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A Functional Polymorphism in the RANTES Gene Promoter Is Associated with the Development of Late-Onset Asthma

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The CC chemokine regulated upon activation, normal T-cell expressed and secreted (RANTES) attracts eosinophils, basophils, and T cells during inflammation and immune response, indicating a possible role for this chemokine in asthma. Both the $-403A$ and $-28G$ alleles of the RANTES promoter region exhibit significantly enhanced promoter activity in reporter constructs *in vitro*. We therefore investigated the genetic influence of these alleles on the development of asthma using case-control analysis in a Japanese population (298 patients with asthma and 311 control subjects). Given the evidence for heterogeneity of asthma according to age at onset, we divided patients with asthma into three subgroups: 117 late-onset patients with asthma (onset at more than 40 years of age), 83 middle-onset patients with asthma (onset at 20 to 40 years of age), and 98 early-onset patients with asthma (onset at less than 20 years of age). The $-28G$ allele was significantly associated with late-onset asthma (odds ratio = 2.033; 95% confidence interval, 1.379–2.998; corrected $p < 0.0025$) but was not associated with the other two asthma subgroups. The $-403A$ allele was not associated with any of the asthma subgroups. Further evidence of the importance of the $-28G$ allele was a significant increase in the production of RANTES *in vitro* in individuals who carried this allele. Our findings suggest that, among Japanese, the $-28G$ allele of the RANTES promoter region confers susceptibility to late-onset asthma.

Keywords: late-onset asthma; RANTES; single nucleotide polymorphism (SNP)

Regulated upon activation, normal T-cell expressed and secreted (RANTES) is a CC chemokine that has been shown to be a potent chemoattractant for T cells, eosinophils, basophils, monocyte/macrophages, and mast cells (1). Both atopic asthma and nonatopic asthma are associated with increased levels of RANTES in bronchoalveolar lavage fluid (2) and bronchial mucosal expression of RANTES (together with eosinophil-active cytokines such as interleukin-5, granulocyte macrophage colony-stimulating factor, and interleukin-3), which contributes to the bronchial mucosal accumulation of activated eosinophils (3). The potential role of RANTES in asthma is also supported by observations that many cell types present in asthmatic airways, such as T cells, platelets, macrophages, endothelial cells, fibroblasts, epithelial cells, and mast cells, have the capacity to generate RANTES (4).

Previous linkage studies have found that human chromo-

some 17q (5, 6), where the gene encoding RANTES is located, contains loci linked with asthma. Polymorphisms with potential functional relevance have also been identified in the RANTES promoter (7, 8); both the $-403A$ and $-28G$ alleles have been associated with increased transcription of the RANTES gene. Further evidence of the functional importance of these variants is the significantly increased risk of atopic dermatitis (7) or human immunodeficiency virus-1 disease progression (8) in individuals carrying the $-403A$ or $-28G$ alleles, respectively. Two genetic studies of children with asthma failed to show an association of asthma with functional polymorphisms in the RANTES promoter region (7, 9), whereas another genetic study found a significant association of the $-403A$ allele with atopy and asthma in adults (10).

The age at onset of asthma covers a wide range. Almost 50% of all subjects with asthma experience onset before the age of 10 years, and 25% of all subjects experience onset after age 40 (11). Respiratory infectious agents such as viruses and *Chlamydia pneumoniae* may be more strongly involved in the pathophysiology of asthma that develops in later life (12). Furthermore, the age at onset of asthma correlates with reactivity of basophils; early-onset asthma is significantly associated with greater levels of anti-immunoglobulin E (IgE)-induced histamine release from basophils (13). In a previous study, we found that the age at onset of asthma affects alteration of total serum IgE levels by the FCER1B promoter polymorphism (14). Although the characteristic features of the histopathology of asthma generally include persistent airway inflammation and tissue eosinophilia, asthma is a heterogeneous condition with a number of overlapping phenotypes, typified at the extremes by early-onset asthma (in which atopy is a prominent feature) and late-onset asthma (in which atopy is often not present). Interactions of environmental factors with specific genes may influence clinical presentation of asthma, including the clinical course (early onset or late onset), the severity of the disease, and therapeutic responses. We hypothesized that two functional alleles at the RANTES promoter region influence susceptibility to asthma and that genetic manifestations of these alleles may be confounded by the age at the onset of asthma. Using case-control analysis, we evaluated the association of these two functional polymorphisms with asthma, dividing subjects into three subgroups according to the age at onset (early, middle, and late onset).

METHODS

Study Population

The study was comprised of 298 patients with asthma and 311 healthy control subjects. Total serum IgE levels (IU/ml) and specific IgE responses to 10 common inhaled allergens, including *Dermatophagoides farinae*, grass pollens, animal dander, and molds, were determined. We defined atopy as having a positive response to at least 1 of the 10 allergens. All participants ($n = 609$) were Japanese, and all gave written informed consent for enrollment in the study and all associated proce-

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TABLE 1. CHARACTERISTICS OF THE STUDY POPULATION

	Healthy Control Subjects	Patients with Asthma			p Value*
		Early Onset	Middle Onset	Late Onset	
No. of subjects	311	98	83	117	
Sex, male/female	191/120	54/44	29/54	45/72	< 0.0001
Age, yr [†]	38.3 (13.4)	31.7 (14.4)	39.9 (11.6)	59.2 (8.1)	< 0.0001
Current smoker [‡]	93 (29.9%)	25 (25.5%)	22 (26.5%)	17 (14.5%)	< 0.05
Ex-smoker [‡]	4 (1.3%)	14 (14.3%)	14 (16.9%)	33 (28.2%)	< 0.0001
Atopy [‡]	153 (49.2%)	92 (93.9%)	65 (78.3%)	58 (49.6%)	< 0.0001
Serum IgE [†]	1.83 (0.62)	2.63 (0.57)	2.41 (0.67)	2.25 (0.58)	< 0.0001
FEV ₁ /FVC [†]	–	69.5 (13.9)	72.5 (13.6)	65.7 (11.2)	< 0.05
Age at onset, yr [†]	–	8.38 (5.99)	31.2 (5.75)	54.2 (8.09)	< 0.0001

* Chi-square test or analysis of variance was used where appropriate.

[†] Values are mean (SD) or [‡] number (%).

dures. The ethics committee of the School of Medicine, Hokkaido University, approved the study.

Allele-Specific Polymerase Chain Reaction and Detection of Fluorescence-labeled Polymerase Chain Reaction Fragments

Using kinetic thermal cycling (15), we determined the genotypes of all 609 subjects with respect to two RANTES promoter polymorphisms: –28C/G and –403G/A. Genotyping was performed by allele-specific polymerase chain reaction; incorporation of SYBR Green I (Applied Biosystems, Foster City, CA) into the real-time polymerase chain reaction allowed discrimination between specific and nonspecific products.

Quantitation of RANTES Protein Levels

Using enzyme-linked immunosorbent assay, we measured RANTES protein levels in supernatants of phytohemagglutinin (PHA)-stimulated mononuclear cells taken from 70 healthy subjects.

Statistical Analysis

We divided patients with asthma into three subgroups according to the age at onset (Group 1, 0–19 years; Group 2, 20–40 years; and Group 3, 41 years or more). The chi-square test was used to compare qualitative risk factors (sex, smoking status, and atopic status) among the three subgroups and the controls. One-way analysis of variance was used to compare quantitative risk factors (age and serum IgE levels). We tested agreement with Hardy-Weinberg equilibrium using a goodness-fit chi-square test.

Univariate logistic regression analysis was initially used to calculate odds ratios with 95% confidence intervals as estimates of relative risk for development of asthma (early, middle, and late onset). As results of case-control analysis indicated association of the –28C/G polymor-

phism with late-onset asthma, further analyses were limited to the 117 patients with late-onset asthma and the 311 control subjects. To evaluate association of the –403G/A and –28C/G polymorphisms with late-onset asthma in the context of haplotype, we assessed differences in haplotype distributions using the log-likelihood ratio test (16).

To control for potential confounding effects of the –403G/A genotypes, chi-square analysis was performed using the Mantel-Haenszel test and stratification according to –403G/A genotype. Also, using multiple regression, we adjusted measurements for possible confounding effects of the –403G/A genotypes, age, sex, smoking status (never smoked, ex-smoker, or current smoker), and atopic status on the odds ratios for the –28C/G polymorphism.

To examine further the relationship between the age at onset of asthma and genetic effects of the –28G allele, we performed survival analyses with the age at onset of asthma as the primary outcome, using all subjects (patients with asthma and control subjects, $n = 609$) or using only patients with asthma ($n = 298$). The subgroups stratified according to the RANTES –28C/G genotypes were analyzed for time to the development of asthma using the standard Kaplan-Meier method.

We compared RANTES protein production of mononuclear cells in response to PHA stimulation between the –403G/A genotypes (–403A carriers versus noncarriers) and between the –28C/G genotypes (–28G carriers versus non-carriers), using an unpaired t test.

RESULTS

Patients with early-onset asthma were highly atopic as a population, compared with patients with late-onset asthma and healthy controls (Table 1). Of the three asthma subgroups, patients with late-onset asthma had the lowest levels of mean total serum IgE,

TABLE 2. ASSOCIATION OF THE RANTES PROMOTER POLYMORPHISMS WITH ASTHMA ACCORDING TO AGE AT ONSET: UNIVARIATE ANALYSIS

				OR	95% CI	p Value
–403G/A Genotype*	GG	GA	AA			
Number of subjects, %						
Healthy control subjects, $n = 311$	140	137	34			
Early-onset asthma, $n = 98$	54	35	9	0.759	0.533, 1.081	0.130
Middle-onset asthma, $n = 83$	45	26	12	0.883	0.615, 1.268	0.490
Late-onset asthma, $n = 117$	47	47	23	1.323	0.976, 1.793	0.070
–28C/G Genotype [†]	CC	CG	GG			
Healthy control subjects, $n = 311$	243	62	6			
Early-onset asthma, $n = 98$	77	18	3	1.031	0.642, 1.657	0.890
Middle-onset asthma, $n = 83$	67	14	2	0.906	0.533, 1.540	0.720
Late-onset asthma, $n = 117$	72	38	7	2.033	1.379, 2.998	0.00034

Definition of abbreviations: CI = confidence interval; OR = odds ratio.

Odds ratios (95% confidence intervals) were calculated for the presence of –403A* and –28G[†] alleles, using univariate logistic regression models. Odds ratios (95% confidence intervals) for the development of asthma (all of the three asthma groups together) were 0.99 (0.79, 1.25) and 1.35 (0.98, 1.85) for the presence of –403A and –28G alleles, respectively.

TABLE 3. LINKAGE DISEQUILIBRIUM BETWEEN RANTES PROMOTER POLYMORPHISMS AND LATE-ONSET ASTHMA

-403G/A	-28C/G	Haplotype Frequency*	
		Healthy Controls	Patients with Late-Onset Asthma
G	C	0.574	0.054
G	G	0.003	0.016
A	C	0.186	0.029
A	G	0.086	0.052

* In the likelihood ratio test of linkage disequilibrium, the statistic has a value of 15.62 with an associated p value of 0.00136. Therefore, haplotype frequencies were estimated under the hypothesis of association between two RANTES promoter polymorphisms and late-onset asthma using the EH program (16). The strongest association is between late-onset asthma and the haplotype comprising both the -403G and -28G alleles.

the lowest frequency of atopic individuals, and the lowest levels of mean FEV₁/FVC. In addition, the frequency of ex-smokers was highest in the late-onset asthma group (Table 1), and cumulative tobacco exposure before the onset of asthma was highest among patients with late-onset asthma. Four early-onset, 33 middle-onset, and 41 late-onset patients with asthma were exposed to tobacco smoke before the onset of asthma; mean \pm SD of log (cigarettes \times years) for these smokers was 1.79 ± 0.54 , 2.25 ± 0.37 , and 2.78 ± 0.39 , respectively ($p < 0.0001$, analysis of variance).

The allelic frequencies and distribution of genotypes among the healthy control subjects (-403A frequency, 0.670; -28G, 0.881) were similar to those reported for control subjects in a previous study (8) of Japanese subjects (-403A, 0.624; -28G, 0.860). Both polymorphisms fulfilled Hardy-Weinberg expectations for these control subjects.

We found that the -28C/G promoter polymorphism is significantly associated with late-onset asthma (odds ratio = 2.033; 95% confidence interval, 1.379–2.998; $p = 0.000341$). In contrast, there were no significant differences in genotype distribution of the -28C/G promoter polymorphism between healthy control subjects and patients with early- or middle-onset asthma (Table 2). Also, in none of the asthma subgroups was the -403G/A promoter polymorphism associated with development of asthma (Table 2). Because we initially studied two markers (-403G/A and -28C/G polymorphisms) in three asthma subgroups (early, middle, and late onset), we multiplied our significance levels by six (two markers \times three subgroups). Using this stringent correction, the association between -28C/G polymorphism and late-onset asthma had a (corrected) p value of less than 0.0025.

The -403G/A and -28C/G polymorphisms were in a significant linkage disequilibrium, with the -403G allele strongly associated with the -28C allele ($p < 0.001$). The frequencies of two-locus haplotypes of the -403G/A and -28C/G polymorphisms differed significantly between patients with late-onset asthma and control subjects ($p = 0.00136$, log-likelihood test; Table 3). This finding is consistent with the single-locus data and supports the association of late-onset asthma with the -28C/G polymorphism within the haplotype context.

The Mantel-Haenszel test, stratified by the -403G/A genotype, showed a significant association between the -28C/G genotype and the development of late-onset asthma (Mantel-Haenszel chi-square = 10.41 with three degrees of freedom, $p = 0.00125$). In addition, the relative risk of asthma for the -28G carriers did not change after adjustment for potential confounding factors such as -403G/A genotype, age, sex, smoking status, and atopic status; multiple logistic regression analysis revealed that the presence of the -28G allele was a significant risk factor

TABLE 4. ASSOCIATION OF LATE-ONSET ASTHMA AND ATOPY WITH TWO RANTES PROMOTER POLYMORPHISMS: MULTIVARIATE ANALYSIS*

		OR	95% CI	p Value
Atopy	Nonatopic	1.00		
	Atopic	1.62	1.14, 2.31	0.00689
403G/A	GG	1.00		
	GA or AA	0.52	0.25, 1.08	0.0802
28C/G	CC	1.00		
	CG or GG	3.88	1.74, 8.64	0.000926

Definition of abbreviations: CI = confidence interval; OR = odds ratio.

* Multivariate analysis for odds of late-onset asthma was performed by logistic regression, with atopy and the RANTES promoter genotypes coded according to the presence or absence of the -403A or -28G alleles. In addition to atopy and the two polymorphisms, sex, age, and smoking status were also adjusted in the multivariate logistic regression model.

for the development of late-onset asthma (adjusted odds ratio = 3.88; 95% confidence interval, 1.74, 8.64; $p = 0.000926$; Table 4). Taken together, these results strongly suggest that the genetic effect of the -28G allele contributes to the development of late-onset asthma, which is independent of atopy and the genetic effect of the -403A allele.

Figure E3 in the online supplement shows the plots of the estimates of the log cumulative hazard for the development of asthma. In each group of subjects (Figures E3A and E3B), the curves for the groups are parallel until the age of 40 years; the slope of the log cumulative hazard plot of the -28G carrier becomes steeper after the age of 40 years. Although none of the statistical tests are effective at detecting this kind of difference between two groups, a visual comparison of the estimated survival curves supported our finding that there is a significant association between late-onset asthma and the RANTES-28G allele.

Mononuclear cells from control subjects who carried the -28G allele ($n = 20$) produced significantly greater levels of RANTES protein than cells from controls who did not carry this allele ($n = 50$): $9,440 \pm 565$ (mean \pm SD) versus $7,978 \pm 358$ pg/ml ($p = 0.012$; Figure 1). In contrast, mean concentrations of RANTES protein did not differ between -403G/A genotypes (Figure 1).

DISCUSSION

This case-control study provides evidence indicating that the -28C/G polymorphism in the RANTES gene is associated with

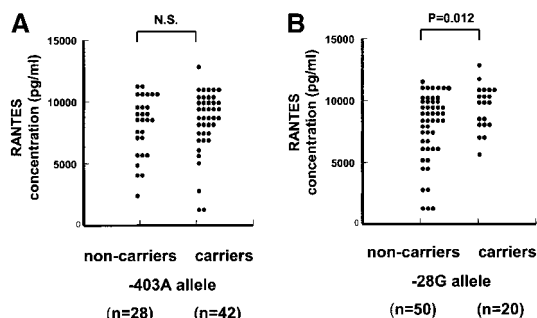


Figure 1. RANTES concentrations in supernatants of PHA-stimulated mononuclear cells. RANTES protein levels were compared between -403A allele carriers and noncarriers (A) and between -28G allele carriers and noncarriers (B).

susceptibility to development of late-onset asthma in Japanese. The association that we observed between the $-28C/G$ polymorphism and RANTES protein levels *in vitro* supports the hypothesis that this specific locus is involved in development of late-onset asthma. Four binding sites for nuclear factor- κB in the RANTES promoter are critical for induction by the proinflammatory cytokines tumor necrosis factor- α and interleukin- 1β and induction through the CD28 costimulatory pathway (17). The $-28C/G$ polymorphism is located immediately downstream of the first of these nuclear factor- κB binding sites (-40 to -31). However, as with any genetic association, it is theoretically possible that the $-28C/G$ polymorphism is merely in linkage disequilibrium with alleles that cause the effects that we observed. Although the $-403G/A$ polymorphism did not appear to influence risk of asthma in these subjects, it is associated with atopic dermatitis (7) in African Americans and with atopy and asthma in white individuals (10). This suggests that phenotypic effects of the $-403G/A$ and $-28C/G$ polymorphisms and their interaction may vary according to environmental and genetic backgrounds, resulting in a complex system of RANTES regulation in humans.

These findings indicate that genetic manifestations of the $-28G$ allele of the RANTES promoter vary with the age at onset of asthma. The correlation between genetic heterogeneity and age at onset is evident in several complex diseases, including Alzheimer's disease (18), breast cancer (19), and Parkinson disease (20). Genetic effects of the $-28G$ allele of the RANTES promoter may be causative factors of late-onset asthma. Although bronchial asthma is caused by complex interactions among genetic and environmental factors, atopy (the propensity to generate IgE in response to common environmental allergens) is by far the strongest risk factor for asthma that has been identified. In this study, adjusted odds ratios for atopy in the development of asthma were 4.47 (95% confidence interval, 2.90–6.89), 2.59 (1.84–3.48), and 1.62 (1.14–2.31) for early-, middle- and late-onset asthma, respectively. The $-28G$ allele of the RANTES promoter region was not associated with early- or middle-onset asthma; effects of this allele may be masked by the effects of other genetic risk factors related to atopy, including genes on chromosomes 5q31 (21) and 11q13 (22). Conversely, in patients with late-onset asthma (in whom genetic predisposition for atopy is generally less important as a risk factor), the genetic influence of the $-28G$ allele was evident.

In general, Th2-derived cytokines modulate expression of chemokines in airway epithelia (23) and play a crucial role in the development of airway inflammation in asthma (24). In response to interleukin-9 (a Th2 cytokine), human bronchial epithelial cells released RANTES in a dose-dependent fashion (25). However, it should not be assumed that asthmatic inflammation is only induced by Th2 cytokines. This issue has recently been examined using experimental models in which investigators co-transferred Th1- and Th2-type specific lymphocytes into a naive mouse. They found that the presence of Th1-type cells did not suppress development of pulmonary disease but rather intensified the inflammation and exacerbated the lung pathophysiology (26). These data, together with the fact that viral respiratory infections remain the leading trigger of asthma attacks, suggest a role for Th1 cells in pathophysiology of airway inflammation in asthma. RANTES is induced by interferon- γ and appears to be closely associated with Th1 response (27). In addition, although increased production of RANTES within the bronchial mucosa is found in all cases of bronchial asthma, regardless of atopic status, production levels tend to be greater in nonatopic asthmatics than in atopic asthmatics (3, 28). Thus, increased expression of RANTES, especially in patients with late-onset nonatopic asthma, may reflect interferon- γ production in response to various stimuli, including viral infections, or (perhaps more likely)

may suggest hereditary propensity for overexpression of the RANTES gene in response to a wider variety of inhaled stimuli.

Viral infections induce a number of cytokine mediators, including RANTES, in both local macrophage populations and airway epithelial cells (29). These mediators upregulate airway inflammation and increase airway responsiveness. Indeed, there is the possibility of a pathogenic link between asthma and viral infection (30). Therefore, in individuals possessing the potentially functional $-28G$ allele, RANTES may be expressed to a greater extent in response to exogenous stimuli, including viral antigens, leading to exaggerated inflammation in the airways. Given the relatively small differences in induced luciferase activity previously observed for different alleles of the $-28C/G$ polymorphism (8), prolonged and repeated exposure to exogenous stimuli may be required for the $-28G$ allele to manifest genetic effects in airways, which may in part explain why this allele is associated with late-onset asthma but not early- or middle-onset asthma.

Specific chemokines are involved in different cellular and molecular pathways that contribute to the complex pathophysiology of asthma in a coordinated fashion, and the role of RANTES in asthma pathogenesis is also known to be extremely complex (31). It is, therefore, necessary to be cautious about the clinical implications of these results until the effects of the $-28G$ allele *in vivo* are understood in greater detail. It is also important to note that the case-control approach has an inherent potential for false-positive results caused by population stratification. Family-based association studies that include the transmission disequilibrium test may eliminate the possibility of false-positive results caused by differences in population stratification between cases and control subjects (32). However, recruiting a sufficient number of father–mother–child trios is considerably more difficult, particularly for late-onset disease. In addition, the case-control approach was recently successfully used to find common haplotypes that display significant population-attributable risk for non–insulin-dependent diabetes mellitus (33), thus indicating the usefulness of the case-control approach for study of adult-onset disease. Nevertheless, these findings are preliminary, and corroborating evidence from an independent study with a more rigorous study design is required.

Case-control studies based on questionnaires are susceptible to recall bias. Definitions of physician-diagnosed asthma have been shown to be specific but somewhat lacking in sensitivity; they probably exclude milder cases of asthma (34). In this study, to judge age at onset of asthma as accurately as possible, patients were asked about episodes of dyspnea, wheezing, or coughing during childhood and puberty. In cases of uncertainty, the time of earliest respiratory symptoms was designated as the age at onset of asthma symptoms, which was used for the calculation of the duration of asthma. A recent study of age at onset of asthma in 4,335 Japanese adult patients with asthmas found that 26.3% developed the disease before the age of 20 years, and 73.8% developed the disease at 20 years or older (35). This variation in age at onset of adult asthma is very similar to that found in this study (Figure E1 in the online data supplement) and is evidence that the self-reported data on age at onset of asthma obtained in this study is reliable. However, we must point out that it is virtually impossible to distinguish recrudescence asthma cases from incident asthma cases in an adult population with any certainty. It seems unlikely that the $-28C/G$ polymorphism contributed to this recall bias, even though a number of patients with asthma who we designated as late-onset may have developed the disease in their childhood. Therefore, we believe that the differences we observed in frequency of the $-28G$ allele among the asthma subgroups cannot be attributed to the recall bias.

In summary, it has previously been suggested that RANTES

is a key mediator of bronchial asthma, and the $-28G$ allele of the RANTES promoter region has previously been shown to be associated with a higher rate of transcription of the RANTES gene. In this study, the $-28G$ allele was significantly associated with late-onset asthma ($p_{\text{corrected}} < 0.0025$). Further evidence of the importance of the $-28G$ allele in development of late-onset asthma is the significant association of this allele with increased production of RANTES protein *in vitro* ($p < 0.05$). Late-onset asthma may provide interesting models of human asthma for comparison with atopic early-onset asthma, which is the most frequent type of asthma. These findings may lead to new insights into ways in which different mechanisms can produce the same phenotype. Clarification of the different underlying mechanisms of the different forms of asthma could result in better targeting for prevention and treatment.

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