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Gene–Gene Interaction Between CCR3 and Eotaxin Genes: The Relationship With Blood Eosinophilia in Asthma

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Purpose: Eosinophils function as an effector cell in the development of asthma and allergic disease. Eotaxins are cytokines that promote pulmonary eosinophilia via the receptor CCR3. Single-nucleotide polymorphisms (SNPs) in CCR3 and eotaxin genes are associated with asthma. In this study, genetic interactions among SNPs of several eotaxin genes and CCR3 were assessed and their relationship with blood eosinophilia in asthma was examined. **Methods:** A total of 533 asthmatics were enrolled in this study. Asthmatics with eosinophilia ($>0.5\times10^9$/L) were compared with those without eosinophilia ($\leq0.5\times10^9$/L). Chi-square tests were used to compare SNP frequencies. Two different models were used to evaluate gene-gene interactions: logistic regression and generalized multifactor dimensionality reduction (GMDR). **Results:** *EOT2+304C>A* (29L>I) was significantly associated with 3 of the 4 CCR3 SNPs among asthmatics with eosinophilia ($P=0.037$-0.009). *EOT2+304C>A* (29L>I) and the CCR3 SNPs were also significantly associated with blood eosinophilia in an interaction model constructed by logistic regression ($P=0.0087$). GMDR analysis showed that the combination of *EOT2+304C>A* (29L>I) and *CCR3-174C>T* was the best model (accuracy=0.536, $P=0.005$, CVC 9/10). **Conclusions:** The epistatic influence of CCR3 on eotaxin gene variants indicates that these variants may be candidate markers for eosinophilia in asthma.

Key Words: Asthma; epistasis; polymorphisms; CCR3; eotaxin

INTRODUCTION

Eosinophils play an important role in the development of asthma and allergic disease as an effector cell. The extent of eosinophilic inflammation is a determinant of the severity of asthma symptoms,[1] and it usually correlates with airflow limitations.[2] Relationships between eosinophilic infiltration and Th2 cytokines have been identified within the target tissues of allergic disorders.[3] The processes of eosinophilic infiltration and peripheral eosinophilia are dependent on eosinophil-specific cytokines and chemokines (*e.g.*, IL-5; the eotaxin family; RANTES; and MCP-2, -3, and -4), which provoke an eosinophilic response in the peripheral blood and airways via CCR3.[4,5] CCR3 mRNA and protein levels are elevated in the bronchial mucosa of asthmatics, and this elevation is associated with airway hyperresponsiveness.[6] The participation of CCR3 in airway eosinophilic infiltration has been demonstrated in a study of CCR3-deficient mice, which showed that most eosinophils are arrested in the subendothelial space.[7] Eosinophils are derived from CD34(+) hematopoietic progenitor cells.[8] Eotaxin-1 and -2 induce the migration of bone marrow and blood CD34(+) CCR3(+) cells *in vitro*.[9] Therefore, the CCR3-eotaxin pathway is important in the regulation of allergen-induced hematopoiesis and in the accumulation and mobilization of eosinophil lineage-committed progenitor cells in the lung.[9] The human CCR3 gene (MIM #601268) is located on chromosome 3p21.3.[10] The single-nucleotide polymorphisms (SNPs) *CCR3-22557G>A* and *-174C>T* are associated with the number of eosinophils in asthmatic patients. It has previously been reported that CCR3 expression is higher on the eosinophils of asthmatic patients lacking a haplotype composed of rare alleles of 4 CCR3 SNPs.[11]

Eotaxin-1 is important for eosinophilic inflammation in early stages of the asthmatic response, while eotaxin-3 may account for eosinophil recruitment to the airways in late stages of the asthmatic response.[12] Three eotaxin family members, eotaxin-1,

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-2, and -3, are selectively bound by CCR3. Eotaxin-1 and -2 show different pathophysiologic responses in relation to eosinophils. Disruption of the eotaxin-1 gene leads to a slight reduction in the eosinophil count in the blood and airways.[13] Eotaxin-2-deficient mice have normal baseline eosinophil levels in their target tissues, and they do not develop airway eosinophilia in response to IL-13.[14] Two eotaxin polymorphisms, *EOT2+1272A>G* and *EOT1+123G>A*, are associated with asthma and high serum total IgE levels, respectively.[15]

Because asthma is a multifactorial disease, the genetic component may be derived from the combined effect of numerous genes. Individual genes may act independently or in combination with other genes in the same biological pathway, resulting in variable effects.[16] The CCR3 gene and eotaxin gene family are possible contributors to the development of asthma or other allergic phenotypes via the receptor-ligand interaction.

In this study, we evaluated gene-gene interactions between CCR3 and eotaxin in relation to eosinophilia in patients with asthma.

MATERIALS AND METHODS

Subjects

We enrolled 533 Korean asthmatics in our study. The Institutional Review Board of [Soonchunhyang University Bucheon Hospital] approved the study. All of the patients had current symptoms, including wheezing, dyspnea, and cough, and met the criteria for asthma as defined by the American Thoracic Society.[17] Each patient showed airway reversibility as documented by an inhalant bronchodilator-induced improvement in the forced expiratory volume in one second (FEV1) of >15%[18] and/or airway hyperresponsiveness as shown by a provocative concentration of methacholine required to cause a 20% decrease in the FEV1 (PC20) of <8 mg/mL.[18] The asthma subjects were divided into 2 groups according to the presence of eosinophilia. Eosinophilia was defined as a blood eosinophil count exceeding 0.5×10^9/L.[19] No subject had used systemic or inhaled steroids for 4 weeks before peripheral blood eosinophil counting. No subject had a history of medication or the ingestion of raw foods that could produce blood eosinophilia. ELISAs for 4 common parasites (*Paragonimus*, *Clonorchis*, *Cysticercus*, and *Sparganum*) were negative in the enrolled patients. The clinical characteristics of the subjects are summarized in Table 1.

Genotyping by single-base extension and electrophoresis

To genotype polymorphic sites, amplifying and extension primers were designed for single-base extension. All primer extension reactions were performed using a SNaPshot ddNTP Primer Extension Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Detailed methods are described in the online supplement.

Table 1. Clinical characteristics of asthma subjects according to the presence of eosinophilia

	Asthma with eosinophilia	Asthma without eosinophilia	*P* value
No. of subjects	155	378	
Median age (range)	37 (8-75)	43 (8-81)	<0.0001
Sex (males/females)	92/63	152/226	<0.0001
Smokers (%)	20.4	23.8	0.42
FVC%, pred.	88.8±1.4	88.0±0.9	0.63
FEV1%, pred.	81.1±1.8	80.8±1.1	0.86
% change in FEV1 by bronchodilator	11.5±1.4	10.5±0.6	0.54
PC20 (mg/mL)	1.7±0.2	3.0±0.3	0.0001
Total IgE (IU/mL)	560±64.1	349±27.2	0.003
Blood eosinophil count ($\times10^9$/L)	0.891±0.04	0.223±0.01	<0.0001
Blood eosinophil count (%)	10.9±0.4	3.4±0.1	<0.0001

Eosinophilia was defined as a blood eosinophil count $>0.5\times10^9$/L. *P* values were calculated for comparisons between asthmatics with eosinophilia and without eosinophilia. Chi-square tests were used to compare categorical variables; Student's *t*-test was used to compare continuous variables. Mean±SEM.

Analysis of polymorphisms, Hardy-Weinberg equilibrium, and linkage disequilibrium

A total of 14 SNPs in eotaxin-1, -2, and -3 (6 SNPs in eotaxin-1, 5 in eotaxin-2, and 3 in eotaxin-3), and 4 SNPs in CCR3 were included in our analysis. Testing for Hardy-Weinberg equilibrium and the calculation of D' for the identification of linkage disequilibrium were performed using PHASE v2.0.2[20] and Arelquin v2.0.[21] Continuous variables, such as the blood eosinophil count and percentage, serum total IgE level, and PC20 methacholine level, were log-transformed to approximate normal distributions. Differences in the log-transformed values among genotypes were examined using a generalized linear model type III SS. Statistical significance was defined at the standard 5% level.

Dependency of allele frequency and logistic regression interaction modeling

The dependency between each pair of CCR3 and eotaxin SNPs in asthmatics with and without eosinophilia was tested by Chi-square analysis.

An epistatic model was designed based on the logistic regression model.[22] "$CCR3_{eff}$," a new numerical variable as an effect of CCR3 SNPs on blood eosinophilia, was derived according to the extent of contribution to eosinophilia in asthma using logistic regression. Odds ratios (ORs) for eosinophilia for each genotype at each locus were calculated in asthmatics with eosinophilia. Each genotype was coded according to the ORs as follows: homozygote with the highest OR, 2; heterozygote, 1; and the other homozygote, 0.

In $CCR3_{eff}=\alpha_1$ (*-22557G>A*)$+\alpha_2$ (*-520T>G*)$+\alpha_3$ (*-174C>T*)$+\alpha_4$ (*+51T>C*),

α denotes the coefficient fitted by logistic regression (α_1=0.3601, α_2=0.3525, α_3=0.4204, and α_4=0.1867). Epistasis was subsequently tested independently for each SNP in eotaxin by fitting the following logistic regression model:

P (Eosinophilia)=exp (X)/[1+exp (X)]

where $X=\beta_0+\beta_1 CCR3_{eff}+\beta_2 SNP_{Eotaxin}+\beta_3$ ($CCR3_{eff} \times SNP_{Eotaxin}$), β_0 is the intercept, β represents the parameter estimates, and $SNP_{Eotaxin}$ is each eotaxin genotype (0, 1, or 2).

The Bonferroni correction for multiple comparisons was used for each eotaxin gene (the global significance level was adjusted to $P<0.0083$ for 6 eotaxin-1 SNPs, $P<0.01$ for 5 eotaxin-2 SNPs, and $P<0.017$ for 3 eotaxin-3 SNPs). Statistical significance was defined at the standard 5% level.

Generalized multifactor dimensionality reduction (GMDR)

To validate the results of our logistic model, we performed GMDR analysis. First, the data were randomly split into 10 equal parts for cross-validation. Nine of these were used as a training set; the remaining set was used for independent testing. Cross-validation is a measure of the number of times a particular set of loci is identified in each possible 9/10 of the subjects. Second, a set of *n* genetic factors was selected. Third, all possible multifactor classes or cells were represented in *n*-dimensional space and a cumulative score was calculated within each cell.[23] Fourth, each multifactor cell in *n*-dimensional space was labeled as "high-risk" if the average score met or exceeded the threshold (0) or as "low-risk" if the threshold was not exceeded. This process was repeated for each possible cross-validation interval. Fifth, all potential combinations of *n* factors were evaluated sequentially for their ability to classify cases and controls in the training data, and the best *n*-factor model that yielded the minimum misclassification error was chosen. Sixth, the independent testing set was used to estimate the prediction error of the best model selected in the fifth step. Finally, among this set of the best models, we pick the model with minimum prediction error and/or maximum cross-validation consistency.[23,24] A covariate analysis was performed as described above[23] with age (continuous variable) and sex (discrete variable) as covariates.

RESULTS

The genotype distributions of the four SNPs in CCR3 and 14 SNPs in the eotaxin genes were in the Hardy-Weinberg equilibrium (data not shown). No pair of SNPs showed strong linkage disequilibrium (>0.8) between the eotaxin gene SNPs and CCR3 SNPS (see Supplementary Table S2).

Epistasis was evaluated to access whether the eotaxin SNPs modified the effect of the CCR3 SNPs on blood eosinophilia susceptibility. We examined whether dependence existed between the CCR3 SNPs and eotaxin gene SNPs before the epistasis tests. *EOT2+304C>A* was dependent on 3 intronic SNPs (*CCR3-22557G>A*, *CCR3-520T>G*, and *CCR3-174C>T*) in CCR3 among asthmatics with eosinophilia by Chi-square analysis (Table 2).

The epistatic model constructed by logistic regression that tested the effect of the combined CCR3 SNPs on blood eosinophilia differed depending on the eotaxin SNP: *EOT2+304C>A* (29L>I) with the CCR3 SNPs was significantly associated with blood eosinophilia (P=0.0087; Table 3). Thus, genetic susceptibility to blood eosinophilia in asthmatics is conferred by both CCR3 and eotaxin.

According to our GMDR results, the combination of *CCR3-*

Table 2. Tests for dependence between the CCR3 gene SNPs and eotaxin gene SNPs

Eotaxin	CCR3	With eosinophilia				Without eosinophilia			
		-22557G>A	*-520T>G*	*-174C>T*	*+51T>C*	*-22557G>A*	*-520T>G*	*-174C>T*	*+51T>C*
Eotaxin-1	*-1329G>A*	0.13	0.37	0.91	0.97	0.41	0.24	**0.043**	0.45
	-329A>G	0.43	0.71	0.8	0.84	0.54	0.84	0.95	0.49
	+123G>A	0.61	0.93	0.77	0.17	0.12	**0.042**	0.47	0.23
	+371T>A	0.17	0.52	0.85	0.5	0.40	0.84	0.51	0.8
	+1654G>A	0.3	0.29	0.77	0.83	0.83	0.43	0.43	0.69
	+1671T>C	0.99	**0.037**	0.26	0.62	0.66	0.71	0.87	0.55
Eotaxin-2	*+96G>A*	0.83	0.75	0.67	0.92	0.33	0.12	0.41	0.61
	+304C>A	**0.037**	**0.016**	**0.009**	0.53	0.65	0.74	0.41	0.54
	+447C>T	0.85	0.85	0.69	0.37	0.84	0.92	0.58	0.14
	+1272A>G	0.64	0.16	0.09	0.45	0.77	0.59	0.39	0.86
	+1923A>C	0.91	0.57	0.83	0.47	0.87	0.36	0.4	0.77
Eotaxin-3	*+77C>T*	0.24	0.94	0.94	0.95	0.83	0.39	0.39	0.15
	+7164A>G	0.33	0.2	0.1	0.52	0.42	0.64	0.75	0.87
	+1579G>A	0.61	0.12	0.35	0.15	0.93	0.59	0.47	0.41

P values calculated from chi-square tests are presented for asthmatics with and without eosinophilia. *P* values in boldface are significant ($P<0.05$).

Table 3. Gene-gene interactions between the CCR3 SNPs and eotaxin gene SNPs in blood eosinophilia in asthmatics

Model	β_3	SE	Chi-square	*P* value
Eotaxin-1 SNP×CCR3 effect				
-1328G>A×CCR3$_{eff}$	0.11	0.46	0.06	0.81
-329A>G×CCR3$_{eff}$	-0.31	0.24	1.70	0.19
+123G>A×CCR3$_{eff}$	-0.62	0.36	3.07	0.08
+371T>A×CCR3$_{eff}$	-0.15	0.26	0.34	0.56
+1654G>A×CCR3$_{eff}$	0.26	0.42	0.40	0.53
+1671T>C×CCR3$_{eff}$	0.06	0.33	0.03	0.87
Eotaxin-2 SNP×CCR3 effect				
+96G>A×CCR3$_{eff}$	0.04	0.42	0.01	0.93
+304C>A(29L>I)×CCR3$_{eff}$	-0.68	0.26	6.89	**0.0087***
+447C>T×CCR3$_{eff}$	0.19	0.23	0.68	0.41
+1272A>G×CCR3$_{eff}$	-0.17	0.35	0.23	0.63
+1923A>C×CCR3$_{eff}$	-0.14	0.33	0.17	0.68
Eotaxin-3 SNP×CCR3 effect				
+77C>T×CCR3$_{eff}$	-0.10	0.25	0.15	0.70
+7164A>G×CCR3$_{eff}$	0.47	0.34	1.94	0.16
+1579G>A×CCR3$_{eff}$	-0.36	0.46	0.60	0.44

P values were calculated by logistic regression analysis. *Significant after the Bonferroni correction ($P<0.01$ for eotaxin-2). SE, standard error.

174C>T and *EOT2+304C>A* was the best model (accuracy=0.536, CVC 9/10; Table 4). This combination was also the best model after adjustment for age and sex as covariates. The combination of the CC genotype of *CCR3-174C>T* and AA or AC genotype of *EOT2+304C>A* was detected in the high-risk group. The combination of CT of *CCR3-174C>T* and CC of *EOT2+304C>A* was also detected in the high-risk group (Figure).

DISCUSSION

Given the wealth of data showing that CCR3 and eotaxin participate in eosinophilic infiltration, the cooperative effect of the SNPs of CCR3 and eotaxin on eosinophilia in asthmatics was analyzed in this study. Three SNPs (*CCR3-22557G>A*, *CCR3-520T>G*, and *CCR3-174C>T*) in CCR3 were significantly associated with the number of eosinophils in patients with asthma.[11] Associations between *EOT2+1272A>G* and the risk of asthma, and between *EOT1+123G>A* and high serum total IgE levels, were reported in Korean asthmatics.[15] However, these SNPs were not found as a best single-locus model in relation to blood eosinophilia in this study.

Gene-gene interactions in various diseases have been studied using a variety of approaches. These studies demonstrated that the overall disease risk can be modeled as the product of the risk conferred by many independent risk factors.[16] In this study, gene-gene interactions were examined using 3 methods. First, a Chi-square test was conducted. Using this approach, *EOT2+304C>A* was found to be significantly associated with the 3 intronic SNPs of CCR3 in patients with eosinophilia (Table 2). Next, we used logistic regression analysis and designed an epistatic model as described previously.[22] Three of the 4 CCR3 SNPs contributed independently to the eosinophilic phenotype in asthma. This model can be used to evaluate the mean effect of all SNPs in CCR3. Of the 14 SNPs in the eotaxin gene family, only 1 in eotaxin-2 (*EOT2+304C>A* [29L>I]) was significantly associated with blood eosinophilia; however, the association was modified by the effect of CCR3 SNPs ($P=0.0087$; Table 3). Finally, we constructed an epistasis model using GMDR with and without covariate adjustment. Because the number of subjects in the 2 groups (asthmatics with or without eosinophilia) was different, GMDR was used instead of regular MDR. The combination of *EOT2+304C>A* and *CCR3-174C>T* was the best model. This model was still the best after adjustment for age and sex as covariates (Table 4). We successfully identified genotype combinations that contribute to eosinophilia in asthma using this model. Asthmatics, who had the C genotype of *CCR3-174C>T* and A or AC of *EOT2+304C>A* and who had the CT genotype of *CCR3-174C>T* and C of *EOT2+304C>A* (Figure),

Table 4. Analysis of gene-gene interactions by GMDR

Model	Accuracy	Sign test (*P*)	CVC
CCR3-174C>T	0.505 (0.476)	0.62 (0.828)	7/10
CCR3-174C>T, EOT2+304C>A	0.536 (0.549)	0.005 (0.011)	9/10
CCR3-520T>G, EOT1+361T>A, EOT2+304C>A	0.559 (0.544)	0.011 (0.011)	4/10
EOT1+361T>A, EOT2+304C>A, EOT2+447C>T, EOT2+1272A>G	0.525 (0.576)	0.172 (0.001)	3/10

The numbers in parentheses are the results after adjustment for age and sex as covariates. Age was used as a continuous variable and sex as a discrete variable. CVC, cross-validation consistency.

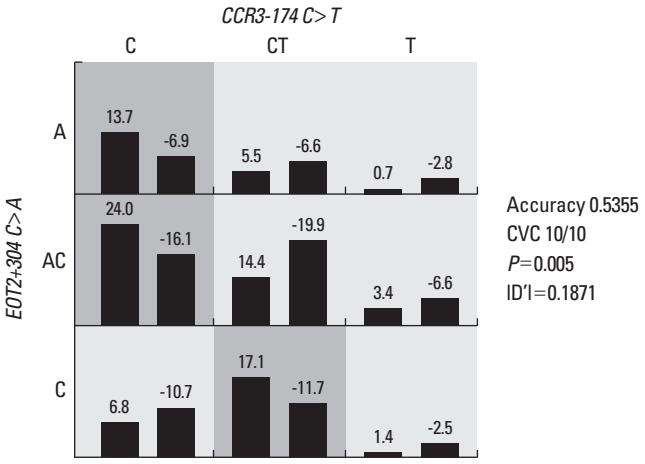

Figure. The best model was composed of *CCR3-174C>T* and *EOT2+304C>A*. In each cell, the left bar represents a positive score and the right bar a negative score. High-risk genotype combinations are shaded dark grey, while low-risk genotypes are shaded light grey. Each *P*-value was obtained from a sign test, CVC, cross-validation consistency; |D'|: Lewontin's |D'| for linkage disequilibrium.

were included in the high-risk group. Thus, the number of blood eosinophils is higher in asthmatics bearing these genotype combinations.

No single SNP in the eotaxin gene family was associated with blood eosinophilia in asthma (data not shown). However, *EOT2+304C>A* in which leucine is changed to isoleucine interacted significantly with the effect of CCR3 SNPs on blood eosinophilia in asthmatics. This finding remained significant after correction with the Bonferroni test ($P<0.01$). The eotaxin and CCR3 pathways are thought to interact biologically, suggesting a gene-gene interaction, or epistasis. The term "epistasis" has been used to describe a "non-independence of effect". In essence, if the effect of one unit is not predictable unless the value of another unit is known, the effect is epistatic.[25] In this regard, the effect of *EOT2+304C>A* may not be disclosed if the effect of CCR3 SNPs is not considered. Moreover, there is no definite evidence that gene-gene interactions can be identified only among loci that show a significant association with phenotype.

There are some limitations to this study. First, blood eosinophilia was included in our models instead of sputum eosinophilia; however, a small number of patients had sputum eosinophilia. Second, it is difficult to confirm the results of interaction models biologically. Eosinophils are further decreased in the lungs and peribronchial tissues of eotaxin-1/-2 double-knockout mice, compared with CCR3-deficient mice.[26] However, the knockout of eotaxin and CCR3 in animals has not been reported.

In summary, novel gene-gene interactions between SNPs of CCR3 and an SNP of eotaxin-2 in the same phenotype were identified in this study. Our analysis of the epistatic influence of CCR3 and eotaxin gene variants suggests that these variants may be candidate markers for eosinophilia in asthma and could be important in understanding the genetics underlying allergic diseases.

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