

Original article

The polymorphisms of Eotaxin 1 and CCR3 genes influence on serum IgE, Eotaxin levels and mild asthmatic children in Taiwan

Background: Asthma is a complex disorder, which is known to be affected by interactions between genetic and environmental factors. The human Eotaxin 1 and CCR3 attract eosinophils and Th2-lymphocytes to migrate to the inflammatory foci that could represent a key mechanism in allergy and asthma.

Objective: We hypothesized that Eotaxin1 gene Ala23Thr and A-384 G, and CCR3 gene T51C polymorphisms are associated with plasma Eotaxin levels and predispose individuals to asthma pathogenesis.

Methods: One hundred seventy-eight hospital-based asthmatic children and 277 community-based controls aged from 5 to 12 years were recruited in southern Taiwan. Whole blood samples and questionnaires were collected. In this study, we addressed genetic effects of Eotaxin 1 and CCR3 genes on asthma, plasma IgE and Eotaxin 1 levels.

Results: In comparison with subjects with Ala23Ala genotype, Ala23Thr polymorphism of the Eotaxin 1 gene showed a significant protective effect on asthma (AOR = 0.58, 95% CI = 0.37–0.92). We demonstrated that the mean Eotaxin 1 concentration was significantly higher in subjects with Ala23Ala than in subjects with Thr23Thr ($P = 0.005$) or Ala23Thr ($P = 0.07$), which showed a gene-dose dependent relationship. But, we observed that the A-384G polymorphism of Eotaxin 1 gene and T51C polymorphism of CCR3 gene are not associated with asthma.

Conclusion: This study finding provide a strong evidence that Eotaxin 1 Thr23Thr homozygote has a protective effect on asthma and significantly decreases plasma Eotaxin 1 concentrations in asthmatics in Taiwan.

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Asthma is a common disease in children that is caused by acute and chronic inflammation in the airways. Asthma evokes chronic inflammatory reactions mediated when a number of chemokines are produced. Eotaxin 1 is a member of the CC chemokine family. It can recruit circulating eosinophils, basophils and Th2-lymphocytes to the inflammatory foci from the blood stream. CCR3 is a G protein-coupled receptor of Eotaxin 1 and is expressed in eosinophils and Th2 lymphocytes (1). These chemotaxis effects of Eotaxin 1 are mediated via CCR3 and could represent a key mechanism in allergy and asthma (1–5).

The human Eotaxin 1 and CCR3 genes are located on chromosome 17q21.1-q21 with three exons (~8 kb) (6, 7) and chromosome 3p21.3 (8) respectively. The expression of Eotaxin 1 mRNA and protein was found to be increased in the bronchial epithelium and endothelial cells of the airways of atopic asthmatics. Furthermore, the elevation of Eotaxin 1 levels was

proportional to eosinophil infiltration and bronchial hyper-reactivity (2, 9) and associated with impaired lung function of the asthmatics (10). Stellato et al. (11) detected expression of CCR3 in eosinophils and airway epitheliums from asthmatic patients. An animal study showed that CCR3 disruption significantly curtails eosinophil recruitment to the lung after allergen challenge (12). Ma et al. (13) found CCR3^{-/-} mice repeatedly sensitized by epicutaneous application of ovalbumin failed to attract eosinophils to the skin. Therefore, CCR3 is critical for eosinophil recruitment to both the skin and the lung and in the development of airway hyper-responsiveness.

In this study, Ala23Thr and A-384G polymorphisms of Eotaxin 1 gene and T51C polymorphism of CCR3 gene have been tested. This genetic association study was conducted to address inherited predisposition to Eotaxin 1 and CCR3 gene in Taiwan and their effects on plasma IgE, Eotaxin 1 levels and asthma.

Methods

Subjects

A genetic association study was conducted from October 2003 to October 2006. One hundred seventy-eight hospital-based asthmatic children were recruited from the Department of Pediatrics in the Memorial Hospital of Kaohsiung Medical University in Taiwan. Asthma was defined by an interview of all subjects with our pediatricians according to GINA guidelines (14). The control group was recruited from an elementary school in Kaohsiung city of Taiwan. In total, 910 grade 4–6 students were assessed for respiratory symptoms and atopic diseases using ISAAC video and a Chinese version of a questionnaire (15) and parents of 424 students agreed to their offspring's blood test. Our community-based control group was defined as those who were free from physician-diagnosed asthma reported by parents and any positive response to ISAAC video questionnaire. But 147 students did not meet the above criteria. Therefore, only 277 students who were free from any respiratory symptoms were selected as community-based controls. The protocol was approved by the IRB of Kaohsiung Medical University. Informed written consent was obtained from the parents of all children.

Written questionnaires for children were completed by their parents, ISAAC video questionnaires were completed by the students after watching the video tape at school. The case and control groups were all aged from 6 to 12 years, living in the Kaohsiung area. The ethnicity of all case and control subjects is Fukein Taiwanese in this study. We assumed that the subjects in our study have the same ethno-geographic origin. Questionnaires for all subjects included demographics (age, sex, ethnicity, education), parents' life styles (cigarette smoking and alcohol use at least once a week persisting regularly for 1 year), and medical history (The age at first time diagnosis, history of asthma, allergic rhinitis and atopic dermatitis diagnosed by a doctor).

Phenotype analysis

Blood samples were collected in heparin-containing venipuncture tube for total plasma IgE and Phadiatop test. Total plasma IgE was measured by using a Microparticle Enzyme Immunoassay (IMX System Abbot, Tokyo, Japan). Phadiatop test was analyzed using immunoassay system Phamarcia UniCAP (Phamarcia, Uppsala, Sweden), an *in vitro* test detecting specific IgE production in response to a mixture of common inhalant allergens and expressed as a positive or negative response with respect to a reference sample, was carried out.

Plasma Eotaxin levels were analyzed by using a commercial kit, which was a human Eotaxin immunoassay (Quantikine, R & D systems, Minneapolis, MN, USA). Blood samples were collected by venipuncture, with EDTA as an anticoagulant. The assay employed the quantitative sandwich ELISA technique using *E. coli*-expressed recombinant human Eotaxin and antibodies raised against the recombinant factor. Its minimum detectable dose of Eotaxin is <5 pg/ml. Both within- and between-run precision levels were <6.7% of CVs.

Mutation analysis

DNA was extracted using a commercial Purgene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN, USA). The A-384G polymorphism of Eotaxin 1 gene and T51C polymorphism of CCR3 gene were detected by using the TaqMan assay of real-time PCR and genotyping analyzed by SDS 2.1 package (Applied Biosystem

SNP genotyping products, <http://www.appliedbiosystems.com/catalog>). The Ala23Thr (G67A) polymorphisms of Eotaxin 1 gene were carried out in a volume of 50 µl containing 200 ng of genomic DNA, 5 pmole of each primer with 2.5 mM of each of the deoxynucleotide triphosphates, Tris-HCl (67 mmol/l, pH 8.8), $[(\text{NH}_4)_2\text{SO}_4]$ (16.6 mmol/l), and 1.25 U of Taq DNA polymerase (Promega Corporation, Madison, WI, USA). The forward primer was, 5'-GAGGAGCAGAGAGGCTGAGA -3' and the reverse primer was 5'-AGCCTGGAGGTGGTTACCTTAC-3'. Following amplification, 8 µl of PCR products of two genes was digested with 1 U of *Bsr* I endonuclease (restriction site 5'-ACTGG-3') at 37°C for 16 h. The Ala23Thr of Eotaxin 1 gene containing allele Ala23 was identified by a single 200 bp band as a wild type, and allele Thr23 was identified by 146 and 54 bp fragments as a variant type. Resultant products were analyzed on 3.5% agarose gel with ethidium bromide (1.5 µg/ml).

Statistical analysis

The chi-squared test was performed to evaluate the differences in genotypes and allele frequencies for obese and normal weight groups (shown in Table 1). A goodness of fit chi-squared test was used to test for Hardy-Weinberg equilibrium (HWE) by comparing the observed number of subjects for each genotype with the expected number of subjects assuming HWE. Two-way analysis of variance (ANOVA) and least significant difference multiple comparisons were used to compare IgE and Eotaxin levels between genotypes, conditional upon control asthma status. IgE and Eotaxin 1 levels were logarithmically transformed before statistical testing to meet the assumption of normal distribution. Adjusted odds ratio

Table 1. Descriptive characteristics of allergies in asthmatic and control children

	Asthmatic children	Control children	P-value
Sample sizes	178	277	
Sex (M/F)	115/63	137/140	0.01
Age ± SD	10.6 ± 2.8	10.4 ± 1.3	0.46
Asthma			
The age at first time diagnosis ± SD	5.02 ± 2.75	–	
Nocturnal waking with wheezing	53/178 (29.8)	–	
Wheezing and shortness of breath after exercise	48/178 (27.0)	–	
>3 asthma attack, in the past 12 months	3/178 (1.8)	–	
Allergy			
IgE ± SD*	831.2 ± 1687.4	275.0 ± 511.7	0.00
IgE ≥ 100U/ml, %	134 (75.3)	125 (45.1)	0.00
Eotaxin ± SD* (pg/ml)	50.4 ± 20.7	51.5 ± 24.0	0.76
Phadiatop, %	127 (71.3)	131 (47.3)	0.00
Allergic rhinitis, %	105 (59.0)	93 (33.6)	0.00
Atopic dermatitis, %	32 (18.0)	18 (6.5)	0.00
Indoor environmental factors			
Pets (dogs, cats, birds), %	33 (18.5)	50 (18.1)	0.63
Mud in walls, %	51 (31.9)	72 (26.7)	0.06
Air cleaner, %	46 (28.8)	52 (19.3)	0.03
Appearance of cockroaches (frequent), %	32 (18.0)	43 (15.5)	0.24
Filled toys, %	86 (48.3)	143 (51.6)	0.83

*IgE and Eotaxin 1 levels were logarithmically transformed before statistical testing to meet the assumption of normal distribution.

Values in parentheses are indicated as percentages.

(AOR) and 95% CI for each polymorphism were calculated by logistic regression, after adjusting for covariates. Regression coefficients (β) and standard errors for Eotaxin1 and IgE concentrations were determined by a linear regression. The statistical analyses were performed with SPSS, release 12.0. Haplotype analysis was conducted and haplotype frequencies were estimated using the expectation-maximization algorithm (16). The extent of disequilibrium was expressed in terms of $D' = D/D_{\max}$. The COCAPHASE program of the UNPHASED software version 2.403 (RFCGR, Cambridge, UK) was used for association analysis of multilocus haplotypes from unphased genotype data (17).

Results

In this study, the mean age of the asthmatic children (10.6 ± 2.8 years) was similar to that of the control children (10.4 ± 1.3 years respectively) ($P > 0.05$) and sex distribution was different between the two groups ($P = 0.01$). The levels of serum total IgE and positive rate of phadiatop test were both significantly higher in the asthmatic children than in control children ($P < 0.01$ for both). But the Eotaxin 1 concentrations were similar in both asthmatic children and controls ($P > 0.05$).

We genotyped these polymorphisms and determined whether they were associated with asthma before and after adjusting for sex distribution. All polymorphisms met HWE in the asthma and control groups (data not shown). The A-384G polymorphism of Eotaxin 1 gene and T51C polymorphism of CCR3 gene are not associated with asthma. In comparison with subjects with

Ala23Ala genotype, Ala23Thr polymorphism of the Eotaxin 1 gene showed a significant protective effect with asthma (AOR = 0.58, 95% CI = 0.37–0.92). A-384G and Ala23Thr variants in Eotaxin1 gene are in strong linkage disequilibrium ($D' = 0.95$ and 1.0 for case and control groups respectively). The haplotype analysis found G-A pairwise (OR = 0.74, $P = 0.03$) had a significantly decreasing and G-G pairwise (OR = 1.61, $P = 0.001$) had a significantly increasing asthma risk in comparison with A-G haplotype. We excluded haplotype A-A from the data because of rare frequency ($< 1\%$). Our results represented that the three haplotype combinations (A-G, G-G, G-A) are significantly related to asthma after adjustment for Bonferroni multiple corrections by multiplying P -values by 3 ($P = 0.006$) (please see Table 2).

The effects of Eotaxin 1 and CCR3 genes polymorphisms on serum Eotaxin 1 concentrations and serum IgE levels were tested using two-way ANOVA adjusting for asthma (Table 3). The mean Eotaxin 1 concentration was significantly higher in subjects with Ala23Ala of Eotaxin 1 gene (53.1 ± 24.2 pg/ml) than in subjects with Ala23Thr (48.6 ± 22.1 pg/ml) ($P = 0.07$) or Thr23Thr (37.6 ± 12.1 pg/ml) ($P = 0.005$), but not for serum IgE levels. The polymorphisms of Eotaxin 1 A-384 G and CCR3 T51C do not have a significant effect on Eotaxin 1 and IgE levels.

By using a multiple logistic regression, we found Eotaxin Thr23Thr genotype, log IgE concentration and history of rhinitis and dermatitis have a significant effect

Table 2. Genotype and allele distributions of Eotaxin 1 and CCR3 genes and associated risk with asthma

Genes	Genotype/allele	Asthma (%)	Control (%)	AOR†	95% CI
CCR3 T51C	TT	153 (92.7)	235 (90.4)	1	
	TC	11 (6.7)	23 (8.8)	0.73	0.34–1.54
	CC	1 (0.6)	2 (0.8)	0.72	0.06–8.12
	T	317 (96.1)	493 (94.8)	1	1
	C	12 (3.9)	27 (5.2)	0.73	0.37–1.45
Eotaxin1 A-384G	AA	61 (35.1)	109 (40.2)	1	1
	AG	83 (47.7)	125 (46.1)	1.12	0.74–1.72
	GG	30 (17.2)	37 (13.7)	1.40	0.79–2.51
	A	205 (58.9)	343 (63.3)	1	1
	G	143 (41.1)	199 (36.7)	1.17	0.89–1.55
Eotaxin1 Ala23Thr	GG	139 (78.5)	189 (68.5)	1	1
	GA	33 (18.6)	77 (27.9)	0.58*	0.37–0.92
	AA	5 (2.8)	10 (3.6)	0.71	0.24–2.16
	G	311 (87.9)	455 (82.4)	1	1
	A	43 (12.1)	97 (17.6)	0.65*	0.44–0.95
Eotaxin 1	Haplotype	Asthma (%)	Control (%)	Haplotype-OR	P -value§
A-384G/Ala23Thr	A/G	203 (58.9)	341 (63.4)	1	
	A/A	1 (0.38)	0 (0)	—	—
	G/A	41 (11.8)	93 (17.3)	0.74*	0.028
	G/G	99 (28.9)	104 (19.3)	1.61	0.001
	LD*‡	0.95	1.0		

* $P < 0.05$.

†AOR, adjusted odds ratio was adjusted for sex distribution.

‡LD*, standardized linkage disequilibrium.

§Multiple comparisons were corrected by using Bonferroni corrections ($P = 0.006$).

Table 3. Effects of Eotaxin 1 and CCR3 polymorphisms on IgE and eotaxin 1 concentrations

Variables	n	IgE (U/ml)*			Eotaxin 1 (pg/ml)*		
		Mean	SD	P†	Mean	SD	P†
Eotaxin1 A-384G							
AA	161	509.6	806.0		52.8	24.0	
AG	202	405.1	870.5		50.7	19.7	
GG	62	463.2	741.6	0.18	49.6	32.1	0.33
Eotaxin1 Ala23Thr							
GG	314	489.4	903.6		53.1 ^c	24.2	
GA	105	514.4	1743.4		48.6	22.1	
AA	15	378.2	584.1	0.74	37.6	12.1	0.007
CCR3 T51C							
TT	382	449.3	838.6		52.1	24.4	
TC	32	466.2	802.5		45.3	12.9	
CC	3	194.0	69.3	0.62	47.6	2.0	0.49

*P-values were from two-way ANOVA adjusting for asthma.

†IgE and eotaxin 1 levels were logarithmically transformed before statistical testing to meet the assumption of normal distribution.

‡Statistical significance of gene-dose trend ($P = 0.003$) was calculated by treating genetic variable as a continuous variable and analyzed by a linear regression.

on asthma. After controlling for age and sex, we found that Thr23Thr genotype plays a strong protective effect on log Eotaxin 1 concentrations ($P = 0.008$). We found phadiotop test, history of asthma and atopic dermatitis were associated with log total IgE levels by using a multiple linear regression (Table 4).

Discussion

At least five case-control studies suggested no association between Eotaxin 1 variants with asthma (18–22), although relationships with asthma-related intermediate phenotypes were observed, as serum total IgE, plasma Eotaxin levels and circulating eosinophil counts. In this study, we found children who were homozygote and heterozygote for threonine form (Thr23Thr and

Ala23Thr) of Eotaxin 1 had a significantly lower risk of asthma than children with two alanine form (Ala23Ala). Eotaxin 1 A-384G and Ala23Thr (G67A) variants close to each other and their disequilibrium coefficients larger than 0.95, should be discussed by haplotype analysis. In this study, we demonstrated G-A haplotype ($OR = 0.74$, $P = 0.028$) has a lower risk, but G-G haplotype ($OR = 1.61$, $P = 0.001$) has a stronger effect on asthma in comparison with A-G haplotype. We also demonstrated Thr23Thr is related to lower plasma Eotaxin 1 concentrations and showed in a gene-dose dependent relationship ($P = 0.003$). In this study, we found that the total IgE levels were correlated with phadiotop test, history of asthma and atopic dermatitis, but not associated with gene polymorphisms of Eotaxin 1 Ala23Thr, A-384G and CCR3 T51C. Raby et al. (23) also did not find Eotaxin 1 Ala23Thr and A-384G associate with total IgE by a family-based association study.

Functional studies of Eotaxin 1 gene for asthma have been performed. Eotaxin mRNA expression correlated significantly with eosinophils and symptom score in asthmatic patients (24). Eotaxin 1 Ala23Thr polymorphism substitutes a polar threonine residue for a hydrophobic alanine residue and alters the Eotaxin1 amino acid sequence (25, 26). Eotaxin 1 threonine variant reduces the predicted signal peptidase activity (22, 26) and secretes Eotaxin 1 protein and eosinophil significantly less than the alanine form (22). Therefore, we propose that this polymorphism would have impaired ability to secrete Eotaxin and is related to the pathogenesis of asthma. Shin et al. (20) genotyped 550 asthmatics and 171 controls, and revealed that Eotaxin 1 gene Ala23Thr is significantly associated with total serum IgE levels ($P = 0.002–0.02$), but not asthma. A large case-control study genotyped 806 asthmatics and 505 controls, and suggested that Eotaxin 1 gene Thr23Thr had lower plasma Eotaxin 1 concentrations and higher levels of lung functions, but was not associated with asthma and IgE (22). Raby et al. evaluated nine common CCL11 polymorphisms and

Table 4. Multivariate analysis for asthma, plasma Eotaxin1 and total IgE levels

Variable	Asthma			log Eotaxin 1†			log IgE†		
	AOR	95% CI	P	β‡	SE (β)	P	β‡	SE (β)	P
Age	0.96	0.85–1.08	0.51	0.029	0.012	0.013*	−0.033	0.038	0.392
Sex (M to F)	1.52	0.96–2.41	0.09	−0.058	0.021	0.007*	−0.041	0.070	0.562
Ala23Thr									
AA, AG to GG	0.58	0.34–0.98	0.047*	−0.062	0.023	0.008*	0.094	0.077	0.225
log IgE	3.10	1.96–4.92	0.001*	−0.009	0.021	0.69	—	—	—
Phadiotop									
Positive to negative	0.87	0.47–1.62	0.66	−0.048	0.031	0.12	0.940	0.077	0.001*
Asthma	—	—	—	—	—	—	0.317	0.099	0.002*
Allergic rhinitis	2.17	1.36–3.46	0.001*	0.014	0.023	0.55	0.067	0.040	0.092
Atopic dermatitis	2.95	1.31–6.65	0.009*	−0.002	0.051	0.98	0.145	0.071	0.043*

* $P < 0.05$.

†Eotaxin 1 and IgE levels were logarithmically transformed before statistical testing.

‡β coefficient.

found an association between A-384G and asthma among African-American families ($P = 0.01$), but not among white and Hispanic families. They also found no association between Ala23Thr and asthma (23). In this multi-racial study, there was statistical heterogeneity in nine common CCL11 polymorphisms of asthma and total IgE (23). Otherwise, several genetic polymorphisms of allergic studies also suggested that there were inconsistent genetic effects between asthma and IgE, included in this study. It is plausible that asthma and IgE could be partially controlled by different genetic factors.

The expression of Eotaxin is upregulated by a Th2 cytokine (27, 28) and down regulated by a Th1 cytokine (29), suggesting the enhanced Eotaxin production may be associated with Th2 polarized allergic inflammation. In this study, we could not detect the differences of Eotaxin 1 concentrations between the asthmatic and control groups. This may be related to the characteristics of our patients. All our cases came from a pediatric outpatient clinic, and most were stable and mild asthmatic patients. Patients (75.3%) were atopic with total IgE >100 U/ml and only three (1.8%) children had an asthma attack more than three times in the past 12 months. From previous studies, Eotaxin 1 levels are proportional to asthma severity (30). Plasma Eotaxin 1 levels were not only at higher levels in clinical patients with asthma compared with controls (31, 32), and during asthma exacerbations are significantly increased compared with levels in patients with stable asthma (33) and have been associated with various measures of asthma severity (33). Symptoms controlled by medical use also influenced the plasma Eotaxin1 concentrations in asthmatic patients. These are important reasons why we cannot find the difference of plasma Eotaxin 1 levels between asthmatic and control groups.

CC chemokine receptors CCR3 was originally identified as an Eotaxin receptor on human eosinophils (6, 34),

and plays an important role in the pathogenesis of asthma (1–4). Linkage analysis of bronchial hyper-responsiveness to chromosome 3 in Dutch and Hutterite populations were demonstrated by genome scan studies (35). A silent mutation T51C in the CCR3 gene is only associated with asthma in a British population, but failed in a Japanese population (36). Our finding was similar to that in Japanese population and both countries are Asian populations.

In this study, several advantages were suggested. Spurious association will not occur because the ethnicity of all subjects was Fukein Taiwanese, which is assumed to be a homogenous population. We performed a genetic association study, which is useful in the evaluation susceptibility genes for complex diseases with modest genetic effects, and they are more powerful than linkage analysis (37, 38). Haplotype analysis was provided, which is more likely to identify disease associations than a single polymorphism, because they reflect complete gene structure. Multiple comparisons were corrected by using Bonferroni corrections to reduce the false positive results in this study. The weakness of this study was the sample-size limitation of haplotype analysis, which made it difficult to estimate the effect of A-A haplotype.

These findings provide strong evidence that Eotaxin 1 Thr23Thr variant has a protective effect on asthma and decreases plasma Eotaxin 1 concentrations. This haplotype analysis also provides evidence that Eotaxin 1 haplotype has an impact in determining susceptibility to asthma in a Taiwanese population.

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