriguingly, the 3 SNPs associated with eosinophil number in our study locate in introns. It remains to be determined whether the alternative splicing is dependent on the SNPs ($-22557G>A$ and $-174C>T$) in the *CCR3* introns. Additionally, Zimmermann et al⁴¹ recently reported areas of active chromatin remodeling in exon 1 of *CCR3*. Given that $-22557G>A$ is located only 63 bp downstream of exon 1 and between *HindIII* restriction sites, which is the location of a fragment showing DNase I hypersensitivity,⁴¹ the effect of the $-22557G>A$ polymorphism on chromatin remodeling in the *CCR3* gene should be investigated.

The present association analysis revealed a trend in associations of 2 SNPs ($-22557G>A$ and $-174C>T$) with eosinophil number in asthmatic patients (Table II). The *ht2* haplotype displayed a negative gene dose effect on the eosinophils number, whereas *ht1* or *ht3* showed a positive effect (Table II). This result indicates the strong genetic association among these 3 completely linked SNPs ($-22557G>A$, $-520T>G$, and $-174C>T$) and peripheral eosinophilia, which we believe is a novel finding. In contrast, we could not find the association in healthy control subjects. A possible explanation for the lack of association might be that they have relatively small numbers of circulating eosinophils because of the low level of IL-5 in healthy subjects.

In our previous study on *IL5RA*, the G allele of *c.-5993A>G* showed a lower frequency in asthmatic patients than in healthy control subjects (0.48 vs 0.51, $P = .04$).³⁷ Among the 10 SNPs in *IL5RA*, the *c.-5091G>A* polymorphism and *c.-480_482ins/del* had a marginal association with the number of peripheral blood eosinophils (Table III).

To predict the effects of *c.-5091G>A* on the function of the 5'-untranslated region (UTR), we searched for functional elements in the 5'-UTR of *IL5RA* using UTRScan (<http://www.ba.itb.cnr.it/BIG/UTRScan>) but found no

patterns of functional elements in the region. Furthermore, there were no putative binding sites for transcription factors (TFSEARCH: V1.3; putative score, >0.87). *c.-5091G>A* might affect the level of IL-5R α expression on peripheral eosinophils through an interaction with an unknown pattern in the 5'-UTR followed by translational controls or through a linkage disequilibrium with other functional SNPs not identified in this study or located on other nearby genes.

On the basis of the association of eosinophil number with polymorphisms of key regulatory genes, as shown in this study, we evaluated the interactions between the *CCR3* and *IL5RA* genes. As shown in Table IV, there was an intergenetic association between *CCR3 ht2* and *c.-5091G>A* of *IL5RA*, such that the genetic effect of this combination lowered *P* values more than the effect of either condition alone. These results strongly suggest that different gene polymorphisms might act additively to influence the development of eosinophilia in asthmatic patients. To evaluate whether these interactions are real or rather the result of fluctuations caused by the small size of the population, we tested the statistical interaction by using multiple regression analysis with interaction terms and could find significant interaction ($P = .001$, data not shown). We cannot exclude the chance of type I error in our positive results of gene-gene interaction using stratification methods because of the small numbers of subjects. However, considering the biologic roles of *CCR3* and IL-5R α , it seems obvious that there are biologic interactions on eosinophil differentiation and infiltration between them.^{8,9}

The expression of the *CCR3* and IL-5R α proteins was measured by means of flow cytometric analysis and was compared among asthmatic patients according to genotype to validate the genetic effects of *CCR3 ht2* and *IL5RA c.-5091G>A* (Fig 3). *CCR3* expression was higher on eosinophils in asthmatic patients lacking *ht2* than in those with *ht2*. Asthmatic patients with the *IL5RA c.-5091A* allele showed higher IL-5R α expression than those with the G allele. Similar trends were observed in subjects with eosinophilia of more than 10% caused by other diseases than asthma (Fig E1). In contrast, *c.-480_482ins/delGTT* had no effect on the IL-5R α expression level on eosinophils (data not shown). These findings demonstrate that *CCR3* and IL-5R α protein expression is dependent on the *ht2* haplotype of *CCR3* and *c.-5091A* of *IL5RA*.

In summary, the genetic association between *CCR3* gene polymorphisms and the number of peripheral blood eosinophils was revealed as a novel finding. In addition, these associations were more pronounced when the *CCR3* polymorphisms were paired with polymorphisms in the *IL5RA* gene. The protein expression levels of *CCR3* and IL-5R α on peripheral blood eosinophils are associated with the polymorphisms on their own genes. This information on the genetic polymorphisms in the *CCR3* and *IL5RA* genes might be useful in developing markers for the intermediate phenotypes of peripheral blood eosinophil number and in designing new strategies for the control of diseases related to hypereosinophilia.

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METHODS

Determining clinical characteristics of individuals

Each patient had airway reversibility, as documented by an inhalant bronchodilator-induced (two 0.1-mg inhalations of salbutamol; Ventolin MDI; GlaxoSmithKline, Greenford, United Kingdom) improvement in FEV₁ by more than 15%^{E1} or airway hyperresponsiveness, as revealed by a PC₂₀ of less than 10 mg/mL methacholine.^{E1} The healthy subjects were spouses of the patients or were recruited from the general population. They gave negative answers to a screening questionnaire for respiratory symptoms^{E2} and had FEV₁ values of 75% of predicted value or greater, a PC₂₀ methacholine value of 10 mg/mL or greater, a total serum IgE level of less than 1000 IU/mL, and a normal chest radiograph. Total cells and a differential cell count of peripheral blood were automatically measured by using a Coulter counter. No subject had used systemic steroids during the 4 weeks before the examination of the peripheral blood eosinophil count. The subjects were skin prick tested for 24 common inhalant allergens, including house dust mites, *Alternaria* species, *Aspergillus* species, pollens, dog, cat, and cockroach. Atopy was defined as having a wheal reaction with the allergen extract equal to or greater than that with histamine (1 mg/mL) or 3 mm in diameter.

Measurement of CCR3 and IL-5R α expression on peripheral blood eosinophils of asthmatic patients

Eosinophil-rich polymorphonuclear leukocytes were purified from the heparinized peripheral blood of the asthmatic patients or subjects with other lung diseases by using Percoll gradient. Purified

cells were stained with Diff-Quik (Baxter Diagnostics, Dudingen, Switzerland) to determine eosinophil purity and number. Eosinophils typically accounted for 60% to 90% of the total cells, as determined by means of microscopic examination. Rat anti-human CCR3 mAb (0.5 μ g, R&D Systems) or mouse anti-human IL-5R α mAb (10 μ g; BD Biosciences, San Jose, Calif) was incubated with 5×10^5 cells, followed by treatment with 50 ng of fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat IgG2a heavy chain (Serotec, Raleigh, NC) for CCR3 staining or 0.5 μ g of FITC-conjugated goat anti-mouse IgM plus IgG plus IgA F(ab')₂ (Southern Biotech, Birmingham, Ala) for IL-5R α staining. The fixed and stained cells were analyzed by using a flow cytometer (FACScan; Becton-Dickinson, Mountain View, Calif). Eosinophils were distinguished from neutrophils by using gated autofluorescence/forward-scatter methods,^{E3,E4} and the FITC intensity was measured on the FL-1 fluorescence channel.^{E4}

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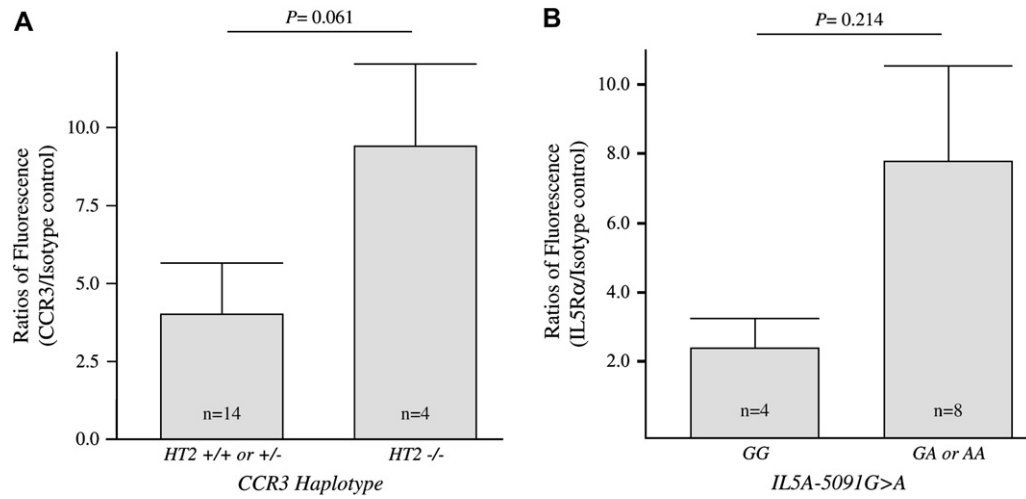


FIG E1. The levels of CCR3 and IL-5R α expression were compared between eosinophils from subjects with peripheral blood eosinophils higher than 10% because of other diseases than asthma. **A**, CCR3 expression between haplotype 2 (*HT2*) and other haplotypes. **B**, IL-5R α expression between G and A allele homozygotes on *IL5RA-5091G>A*. Each datum was presented as the ratio of fluorescence intensities for each molecule to the values of the isotype-matched control.

TABLE E1. Primer sequences for *CCR3* SNP screening

Primer	Locus		Sequences (5'-3')
CCR3-p1	Exon 1	Forward	TTCCTGGCACCTCTGATATCC
		Reverse	TGGCCAAAAACCCAGTTATG
CCR3-p2	Exon 3	Forward	CCTCGTCACCCTTCCATTCT
		Reverse	TGACAAAAATGAGCCGGATG
CCR3-p1	Exon 3	Forward	TCGTTCTCCCTCTGCTCGT
		Reverse	TGGTCCTTCCTCTTTAGGCAA
CCR3-p4	Exon 3	Forward	AATTATAACAACCCCCACCA
		Reverse	GATCCAAGAGCACTGCTGG

TABLE E2. Sequences of amplifying and extension primers for *CCR3* SNP genotyping by using the single-base extension method

Locus		Sequences (5'-3')
-22557G>A	Forward	GTAGGTGTCGCTTTTCCAG
	Reverse	TGGCCCCATACTACCTCTA
	Extension	AATCAATGATGATGGTCCAGGCAGGTGGAAGTA
-520T>G	Forward	TGACAGGAGAAAATGGACATGG
	Reverse	TACTGTCTCTTACGGCATTGTC
	Extension	TATAATCAATGATGATCAATTTCCCCATTA ACTATAATGAATG
-174C>T	Forward	ACCCACAACATTTCTGCT
	Reverse	CCTTCGTAAAAACACACCAGTT
	Extension	ATTATAATCAATGATGATGCATATCAGAGGTGCCAGGA
+51T>C (Y17Y)	Forward	GGGAGAAGTGAAATGACAACCT
	Reverse	AAGAGGCCACAGTGAACAC
	Extension	TTGAGACCTTTGGTACCACATCCTACTA

TABLE E3. Hardy-Weinberg equilibrium of *CCR3* genotypes and haplotypes

Locus	Observed heterozygote	Expected heterozygote	<i>P</i> value
−22557G>A	0.344	0.580	.071
−520T>G	0.469	0.633	.572
−174C>T	0.454	0.622	.720
+51T>C	0.116	0.388	.722
<i>ht1</i>	0.450	0.475	.168
<i>ht2</i>	0.280	0.300	.070
<i>ht3</i>	0.244	0.251	.468
<i>ht4</i>	0.099	0.097	.715

TABLE E4. Comparisons of allele frequencies of SNPs on *CCR3* between asthma and control by using logistic regression

Locus	Reference SNP ID	Genotype	Status of asthma (N [%])		Odd ratio (95% CI)	P value* (P _{corr} value)†
			Asthmatic patients	Healthy control subjects		
<i>CCR3</i> -22557 G>A	rs9853223	GG	334 (59.0%)	109 (61.6%)	0.92 (0.68-1.25)	.583 (1.000)
		GA	193 (34.1%)	57 (32.2%)		
		AA	39 (6.9%)	11 (6.2%)		
<i>CCR3</i> -520 T>G	rs3091250	TT	204 (36.9%)	68 (38.9%)	0.98 (0.75-1.28)	.863 (1.000)
		TG	258 (46.7%)	76 (43.4%)		
		GG	91 (16.5%)	31 (17.7%)		
<i>CCR3</i> -174 C>T	rs3091311	CC	249 (44.7%)	79 (44.4%)	0.97 (0.74-1.29)	.847 (1.000)
		CT	245 (44.0%)	78 (43.8%)		
		TT	63 (11.3%)	21 (11.8%)		
<i>CCR3</i> +51 T>C	rs4987053	TT	513 (89.7%)	159 (90.3%)	1.15 (0.64-2.09)	.642 (1.000)
		TC	59 (10.3%)	16 (9.1%)		
		CC	0 (0.0%)	1 (0.6%)		
<i>CCR3</i> <i>ht1</i>	—	<i>ht1/ht1</i>	222 (38.5%)	71 (39.4%)	1.01 (0.77-1.31)	.963 (1.000)
		<i>ht1/-</i>	263 (45.7%)	78 (43.3%)		
		<i>-/-</i>	91 (15.8%)	31 (17.2%)		
<i>CCR3</i> <i>ht2</i>	—	<i>ht2/ht2</i>	27 (4.7%)	6 (3.3%)	1.16 (0.83-1.62)	.401 (1.000)
		<i>ht2/-</i>	162 (28.1%)	50 (27.8%)		
		<i>-/-</i>	387 (67.2%)	124 (68.9%)		
<i>CCR3</i> <i>ht3</i>	—	<i>ht3/ht3</i>	13 (2.3%)	6 (3.3%)	0.90 (0.64-1.29)	.576 (1.000)
		<i>ht3/-</i>	139 (24.1%)	46 (25.6%)		
		<i>-/-</i>	424 (73.6%)	128 (71.1%)		
<i>CCR3</i> <i>ht4</i>	—	<i>ht4/ht4</i>	0 (0.0%)	1 (0.6%)	0.89 (0.49-1.61)	.699 (1.000)
		<i>ht4/-</i>	59 (10.2%)	16 (8.9%)		
		<i>-/-</i>	517 (89.8%)	163 (90.6%)		

*P values of codominant models.

†To achieve the optimal correction for multiple testing of SNPs in linkage disequilibrium with each other, the effective number of independent marker loci was calculated by using the software SNPSpD (<http://www.genepi.qimr.edu.au/general/daleN/SNPSpD/>) on the basis of the spectral decomposition of matrices of pairwise linkage disequilibrium between SNPs.

TABLE E5. The haplotype analysis for the association of *CCR3* or *IL5RA* haplotypes with numbers of blood eosinophil of asthmatic patients

Haplotype	No. of eosinophils (GM [95% CI])*	P value†
<i>IL5RA</i> Block 1- <i>ht1</i>	232.4 (204.8-263.7)	.240
<i>IL5RA</i> Block 1- <i>ht2</i>	208.8 (184.1-236.9)	.012
<i>IL5RA</i> Block 1- <i>ht3</i>	188.0 (162.3-217.8)	.241
<i>IL5RA</i> Block 1- <i>ht4</i>	201.1 (169.7-238.2)	.583
<i>IL5RA</i> Block 2- <i>ht1</i>	211.6 (193.1-231.8)	.084
<i>IL5RA</i> Block 2- <i>ht2</i>	196.1 (175.6-218.9)	.187
<i>IL5RA</i> Block 2- <i>ht3</i>	212.3 (187.6-240.1)	.772
<i>IL5RA</i> Block 2- <i>ht4</i>	185.4 (157-218.9)	.200
<i>CCR3 ht1</i>	281.9 (263.3-301.8)	.036
<i>CCR3 ht2</i>	238.8 (214.1-266.4)	.001
<i>CCR3 ht3</i>	254.5 (219.4-295.2)	.998

*Values are presented as geometric means.

†P values were obtained by means of comparison with other haplotypes by using general linear model type III SS, controlling age and sex as covariables.

TABLE E6. The association of *CCR3 ht2* and *IL5RA c.-5091G>A* with the number of eosinophils in healthy control subjects and atopic and nonatopic asthmatic patients

Subjects	Locus		N	Geometric mean (95% CI)	<i>P</i> _{Cod} value	<i>P</i> _{Dom} value	<i>P</i> _{Rec} value
Healthy control subjects	<i>CCR3 ht2</i>	+/+	4	204.8 (109.5-383.1)	.587	.721	.304
		-/+	35	123.5 (99.7-153.0)			
		-/-	82	120.2 (99.5-145.3)			
	<i>IL5RA c.-5091</i>	GG	85	111.5 (98.6-126.0)	.640	.556	.618
		GA	57	118.5 (102.8-136.6)			
		AA	9	94.3 (64.4-138.0)			
Atopic asthmatic patients	<i>CCR3 ht2</i>	+/+	12	252.1 (164.1-387.3)	.047	.017	.205
		-/+	99	284.3 (239.4-337.7)			
		-/-	214	364.5 (322.2-412.3)			
	<i>IL5RA c.-5091</i>	GG	134	259.5 (212.1-317.4)	.328	.177	.272
		GA	81	371.4 (286.7-481.0)			
		AA	24	415.1 (105.3-1636.2)			
Nonatopic asthmatic patients	<i>CCR3 ht2</i>	+/+	8	142.3 (75.9-267.0)	.017	.340	.004
		-/+	47	297.2 (233.5-378.4)			
		-/-	115	315.1 (265.6-373.7)			
	<i>IL5RA c.-5091</i>	GG	71	328.6 (280.9-384.3)	.073	.025	.291
		GA	46	371.6 (303.1-455.6)			
		AA	6	432.9 (311.4-601.9)			

Cod, Codominant model; *Dom*, dominant model; *Rec*, recessive model.

TABLE E7. Gene-gene interaction between *CCR3 ht2* and *IL5RA-5091G>A* for the number of blood eosinophils in healthy control subjects, atopic asthmatic patients, and nonatopic asthmatic patients

		<i>IL5RA</i> c.-5091G>A				
		<i>GG</i>		<i>GA or AA</i>		
		N	GM (95% CI)	N	GM (95% CI)	<i>P</i> value*
Healthy control subjects						
<i>CCR3 ht2</i>	+/+	1	272	2	177.7 (87.0-363.1)	—
	-/+	18	130.2 (93.4-181.4)	16	110.3 (81.9-148.5)	.574
	-/-	47	102.5 (80.9-129.7)	26	138.7 (96.2-200.0)	.157
<i>P</i> value†	Cod		.190		.601	
	Dom		.248		.354	
	Rec		.104		.867	
Atopic patients with asthma						
<i>CCR3 ht2</i>	+/+	3	333.6 (64.1-1735.4)	5	232.2 (98.3-548.6)	.403
	-/+	36	301.1 (235.0-385.9)	32	293.6 (210.2-409.9)	.877
	-/-	95	339.4 (277.5-415.2)	67	451.3 (366.3-556.1)	.054
<i>P</i> value†	Cod		.913		.047	
	Dom		.670		.017	
	Rec		.916		.176	
Nonatopic BA						
<i>CCR3 ht2</i>	+/+	1	69.0	5	136.2 (54.1-343.0)	.803
	-/+	20	233.2 (166.3-326.9)	11	385.0 (244.8-605.4)	.058
	-/-	50	278.1 (216.1-357.9)	36	430.1 (314.1-588.9)	.027
<i>P</i> value†	Cod		.233		.037	
	Dom		.343		.123	
	Rec		.113		.011	

GM, Geometric mean; Cod, codominant model; Dom, dominant model; Rec, recessive model.

**P* values of the association of *IL5RA c.-5091G>A* were obtained by general linear model type III SS controlling age and sex as covariables.

†*P* values of the association of *CCR3 ht2* were obtained by general linear model type III SS controlling age and sex as covariables.