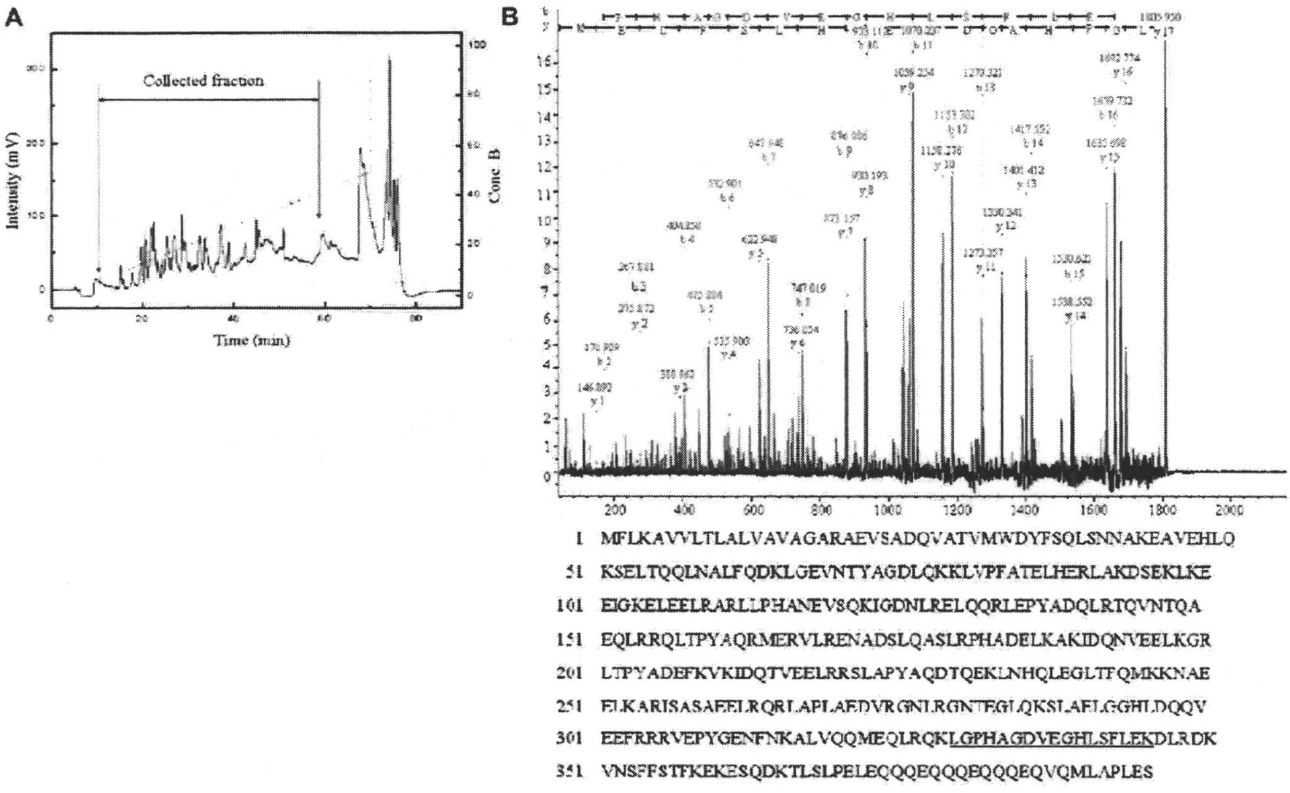

FIG E1. An example of nano-HPLC sample separation and MALDI-TOF/TOF analysis (spot 10). **A,** An example of a chromatograph of nano-HPLC. The *y-axis* represents intensity, which shows the relative abundance of the separated peptide at each separation time. **B,** An example of MALDI-TOF/TOF analysis. The MS/MS data show *underlined* amino acid sequences, which form part of apoA-IV.

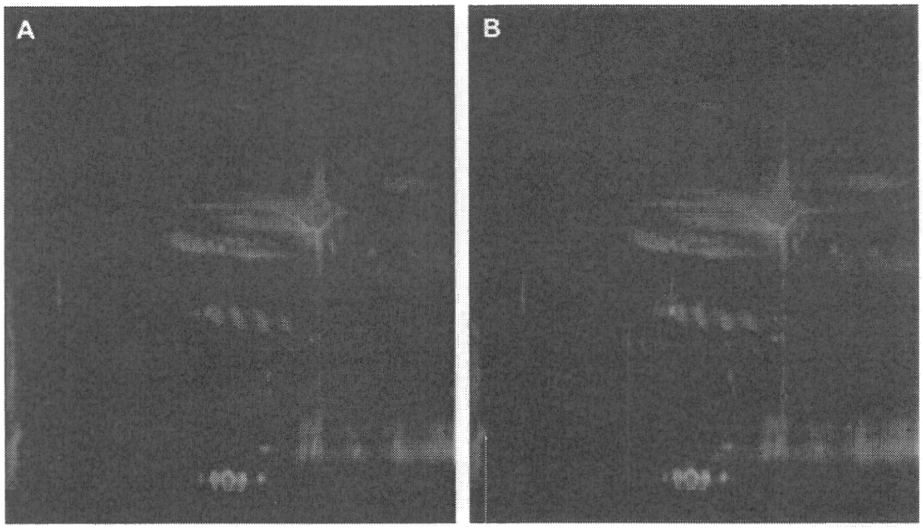

FIG E2. Western blotting with apoA-IV–specific antibody. **A,** 2-DE image of the total protein stained with *Deep Purple* total protein stain. **B,** 2-DE image of the total protein merged with the image detected with the apoA-IV–specific antibody. The *arrow* indicates the spot corresponding to apoA-IV.

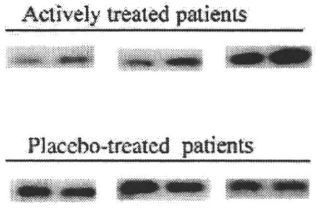

FIG E3. Representative Western blot image of apoA-IV in actively treated and placebo-treated groups.

TABLE E1. Characteristics of patients

	Patient no.	IgE RIST (IU/mL)	IgE RAST (UA/mL)						
			JC	*Dermatophagoides pteronyssinus*	*Dermatophagoides farinae*	*D glomerata*	*A artemisiifolia*	*C albicans*	*Aspergillus*
Placebo	1	7	3.69	<0.34	<0.34	<0.34	<0.34	<0.34	<0.34
	2	290	6.57	19.10	18.20	2.88	<0.34	<0.34	<0.34
	3	68	11.30	<0.34	<0.34	<0.34	<0.34	<0.34	<0.34
	4	190	44.90	1.51	1.51	0.63	<0.34	<0.34	<0.34
	5	82	12.00	<0.34	<0.34	<0.34	<0.34	0.52	<0.34
	6	24	5.00	<0.34	<0.34	<0.34	<0.34	<0.34	<0.34
	7	70	1.95	0.54	0.53	<0.34	<0.34	<0.34	<0.34
	8	21	1.25	<0.34	<0.34	<0.34	<0.34	<0.34	<0.34
	9	370	73.50	2.89	0.49	1.60	<0.34	<0.34	<0.34
Actively treated	1	210	9.65	<0.34	<0.34	0.79	3.85	<0.34	<0.34
	2	180	11.60	<0.34	<0.34	<0.34	<0.34	<0.34	<0.34
	3	99	14.50	<0.34	<0.34	0.53	<0.34	<0.34	<0.34
	4	29	19.90	<0.34	<0.34	<0.34	<0.34	<0.34	<0.34
	5	120	13.40	0.40	<0.34	<0.34	<0.34	<0.34	<0.34
	6	48	1.92	<0.34	<0.34	<0.34	<0.34	<0.34	<0.34
	7	60	5.18	<0.34	<0.34	5.91	<0.34	<0.34	<0.34
	8	240	25.10	0.45	<0.34	0.60	<0.34	0.72	<0.34
	9	72	3.52	<0.34	<0.34	<0.34	<0.34	<0.34	<0.34
	10	25	5.39	<0.34	<0.34	<0.34	<0.34	<0.34	<0.34
	11	11	1.18	<0.34	<0.34	<0.34	<0.34	<0.34	<0.34
	12	150	8.09	12.40	18.10	<0.34	0.38	<0.34	<0.34
	13	84	5.23	<0.34	<0.34	<0.34	<0.34	<0.34	<0.34
	14	470	27.10	0.46	<0.34	<0.34	0.70	<0.34	<0.34
	15	250	7.68	18.60	24.80	3.14	0.58	<0.34	<0.34

Specific IgE	Class
<0.34	0
0.35-0.69	1
0.70-3.49	2
3.50-17.49	3
17.50-49.99	4
50.00-99.99	5
>100	6

Original Paper

International Archives of Allergy and Immunology

Int Arch Allergy Immunol 2010;151:255–261
DOI: 10.1159/000242363

Received: July 7, 2008
Accepted after revision: May 25, 2009
Published online: September 29, 2009

Prevalence of Allergic Rhinitis and Sensitization to Common Aeroallergens in a Japanese Population

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Key Words
Aeroallergen · Allergic rhinitis · Dust mite · Specific human IgE · Japanese cedar pollen

Abstract
Background: Allergic rhinitis (AR) is recognized as a major health problem worldwide, and its prevalence depends on the age range of the subjects. The aims of this study were to determine the current prevalence of AR, effects of age on the prevalence of IgE sensitization to inhalant allergens, and serum total IgE levels in Japanese subjects. ***Methods:*** We conducted a survey of 1,540 subjects between 20 and 49 years of age in 2006 and 2007 and examined the prevalence of AR and sensitization to 7 common aeroallergens. We measured serum total IgE and specific IgE to 7 aeroallergens. AR was determined based on symptoms, predominantly in the nose and eyes, caused by aeroallergens as mentioned in a questionnaire and sensitization to any of the 7 aeroallergens as assessed by measurement of serum specific IgE. ***Results:*** The prevalence of AR was 44.2% (681 of the 1,540 subjects) and there was no difference among age decades. Of the 1,540 subjects, 1,073 (69.7%) were sensitized to at least 1 of the 7 aeroallergens. The most common allergen in AR was Japanese cedar pollen (89.6%, 610 of the 681 with AR) in all the age decades examined. The sensitization rate to mites was significantly higher in the younger subjects. ***Conclusion:*** Our data suggest that the prevalence of AR between 20 and 49 years of age has increased by nearly 10% during the last 10 years. Cedar pollen and mites were predominant allergen sources among the 7 aeroallergens in the Japanese population.

Introduction

Allergic rhinitis (AR), the most common type of rhinitis, is a heterogeneous disorder that significantly impairs the patient's quality of life, and its prevalence has markedly increased in recent decades [1, 2]. Epidemiologic and serological studies have provided valuable information to develop effective strategies for the prevention and treatment of the disease [3–6]. Japanese cedar

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pollinosis (JCP) is a common allergic disease, and the increase in its prevalence is a major public health problem in Japan [7]. Several epidemiologic studies have been conducted on JCP [8–11]. Sakurai et al. [9] reported the prevalence and risk factors of AR and JCP among 2,307 Japanese men; the prevalence rates of AR, seasonal rhinitis and JCP were 35.5, 28.8 and 11.0%, respectively, in 1998. Kaneko et al. [10] conducted a meta-regression analysis of 38 population-based surveys in Japan. The prevalence of JCP among adolescents in the general population was estimated at 28.7% in metropolitan areas and 24.5% in urban areas in the year 2004. The study also reported that the prevalence of JCP increased 2.6-fold between 1980 and 2000. To monitor the prevalence of sensitization is useful for understanding AR and developing preventive measures.

In AR, an IgE-mediated response to allergens is triggered and characterized by type-2-helper-T-cell-dependent inflammation [12]. Allergen-specific IgE is a critical factor in the mechanism of AR. Serum allergen-specific IgE results closely correlate to those of skin tests and nasal challenges. Allergen-specific IgE tests are highly specific and sensitive. One of their advantages is that drugs and skin diseases do not influence the measurement [1].

Sensitization is an important risk factor for developing allergic disease [13]. Epidemiological investigation of AR is important to clarify its etiology and develop appropriate preventive and therapeutic techniques. There have been few epidemiological studies on the age effect on the prevalence of AR and IgE sensitization to inhalant allergens, and serum total IgE levels in Japanese subjects. Therefore, we conducted an epidemiological study on a total of 1,540 subjects aged 20–49 years. The protocol comprised a questionnaire, measurement of total serum IgE antibodies and allergen-specific IgE antibodies against 7 aeroallergens in 2006 and 2007. The major findings of this study are the prevalence of allergic sensitization and AR, the age effect on them, and total serum IgE and AR, and the related age effect.

Material and Methods

Study Subjects

A total of 1,540 subjects were recruited from residents of Eiheiji-cho and the cities of Fukui, and Echizen in Fukui prefecture, in the central Hokuriku area of Japan in May and June of both 2006 and 2007. In that area, Japanese cedar pollen counts are at the average level of the islands of Honshu, Shikoku and Kyushu [7]. The 1,540 subjects were workers of 4 hospitals and students of nursing and medical colleges in the University of Fukui. The number of females was higher than that of males (mean age, 32.1 years; range, 20–49 years; male:female ratio, 1.0:2.40; mean serum IgE level, 233.8 IU/ml; median serum IgE level, 73.5 IU/ml). The participants were recruited during their annual health check-up in 2006 or 2007; 13 subjects did not agree to participate in this survey. Reasons for nonparticipation were lack of interest or time. All of the 1,540 participants agreed to measurement of serum total IgE and specific IgE to 7 aeroallergens and to answer a questionnaire. Blood collection and the questionnaire survey were performed at the same time after informed consent was received. We did not conduct a follow-up survey in this study. The diagnosis of AR was confirmed by seasonal or perennial symptoms of rhinitis consisting of any combination of the following: nasal itching, sneezing, discharge and stuffiness caused by inhalation of aeroallergens, reported on a questionnaire. All of the subjects with AR were also positive for serum-specific IgE to 1 or more of the 7 aeroallergens. All individuals were unrelated Japanese individuals and gave written informed consent to participate in the study according to the rules of the ethics committees of the Faculty of Medical Science, University of Fukui and the Institute of Physical and Chemical Research (RIKEN).

Measurement of Serum Levels of Specific IgE Antibodies

Specific IgEs to 7 aeroallergens, *Cryptomeria japonica*, *Dermatophagoides pteronyssinus* (Der p), *Dermatophagoides farinae* (Der f), *Dactylis glomerata*, *Ambrosia artemisiifolia*, *Candida albicans* and *Aspergillus fumigatus* were measured with a Pharmacia CAP System (Pharmacia CAP, Upsala, Sweden) (table 1). Allergen sensitization was classified as positive if the allergen-specific serum IgE level was above 0.7 (CAP RAST score of 2).

Statistical Analysis

To clarify the age-specific prevalence of AR and sensitization to the 7 aeroallergens examined, patients were divided into 3 age groups, the 20s (20 to <30 years), 30s (30 to <40 years) and 40s (40 to <50 years). We then compared differences in frequencies of sensitization to each of the 7 aeroallergens among these age groups by using the Kruskal-Wallis test and then by individual testing using the Mann-Whitney U test if significant. Serum total IgE was analyzed at a quantitative level, and log-transformed individual serum IgE levels were used in the figures. Correlations of total IgE levels and age were analyzed by Spearman's test. $p < 0.05$ was considered statistically significant. Logistic regression analysis was implemented for the AR and sensitization to assess the effects of gender, age and total serum IgE (SPSS 14.0J, SPSS, Inc., Chicago, Ill., USA).

Results

Prevalence of Allergic Sensitization and AR

Positive sensitization refers to an allergen-specific serum IgE level >0.7 (CAP RAST score of 2). The prevalence of allergic sensitization to each allergen tested is presented in table 1. Of the 1,540 subjects, 1,073 (69.7%) exhibited positive sensitization to at least 1 aeroallergen (fig. 1). A total of 467 of the 1,540 subjects (30.3%) showed

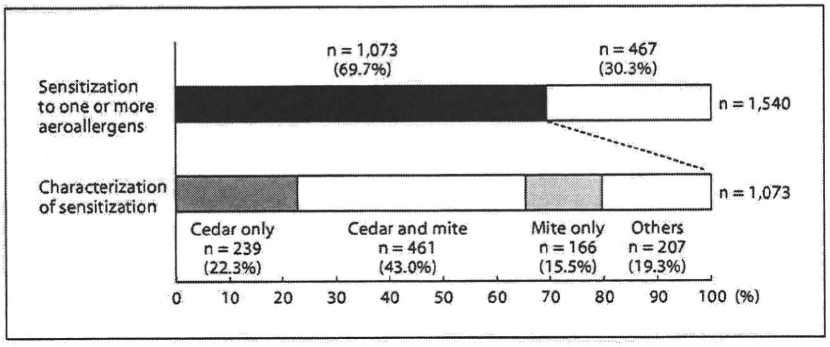

Fig. 1. The prevalence of sensitization to 7 test aeroallergens and characterization of sensitization.

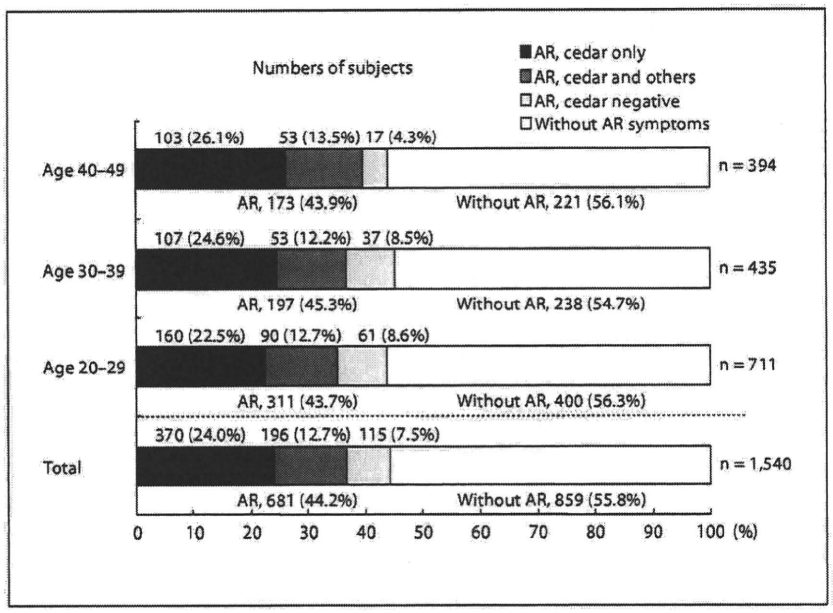

Fig. 2. Age effects on the prevalence of AR and sensitization to Japanese cedar pollen.

no sensitization to any of the 7 aeroallergens examined (fig. 1). Seven hundred subjects (45.3%) were sensitized to *C. japonica*, (Japanese cedar, JC) pollen, thus accounting for 65.3% of the 1,073 subjects with positive sensitization to aeroallergens. Of the 1,073 subjects, 627 (58.5%) were sensitized to mites. Thus, JC pollen and mites were the two predominant aeroallergens among the 7 tested aeroallergens (fig. 1).

Of the 1,540 participating subjects, 681 (44.2%) had symptoms of AR at the time of the survey (fig. 2). The prevalence of JCP was 36.7% (566 of the 1,540 subjects) in this study (fig. 2). The positive rates for specific IgE antibodies to Japanese cedar pollen were 89.6% (610 of 681) in the AR group and 28.5% (245 of 859) in the no-symptom group (fig. 3). Of the 681 AR subjects, 167 (24.5%) were sensitized to only Japanese cedar pollen (fig. 3).

Age Effect on the Prevalence of Allergic Sensitization and AR

We found significant associations between the allergic sensitization to the 7 aeroallergens and the age groups (table 1) ($p = 0.0019$ by the Kruskal-Wallis test). More subjects were sensitized to Japanese cedar pollen than to any other of the 7 tested allergens in each age group (table 1). The sensitization rates to Japanese cedar pollen were 59% (421 of 711 subjects), 52% (226 of 435) and 53% (208 of 394) for subjects in their 20s, 30s and 40s, respectively. We found a significant association between sensitization to Japanese cedar pollen and the age range of the subjects ($p = 0.015$ by the Mann-Whitney U test) (table 2). The sensitization rate against mites, Der p and/or Der f, was higher for those in their 20s (50%, 355 of 711 subjects), than for those in their 30s (41%, 179 of 435) and 40s

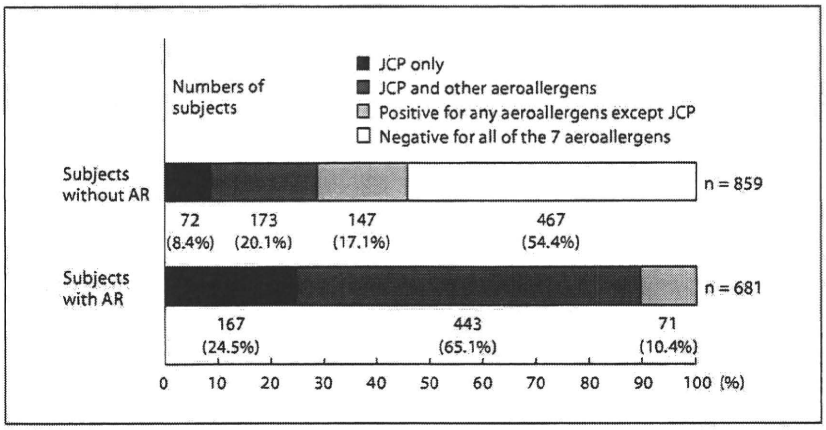

Fig. 3. Prevalence of AR and sensitization to Japanese cedar pollen.

Table 1. Prevalence of sensitization to 7 aeroallergens according to age group

	Total (n = 1,540)	20s (n = 711)	30s (n = 435)	40s (n = 394)
Cryptomeria japonica	855 (56)	421 (59)	226 (52)	208 (53)
Dermatophagoides pteronyssinus	625 (41)	345 (49)	174 (40)	106 (27)
Dermatophagoides farinae	622 (40)	342 (48)	168 (39)	112 (28)
Dactylis glomerata	352 (23)	198 (28)	90 (21)	64 (16)
Ambrosia artemisiifolia	137 (9)	67 (9)	45 (10)	25 (6)
Candida albicans	82 (5)	43 (6)	24 (6)	15 (4)
Aspergillus fumigatus	34 (2)	25 (4)	8 (2)	1 (0.3)

Figures in parentheses are percentages.

Table 2. Age effects on sensitization to JCP, dust mites and *Dactylis glomerata*

Aeroallergen	Sensitization	20s (n = 711)	30s (n = 435)	40s (n = 394)	p value
Cryptomeria japonica	positive	421 (59)	226 (52)	208 (53)	0.015
	negative	290 (41)	209 (48)	186 (47)	
Dust mites	positive	355 (50)	179 (41)	115 (29)	3.9×10^{-11}
	negative	356 (50)	256 (59)	279 (71)	
Dactylis glomerata	positive	198 (28)	90 (21)	64 (16)	4.8×10^{-6}
	negative	513 (72)	345 (79)	330 (84)	

Figures in parentheses are percentages. p value as obtained by the Mann-Whitney U test.

(29% 115 of 394), ($p < 0.001$ by the Mann-Whitney U test) (table 2). The prevalence of sensitization to *D. glomerata* was also higher in those in their 20s (28%, 198 of 711 subjects) than in those in their 30s (21%, 90 of 435) and 40s (16%, 64 of 394) ($p < 0.001$ by the Mann-Whitney U test) (table 2). AR was confirmed in 311 of the 711 subjects (43.7%) in their 20s, 197 of the 435 (45.3%) in their 30s and 173 of the 394 (43.9%) in their 40s (fig. 2). There was no significant difference in the prevalence of AR among the age groups.

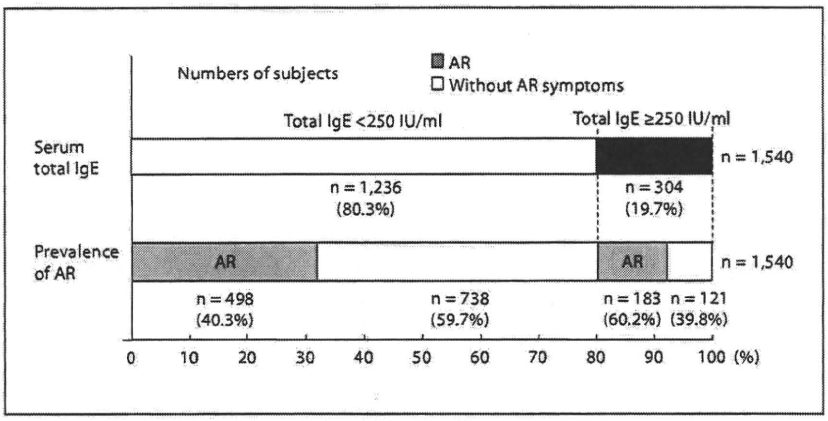

Fig. 4. Serum total IgE levels and prevalence of AR.

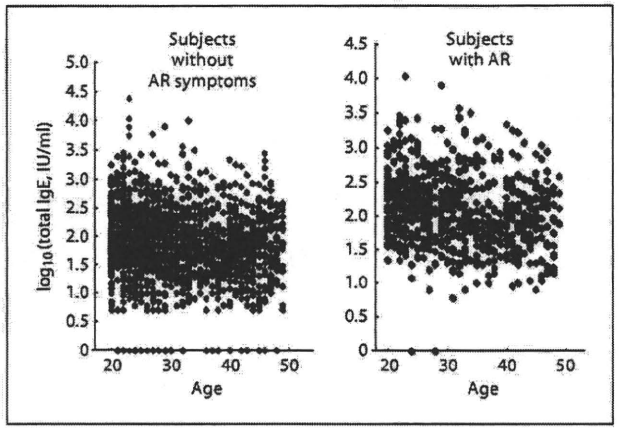

Fig. 5. Age effects on serum total IgE levels in subjects with AR and non-AR.

Total Serum IgE and AR, Sensitization, and Age Effect

There were 304 subjects (19.7%) who had high total IgE levels (≥250 IU/ml), and the prevalence of AR in this group was 60.2% (183 of the 304). However, the prevalence of AR of subjects with normal total IgE (<250 IU/ml) was 40.3% (498 of 1,236) (fig. 4).

The serum total IgE level was analyzed at a quantitative level (fig. 5). The means of $\log_{10}$ [total IgE (IU/ml)] and standard deviations of all 1,540 subjects, subjects without AR and subjects with AR were 1.87 [= $\log_{10}$ (74.1 IU/ml)] ± 0.65, 1.69 [= $\log_{10}$ (49.0 IU/ml)] ± 0.67 and 2.09 [= $\log_{10}$ (123.0 IU/ml)] ± 0.53, respectively.

We investigated the correlation between this level and age using Spearman's rank correlation coefficient (fig. 5). Although we could not find any significant correlation between the serum total IgE level and the age range of the 1,540 subjects, an inverse correlation was found between the total IgE level and age in the AR group ($r_s = -0.21$, $p < 0.01$) (fig. 5). Total IgE levels were higher in younger subjects than in older subjects in the AR group. The results of the stepwise logistic regression analysis for positive sensitization to 1 or more of the 7 aeroallergens showed significant effects of total IgE (Wald statistic = 153.5, d.f. = 1, $p < 0.001$) and age (Wald statistic = 9.5, d.f. = 1, $p = 0.002$), but no effect of gender. There was no significant effect of age, gender, or total IgE on AR by logistic regression analysis.

Discussion

Estimates of the latest prevalence provide valuable information to develop effective strategies for the prevention and treatment of disease. We conducted an epidemiologic survey of AR and examined the sensitization rates against 7 aeroallergens by measuring the serum-specific IgE of 1,540 subjects aged between 20 and 49 years in a Japanese population in 2006 and 2007. The population aged between 20 and 49 years represented 38.8% of the population of Japan in 2008 according to current population estimates by the Ministry of Internal Affairs and Communications (http://www.stat.go.jp/english/data). We also examined the role of age effects on the prevalence. In this study, 681 of the 1,540 subjects (44.2%) were diagnosed as having AR. Increases in prevalence of AR and asthma have been reported by studies of relatively large populations in the United States, Great Britain, Australia and New Zealand, with cross-referenc-

es to earlier relevant studies, and the recent AR prevalence in these studies ranged from 23 to 28% [14]. The International Study of Asthma and Allergies in Childhood in 1997 reported that the prevalence of rhinoconjunctivitis varied across centers from 0.8 to 14.9% in 6-to 7-year-olds and from 1.4 to 39.7% in 13- to 14-year-olds [15]. In an Aberdeen population study on 3,537 subjects, the prevalence of hay fever increased significantly from 1994 (13%) to 1999 (15%) [16]. In Japan, Sakurai et al. [9] showed that the prevalence rates of AR, seasonal rhinitis and JCP were 36, 29, and 11%, respectively, and age was a negative risk factor for all allergic conditions. The subjects of the study consisted of 2,307 male railway employees who underwent a health examination from February to May 1995 (mean age, 41.4 years; range, 19–65 years). In the study, AR was determined from self-reported AR or from the seasonal nasal symptoms, and JCP was defined as the presence of cedar-specific IgE positivity among subjects with seasonal rhinitis. The prevalence of AR in this study was 44.2% (681 of the 1,540 subjects), which is higher than in previous reports. However, there was no difference of prevalence between 20 and 49-year-olds. Interestingly, the prevalence of AR in subjects aged 30–39 years was 42.7% in a study conducted in 1995 [9]. These subjects aged 30–39 years in 1995 were 40–49 years old in 2005. The prevalence of AR in this study for subjects from 40 to 49 years of age was 43.9%, and there was no difference in the prevalence between the studies. The prevalence among this age group did not markedly increase during the last 10 years. Further etiological studies in independent populations or those aged less than 20 years and elderly populations are needed to determine the effects of age on the susceptibility to AR.

In the present study, a total of 859 subjects (859/1,540, 55.8%) had no symptoms of AR; however, among them, 392 subjects (392/859, 45.6%) were already sensitized to one or more of the 7 test aeroallergens. It is generally recognized that sensitization to any allergen is an important risk factor for developing allergic diseases; however, those sensitized subjects had no symptoms of AR.

The present study has shown that a total of 167 of 681 subjects with AR (24.5%) were sensitized to JC pollen and not to the other 6 test aeroallergens. Allergen-specific immunotherapy is established as an effective treatment for patients with IgE-mediated reactions, and it has been widely used as a desensitizing therapy for AR [17, 18]. Specific immunotherapy retrospectively reduces new sensitization in monosensitized subjects suffering from AR [19]. Subjects with monoallergen sensitization appear to be good candidates for immunotherapy.

Among the 681 subjects with AR, 451 (66.2%) were sensitized to multiple (two or more) aeroallergens, and 385 (56.5%) were sensitized to dust mites. Although our data strongly indicated an important role of JCP in AR, a significantly higher prevalence of sensitization to dust mites was observed in younger subjects. Dust mites, an indoor allergen, have a predominant impact on asthma, and a recent population-based study has shown that dust mite sensitization is a significant risk factor for developing the disease [20]. Another recent study, a long-term (23-year) follow-up study of university students, has shown that sensitization to pollen leads to an increased risk of developing asthma [21]. A limitation of our study was the lack of longitudinal data. To clarify factors that increased the risk of developing new AR or bronchial asthma, further cohort analyses should be conducted regarding the involvement of the sensitized allergens in airway allergic inflammation.

A recent etiological study in an unselected rural Chinese population tested sensitization to 14 allergens, including 5 aeroallergens (dust mite, cockroach, *Alternaria tenuis*, dog epithelia, and cat hair) by skin prick tests. 2,118 subjects whose ages ranged from 11 to 71 years were tested (43.3% were children between 11 and 17 years old) [22]. The study showed that 41.1% of the children were sensitized to 1 or more aeroallergens, and 36.5% of the adult subjects aged ≥18 years were sensitized [22]. The most common sensitizing aeroallergen in the Chinese study was dust mites (30.6%) [22]. In meta-analyses using data from 12,687 subjects aged 20–44 years in the European Community Respiratory Health Survey conducted in 2002, the highest prevalence of sensitization was found for the house dust mite (20.2%) [23]. In the present study, of the 1,540 subjects, 1,073 (69.7%) were sensitized to at least 1 of the 7 aeroallergens, and 855 (55.5%) and 649 (42.1%) were sensitized to Japanese cedar pollen and dust mites, respectively.

Several limitations of this survey should be mentioned. The survey is likely to be fraught with a certain recruitment bias. In general, individuals affected by a specific disease are more willing and interested in a study. However, only 13 subjects (0.84%) did not agree to participate in this survey whereas 1,540 subjects agreed to assays of serum total IgE and specific IgE for the 7 aeroallergens and to answer the questionnaire in the present study. Hospital workers, nursing and medical students might not be representative of the general population and there might have been a population selection bias with regard to socioeconomic status and higher education. Previous studies in various countries have reported an increased

occurrence of asthma among specific groups of healthcare workers [24–26]. Thus, selection bias might have had an influence on the higher prevalence of sensitization to 1 or more aeroallergens (69.7%) and of AR (44.2%) in our study.

Although a population selection bias might reduce the generalizability of the study, we showed here that the prevalence of AR has increased and that Japanese cedar pollen and dust mites were the predominant allergen sources among the 7 tested allergen sources in the Japanese population. However, further study is needed using larger, more representative samples.

Acknowledgments

We thank all the participants in the study. We also thank Makiko Shimizu-Terada, Hiroshi Sekiguchi, Aya Jodo-Ito, Nami Kawaraichi, Yuko Taki, Kazumi Uno and Yukako Ishikawa. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare of Japan.

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doi: 10.1111/j.1365-2222.2008.03114.x

ORIGINAL PAPER Asthma and Rhinitis

Clinical and Experimental Allergy, 38, 1875–1881

Association of serum interleukin-33 level and the interleukin-33 genetic variant with Japanese cedar pollinosis

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Cite this as: M. Sakashita, T. Yoshimoto, T. Hirota, M. Harada, K. Okubo, Y. Osawa, S. Fujieda, Y. Nakamura, K. Yasuda, K. Nakanishi and M. Tamari, *Clinical and Experimental Allergy*, 2008 (38) 1875–1881.

Summary

Background IL-33, an IL-1-like cytokine, is a ligand for IL1RL1, which is an important effector molecule of type 2 T helper responses. Although IL-33/IL1RL1 interaction has been suggested to be important in induction of allergic airway inflammation, serum levels of IL-33 and the genetic influences of the polymorphisms of *IL-33* in human allergic diseases are unclear.

Objective The aim of this study was to examine whether the serum IL-33 level and polymorphisms in *IL-33* are associated with Japanese cedar (JC) pollinosis, the most common form of allergic rhinitis, and a major public health problem, in Japan.

Methods We performed linkage disequilibrium (LD) mapping of the gene using the HapMap database, and two selected tag single nucleotide polymorphisms were genotyped. We conducted an association study of IL-33 (JC pollinosis, $n = 170$; normal controls, $n = 100$) and measured the IL-33 levels in sera of the 270 subjects by ELISA.

Results Serum levels of IL-33 were significantly higher in patients with JC pollinosis ($P = 0.0018$) than in controls. In genetic association analysis, we found a positive association between the polymorphism and JC pollinosis ($P = 0.048$).

Conclusion Our results support a role for IL-33 in the pathogenesis of JC pollinosis.

Keywords association, IL-33, JC pollinosis, polymorphism, serum level
Submitted 28 August 2007; revised 3 February 2008, 31 July 2008; accepted 3 September 2008

Introduction

Allergic diseases are increasing world-wide, and Japanese cedar (JC) pollinosis, which is a disease of allergic rhinitis and allergic conjunctivitis caused by exposure to JC pollen, is one of the most common allergic diseases in Japan [1]. The increase of JC pollinosis in Japan has become a social problem, with a prevalence of > 16% [1]. Allergic rhinitis is the result of an inflammatory reaction triggered by type 2 T helper (Th2) cell-mediated immune responses against allergens [2]. Th2 cytokines induce B cells to produce high amounts of IgG4 and IgE in humans, and promote the growth and differentiation of mast cells and eosinophils [3, 4]. IL1RL1 belongs to the IL-1 receptor family and functions as an important effector molecule of Th2 responses [5–8]. IL-33, an IL-1-like cytokine, has been identified as a ligand for IL1RL1, and can be detected in epithelial cells from the bronchi and small airways, which indicates a possible role in the regulation of mucosal function [9]. It activates NF-κB and mitogen-activated protein kinases, and drives production of Th2-associated cytokines from *in vitro* polarized Th2 cells via IL1RL1 [9]. *In vivo* analysis has demonstrated that IL-33 strongly induces gene expression of Th2-associated cytokines such as IL-4, IL-5 and IL-13, and IL-33-treated mice have significantly higher serum levels of IgE. In addition, IL-33 induces pathological changes in mucosal organs such as the lung, resulting in hypertrophied epithelial lining of the airways with large amount of mucus,

and those changes are primarily restricted to the bronchi and larger bronchioles [9].

Although very little work has been done with IL-33, intensive studies of IL-33 receptor IL1RL1 have shown its regulatory functions in the development and effector phases of Th2 responses [10]. The *IL1RL1* gene encodes a soluble-secreted protein, IL1RL1, and a transmembrane protein, ST2L [11]. In murine models of allergic airway inflammation, increases in endogenous IL1RL1 protein after allergen exposure modulate Th2-mediated airway inflammation [12], and blockade of the binding of the ligand for ST2L using a recombinant IgG fusion protein inhibits allergic inflammation [6, 7]. Other studies have reported that ST2L is a reliable selective marker of both murine and human Th2 lymphocytes in allergic airway inflammation [7, 13]. Moreover, a study has revealed that soluble IL1RL1 acts as a negative regulator of Th2 cytokine production via IL-33 signalling in allergic airway inflammation. In asthmatic patients, serum levels of soluble IL1RL1 are markedly elevated during acute attacks and the magnitude of the elevation correlates with the reduction of pulmonary functions and increased levels of serum IL-5 [14]. These findings imply that IL-33 is a good candidate for involvement in JC pollinosis, an allergen-induced upper airway inflammation.

A large number of association studies using polymorphic markers have been performed to discover genetic components in the pathogenesis of allergic diseases [15–17]. Recently, we have reported that functional single nucleotide polymorphisms (SNPs) in the IL1RL1 distal promoter region are associated with atopic dermatitis. The genetic variants regulate IL1RL1 expression, and immunohistochemical staining of a skin biopsy specimen from an atopic dermatitis patient showed IL1RL1 staining in keratinocytes as well as in cells infiltrating the dermal layer [18]. However, there have been no genetic association studies with IL-33.

In this study, to test whether genetic variations of IL-33 contribute to susceptibility to JC pollinosis, we first selected a genetic polymorphism of IL-33 using HapMap linkage disequilibrium (LD) data and conducted association studies. In addition, we examined the associations between serum IL-33 levels and JC pollinosis and serum total IgE levels.

Methods

Study subjects

All subjects were recruited from residents of Eiheiji-cho, in Fukui prefecture, in the central area of Japan between May and June 2006. Because these participants were workers of the Fukui University hospital and students of nursing and medical colleges in Fukui, the number of females was higher than that of males. Specific IgE to seven aeroallergens, *Cryptomeria japonica, Dermatophagoides pteronyssinus, Dermatophagoides farinae, Candida albicans, Aspergillus fumigatus, Dactylis glomerata* and *Ambrosia*, were measured with a Pharmacia CAP System (Pharmacia CAP, Uppsala, Sweden) (Table 1). Positive sensitization refers to an allergen-specific serum IgE level >0.7 (CAP RAST score of 2). Diagnosis of JC pollinosis was confirmed by symptoms of allergic rhinoconjunctivitis during the JC pollinosis season and positive serum-specific IgE towards JC pollinosis. A total of 170 patients with JC pollinosis were recruited (Table 1). One hundred healthy subjects who had never had symptoms of allergic rhinitis and showed no sensitization to any of the seven aeroallergens were recruited as controls (Table 1). We recruited 29 subjects with infectious rhinitis who were diagnosed by otolaryngologists and showed no sensitization to any of the seven aeroallergens. All individuals were unrelated Japanese and gave written informed consent to participate in the study according to the rules of the process committees at the School of Medicine, University of Fukui, the Nippon Medical School and The Institute of Physical and Chemical Research.

Selection of polymorphisms for genotyping

Genomic DNA was prepared from peripheral blood samples, using standard protocols. There were 22 SNPs in the *IL-33* gene with a minor allele frequency (MAF) of $>10\%$ in the HapMap Japanese data set (URL: http://www.hapmap.org/index.html.en) (Table 2). Pairwise LD was calculated as r^2 by using the Haploview 3.2 program (http://www.broad.mit.edu/mpg/haploview/). Genotyping of SNPs was performed by the TaqMan™ allele-specific amplification (TaqMan-ASA) method (Applied Biosystems, Foster City, CA, USA). rs1929992 was genotyped by Custom TaqMan® SNP Genotyping Assay Service with primers 5′-GGAAAAAAACACATTTTCCCCCCAA-3′ and 5′-AAACCATCTTAACTACTACTTAAAATGTATAAAGTGT TAGAATTAT-3′. The probes used were VIC-TCATGGT CAAAATATTGAAAT and FAM-ATGGTCAAAATGTTGAA AT. rs10975519 was genotyped by TaqMan(R) Pre-Designed SNP Genotyping Assays, C___2762153_10.

Reagents for human interleukin-33

Recombinant human IL-33 (rhIL-33) and a rabbit-neutralizing anti-hIL-33 IgG antibody were made by Hokudo Co., Ltd. (Sapporo, Japan). Briefly, rhIL-33 (mature form) was amplified from human lung cDNA (BioChain Institute, Hayward, CA, USA) as a template, and subcloned into pET28a vector (Novagen, Madison, WI, USA). BL21 (DE3) RIL was transformed and the expressed recombinant protein was purified with Ni-NTA resin. Endotoxin was removed by filtration through Zetapor (Cuno, Meriden, CT, USA). For establishment of a polyclonal antibody to hIL-33, rabbits

were immunized with rhIL-33 (200 μg/body) with CFA, and boosted with rhIL-33 (200 μg/body) with IFA three times every 2 weeks. Seven weeks later, serum was collected and the antibody was purified using a Protein-A sepharose column. This IgG antibody (R2) was further purified with an rhIL-33 sepharose column and was biotinylated with NHS-biotin (Sigma, St Louis, MO, USA) in our laboratory. This purified anti-hIL-33 antibody could completely neutralize 50 ng/mL of IL-33 at the concentration of 10 μg/mL *in vitro*.

Table 1. Characteristics of the patients with Japanese cedar (JC) pollinosis and controls

Characteristics	Case	Control
Age (year, median with range)	30 (20–49)	32.5 (20–49)
Total subjects and sex (% male subjects)	170 (14)	100 (9.0)
Serum total IgE (IU/mL, mean±SEM)	280.2±879.2	42.9±51.5
Atopic sensitization (RAST) (number (%))		
Japanese cedar pollen positive	170 (100)	0 (0)
Dermatophagoides pteronyssinus positive	80 (47.1)	0 (0)
Dermatophagoides farinae positive	78 (45.9)	0 (0)
Candida albicans positive	10 (5.9)	0 (0)
Aspergillus positive	3 (1.8)	0 (0)
Dactylis glomerata positive	61 (35.9)	0 (0)
Ambrosia positive	23 (13.5)	0 (0)

Enzyme-linked immunosorbent assay of serum levels of interleulun-33

To elucidate the biological roles of the *IL-33* gene, we constructed an ELISA system to quantify human IL-33 protein in sera of subjects with JC pollinosis and controls. A 96-well plate (Costar, Cambridge, MA, USA) was coated with the anti-hIL-33 IgG antibody (R2) and blocked with StartingBlock™ blocking buffer (PIERCE, Rockford, IL, USA). Human IL-33 was detected with the biotinylated-anti-IL-33 antibody and streptavidin-HRP. The ELISA system was specific for hIL-33 and did not cross-react with other cytokines tested, which included IL-1β, IL-2, IL-4, IL-12, IL-18, TNF-α, IFN-γ and GM-CSF. Serum samples were collected, and then they were stored at −80 °C until measurement. IL-33 was assayed by ELISA with reference standard curves using known amounts of hIL-33. The lower limit of ELISA sensitivity for serum IL-33 was 30 pg/mL. A value of 0 was assigned to results that were below the assay's lower limit of detection for non-parametric statistical calculations in Fig. 2.

Statistical analysis

We calculated allele frequencies and tested agreement with Hardy–Weinberg equilibrium using a χ^2 goodness-of-fit

Table 2. Locations and allele frequencies of polymorphisms in *IL-33* based on the HapMap JPT data set

SNP*	Location	Amino acid	MAF (%)‡	NCBI§
−5345 G/A	5′-Flanking region		0.477	rs928414
−5194 T/G	5′-Flanking region		0.477	rs4237164
−4432 G/A	5′-Flanking region		0.477	rs10975509
−1611 C/T	5′-Flanking region		0.466	rs7025417
−1037 T/C	Intron 1		0.467	rs10975511
1256 C/T	Intron 1		0.455	rs4742170
2241 C/G	Intron 1		0.455	rs7019575
4450 G/A	Intron 1		0.455	rs10975514
5999 G/A	Intron 1		0.443	rs10975516
9318 C/A	Intron 2		0.443	rs1317230
9813 G/T	Intron 3		0.455	rs1330383
9894 T/C†	Intron 3		0.455	rs1929992
11607 T/C	Intron 4		0.432	rs1113573
11877 C/T†	Exon 5	Tyr163Tyr	0.433	rs10975519
12016 G/C	Intron 5		0.422	rs10975520
12514 T/C	Intron 5		0.427	rs7044343
13206 A/G	Intron 6		0.487	rs7871381
13316 C/A	Intron 6		0.371	rs1412421
13625 G/A	Intron 6		0.422	rs7047921
14187 G/T	Intron 6		0.420	rs1332290
14598 G/A	Exon 7	3′-UTR	0.409	rs1048274
23562 G/C	3′-Flanking region		0.455	rs10815397

*Numbering according to the genomic sequence of IL-33 (AL353741.16) and position 1 is the A of the initiation codon. Major allele/minor allele.
†SNPs were genotyped in this study.
‡Minor allele frequencies
§NCBI, number from the dbSNP of NCBI (http://www.ncbi.nlm.nih.gov/SNP/).
SNP, single nucleotide polymorphisms; MAF, minor allele frequency.

test at each locus. We then compared differences in allele frequencies and genotype distribution of the polymorphism between case and control subjects by using a 2×2 contingency χ^2 test with one degree of freedom, and calculated odds ratios (ORs) with 95% confidence intervals (CIs). Serum total IgE and IL-33 levels were analysed as quantitative levels, and we investigated associations between these levels and genetic variations. Log-transformed individual serum IgE levels were analysed by one-way ANOVA. When the data for IL-33 levels were not distributed normally after log-transformation, they were analysed using non-parametric equivalents and summarized using the median. Multiple comparisons were first analysed by the Kruskal–Wallis test and then by individual testing by the Mann–Whitney *U*-test if significant. Correlations were analysed by Spearman's test. A *P* value of less than 0.05 was considered statistically significant.

Results

Linkage disequilibrium of the IL-33 *gene*

A total of 22 polymorphisms with a frequency >0.10 in IL-33 were contained in the public databases available at the NCBI dbSNP website (http://www.ncbi.nlm.nih.gov/SNP/) (Table 2). Two variants including a synonymous substitution (Tyr163Tyr) were in the exons, and four variants were in the 5′-flanking region of the *IL-33* gene. Pairwise LD among the 22 SNPs was measured by different parameters, r^2 using the Haploview 3.2 program (http://www.broad.mit.edu/mpg/haploview/) (Fig. 1), and all the 22 SNPs were in strong LD ($r^2>0.75$). We finally selected polymorphism rs1929992 and rs10975519 (Tyr163Tyr) for association studies using tagger in the Haploview 3.2 program, and these two SNPs captured 22 of 22 alleles with a mean r^2 of 0.95 ($r^2>0.91$).

Association between polymorphisms in the IL-33 *gene and susceptibility of Japanese cedar pollinosis*

The locus was in Hardy–Weinberg equilibrium in the entire group. To test the association between the SNP and JC pollinosis, we compared differences in the allele frequency and genotype distribution of each polymorphism between case and control subjects by using contingency chi-square tests with one degree of freedom. ORs with 95% CIs were also calculated. In the population genotyped in this study, the MAF of rs1929992 (C=0.49) was higher than those in the HapMap JPT data set (C=0.46). We found a significant association between rs1929992 (T>C) and JC pollinosis (TT+TC vs. CC: OR, 1.82; 95% CI, 1.00–3.31; $P=0.048$) (Table 3). The serum total IgE level was analysed as a quantitative level, and we investigated the association between this level and genetic variation. However, we could not find any association between the SNP and serum IgE level in this study ($P=0.46$ by ANOVA).

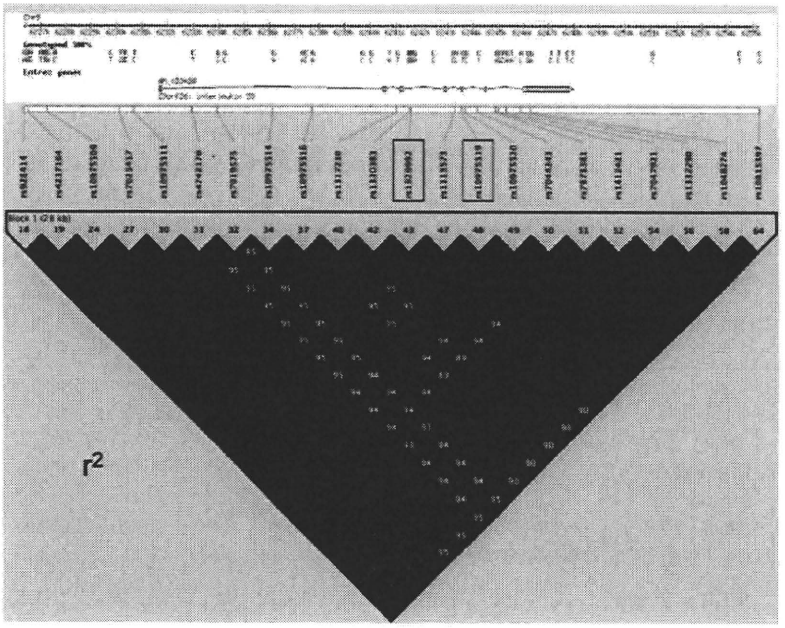

Fig. 1. Pairwise linkage disequilibrium between 22 SNPs as measured by r^2 estimated by the Haploview 3.2 program using the HapMap JPT data set. The boxed polymorphisms, rs1929992 and rs10975519, were genotyped in this study.

Patients with Japanese cedar pollinosis display higher interleukin-33 serum levels than healthy control subjects

To evaluate whether dysregulation at the IL-33 protein level might be a characteristic feature of JC pollinosis, we conducted ELISA assays of sera of patients with JC pollinosis ($n = 170$) and healthy control subjects ($n = 100$). Patients with JC pollinosis exhibited significantly higher serum levels of the IL-33 protein ($P = 0.0018$) (Fig. 2). The median serum IL-33 concentration of JC pollinosis patients was 549 pg/mL, compared with 361.8 pg/mL for controls. In addition, we examined the serum IL-33 level in infectious rhinitis as non-allergic rhinitis. The median serum IL-33 concentration of subjects with infectious rhinitis was 241.3 pg/mL. There was no significant difference of the serum IL-33 level between healthy control subjects and those with infectious rhinitis. Although total serum IgE and IL-33 levels were analysed as quantitative phenotypes, there was no significant association between the total serum IgE level and serum IL-33 level ($P = 0.095$ by Spearman's test). We also examined whether the *IL-33* genotype affected the serum level of IL-33, but we could not find any significant association between the genotype and serum IL-33 level ($P = 0.58$ by the Kruskal–Wallis test).

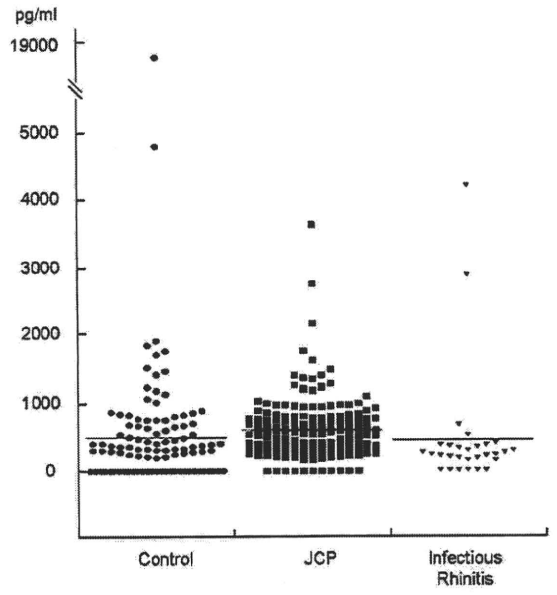

Fig. 2. Comparison of serum IL-33 levels among controls, patients with JC pollinosis and those with infectious rhinitis. Horizontal bars indicate the median value of each group. JCP, Japanese Cedar pollinosis.

Discussion

To determine the role of the *IL-33* gene in the pathogenesis of JC pollinosis, we conducted an association study using the sequence variation of the *IL-33* gene and compared serum IL-33 levels between subjects with JC pollinosis and controls. We found a significant association between JC pollinosis susceptibility and IL-33 polymorphism and higher serum IL-33 levels in subjects with JC pollinosis. Although IL-33 has been thought to play an important role in allergic diseases, this is the first study providing evidence for its involvement in such a disease. We consider the results to be hypothesis generating as the findings in this study need to be confirmed in another population with a larger size.

Recent studies have reported important roles of non-lymphoid cell-derived cytokines such as IL-33 and TSLP in the induction of Th2 differentiation [9, 19]. IL-33 is highly expressed in normal human bronchial epithelial cells and airway smooth muscle cells [9]. It induces Th2-type responses and Th2-associated cytokines IL-4, IL-5 and IL-13 by signalling through IL1RL1 [9, 20]. A recent study has shown that IL-33 induces IL-13 production by mast cells independently of IgE–FcεRI signals in mice. These findings suggest important roles for IL-33 in mast cell- and Th2 cytokine-associated immune disorders [21].

Table 3. Association between polymorphisms of *IL-33* and Japanese cedar (JC) pollinosis

Genotype	Cases ($n = 170$)	Controls ($n = 100$)	Allele	Cases ($n = 170$)	Controls ($n = 100$)	Genotype P	Dominant* P	Recessive† P	Allelic‡ P
rs1929992									
TT	44 (26.0)	32 (32.3)	T	162 (47.9)	112 (56.6)	0.13	0.27	0.048	0.053
TC	74 (43.8)	48 (48.4)	C	176 (52.1)	86 (43.4)				
CC	51 (30.2)	19 (19.2)							
rs10975519									
CC	52 (30.6)	36 (36.0)	C	177 (52.1)	119 (59.5)	0.20	0.36	0.074	0.093
CT	73 (42.9)	47 (47.0)	T	163 (47.9)	81 (40.5)				
TT	45 (26.5)	17 (17.0)							

*Dominant model (TT vs. CC+TC in rs1929992, CC vs. CT+TT in rs10975519).

†Recessive model (TT+TC vs. CC in rs1929992, CC+CT vs. TT in rs10975519).

‡Allelic model (T allele vs. C allele in rs1929992, C allele vs. T allele in rs10975519).

Structurally, IL-33 is related to IL-18, and intensive studies of the relationship between allergic inflammation and IL-18 have been conducted. IL-18 was the first cytokine demonstrated to activate T cells to produce abundant IFN-γ without T cell receptor (TCR) engagement [22]. Furthermore, genetic association studies of the *IL-18* gene have provided evidence for an association with atopic diseases [23–26]. Verhaeghe et al. reported the up-regulation of IL-18 in nasal secretions in allergic rhinitis and the persistence of elevated IL-18 concentrations until after the season [27]. Increased IL-33 concentrations were observed in subjects with JC pollinosis in the present study; however, there was no significant difference in the serum IL-33 level between controls and subjects with infectious rhinitis. Up-regulation of the IL-33 level appears to be characteristic of JC pollinosis. Further analyses of the involvement and interactions of those structurally similar cytokines in allergic inflammation should also be conducted.

Recent reports have shown that IL1RL1 is a reliable marker of Th2 lymphocytes in allergic airway inflammation [7, 13, 28]. Elevated levels of the soluble form of IL1RL1 in the circulation of patients with asthma with acute exacerbation have been reported [14]. The study has also shown that a differential rise of serum IL1RL1 level that correlates well with the severity of asthma exacerbation [14]. In a murine model of allergic airway inflammation, serum murine (m) IL1RL1 protein levels increased after allergen exposure, and pre-treatment with soluble mIL1RL1 protein significantly inhibited the Th2 cytokine production [12]. Other studies have shown that administration of either a monoclonal antibody against IL1RL1 or a recombinant IL1RL1 fusion protein attenuates eosinophilic inflammation of the airways and suppresses IL-4 and IL-5 production *in vivo* following adoptive transfer of Th2 cells [6, 7]. These findings suggest that blocking IL1RL1 pathways would be therapeutically efficacious as a new treatment for allergic diseases, and expression of soluble IL1RL1 could serve as a physiological mechanism to down-regulate Th2-driven immunopathology [10]. In this study, we did not measure the serum soluble IL1RL1 levels, and further examination of the relationship between serum IL-33 and soluble IL1RL1 is needed to clarify their functions in Th2 inflammation. The genetic factors of the *IL-33* gene or serum IL-33 level might provide valuable information for selecting appropriate therapeutic options.

We showed here a significant association between susceptibility to JC pollinosis and a polymorphism. In this study, we selected polymorphisms using HapMap information, and did not examine the functional effects of polymorphisms in strong LD with the related variant. Previous studies have shown that polymorphisms in exons often contribute to their transcript stability [29, 30]. Variants rs10975519 (Tyr163Tyr) and rs1048274 in the exon might affect the expression level or mRNA stability of the *IL-33* gene. In addition, four genetic variations were in the 5′-flanking region, which is often involved in transcriptional regulation of the gene. Several transcription factors are involved in asthmatic inflammation, including NF-κB, activator protein-1 (AP-1), nuclear factor of activated T cells (NF-AT), cyclic AMP response element-binding protein (CREB) and signal transduction-activated transcription factors (STAT) [31]. Using the TRASFAC system, we surveyed whether SNPs in the 5′ region of the *IL-33* gene create transcription factor binding sites. However, we could not find any SNP that changed the affinity of those transcription factors. The functions of these linked polymorphisms remain to be elucidated. Demonstrating the alteration of gene functions as the result of polymorphisms is necessary to further validate the involvement of the *IL-33* gene in the pathogenesis of JC pollinosis. Furthermore, there were gender differences in the population in this study, and several studies have suggested that sex affects the asthma phenotype, possibly via hormone-related events [32, 33]. If there is a sex-related difference in the association of IL-33 with JC pollinosis, looking at females only might be informative.

Our data strongly support the important role of IL-33 in JC pollinosis. Further investigation of the connections between genotypes and the functional role of IL-33 during allergic events may provide additional targets for therapeutic interventions and would be helpful to clarify the aetiology of allergic diseases.

Acknowledgements

We thank all the participants in the study. We also thank Makiko Shimizu-Terada, Hiroshi Sekiguchi, Aya Jodo-Ito, Yuko Taki and Nami Kawaraichi. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare of Japan.

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doi: 10.1111/j.1365-2222.2009.03316.x
Clinical & Experimental Allergy, 39, 1508–1514

ORIGINAL ARTICLE **Asthma and Rhinitis**

Associations between decay-accelerating factor polymorphisms and allergic respiratory diseases

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Clinical & Experimental Allergy

Summary

Background Allergic diseases such as asthma and allergic rhinitis are major causes of morbidity in developed countries. The pathology underlying allergic respiratory diseases is considered to be IgE-mediated type I allergy characterized by mucosal inflammation that occurs in response to allergen exposure. They are common diseases involving a complex inheritance. Complement systems are known to play an important role in allergic diseases. Decay-accelerating factor (DAF) is important for the regulation of the complement system and is a good candidate for determining the susceptibility to allergic diseases.

Objective The present study aimed to investigate whether polymorphisms in the *DAF* gene are associated with allergic respiratory diseases in the Japanese population.

Methods We performed mutation screenings of DAF and conducted a tag single-nucleotide polymorphisms (SNP) association analysis for 684 unrelated adult individuals with seasonal allergic rhinitis (SAR) with Japanese ceder pollen, 188 mite-sensitive adults with asthma, and 346 unrelated non-allergic healthy controls.

Results DAF is located in the tight linkage disequilibrium (LD) block spanning 62 kb. The tag SNP analysis revealed that rs10746463 was significantly associated with SAR ($P = 0.00033$) and mite-sensitive adult asthma ($P = 0.044$). The rs2564978 and rs3841376 haplotypes, which are located in the promoter region of *DAF*, were in complete LD with rs10746463 ($r^2 = 1$). Luciferase reporter assays with constructs containing the 5′ flanking regions of *DAF* showed that the plasmid with rs2564978 C/rs3841376 deletion (the risk haplotype) had a statistically significantly lower transcriptional activity than that containing the rs2564978 T/rs3841376 insertion.

Conclusions Our results suggest that *DAF* is one of the genes involved in conferring susceptibility to allergic respiratory diseases and show that decreased levels of *DAF* may be associated with the enhanced specific IgE responses occurring in allergic diseases in the Japanese population.

Keywords Japanese cedar, luciferase assay, polymorphism
Submitted 3 February 2009; revised 7 May 2009; accepted 11 May 2009

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Cite this as: T. Kawai, S. Takeshita, Y. Imoto, Y. Matsumoto, M. Sakashita, D. Suzuki, M. Shibasaki, M. Tamari, T. Hirota, T. Arinami, S. Fujieda and E. Noguchi, *Clinical & Experimental Allergy*, 2009 (39) 1508–1514.

Introduction

Allergic diseases such as asthma and allergic rhinitis are major causes of morbidity in developed countries, and their incidence is increasing. Seasonal allergic rhinitis (SAR) by Japanese cedar (*Cryptomeria japonica*; JC) is an IgE-mediated type I allergy affecting the nasal mucosa. It occurs after an exposure to JC pollen. It is one of the most common allergic diseases in Japan, affecting 19.4% of the Japanese population [1]. It is thus a major public health issue in Japan. According to a national survey, the prevalence of rhinitis in Japan was 0.16 in 1992 and 0.21 in 2002 [2]. Over 16% of the Japanese population suffers from the allergy during JC seasons. SAR therefore contributes to the undermined quality of life and decline in labour productivity [3].

Epidemiologic studies have consistently shown that asthma and rhinitis often coexist in the same patients in

every region of the world [4], suggesting the concept of 'one airway, one disease' [5]. Currently, approximately 300 million people worldwide have asthma, and this disease claims the lives of 180 000 people every year [6]. Increased levels of IgE against common environmental allergens are considered as the strongest predisposing factor for asthma, and dust mite allergy is strongly associated with asthma [7].

It is generally accepted that allergic diseases such as asthma and rhinitis are the result of inappropriate immunological responses to common environmental allergens in genetically susceptible individuals [8]. Twin and family studies have confirmed the existence of a genetic predisposition to the development of asthma and allergic rhinitis, with a heritability of 0.71 and 0.69, respectively [9], although a clear Mendelian pattern of inheritance has not been established.

It is well known that patients with allergic rhinitis and asthma develop T helper type 2 (Th2) polarized immune responses and high IgE responsiveness against allergens. However, the important role of other immune systems such as the complement system in allergic inflammation has also been demonstrated [10, 11]. Genetic studies have reported the association of polymorphisms in complement 3 (C3) with asthma in various populations [12, 13]. Activation of the complement system produces proteins called anaphylatoxins, which are the cleavage products of C3 (C3a) and C5 (C5a). Anaphylatoxins have a variety of effects on cells: they cause smooth muscle contraction, enhance vascular permeability, and act as chemotactants for a wide variety of leucocytes [11]. Thus, inappropriate regulation of the complement system may damage the host tissues [14]. A number of membrane-bound proteins such as the decay-accelerating factor (DAF) play an important role in the regulation of the complement system. The physiological role of DAF is to inhibit the complement cascade at the critical C3 convertase stage by binding to the C3 and C5 convertases, and consequently accelerating the decay of the enzyme subunit [15]. In this study, to elucidate the role of DAF in the development of allergic respiratory diseases, we performed a single-nucleotide polymorphism (SNP) association study with unrelated patients having JC-induced SAR or mite-sensitive asthma, and unrelated Japanese non-allergic healthy controls.

Materials and methods

Subjects

Patients with JC-induced SAR were recruited from among patients who visited the otolaryngology department of the University Hospital of Fukui, Japan, and from among hospital workers and students of the same university hospital. Healthy, non-allergic controls were also recruited from among the hospital workers and students of the University Hospital of Fukui. The recruitment was carried out between 2005 and 2007; all the subjects were residents of Fukui City, Japan. The diagnosis of JC-induced SAR was based on a positive history of rhinitis during the cedar pollen season and high levels of allergen-specific IgE antibodies in the serum (RAST score $\geqslant$ class 2); the diagnosis was confirmed by the participating otolaryngologists. The control group comprised healthy adult subjects without any allergic symptoms and with no allergen-specific IgE antibodies against common inhaled allergens [JC pollen, orchard grass, and house dust mite (HDM)].

We recruited 188 adult atopic asthma patients sensitive to HDM (*Dermatophagoides farina* or *Dermatophagoides pteronyssinus* RAST score $\geqslant$ class 2) from Miyatake asthma clinic (mean age, 46.8±14.8 years; male : female ratio, 0.97).

All participants gave their written informed consent to participate in the study. The re-sequencing panel consisted of 32 SAR patients. This study was approved by the Ethical Committee of University of Tsukuba and University of Fukui, Japan.

Tag single-nucleotide polymorphisms analysis of decay-accelerating factor

The genotype data of the *DAF* region of an Asian population (Japanese from Tokyo, Japan, and Han Chinese from Beijing, China) were downloaded from the HapMap website (http://www.hapmap.org/, data release #21), and tag SNPs were selected using the Tagger software [16] implemented in the Haploview software [17], with an r^2 threshold of 0.8 and allele frequencies of 0.1. In 32 patients, we performed re-sequencing of the 5′ flanking region in *DAF* using the primer pair 5′-ATTGTTATCC CACCCCACAC-3′ and 5′-GACAAACAAGACGGGTGGA-3′ that amplified the region located from −633 to +126 bp relative to the transcription initiation site. Next, we genotyped tag SNPs as well as the newly identified SNPs that were not in a strong linkage disequilibrium (LD) with other typed SNPs. The SNPs were genotyped using the TaqMan Assay-on-Demand™ SNP Typing Systems (Applied Biosystems, Foster City, CA, USA) by following the manufacturer's instructions.

Reporter assay

We generated luciferase reporter gene constructs containing the 5′ upstream region of *DAF* spanning from position −441 to +289 bp relative to the transcription initiation site; this region contained two polymorphisms that were in complete LD ($r^2 = 1$): the rs2564978 T/C polymorphism was located at −401 bp relative to the transcription initiation site and the 21 bp insertion/deletion polymorphism rs3841376 was located at −333 bp relative to the