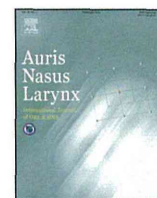


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## Analysis of factors influencing sensitization of Japanese cedar pollen in asymptomatic subjects

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### ABSTRACT

**Objective:** Japanese cedar pollinosis is increasing rapidly in Japan. Although analysis has been made concerning the factors influencing the development of the cedar pollinosis, analysis concerning the risk factors influencing the sensitization in asymptomatic subjects has not been done.

**Methods:** Risk factors for sensitization to Japanese cedar pollen were analyzed among 73 subjects (32 men and 41 women) who do not develop symptoms of pollinosis at the time of Japanese cedar pollen scattering. Their ages ranged from 18 to 60 years with the mean of 34.1 years. Possible factors influencing sensitization were investigated through a written questionnaire and doctors' questioning. Japanese cedar-specific IgE titers and *Dermatophagoides pteronyssinus*-specific IgE titers in the serum were measured by CAP-FEIA (fluorescent enzyme immunoassay).

**Results:** Of the 73 subjects, 26 were sensitized to the Japanese cedar pollen, for a 36% sensitization rate. Among the eleven factors examined, only one factor was shown to significantly influence the sensitization rate to Japanese cedar pollen. It was sensitization to house dust mites (56.5% vs. 26.0%  $\chi^2$  value = 6.27,  $p = 0.012$ ). The sensitization rate to the pollen did not correlate to the presence of other allergic diseases, history of rhinosinusitis, family history of Japanese cedar pollinosis, food preference, presence or absence of cedar trees in the surroundings, present living circumstances, childhood circumstances, age, sex, or smoking habits. We calculated odds ratios in order to estimate how much those factors influence the sensitization to Japanese cedar pollen. Significantly high odds ratio for sensitization to house dust mite (6.63; 95% confidence interval (CI): 1.76–32.2) was found.

**Conclusion:** The present study indicates that sensitization to the pollen in the subjects without pollinosis is influenced by sensitization to house dust mite.

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## 1. Introduction

Japanese cedar pollinosis was first described by Saito [1] in 1964. Since then, the prevalence of pollinosis has been increasing. The prevalence of seasonal allergic rhinitis has increased over the past 10 years due to increased pollen exposure [2]. Sakashita et al. [3] found that the prevalence of allergic rhinitis in adults between 20 and 49 years of age has increased by nearly 10% during the last 10 years. The prevalence rate of pollinosis is 26.5% among Japanese people [4].

It has been reported that genetic factors [5–7], young age [2], fish intake [8], family history of pollinosis [3], and residence along a main street [3] influence the development of Japanese cedar pollinosis.

For people to develop pollinosis, they need to be sensitized. However, it is not clearly known what factors are influencing the sensitization to Japanese cedar pollen in patients without pollinosis. To decrease the prevalence of Japanese cedar pollinosis, it is very important to analyze what factors are playing roles in the sensitization in asymptomatic subjects. The purpose of this study is to elucidate the factors influencing the sensitization to Japanese cedar pollen.

## 2. Materials and methods

### 2.1. Study subjects

The subjects were 73 people (32 men and 41 women) who do not develop symptoms of pollinosis at the time of Japanese cedar pollen scattering. Those who have perennial allergic symptoms were not included in the subjects. Their ages ranged from 18 to 60 years with a mean age of 34.1 years. The age distribution is shown

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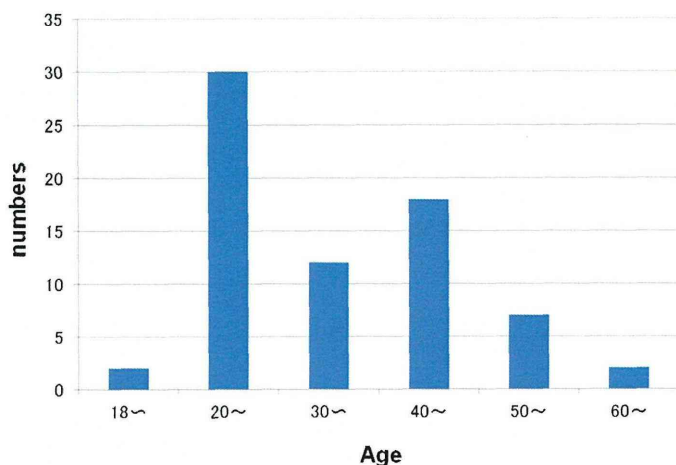


Fig. 1. Age distribution of the subjects examined.

In Fig. 1. In order to recruit subjects for the present study, we mounted posters and utilized Internet. We did not recruited subjects from patients in our outpatient clinic. Written informed consent was obtained from the subjects before their participation in the study. This study was approved by Mie University School of Medicine Ethical Committee (No. 2282).

## 2.2. Questionnaire

The following items were investigated by a written questionnaire and doctors' questioning.

- Past history of rhinosinusitis.
- Presence of other allergic diseases (allergic conjunctivitis, urticaria, drug allergy, food allergy, atopic dermatitis, bronchial asthma): information of the past history alone of other allergic diseases was not included.
- Family history of Japanese cedar pollinosis (siblings, parents, grandparents, children): presence of Japanese cedar pollinosis was defined as development of typical symptoms of nose and eyes at the time of cedar pollen scattering.
- Smoking habit: ex-smoking was not included.
- Food preference (whether they prefer meat or fish).
- Environment of the present residence (urban or rural).
- Environment of their childhood (urban or rural).

## 2.3. Laboratory analysis

Japanese cedar-specific IgE titers and *Dermatophagoides pteronyssinus*-specific IgE titers in the serum were measured by CAP-FEIA (fluorescent enzyme immunoassay). We examined the two IgE titers because cedar pollen and mites were the predominant allergen sources among seven aeroallergens in the Japanese population [3]. Sensitization was defined as serum allergen specific IgE titers with 0.70 UA/mL and over (IgE CAP score  $\geq 2$ ).

## 2.4. Statistical analysis

A chi-square test was used to compare sensitization rates to Japanese cedar pollen according to each factors examined in univariate analysis. The outcome variable of this study was sensitization to cedar pollen. Multivariate regression logistic test was used to assess putative relationship between the factors of interest and sensitization to cedar pollen. The factors of interest were sensitization to house dust mite, presence of other allergic diseases, age (less than 40 years vs. 40 years and over), family

history of cedar pollinosis, history of rhinosinusitis, smoking habit, preference of eating (meat vs. fish), environment lived in childhood (in urban vs. in rural), environment currently living (in urban vs. in rural), exposure to cedar pollen (being surrounded by many cedar trees vs. none), and sex (female vs. male). The magnitude of the relation between these factors and sensitization to cedar pollen was expressed by odds ratios with 95% confidence intervals. To compare the means of Japanese cedar-specific IgE titers and scores between subjects sensitized to house dust mite, Student's *t*-test and Mann–Whitney's *U*-test were used respectively. Statistical significance was defined as  $P < 0.05$ . All statistical analyses were performed with JMP version 5.1.1 (SAS Institute Inc., USA).

## 2.5. Determination of sample size

Prevalence of sensitization to house dust mite was defined as primary factor of interest. According to previous surveys assessed sensitization rate to various airborne antigens in certain populations in Japan, prevalence of sensitization to house dust mite in subjects who were not sensitized to Japanese cedar pollen ('controls') was estimated at about 20%. In this study, whether a subject was sensitized to cedar pollen could not be identified until titer of cedar pollen-specific IgE was measured. We assumed the ratio of subjects not sensitized ('controls') to subjects sensitized to cedar pollen ('cases') to be 2. We also assumed prevalence of sensitization to house dust mite in subjects not sensitized to cedar pollen ('controls') to be 20% and odds ratio of 4 for prevalence of sensitization to house dust mite in subjects sensitized to cedar pollen ('cases'). According to these assumptions, we calculated the sample size with 80% power of obtaining a significant difference at the 5% significance level. Required numbers of subjects sensitized to cedar pollen ('cases') and subjects not sensitized ('control') were 26 and 56, respectively. However, despite of maximum effort, a total of 73 subjects finally participated in this study.

## 3. Results

Of the 73 subjects, 26 were sensitized to Japanese cedar pollen (Fig. 2). The sensitization rate was 36%. The IgE titers in response to Japanese cedar pollen ranged from 0.34 to 84.7 UA/mL and the scores ranged from 0 to 5 (Fig. 2). The mean IgE score of this population was 1.12 and that of the sensitized subjects was 2.8.

Among the eleven factors examined, only one factor was shown to significantly influence the sensitization rate to Japanese cedar pollen. It was sensitization to house dust mites (Table 1).

The subjects who were sensitized to house dust mites had significantly higher sensitization to the Japanese cedar pollen than

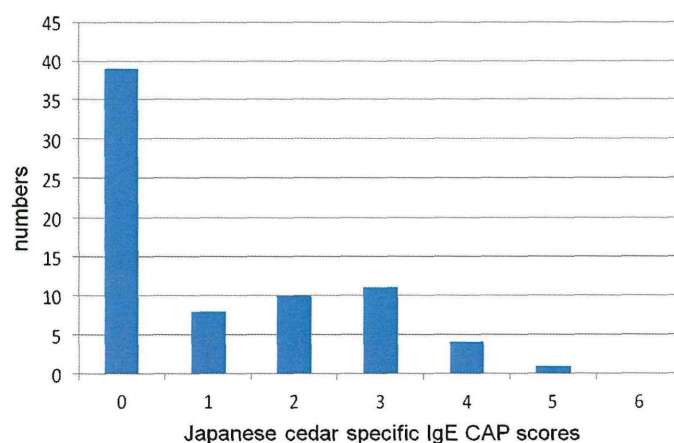


Fig. 2. Distribution of Japanese cedar-specific IgE CAP scores.

**Table 1**  
Comparison of sensitization rates to pollen according to various factors.

Factors	Sensitization rate	$\chi^2$ value	p-Value
Sensitized to house dust mite vs. unsensitized	56.5% vs. 26.0%	6.27	0.012
Other allergic diseases vs. none	52.4% vs. 28.9%	3.53	0.060
Age less than 40 years vs. 40 years and over	43.5% vs. 22.2%	3.48	0.062
Family history of cedar pollinosis vs. none	43.2% vs. 24.1%	2.84	0.092
History of rhinosinusitis vs. none	20.0% vs. 36.8%	0.622	0.430
Smoking habit vs. none	30.0% vs. 36.5%	0.163	0.684
Preference of eating (meat vs. fish)	39.1% vs. 29.6%	0.680	0.410
Lived in the city in childhood vs. in the country	29.4% vs. 37.5%	0.380	0.538
Now living in the city vs. in the country	40.0% vs. 34.0%	0.229	0.633
Surrounded by many cedar trees vs. none	42.8% vs. 32.8%	0.496	0.481
Sex (female vs. male)	36.6% vs. 34.4%	0.038	0.845

those without sensitization to house dust mites (56.5% vs. 26.0%  $\chi^2$  value = 6.27,  $p = 0.012$ ). Those who were sensitized to house dust mite had significantly higher Japanese cedar pollen-specific IgE scores (Fig. 3) and significantly higher Japanese cedar pollen specific IgE titers (Fig. 4).

The sensitization rate did not correlate to presence of other allergic diseases, history of rhinosinusitis, family history of Japanese cedar pollinosis, food preference, presence of cedar trees in the surroundings, present living circumstances, childhood living circumstances, age, sex, or smoking habits (Table 1). We also examined the relationship between pollinosis and the family history of Japanese cedar pollinosis of siblings, parents, grandparents, and offspring, but no relationship was found.

We calculated odds ratios to estimate how much those factors influence the sensitization to Japanese cedar pollen using multivariate regression logistic test (Table 2). Significantly high odds ratio for sensitization to house dust mite (6.63; 95% confidence interval (CI): 1.76–32.2) was found. Odds ratios were high for association with preference of eating meat rather than fish (3.59; 95% CI: 0.93–17.8), age less than 40 years (2.58; 95% CI: 0.69–10.8), presence of other allergic diseases (2.37; 95% CI: 0.77–9.59), and family history of cedar pollinosis (2.09; 95% CI: 0.60–7.92), but they were not significant (Table 2).

#### 4. Discussion

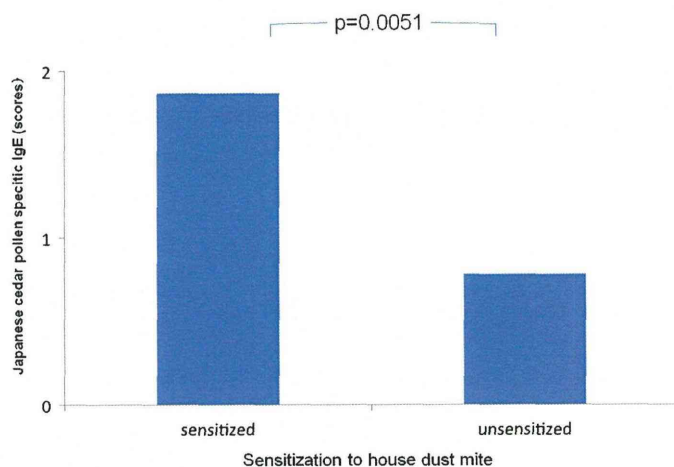
To the best of our knowledge, this is the first report on the analysis of factors influencing the sensitization rate to Japanese cedar pollen in asymptomatic subjects. The mean IgE score of this sensitized population was 2.8. This value is significantly lower than

those of patients with pollinosis alone and those with pollinosis and who are allergic to house dust mites (data not shown). It is known that the percentage of development of pollinosis symptoms depends on the specific IgE titers [9]. Concerning the relationship between specific IgE titers and classes, Class 0 (0.34 UA/ml and under) and Class 1 (0.35–0.69) are considered to be negative (not sensitized). Classes 2–6 (0.79 UA/ml and over) are considered to be positive (sensitized). According to Ohtsuka [9], the rate of development of symptoms increases with increasing classes. For example, the percentage of developing symptoms are 100% in Class 6, 85% in Class 5, 50% in Class 4, 38% in Class 3, 13% in Class 2, 3% in Class 1, and 3% in Class 0.

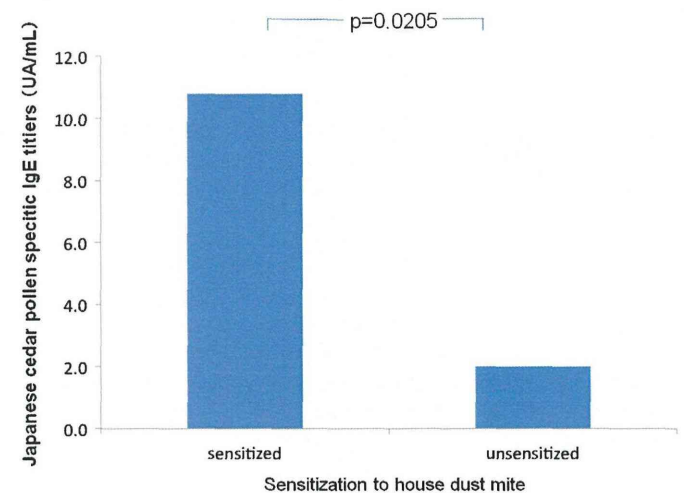
The present study indicates that sensitization to the pollen of the subjects without pollinosis is influenced by their sensitization to house dust mite, not by their family history or their residential environments.

Among the 26 people sensitized to the pollen, 13 were also sensitized to house dust mites. Since it is clearly demonstrated that most of patients with Japanese cedar pollinosis were allergic to house dust mite in children [10], we have excluded those who had perennial allergic symptoms from the subjects in the present study. Although the reason why house dust mite sensitization alone leads to pollen sensitization is not clear, the result might simply show that they are predisposed to producing IgE antibodies to various antigens.

Although statistically not significant, the sensitization rate to cedar was higher in a younger population. Yonekura et al. [2] examined the natural history of seasonal allergic rhinitis and found that the sensitization rates to cedar pollen decreased with age in the same subject groups over 10 years.



**Fig. 3.** Subjects sensitized to house dust mite had significantly higher Japanese cedar pollen-specific IgE scores.



**Fig. 4.** Subjects sensitized to house dust mite had significantly higher Japanese cedar pollen-specific IgE titers.

**Table 2**  
Risk factors influencing sensitization to Japanese cedar pollen.

Factors	Odds ratios	95% confidence interval	p-Value
Sensitized to house dust mite	6.63	1.76–32.2	0.009
Preference of eating meat (rather than fish)	3.59	0.93–17.8	0.082
Age less than 40 years	2.58	0.69–10.8	0.258
Other allergic diseases	2.37	0.77–9.59	0.124
Family history of cedar pollinosis	2.09	0.60–7.92	0.258
Surrounded by many cedar trees	1.59	0.33–7.44	0.551
History of rhinosinusitis	0.80	0.09–6.24	0.835
Lived in the city in childhood	0.64	0.14–2.62	0.541
Smoking habit	0.64	0.10–3.57	0.626
Sex (female)	0.57	0.15–2.06	0.399
Now living in the city	0.55	0.14–1.99	0.383

Although statistically not significant, the sensitization rate to the pollen was higher in the presence of other allergic diseases. Comparing patients with pollinosis to control subjects, Ozasa et al. [11] reported that significantly high odds ratios for a past history of allergic disease [8.80; 95% confidence interval (CI); 3.49–22.2], atopic dermatitis (9.00; 95% CI: 1.14–71.0), and a sibling history of allergic disease (3.25; 95% CI: 1.06–9.97) were found. The mechanism by which other allergic diseases increase the sensitization to pollen is not clear. One possibility is that other allergic diseases increase the interleukin-4 concentration in the blood, which increases the nasal permeability to the pollen [12]. Concerning the atopic dermatitis, it is reported that a null mutation of filaggrin increases the prevalence of both atopic dermatitis and allergic rhinitis [13].

The fact that a family history of pollinosis did not increase the sensitization rate to pollen was somewhat surprising. There are both positive and negative data concerning the relationship between a family history of pollinosis and the development of pollinosis. Ozasa et al. failed to demonstrate the association between the pollinosis of parents and siblings and the pollinosis of the examined subjects [11]. Conversely, Sakurai et al. [3] reported an increased prevalence of pollinosis with a family history of pollinosis.

Many reports concerning the association of pollinosis and gene polymorphisms suggest that Japanese cedar pollinosis may be inherited. Those genes include eosinophil peroxidase and interleukin-4 receptor alpha-chain [5], ADAM metallopeptidase domain 33 [6], and matrix metallopeptidase-9 [7]. However, such an inheritance might be a very weak factor compared with nongenetic factors.

Smoking habit showed no statistically significant relationship. According to Nagata et al. [14], when compared with those who had never smoked, current smokers at baseline were at a significantly decreased risk for cedar pollinosis after controlling for covariates in men (hazard ratio [HR]: 0.64; 95% CI: 0.50–0.83) as well as in women (HR: 0.64; 95% CI: 0.47–0.88). Ozasa et al. [11] reported that the odds ratio for passive smoking from 7 to 15 years of age as a result of the father's smoking habit (0.38; 95% CI: 0.17–0.86) was also significantly low. According to Kurosawa et al. [15], passive smoking was related to a significantly lower prevalence of Japanese cedar pollinosis (adjusted odds ratio: 0.81; 95% CI: 0.74–0.88). The reason why smoking or passive smoking is related to a lower prevalence of Japanese cedar pollinosis is currently unknown. Smoking was suggested to increase the level of antigen-specific IgE in serum [16]. However, smoking and passive smoking might have other effects of decreasing sensitization to Japanese cedar pollen and decreasing the development of cedar pollinosis.

Our study has limitations. First, the number of subjects was small. The sample size was determined by assuming sensitization to house dust mite as primary factor of interest. This study

demonstrated that sensitization to house dust mite is an influencing factor in sensitization to Japanese cedar pollen, however the remains of the factors were not demonstrated to be significant. Some of non-significant factors might be true negative, or some might be false negative due to type II error because of the small sample size. Larger sample sizes are required to reduce type II error, enabling detect factors with slight differences. Second, the subjects' population lacked accurate representation of Japan's current population. This might result from small sample size and recruitment bias of subjects. According to the population estimates published by Ministry of Internal Affairs and Communications (<http://www.stat.go.jp/english/data>), the population aged between 18 and 60 years accounts for 52.5% of the population of Japan in 2011. Furthermore, in this study, subjects aged 20–29 years account for 41.1% of all the subjects, 30–39 years for 16.4%, 40–49 years for 24.7%, and 50–59 year for 9.6%, indicating different distribution pattern between the subjects of the study and age-matched Japan's current population. This difference reduces generalizability of this study.

In conclusion, the present study has shown that the sensitization of subjects without pollinosis is influenced by their sensitization to house dust mite, but not by family history or their environment. Further studies in larger populations are necessary.

#### Conflict of interest

None.

#### Acknowledgements

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#### References

- [1] Horiguchi S, Saito Y. Discovery of Japanese cedar pollinosis in Nikko, Ibaraki Prefecture. *Arerugi* 1964;13:16–8 [in Japanese].
- [2] Yonekura S, Okamoto Y, Horiguchi S, Sakurai D, Chazono H, Hanazawa T, et al. Effects of aging on the natural history of seasonal allergic rhinitis in middle-aged subjects in South Chiba, Japan. *Int Arch Allergy Immunol* 2012;157:73–80.
- [3] Sakashita M, Hirota T, Harada M, Nakamichi R, Tsunoda T, Osawa Y, et al. Prevalence of allergic rhinitis and sensitization to common aeroallergens in a Japanese population. *Int Arch Allergy Immunol* 2010;151:255–61.
- [4] Practical guideline for the management of allergic rhinitis in Japan – perennial rhinitis and pollinosis – 2009 edition. 6th ed. Tokyo: Life Science; 2008 [in Japanese].
- [5] Nakamura H, Miyagawa K, Ogino K, Endo T, Imai T, Ozasa K, et al. High contribution contrast between the genes of eosinophil peroxidase and IL-4

- receptor alpha-chain in Japanese cedar pollinosis. *J Allergy Clin Immunol* 2003;112:1127–31.
- [6] Cheng L, Enomoto T, Hirota T, Shimizu M, Takahashi N, Akahoshi M, et al. Polymorphisms in ADAM33 are associated with allergic rhinitis due to Japanese cedar pollen. *Clin Exp Allergy* 2004;34:1192–201.
- [7] Inoue H, Mashimo Y, Funamizu M, Yonekura S, Horiguchi S, Shimojo N, et al. Association of the MMP9 gene with childhood cedar pollen sensitization and pollinosis. *J Hum Genet* 2012;57:176–83.
- [8] Miyake Y, Sasaki S, Tanaka K, Ohya Y, Miyamoto S, Matsunaga I, et al. Fish and fat intake and prevalence of allergic rhinitis in Japanese females: the Osaka Maternal and Child Health Study. *J Am Coll Nutr* 2007;26:279–87.
- [9] Ohtsuka H. New IgE antibody testing and their results. *Arerugi no rinshou* 1993;13:668–71 [in Japanese].
- [10] Masuda S, Takeuchi K, Yuta A, Okawa C, Ukai K, Sakakura Y. Japanese cedar pollinosis in children in our allergy clinic. *Arerugi* 1998;47:1182–9 [in Japanese].
- [11] Ozasa K, Takenaka H, Takagi N, Aoike A, Kawai K. A case control study of risk factors for Japanese cedar pollinosis. *Nihon Eiseigaku Zasshi* 1995;50:622–30 [in Japanese].
- [12] Di Leo V, Yang PC, Berin MC, Perdue MH. Factors regulating the effect of IL-4 on intestinal epithelial barrier function. *Int Arch Allergy Immunol* 2002;129:219–27.
- [13] Weidinger S, O'Sullivan M, Illig T, Baurecht H, Depner M, Rodriguez E, et al. Filaggrin mutations, atopic eczema, hay fever, and asthma in children. *J Allergy Clin Immunol* 2008;121:1203–9.
- [14] Nagata C, Nakamura K, Fujii K, Kawachi T, Takatsuka N, Oba S, et al. Smoking and risk of cedar pollinosis in Japanese men and women. *Int Arch Allergy Immunol* 2008;147:117–24.
- [15] Kurosaka F, Nakatani Y, Terada T, Tanaka A, Ikeuchi H, Hayakawa A, et al. Current cat ownership may be associated with the lower prevalence of atopic dermatitis, allergic rhinitis, and Japanese cedar pollinosis in schoolchildren in Himeji, Japan. *Pediatr Allergy Immunol* 2006;17:22–8.
- [16] Moerloose KB, Robays LJ, Maes T, Brusselle GG, Tournoy KG, Joos GF. Cigarette smoke exposure facilitates allergic sensitization in mice. *Respir Res* 2006;7:49.

# Expression and Function of Allergin-1 on Human Primary Mast Cells

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## Abstract

Mast cells (MC) play an important role in allergic and non-allergic immune responses. Activation of human MC is modulated by several cell surface inhibitory receptors, including recently identified Allergin-1 expressed on both human and mouse MC. Although Allergin-1 suppresses IgE-mediated, mast cell-dependent anaphylaxis in mice, the expression profile and function of Allergin-1 on human primary MC remains undetermined. Here, we established a seven-color flow cytometry method for assessing expression and function of a very small number of human primary MC. We show that Allergin-1S1, a splicing isoform of Allergin-1, is predominantly expressed on human primary MC in both bronchoalveolar lavage (BAL) fluid and nasal scratching specimens. Moreover, Allergin-1S1 inhibits IgE-mediated activation from human primary MC in BAL fluid. These results indicate that Allergin-1 on human primary MC exhibits similar characteristics as mouse Allergin-1 in the expression profile and function.

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**Competing Interests:** Masamichi Imai and Shiro Shibayama are employees of Ono Pharmaceutical Company. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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## Introduction

Mast cells (MC) are widely distributed throughout vascularized tissues, particularly near surfaces exposed to the external environment such as the skin, airways, and gastrointestinal tract. MC are well positioned to be involved in the first line of immune responses against environmental antigens, toxins, or invading pathogens [1]. MC express FcεRI, a high-affinity receptor for IgE, on their surface and play a central role in IgE-associated allergic responses [2–4]. Crosslinking of FcεRI-bound IgE with multivalent antigen initiates the activation of MC by promoting the aggregation of FcεRI, resulting in the degranulation of MC, along with the concomitant secretion of chemical mediators such as histamine, tryptase, carboxypeptidase A, and proteoglycans that are stored in the cytoplasmic granules of these cells, and the de novo synthesis of pro-inflammatory lipid mediators such as prostaglandins and leukotrienes as well as platelet-activating factor in the early phase. MC also play an important role in innate immune responses against bacteria and parasites through the synthesis and secretion of cytokines and chemokines that recruit neutrophils, eosinophils, and Th2 cells to the site of infection [1,5].

A major problem in MC research is the difficulty of obtaining primary MC, particularly in human, because MC are found not in

the peripheral blood but in the systemic organs. Moreover, MC show a very low frequency in the systemic organs. Therefore, most MC experiments are performed with cultured MC derived from human blood progenitors or mouse bone marrow progenitors. However, the phenotypical and functional characteristics of MC depend on many factors, including species of animal, specific anatomical location, and status of maturation [6]. For example, although MC express IL-3 receptor, CD14, and Toll-like receptors in mouse, these molecules are scarcely detected on human MC [7]. The results of studies utilizing mouse MC are not directly transferable to human MC research. Therefore, it is desirable to be able to analyze human primary MC for research into allergic and non-allergic diseases mediated by MC effector function.

Activation of human MC is modulated by several cell surface inhibitory receptors, including FcγRIIB [8], SIRP-α [9], CD300A (CMRF35) [10–12], and LILR-B2 [13]. We recently identified a novel immunoglobulin (Ig)-like inhibitory receptor, designated Allergy-inhibitory receptor (Allergin)-1, which contains the immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic portion, in both human and mouse MC [14]. Mice deficient in Allergin-1 show significantly enhanced passive systemic and cutaneous anaphylaxis [14], indicating that Allergin-1 suppresses IgE-mediated, mast cell-dependent anaphylaxis

in mice. Although mouse Allergin-1 contains one Ig-like domain in the extracellular portion, we identified three splicing isoforms of human Allergin-1: Allergin-1 long form (Allergin-1L) that contains two Ig-like domains, Allergin-1 short-form 1 (Allergin-1S1) that contains the first Ig-like domain of Allergin-1L, and Allergin-1 short-form 2 (Allergin-1S2) that contains the second Ig-like domain of Allergin-1L in the extracellular portion. However, the expression profile of the Allergin-1 isoforms on human primary MC remains undetermined. Moreover, it remains unclear whether Allergin-1 inhibits the IgE-mediated activation of human primary MC.

In this study, we utilized flow cytometric method to assess the expression and function of Allergin-1 on a small number of human primary MC at a single cell level in bronchoalveolar lavage fluid (BALF) and nasal scratching specimens (NSS). We show that Allergin-1S1 is the major isoform expressed on human primary MC and that it inhibits IgE-mediated activation.

## Methods

### Samples

BALF was obtained from patients with pulmonary diseases at the University of Tsukuba Hospital, Japan. NSS were obtained from patients with non-seasonal allergic rhinitis at the Fukui University Hospital, Japan, and from healthy volunteers (**Table S1**). Peripheral blood and cord blood for the generation of cultured MC were obtained from healthy volunteers and RIKEN BioResource Center (Tsukuba, Japan), respectively. Written informed consent was obtained from the patients and healthy volunteers. This study was approved by the ethical review boards of both the University of Tsukuba and the University of Fukui. BAL was performed by using 150-mL aliquots of isotonic saline. NSS were obtained by brushing the unilateral inferior turbinates with a CytoSoft Cytology Brush (Medical Packaging, Camarillo, CA). BALF and NSS with blood contamination due to traumatic bleeding were excluded from the study. BALF and NSS were filtered through a 70- $\mu$ m cell-strainer and resuspended in phosphate-buffered saline containing 2% fetal bovine serum.

### Antibodies, reagents, and transfectants

Human IL-3, IL-6, and Stem Cell Factor were purchased from PeproTech (London, UK). Monoclonal antibodies (mAbs) used in the flow cytometry analyses are shown in **Table S2**. TNP-specific mouse IgE was purchased from BD Biosciences (San Jose, CA). Anti-CD300A mAb (clone: TX49) was generated in our laboratory [15]. Anti-Allergin-1 mAbs (clones: EX29, EX32, and EX33) were provided by Exploratory Research Laboratories, Tsukuba Research Institute, Ono Pharmaceutical Co., Ltd., Japan. BW5147 cells stably expressing Allergin-1L, Allergin-1S1, or Allergin-1S2 were established by using a retroviral vector, as described [14].

### Flow cytometric analyses

For phenotypical analysis of primary MC, the cells obtained from the BALF or NSS were stained with propidium iodide (PI); Horizon V500-conjugated anti-CD45; a Horizon V450-conjugated mAb cocktail containing anti-CD3, anti-CD19, anti-CD56, anti-CD11b, and anti-CD11c mAbs; PE-Cy7-conjugated anti-c-Kit; FITC-conjugated anti-Fc $\epsilon$ RI $\alpha$ ; and PE and/or APC-conjugated mAbs of interest (**Table S2**). Finally, cells were analyzed by using a FACS Fortessa with either Diva (Becton Dickinson, San Jose, CA) or Flow-Jo software (Tree Star, Inc. Ashland, OR). We purified cells by using a FACS Aria (Becton Dickinson, San Jose, CA) for morphological assessment.

### MC activation assay

After filtration of BALF, the cells were washed with HEPES-Tyrode's Buffer and then incubated with human IgG at 100  $\mu$ g/mL in HEPES-Tyrode's Buffer to block nonspecific binding to Fc $\gamma$  receptors. Cells were then incubated with 2  $\mu$ g/mL trinitrophenol (TNP)-specific mouse IgE (Becton Dickinson) at 37°C for 2 h. After washing, cells were incubated with 200  $\mu$ L of HEPES-Tyrode's Buffer containing various amounts of the TNP-conjugated F(ab')<sub>2</sub> fragment of the mAb against Allergin-1L and Allergin-1S1 (clone: EX33) or the TNP-conjugated F(ab')<sub>2</sub> fragment of rat IgG (TNP-control Ig) at 37°C for 30 min, followed by incubation at 4°C for 10 min. Cells were then stained with APC-conjugated anti-CD107a, in addition to PI and the mAbs described above, to determine the MC population. Activation assay for Cord blood-derived cultured MC (CB-MC) and peripheral blood-derived cultured MC (PB-MC), cultured MC were incubated with 2  $\mu$ g/mL TNP-specific mouse IgE containing culture medium at 37°C in 5% CO<sub>2</sub> incubator for five days. Cells were then stimulated by 200  $\mu$ L of HEPES-Tyrode's Buffer containing TNP-conjugated F(ab')<sub>2</sub> fragment of EX33 or TNP-control Ig at 37°C for 30 min, followed by incubation at 4°C for 10 min as well as BALF cells. After stimulation, cells were stained with APC-conjugated anti-CD107a, in addition to PI and PE-Cy7-conjugated anti-c-Kit to determine the MC population.

### MC culture

PB-MC and CB-MC were generated as described [16]. In brief, CD34<sup>+</sup> cells were isolated from human peripheral blood mononuclear cells or cord blood mononuclear cells by using MACS cell separation system (Miltenyi Biotech) and cultured in serum-free Iscove's modified Dulbecco medium containing 80% methylcellulose medium, Stem Cell Factor (200 ng/mL), IL-6 (50 ng/mL), and IL-3 (1 ng/mL) for 6 to 8 weeks. Methylcellulose medium was then dissolved in phosphate-buffered saline, and the cells were cultured in Iscove's modified Dulbecco medium with 5% fetal bovine serum containing Stem Cell Factor (100 ng/mL) and IL-6 (50 ng/mL). For the phenotypical and functional analyses, PB-MC and CB-MC that had been cultured for more than 8 weeks were used.

### Quantitative RT-PCR analysis

Total RNAs of CB-MC and PB-MC were isolated by ISOGEN (Nippon Gene), and the cDNA was synthesized with The High Capacity cDNA Reverse Transcription Kit (Invitrogen). For quantitative RT-PCR analysis, cDNA fragments were amplified with the TaqMan Universal Master Mix (Applied Biosystem) and quantified with the TaqMan Gene Expression assay (Applied Biosystem). The primers and probes were designed as shown **Fig. 1A**. The sequences were; Allergin-1L-Forward;5'-CCC-AAGTTACCAGCTGTTCAA-3',

Allergin-1L-Reverse;5'-TATATGTCGGTCTGTTTCTGTT-TGAAT-3',

Allergin-1L-Probe;5'-CGATTGTCGACCCGGTG-3',

Allergin-1S1-Forward;5'-TAGGATTATCACCACCAGCAA-CAG-3',

Allergin-1S1-Reverse;5'-AGCTGTATTGGATTGTGAGGC-A-3',

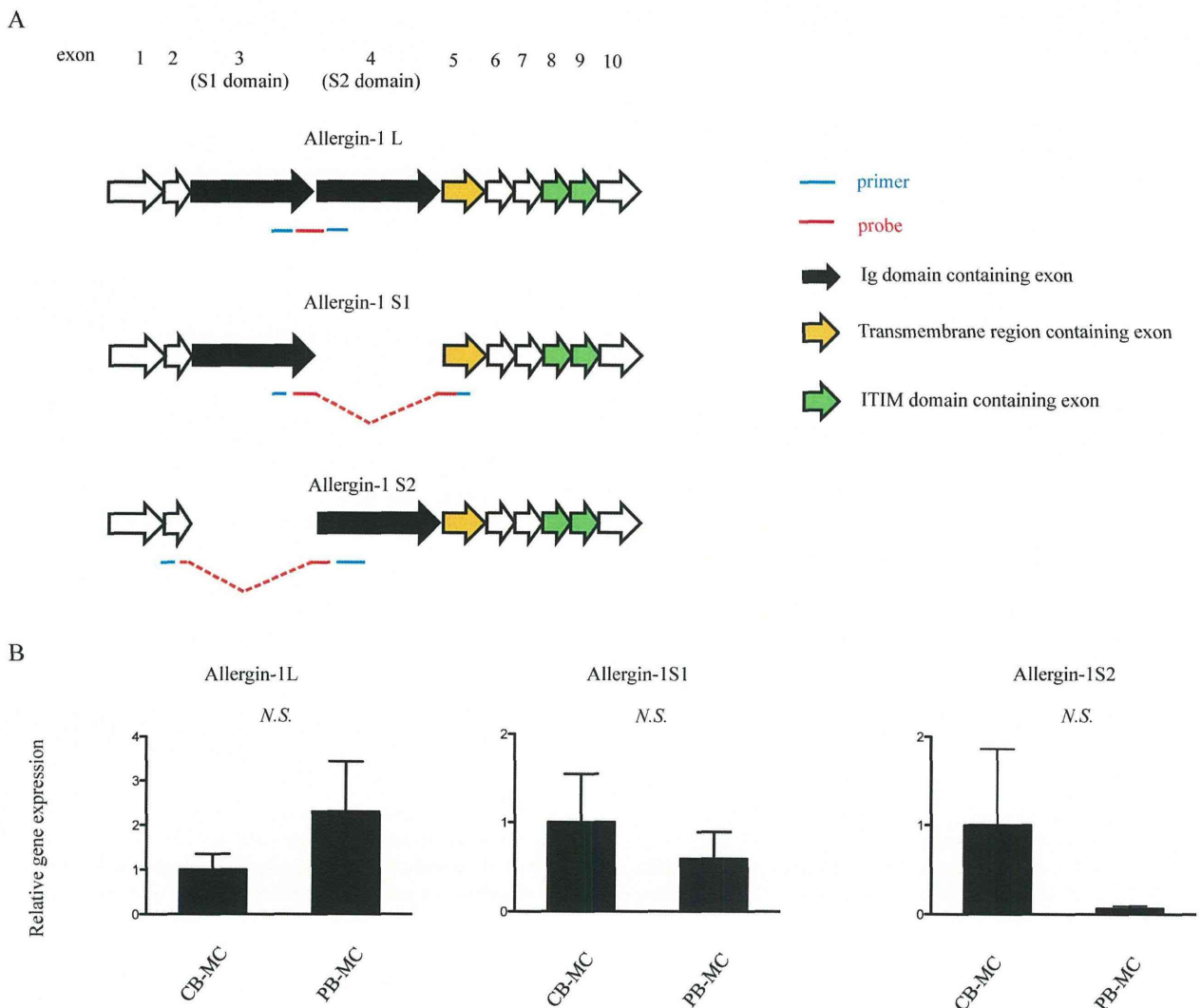
Allergin-1S1-Probe;5'-TCGGCCGACAATC-3',

Allergin-1S2-Forward;5'-AAATGCAAAGCCCAAGTTACCA-3',

Allergin-1S2-Reverse;5'-AATTGATGGGCAGCGAGC-3',

Allergin-1S2-Probe;5'-ACAAATGACCCGGTGACT-3'





**Figure 1. Expression of Allergin-1 isoform transcripts in cultured MC.** A. Primers and probes for qRT-PCR were designed to detect Allergin-1L, Allergin-1S1 or Allergin-1S2 mRNAs. B. CB-MC (n=3) and PB-MC (n=3) cultured for more than eight weeks were subjected to qRT-PCR, as described in Method. doi:10.1371/journal.pone.0076160.g001

### Statistical analysis

All values in the figures and text are expressed as mean  $\pm$  SEM. Statistical significance of the differences among median values was evaluated by using the Mann-Whitney *U* test or Kruskal-Wallis one-way analysis with post-hoc Dunn's test to compare.

### Results

#### Expression of Allergin-1 isoforms on human cultured MC

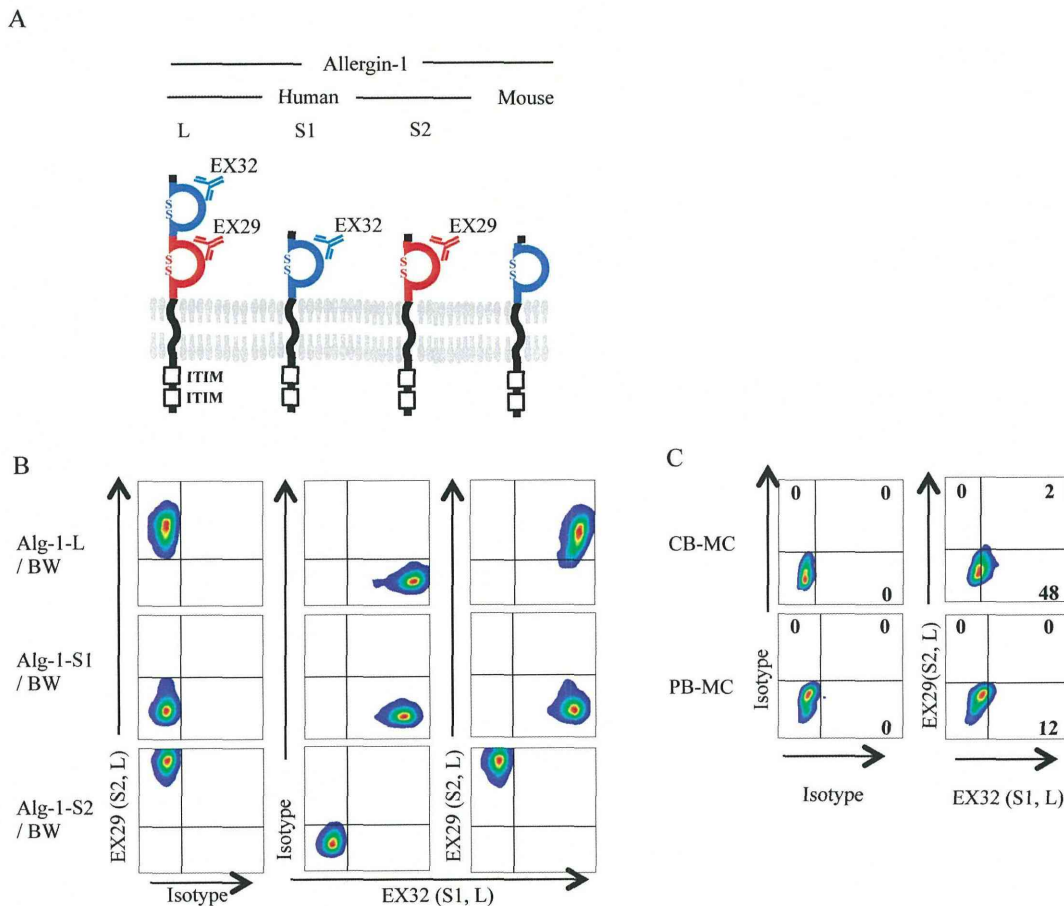
Unlike mouse Allergin-1, human Allergin-1 consists of three splicing isoforms: Allergin-1L, Allergin-1S1, and Allergin-1S2 (**Fig. 1A**) [14]. To analyze the expression profile of the Allergin-1 isoform in human MC, we performed PCR by using primer pairs that specifically detect the mRNA of each isoform (**Fig. 1A**). We detected all the isoforms in both CB-MC and PB-MC but observed no significant difference in the amount of each isoform between CB-MC and PB-MC (**Fig. 1B**).

To examine the protein expression profile of the Allergin-1 isoforms on MC, we generated three mAbs (clones: EX29, EX32,

and EX33) against human Allergin-1 (**Fig. 2A**). To examine the specificities of these mAbs, we generated BW5147 transfectants stably expressing Allergin-1L, Allergin-1S1, or Allergin-1S2 [14]. EX32 and EX33 bound to transfectants expressing Allergin-1L or Allergin-1S1, but not Allergin-1S2. In contrast, EX29 bound to transfectant expressing Allergin-1L or Allergin-1S2, but not Allergin-1S1 (**Fig. 2B, and data not shown**), indicating that EX32 and EX33 recognize an epitope of the first Ig-like domain of Allergin-1L, and EX29 recognizes an epitope of the second Ig-like domain. By using EX32 and EX29, we examined the expression of the Allergin-1 splicing isoforms on cultured MC (CB-MC and PB-MC). EX32 stained both CB-MC and PB-MC, whereas EX29 did not stain CB-MC or PB-MC (**Fig. 2C**), suggesting that these cultured MC express Allergin-1S1, but not Allergin-1L or Allergin-1S2.

#### Isolation and characterization of human primary MC in BALF

To examine the expression of Allergin-1 isoforms on human primary MC, we first developed a flow cytometric method to



**Figure 2. Expression of Allergin-1 isoforms on CB-MC and PB-MC.** A. Structure of human and mouse Allergin-1. Unlike mouse Allergin-1, human Allergin-1 consists of three splice isoforms, Allergin-1L, Allergin-1S1, and Allergin-1S2. The first and second immunoglobulin-like domains in the extracellular portion of Allergin-1L is identical to that of Allergin-1S1 and Allergin-1S2, respectively. B. BW5147 transfectants stably expressing each isoform of Allergin-1 were stained with isotype control, anti-Allergin-1S1-specific EX32, and/or anti-Allergin-1S2-specific EX29 mAbs and were analyzed by using flow cytometry. C. CB-MC and PB-MC were stained with isotype control Abs or with EX32 and EX29 mAbs and were analyzed by using flow cytometry. doi:10.1371/journal.pone.0076160.g002

characterize the human primary MC in the BALF. We obtained the BALF cells from patients with pulmonary diseases and simultaneously stained the cells with PI and mAbs against CD45; the lineage markers CD3, CD19, CD56, CD11b, and CD11c; c-Kit; and FcεRIα and were analyzed by using multicolor flow cytometry. MC were defined as PI<sup>+</sup>CD45<sup>+</sup>Lin<sup>-</sup>c-Kit<sup>+</sup>FcεRIα<sup>+</sup> cells (Fig. 3A), which comprised 0.153% ± 0.041% (*n* = 28) of the total cell population in BALF. No significant differences were observed in the frequency of MC in BALF (BAL-MC) from patients with pulmonary diseases such as idiopathic interstitial pneumonia, sarcoidosis, or eosinophilic pneumonia (Table S1). Staining of BAL-MC sorted from BALF with Toluidine-blue showed a characteristics of MC with rich granules in the cytoplasm (Fig. 3B).

#### Expression of Allergin-1 isoforms on human primary MC in BALF

We then conducted a seven-color flow cytometric analysis in which the cells obtained from the BALF of patients with various pulmonary diseases were simultaneously stained with EX32 and EX29, in addition to PI and mAbs against CD45, lineage markers,

c-Kit, and FcεRIα (Fig. 4A). We found diverse expression profiles on BAL-MC among the patients (Fig. 4B), and, in accordance with the results from PB-MC and CB-MC, Allergin-1S1 was dominantly expressed on BAL-MC, compared with Allergin-1L1 or Allergin-1S2 (Fig. 4C).

#### Expression of Allergin-1 isoforms on human primary MC in the nasal cavity

We also examined MC obtained from the nasal cavity (N-MC) of healthy donors and patients with non-seasonal allergic rhinitis by using multicolor flow cytometry. We detected few N-MC in nasal scratching specimens from healthy donors. In contrast, we clearly observed N-MC in nasal scratching specimens from patients with non-seasonal rhinitis (Fig. 5A). The number of N-MC obtained from rhinitis patients was significantly higher than that from healthy donors (Fig. 5B). Flow cytometric analysis showed that N-MC preferentially expressed Allergin-1S1, rather than Allergin-1L or Allergin-1S2 (Fig. 5C, D), suggesting that Allergin-1S1 is the dominant isoform of Allergin-1 in human primary MC.