

High contribution contrast between the genes of eosinophil peroxidase and IL-4 receptor α -chain in Japanese cedar pollinosis

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Background: Japanese cedar pollinosis is the most common form of hayfever in Japan in spring and has remarkably increased since 1960.

Objective: We sought to clarify the candidate genes for cedar pollinosis using a case-control study.

Methods: After diagnosing 351 subjects on the basis of an intradermal test, nasal provocation test, and questionnaire regarding nasal and conjunctival symptoms, we determined the blood-specific IgE values and genotypes of eosinophil peroxidase (EPO) and interleukin-4 receptor α -chain (IL4RA) in 145 patients with pollinosis and 206 healthy subjects, including 75 healthy subjects with higher specific IgE values.

Results: We found significant differences in the frequencies of Pro358Leu in EPO and of Ile50Val and Glu375Ala in IL4RA between patients and healthy subjects. There was a significantly higher frequency of 358Leu in EPO in patients than in healthy subjects showing a higher specific IgE value. In contrast, we recognized significant changes in the prevalence of Ile50Val and Glu375Ala in IL4RA in healthy subjects with a normal IgE value compared with those in healthy subjects with a higher specific IgE value. The relationship between EPO polymorphisms and the onset of symptoms was exactly opposite that for IL4RA.

Conclusions: These results suggest that Pro358Leu in EPO is strongly involved in the development of cedar pollinosis.

Ile50Val and Glu375Ala in IL4RA seem to be related to cedar pollen sensitization. Subjects with Ile50 or Glu375 might develop cedar pollinosis with increased exposure to cedar pollen. (J Allergy Clin Immunol 2003;112:1127-31.)

Key words: Allergy, association study, cedar pollinosis, eosinophil peroxidase, IgE, IL-4 receptor α -chain, odds ratio, onset, polymorphisms, sensitization

Japanese cedar pollinosis is the most common form of hayfever in Japan in spring and has remarkably increased since 1960. Cedar pollinosis is defined as a type I allergic disease with ocular and nasal symptoms that develop paroxysmally on contact with Japanese cedar pollen.^{1,2} These symptoms, which occur seasonally each year, are typical features of allergic rhinitis, such as sneezing, excessive nasal secretion, nasal congestion, and conjunctival itching. Cross-sectional and ecologic studies suggest that environmental factors such as NO₂ or suspended particulate matter and lifestyle, including smoking, are associated with development of the disease.³⁻⁵ Based on the accumulated evidence, cedar pollinosis is thought to be produced by genetic interaction, exposure to cedar pollen, and other environmental factors.⁶ Candidate genes for cedar pollinosis, a multifactorial allergic disease, remain to be elucidated.⁷

IL-4 is a pleiotropic cytokine produced by mast cells, basophils, and T cells and plays a central role in IgE-dependent inflammatory reactions.⁸ IL-4 is central to B-cell switching to IgE antibody production and to the maturation of T-helper cells to the T_H2 phenotype. IL-4 operates through the IL-4 receptor, a heterodimeric complex comprising the IL-4 receptor α chain (IL4RA) and the γ chain. Deichmann and colleagues⁹ conducted a systematic search for variations in the IL-4RA gene and found 13 polymorphic variants in the coding region, including 7 variants that resulted in amino acid substitution. The prevalence of Ile50 has been demonstrated to be higher than Val50 in individuals with atopic asthma, especially during childhood in the Japanese.¹⁰ The Ile50Val variant of IL-4RA upregulates IgE synthesis and is associated with atopic asthma.¹¹ However, many reports have found no involvement of the IL-4RA genes, including the variant of IL-4RA.¹²⁻¹⁵ Thus, the involvement of the IL-4RA gene in allergic diseases, including cedar pollinosis, remains to be elucidated.

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Abbreviations used:

EPO: Eosinophil peroxidase
 IDT: Intradermal test
 IL-4RA: Interleukin 4 receptor α -chain
 NPT: Nasal provocation test

Multiple independent lines of evidence implicate eosinophils as active participants in the pathogenesis of allergic diseases.¹⁶ On stimulation, eosinophils release cytotoxic granule proteins, including eosinophil peroxidase (EPO), which are considered to play a seminal role as mediators of inflammation and tissue damage in the pathogenesis of parasitic infections and allergic diseases.¹⁷ To date, we know of 2 polymorphisms leading to the substitution of an amino acid that constitutes EPO protein.^{18,19} Asp648Asn is thought to produce unstable EPO proteins that undergo progressive degradation as the cells mature and decreased volume of the granule matrix in eosinophils.²⁰ However, there has been no study reporting the relationship between polymorphisms of EPO and allergies such as atopic diseases or cedar pollinosis. Therefore, we sought to determine the candidate genes for the proteins related to the allergy pathogenesis, especially EPO and IL-4RA, using a case-control study.

METHODS

Subjects

The subjects of this study were recruited from residents of Gotanda, Sinagawa-ku, the central area of metropolitan Tokyo. Four hundred eight people in the area voluntarily participated in this study. All subjects received a self-administered questionnaire regarding their history of allergic diseases, ie, cedar pollinosis, asthma, and atopic dermatitis, as well as nasal and conjunctival symptoms: sneezing, nasal discharge, itching of nasal mucosa or conjunctiva, and watering eyes. They underwent an intradermal test (IDT) and nasal provocation test (NPT) using Japanese cedar (*Cryptomeria japonica*). The diagnosis of cedar pollinosis was based on the questionnaire, IDT, NPT, and the history of cedar pollinosis. The criteria for the diagnosis of cedar pollinosis were as follows: 1) under drug therapy or immunotherapy for cedar pollinosis, and 2) at least 1 positive IDT or NPT result in subjects with symptoms. Forty-five participants with a past history of asthma, atopic dermatitis, or any immunotherapy were excluded from study. In addition, 12 participants positive for either IDT or NPT with no symptoms were excluded owing to the unconfirmed diagnosis of pollinosis. The mean age \pm SD was 45.5 ± 20.7 years and 43.4 ± 17.3 years in 206 healthy subjects and 145 patients with cedar pollinosis, respectively, and these ages were not recognized to be significantly different by Student's *t* test.

All subjects were fully informed of the protocol and gave their informed consent before the experiment. This study was approved by the Ethics Committee on Experimentation of Kanazawa University, Takara-machi Campus.

Specific IgE

A blood sample from each subject was obtained by cubital venous puncture to determine the specific IgE value and genotypes of EPO and IL-4RA. Measurement of the specific IgE antibody level to Japanese cedar pollen was performed by a RAST. The lev-

els of a specific antibody were classified into 2 groups: subjects with a higher specific IgE value who had an RAST score 1 of 0.35 (U/mL) or higher and subjects with a normal IgE value. The specific IgE value was not included in this diagnosis, although all subjects diagnosed with cedar pollinosis had a higher specific IgE value.

Genotype

DNA was extracted from peripheral blood leukocytes using the Automatic DNA isolation system (Kurabo, Tokyo, Japan). Amplification was performed using a Takara LA *Taq* (Takara, Tokyo, Japan). The final reaction mixture (25 μ L) consisted of 50 ng of genomic DNA (1 μ L), 2.5 μ L *Taq* buffer, 2 μ L dNTP, 0.125 μ L *Taq*, 2 μ L $MgCl_2$, and 1 μ L EPO variant-specific primer pairs. The specific primer pairs used were as follows: for exon 7 of EPO, forward CACTGTCTCCTCTCCAT and reverse GTTTCCTGGGAAGACACCA; for exon 3 of IL-4RA, forward 5'-CGGAATCCGAGGCCACACGTGT-3' and reverse 5'-CGCTGGGCTTGAAGGAG-3'; and for exon 9 of IL-4RA, forward 5'-ATCAGCGTGGT-GCGATGTGT-3' and reverse 5'-GAATGAGGTCTTGGAAAGG-3'. For IL-4RA polymorphisms, the PCR protocol consisted of a pre-PCR heat activation step (95°C, 5 minutes); followed by 25 cycles of denaturation (95°C, 1 minute), annealing (55°C, 1 minute), and extension (72°C, 1 minute); and a final cycle extension at 72°C for 7 minutes. PCR products were visualized after agarose gel electrophoresis and ethidium bromide staining. PCR products were purified using Microcon (Millipore, Bedford, Mass).

DNA sequencing

Direct DNA sequencing of PCR products was performed on an ABI Prism 377 genetic analyzer (Perkin-Elmer, Norwalk, Conn) using the ABI Prism Big Dye Terminator DNA sequencing kit (Perkin-Elmer).

Statistics

Allele frequencies of the mutant type are expressed in all tables and compared between the 2 groups of pollinosis patients and healthy subjects, between pollinosis patients and healthy subjects with a higher specific IgE value, between healthy subjects with high and low specific IgE values, and between pollinosis patients with early and late onset of symptoms with the χ^2 test. All statistical tests were 2-tailed. Values of *P* < .05 were regarded as statistically significant.

RESULTS

Allele frequencies

We found 10 and 7 cases with polymorphisms at amino acid 326 in exon 7 of EPO as substitutions of Arg into His (Arg326His) and Leu (Arg326Leu), respectively. The allele frequencies in 326His and 326Leu were 0.746% and 1.23% for healthy subjects and 2.45% and 0.725% for those with pollinosis, showing no significant difference in allele frequencies between the healthy and subjects with pollinosis. We recognized a significantly higher frequency of 358Leu allele in exon 7 of EPO (*P* < .001) in the pollinosis group, with a high odds ratio and 95% CI of 6.92 and 2.14 to 22.4, respectively (Table I). Regarding the relationship between polymorphisms of IL-4RA and pollinosis, the odds ratios (95% CIs) of subjects with pollinosis compared with healthy subjects was 0.616 (0.449-0.847) and 0.352 (0.183-0.676) in Ile50Val and Glu375Ala of IL-4RA, respectively, and were significant (both *P* < .05). There were no significant differences in the prevalence of

TABLE I. Genotype frequencies of exon 7 in EPO in the study group

	Arg326His			Arg326Leu			Pro358Leu		
	Arg/Arg	Arg/His	His/His	Arg/Arg	Arg/Leu	Leu/Leu	Pro/Pro	Pro/Leu	Leu/Leu
Subjects									
Control	198	3	0	198	5	0	203	3	0
Pollinosis	136	7	0	136	2	0	131	14	0
Allele frequency (%)									
of polymorphism									
Control		0.746			1.23			0.728	
Pollinosis		2.45			0.725			4.83	
Odds ratio (95% CI)		3.34			0.585			6.92	
		(0.701-15.9)			(0.212-1.62)			(2.14-22.4)*	

Whole numbers represent numbers of subjects in that group.

*Statistically significant at $P < .001$.

TABLE II. Genotype frequencies of IL-4RA in the study group

	Ile50Val			Ala71Thr			Glu375Ala			Cys406Arg			Ser411Leu			Ser478Pro			Gln551Arg		
	Ile/Ile	Ile/Val	Val/Val	Ala/Ala	Ala/Thr	Thr/Thr	Glu/Glu	Glu/Ala	Ala/Ala	Cys/Cys	Cys/Arg	Arg/Arg	Ser/Ser	Ser/Leu	Leu/Leu	Ser/Ser	Ser/Pro	Pro/Pro	Gln/Gln	Gln/Arg	Arg/Arg
Subjects																					
Control	91	76	39	185	20	1	171	32	3	177	27	2	202	3	1	184	20	2	166	37	3
Pollinosis	81	50	14	133	12	0	135	10	0	131	14	0	141	2	2	132	12	1	112	32	1
Allele frequency (%)																					
of polymorphism																					
Control		37.4			5.34			9.22		7.52			1.21			5.83			10.4		
Pollinosis		26.9			4.14			3.45		4.83			2.07			4.83			11.7		
Odds ratio (95% CI)		0.616			0.765			0.352		0.623			1.72			0.820			1.14		
		(0.449-0.847)*			(0.430-1.36)			(0.183-0.676)*		(0.349-1.12)			(0.284-10.4)			(0.486-1.38)			(0.614-2.11)		

*Statistically significant at $P < .05$.

the Ala71Thr, Cys406Arg, Ser411Leu, Ser478Pro, or Gln551Arg polymorphisms of IL-4RA between healthy subjects and patients with pollinosis (Table II).

Specific IgE value

The allele frequency (4.83%) of 358Leu of EPO in patients with pollinosis was significantly higher than that (0.667%) in healthy subjects showing a high specific IgE value ($P < .05$). There was no significant difference in the prevalence of Ile50Val and Glu375Ala in IL-4RA between subjects with pollinosis and healthy subjects with a high specific IgE value, although the allele frequencies of 28.0% and 4.00% for 50Val and 375Ala, respectively (both $P < .01$), in healthy subjects with a high specific IgE value, were significantly lower than those of 42.7% and 12.2% in healthy subjects showing a normal specific IgE value (Table III).

Onset of symptoms

The onset of symptoms related to pollinosis in 115 patients was clarified by the questionnaire. Fifty-seven and 58 patients had the symptoms before and after graduation from elementary school, respectively. Allele frequencies of Pro358Leu in EPO and of Ile50Val and Glu375Ala in IL-4RA are shown in Table IV, according to the onset of symptoms. The allele frequency of 358Leu in EPO, 8.77% in patients with early onset, was significantly higher than 1.72% in those with late onset

($P < .01$). The indicating frequencies show the direction of the wild type, although we observed significantly higher frequencies of 81.9% and 98.3% in Ile50 and Glu375, respectively, in patients with late onset compared with 68.4% and 93.0% in those with early onset (both $P < .05$).

DISCUSSION

There have been some studies reporting an association between allergic rhinitis and gene polymorphisms, such as Glu237Gly of FcεRIβ⁷ and G127T in exon 1 of IL18,²¹ as well as HLA class II alleles.^{22,23} We found a significant relationship between the Pro358Leu allele in EPO and cedar pollinosis with a high odds ratio. Human EPO is a heme-containing glycoprotein and a member of the peroxidase gene family that includes the closely related neutrophil myeloperoxidase, as well as porcine and human lactoperoxidase and thyroid peroxidase.^{24,25} The similarity of the EPO nucleotide sequence to that of other eukaryotic peroxidases suggests that these peroxidases compose a multigene family evolved by gene duplication.^{24,25} Since the region encompassing Pro358Leu is well conserved among these peroxidases in humans as well as in pigs, the variation might be involved in the hyperactivity of peroxidase, resulting in allergy. This assumption should be confirmed by further function analysis of variants of the EPO gene.

TABLE III. Comparisons of genotype of Pro358Leu (EPO), Ile50Val (IL-4RA), and Glu375Ala (IL-4RA) in the study group between pollinosis and control groups with higher specific IgE values

Number of subjects	Specific IgE value	Pro358Leu (EPO)			Ile50Val (IL-4RA)			Glu375Ala (IL-4RA)		
		Pro/Pro	Pro/Leu	Leu/Leu	Ile/Ile	Ile/Val	Val/Val	Glu/Glu	Glu/Ala	Ala/Ala
Healthy	Normal	129	2	0	48	54	29	101	28	2
	High	74	1	0	43	22	10	70	4	1
Pollinosis	High	131	14	0	81	50	14	135	10	0
Allele frequency (%) of polymorphism										
Healthy	Normal		0.763			42.7			12.2	
	High		0.667			28.0 †			4.00 ‡	
Pollinosis	High		4.83*			26.9			3.45	

No patients with pollinosis had normal specific IgE values. Statistical significance of difference in allele frequencies of polymorphisms between pollinosis and healthy groups with high specific IgE values: * $P < .05$; between healthy subjects with normal and high specific IgE values: † $P < .05$, ‡ $P < .01$.

TABLE IV. Comparisons of genotype of Pro358Leu (EPO), Ile50Val (IL-4RA), and Glu375Ala (IL-4RA) of pollinosis between early and late onset of symptoms

Onset	Pro358Leu (EPO)			Ile50Val (IL-4RA)			Glu375Ala (IL-4RA)		
	Pro/Pro	Pro/Leu	Leu/Leu	Ile/Ile	Ile/Val	Val/Val	Glu/Glu	Glu/Ala	Ala/Ala
Early	47	10	0	26	26	5	49	8	0
Late	56	2	0	44	7	7	56	2	0
Allele frequency (%) of polymorphism									
Early		8.77			31.6			7.02	
Late		1.72**			18.1*			1.72*	

*Statistical significant at $P < .05$.

Many researchers have tried to identify variants of IL-4RA genes and have examined their associations with asthma or atopy.²⁶ Systematic research has demonstrated polymorphic variants in the coding region in IL-4RA, including 7 variants resulting in amino acid substitution. Ile50Val,²⁷ Ser478Pro,²⁸ and Gln551Arg²⁶ have been reported to be involved in the risk for hyper-IgE syndrome, atopic dermatitis, and the asthma phenotype. Izuhara et al¹⁰ have demonstrated a functional relationship between Ile50Val and signal transduction in experiments using transfectants that expressed IL-4A bearing either Ile50 or Val50, showing that germline ϵ -transcription activity and Stat6 activity were upregulated more greatly by the Ile50 variant compared with Val50. On the other hand, some reports have found no relationship between the polymorphisms of Ile50Val or Ala375Glu and atopic dermatitis or asthma.¹²⁻¹⁵ Caggana et al²⁹ have demonstrated that Arg551 and Ile50 alleles are most frequently found in atopy or atopic asthma in blacks, and in whites, respectively, suggesting the importance of determining the frequency of single-nucleotide polymorphisms in different populations before drawing conclusions from allele-association studies, since allele frequencies might differ between populations. Forrest et al³⁰ have demonstrated no involvement of IL-4RA genes in early-onset atopic eczema, suggesting no agreement regarding a candidate gene for allergy because of heterogeneity, including factors such as the early age of onset in the cohort. Therefore, we observed not only different allele frequencies of Ile50Val and Glu375Ala between

healthy subjects and those with pollinosis but also in patients with late onset with Ile50 or Glu375. Late onset suggests that subjects with Ile50 or Glu375 develop cedar pollinosis with increased exposure to cedar pollen. Such a relationship between exposure to cedar and genes seems to be a typical case of the interaction of genetics and environment. Taken together, the contribution of Ile50 or Glu375 in IL-4RA to the disease might be specific to cedar pollinosis in Japanese populations.

The involvement of Pro358Leu in EPO in cedar pollinosis seems to differ from that of Ile50Val and Glu375Ala in IL-4RA, because of the relationship between the mutant prevalence and specific IgE value. Some people sensitized to cedar pollen, showing a high cedar-pollen-specific IgE value in the blood, do not develop symptoms of allergic rhinitis.³¹ Ober and colleagues³² conducted a thorough analysis of all variants in the coding region of the IL-4RA gene in Hutterites and in selected outbred white, black, and Hispanic families, recognizing a strong association between 406Cys-478Ser and asthma. Our results showing no subjects with pollinosis and a normal, specific IgE value support the concept that a higher specific IgE value is necessary but not sufficient for the subsequent development of pollinosis. Based on these facts, our criteria for diagnosis of the disease did not include a specific IgE value but a provocation test, including nasal conjunctival symptoms. It is noteworthy that almost all subjects with the 358Leu allele in EPO, when they had a higher specific IgE value, developed cedar pollinosis, whereas only a few subjects with a higher specific IgE

value whose alleles were Ile50 or Glu375 did not develop the disease. These results suggest that Pro358Leu in EPO is strongly involved in the development of the disease. Ile50Val and Glu375Ala in IL-4RA seem to be related to cedar pollen sensitization. Therefore, the contrast between the involvement of the 2 genes, EPO and IL-4RA, in cedar pollinosis could be a model for atopic diseases, including asthma and atopic dermatitis, in the interaction of genetics and environment.

In conclusion, our results suggest that Pro358Leu in EPO is significantly involved in the development of cedar pollinosis. Ile50Val and Glu375Ala in IL-4RA seem to be related to cedar pollen sensitization. Subjects with Ile50 or Glu375 might develop cedar pollinosis with increased exposure to cedar pollen.

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