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Genetic polymorphisms of CC chemokine receptor 3 in Japanese and British asthmatics

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ABSTRACT: Whole genome scan analyses have revealed that chromosomal region 3p21-24, which contains a gene cluster of CC chemokine receptors such as CCR3, is possibly linked to asthma. Because CCR3 ligands play a pivotal role in the selective recruitment and activation of inflammatory cells in the asthmatic airway, the authors examined whether there is any association between asthma and the CCR3 gene polymorphisms.

Three polymorphisms were identified using the single stranded conformational polymorphism method in Japanese (Asian) and British (Caucasian) subjects; one silent mutation T51C and two missense mutations G824A and T971C. These polymorphisms were examined in 391 Japanese subjects (210 asthmatics and 181 nonasthmatic controls) and 234 British subjects (142 asthmatics and 92 nonasthmatic controls). Asthma diagnosis was based on episodic symptoms, documented wheeze, and the presence of reversible airflow limitation.

CCR3 T51C demonstrated a significant association with the diagnosis of asthma in the British population (odds ratio 2.35, $p < 0.01$), but not in the Japanese population. Multiple logistic regression analysis also showed that CCR3 T51C was associated with asthma (odds ratio 2.83, $p < 0.02$), independent of atopic phenotypes such as high levels of total or house dust mite-specific immunoglobulin-E in serum.

In conclusion, a significant association between asthma and CCR3 T51C polymorphism localized on chromosome 3p21 was found.

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Chemokine receptor expression mediates the composition of cellular infiltrates in different types of inflammatory pathology. For example, CC chemokine receptor 5 (CCR5) and CXC chemokine receptor 3 (CXCR3) are preferentially expressed on human type 1 helper T (Th1) lymphocytes in the synovial fluid from patients with rheumatoid arthritis [1, 2], while CC chemokine receptors CCR3 and CCR4 are expressed on type 2 helper T (Th2) lymphocytes and thus essential for the pathogenesis of an allergic inflammation [3–6]. CCR3 was originally identified as an eotaxin receptor on human eosinophils [7–9], and was subsequently demonstrated to bind to other eosinophilic chemokines such as regulated on activation, normal T-cell expressed and secreted (RANTES), monocyte chemoattractant peptide 2, 3 and 4 (MCP-2, MCP-3, and MCP-4, respectively) [10]. The selective expression of CCR3 on eosinophils, basophils, and Th2 lymphocytes strongly indicates the role of this receptor in the pathophysiology of asthma [3–5, 11, 12].

Chromosomal localization has already been examined for CCR1–5 genes; CCR1, CCR2, CCR3 and CCR5 genes are localized on chromosome 3p21.3, while CCR4 gene is located at a slightly distal region (3p24) on the same chromosome [13]. Interestingly, two of the five genome scans searching for asthma susceptibility loci, reported a positive linkage of asthmatic phenotypes to chromosome 3 [14, 15]. A genome scan of a genetically homogeneous Dutch population demonstrated evidence of linkage of bronchial hyperresporisiveness to chromosome 3 [15]. Another genome scan in the Hutterites, an isolated population of European ancestry, also demonstrated that two adjacent markers on 3p24 showed possible linkage to bronchial hyperresponsiveness or asthmatic symptoms [14]. The biological activities of CCR3 and its

chromosomal localization suggest that there may be asthma-related mutation(s) within CCR3 gene or the CC chemokine receptor gene cluster on chromosome 3.

In the present study, the polymorphisms within the CCR3 gene in the Japanese (Asian) and British (Caucasian) populations were examined, and it was found that a silent mutation (T51C) in the CCR3 gene was significantly associated with asthma in the British population.

Materials and methods

Subjects

Two-hundred and ten consecutive Japanese asthmatic patients visiting Keio University Hospital in Tokyo, were enrolled in this study. They had been diagnosed with asthma by pulmonologists based on: 1) recurrent breathlessness or chest tightness; 2) physician-documented wheeze; and 3) reversible airflow limitation, confirmed either by variability in serial peak flow rates $>20\%$, or by a $>15\%$ increase of forced expiratory volume in one second (FEV_1) after an inhalation of salbutamol (200 μg). The authors also recruited 181 healthy volunteers without a history of asthma, from the same area in Tokyo.

For the British part of the study, 142 patients with asthma were collected from the Chest unit in Oxford, based on similar criteria to those employed in the Japanese study. Ninety-two healthy subjects without a history of asthma were also collected from the same area in England. All the asthmatic and control subjects in the British study were Caucasians.

Total serum immunoglobulin E (IgE) levels and radioallergosorbent test (RAST) scores for house dust mites were determined by the CAP system (Pharmacia, Uppsala, Sweden). Asthma severity was determined as follows: "mild asthma" was designated as episodic asthma that could be controlled with only intermittent or regular use of bronchodilators, and "moderate to severe asthma" was designated as persistent asthma for which daily use of inhaled and/or oral corticosteroids was necessary to control symptoms. This study was approved by the Institutional Ethical Committee in each institute.

Single-stranded conformational polymorphism analysis of the CCR3 gene

Genomic deoxyribonucleic acid (DNA) was extracted from peripheral blood leukocytes of 90 Japanese (60 asthmatic and 30 nonasthmatic) and 60 British (30 asthmatic and 30 nonasthmatic) subjects using a commercially available kit (QIAamp, QIAGEN Inc., Valencia, CA). The intronless CCR3 gene (1717 bp) was amplified as eight overlapped fragments (181–211 bp) in length by polymerase chain reaction (PCR), using appropriate primers designed according to the reported nucleotide sequence [4]. PCR was performed using Taq polymerase (AmpliTaq Gold, Perkin-Elmer Japan, Chiba, Japan), and an attached buffer (including $MgCl_2$ at the final concentration of 1.5 mM) as follows: one cycle of 95 °C for 9 min, 35 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and one cycle of 72 °C for 5 min. A 5-μL volume of the PCR product was diluted in 45 μL of 95% formamide loading buffer, denatured at 80 °C for 5 min, and then loaded onto a nondenaturing polyacrylamide gel (Super Detection Kit, Toyobo, Osaka, Japan). DNA samples with mutant allele(s) were reamplified by PCR, and the nucleotide sequences were directly determined using an automated sequencer (ABI 373S, Applied Biosystems Inc., Foster City, CA, USA).

Genotyping CCR3 T51C polymorphism

Genomic DNA was extracted from all studied subjects by the method described above, and the CCR3 T51C polymorphism was analysed by a restriction fragment length polymorphism (RFLP) method. An Nla III restriction site was introduced during PCR process using a sense primer (5′-CTTTGGTACCA-CATCCTACCA-3′) and an antisense primer (5′-TGA-GAGGAGCTTACACATGC-3′) performed under the same PCR condition described above. Amplified DNA (10 μL) was then digested with Nla III (2 U·20 μL, New England Biolab, Beverly, MA, USA) at 37 °C for 2 h and the fragments were resolved on a 3% agarose gel (NuSieve 3:1 agarose, FMC, Rockland, ME, USA). The wild-type allele yielded 108-bp and 22-bp fragments while the mutant allele remained undigested (130 bp).

Statistical analysis

The Hardy-Weinberg equilibrium was tested by the Chi-squared test for the frequencies of the CCR3 genotypes. Categorical variables among the different CCR3 genotypes were compared by the Fisher's exact test. Odds ratio (OR) and 95% confidence intervals (CI) were calculated from the β-coefficients and their standard errors. Multiple logistic regression analysis was also carried out to assess the relative contribution of the CC chemokine receptor genotypes, using asthma as a dependent variable, and total serum IgE levels and RAST score for house dust mite as independent variables. A p-value $\leqslant 0.05$ was considered statistically significant.

Results

Table 1 demonstrates the data of the atopic status (total serum IgE levels, RAST scores for house dust mite) and the severity of asthma for the studied subjects. PCR single stranded conformational polymorphism identified three point mutations; a silent mutation T51C, and two missense mutations; G824A which substitutes a glutamine residue for an arginine at position 275 and T971C which replaces leucine at residue 324 with proline.

The frequency of CCR3 51C allele was 0.03 in the Japanese control subjects (n=181) and 0.07 in the British controls (n=92), showing a significant difference

Table 1. – Demographic data of studied subjects

		Japanese population		British population	
		Control	Asthmatic	Control	Asthmatic
Total number of subjects		181	210	92	142
Total serum IgE	$\geqslant 200$ IU·mL^{-1}	41	128	1	78
	<200 IU·mL^{-1}	140	82	91	64
RAST (house dust mite)	$\geqslant 2$	54	93	15	91
	<2	127	117	77	51
Severity of asthma	Mild		67		38
	Moderate - severe		143		104

IgE: immunoglobulin E; RAST: radioallergosorbent test.

between these populations ($p<0.05$). Univariate analysis demonstrated a significant association between the CCR3 51C allele and asthma in the British population (OR 2.35, 95% CI 1.27–4.34, p=0.007, table 2). When divided by the asthma severity, the 51C allele showed a significant association with moderate-to-severe asthma (OR 2.37, 95% CI 1.25–4.51, p=0.009). The 51C allele showed an equivalent OR (2.28) for mild asthma, but their association did not reach a significant level (p=0.067).

In order to avoid being confounded with atopic status, the authors performed multiple logistic regression analysis using asthma, CCR3 genotypes, total serum IgE levels, and RAST scores for house dust mite as parameters (table 3). Analysis revealed that the presence of the CCR3 51C allele was an independent risk factor for asthma in the British population (OR 2.83, 95% CI 1.26–6.32, $p<0.02$).

Discussion

CCR3 is the dominant chemokine receptor on eosinophils and basophils [11, 16] and its ligands (eotaxin, RANTES, MCP-2, 3, 4) are important elements for the chemotaxis and activation of eosinophils and basophils at the site of allergic inflammation [4–6]. Selective expression of CCR3 on Th2, but not Th1 lymphocytes, further underscores the role of this receptor on the pathogenesis of asthma and other allergic diseases [3]. Thus, the authors analysed the CCR3 gene in search for host genetic factors that modify the asthma phenotype, and found two missense mutations which may change the function of the receptor and one silent mutation which was associated with asthma in a Caucasian population.

ZIMMERMANN *et al.* [17] examined 109 individuals (80% Caucasian, 20% African-American), and reported four CCR3 gene polymorphisms including two missense mutations (G824A, T1052C) and two silent mutations (T51C, C240T). In the present study, PCR-single stranded conformational polymorphism (SSCP) analysis in 90 Asian (Japanese) and 60 Caucasian (British) subjects was performed, confirming the presence of G824A and T51C in the present population and adding a new missense mutation T971C to the list of CCR3 gene polymorphisms. T971C mutation replaces a leucine residue with proline at position 324, that locates in the C-terminal intracellular domain, and may affect the intracellular signaling of CCR3. T971C has not been identified in the non-Asian subjects of either the study of ZIMMERMANN *et al.* [17] or the present study. It is possible that T971C is specific to the population of Japanese or Asian ancestry, but further study is necessary to confirm the ethnic specificity of this change.

Univariate analysis demonstrated a significant association between CCR3 T51C mutation and asthma in the British population with an OR of 2.35. The multiple logistic regression analysis further supported the association and demonstrated that the association was independent of atopic elements such as total or house dust mite-specific IgE levels in serum. In contrast, the association between CCR3 51C and asthma in the Japanese population could not be confirmed, and ZIMMERMANN *et al.* [17] also failed to demonstrate the

Table 2. – Association of CCR3 T51C with asthma

	No. of cases	Genotype			Allele		OR (95% CI)*	χ^2	p-value
		TT	TC	CC	T	C			
Japanese population									
Control	181	168	13	0	349	13			
Asthma	210	190	20	0	400	20	1.35 (0.66–2.74)	0.67	0.478
Mild	67	62	5	0	129	5	1.04 (0.36–2.98)	0.01	0.999
Moderate – severe	143	127	15	0	271	15	1.49 (0.70–3.17)	1.07	0.334
British population									
Control	92	78	14	0	170	14			
Asthma	142	101	36	5	238	46	2.35 (1.27–4.34)	7.37	0.007
Mild	38	27	10	1	64	12	2.28 (1.02–5.10)	4.00	0.067
Moderate – severe	104	74	26	4	174	34	2.37 (1.25–4.51)	6.34	0.009

OR: odds ratio; CI: confidence intervals; χ^2: Chi-squared; *: allele C *versus* allele T.

Table 3. – Multiple logistic regression analysis for the risk factors of asthma

Independent variables	Categories	OR	95% CI	χ^2	p-value
Japanese population					
CCR3 51C allele	+	1.72	0.79–3.74	1.85	0.172
Total serum IgE	$\geqslant$200 IU·mL^{-1}	5.86	3.53–9.74	46.4	<0.001
RAST (house dust mite)	$\geqslant$2	0.86	0.51–1.44	0.34	0.561
British population					
CCR3 51C allele	+	2.83	1.26–6.32	6.41	<0.02
Total serum IgE	$\geqslant$200 IU·mL^{-1}	72.60	9.6–547.1	17.30	<0.001
RAST (house dust mite)	$\geqslant$2	4.39	2.08–9.28	15.00	<0.001

OR: odds ratio; CI: confidence interval; χ^2: Chi-squared; IgE: immunoglobulin E; RAST: radioallergosorbent test; +: allele present.

association in their population (80% Caucasian, 20% African-American). This may be due to the heterogeneity among the different ethnic populations. CCR3 gene is localized on the short arm of chromosome 3 [13] and two out of five genome scans performed in Caucasian populations showed a possible linkage of asthma with chromosome 3 [15] or 3p24 [14]. Although there are limited genome scan data for non-Caucasian populations, the Collaborative Study on the Genetics of Asthma in the United States [18] could not show linkage with chromosome 3 in African-American families. A genome scan in a Japanese population also failed to demonstrate linkage between chromosome 3 and atopic asthma [19].

The lack of power is another possible explanation why the study of ZIMMERMANN *et al.* [17] and the present Japanese study, failed to demonstrate an association. In the study of ZIMMERMANN *et al.* [17], only 109 subjects were examined (53 control subjects and 56 asthmatic subjects). Given the CCR 51C allele frequency of 0.13 in their population and the expected OR of 2.35, $\sim$300 subjects are necessary to demonstrate a significant association with enough power (β=0.8). The present Japanese study included 391 subjects, but nearly 1,000 subjects would be needed to demonstrate an association due to the lower allele frequency (0.03) in this population.

Because T51C is a silent mutation which does not change the coded amino acid or modify the ribonucleic acid (RNA) splicing site, it should be in linkage disequilibrium with another mutation within or nearby the CCR3 gene. No significant association between CCR3 G824A and asthma was found in the present population (unpublished data), although the current study apparently lacked power due to the low allele frequency of this polymorphism (0.03 for the British population, and 0.00 for the Japanese population). Other CCR3 missense mutations, T971C identified by the present authors and T1024C identified by ZIMMERMANN *et al.* [17], also occurred too rarely (<1% of allele frequencies) to explain the association between asthma and the T51C polymorphism.

CCR3 is localized within a CC chemokine receptor gene cluster including CCR1, CCR2, CCR4, and CCR5 [13]. Thus, there may be an asthma-related gene for Caucasian subjects in one of the CC chemokine receptor genes. The most well-characterized mutation in the CC chemokine receptor genes is the 32-nucleotide deletion (Δ32) within the CCR5 gene. The CCR5 Δ32 encodes a severely truncated peptide which cannot be detected on the cellular surface and does not transduce CCR5-mediated signals [20–22]. Recently, HALL *et al.* [23] reported that children carrying CCR5 Δ32 mutation are at reduced risk of developing asthma (OR 0.36). They found no asthmatic children who were homozygous for CCR5 Δ32 and demonstrated that the prevalence of asthma was markedly lower in children heterozygous for CCR5 Δ32 than in children with only the wild-type receptor genes [23]. This study, however, found no association between CCR5 Δ32 and asthma in the British population, and there was no linkage disequilibrium between CCR3 T51C and CCR5 Δ32 (unpublished data).

In conclusion, three CCR3 gene polymorphisms including a newly-identified missense mutation T971C, have been identified. The biological and clinical consequences of the missense mutations (G824A, T971C) are as yet unknown. There was a significant association between a silent mutation CCR3 T51C and asthma in the British population, suggesting the presence of undetermined mutation(s) in the CC chemokine receptor gene cluster on the chromosome 3p21-24.

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