

Association of Single Nucleotide Polymorphisms in the Eosinophil Peroxidase Gene with Japanese Cedar Pollinosis

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Key Words

Cedar pollinosis · Eosinophil peroxidase · Polymorphisms · Transmission disequilibrium test

Abstract

Background: Japanese cedar pollinosis is the most common form of hay fever in spring in Japan. We have previously demonstrated that single nucleotide polymorphism Pro358Leu of exon 7 in the eosinophil peroxidase (EPO) gene is associated with cedar pollinosis, although the association has not been confirmed by analysis of the whole gene in a different population. **Methods:** We sequenced all exons of the EPO gene in 60 children with pollinosis and their parents using the PCR-restriction fragment length polymorphism method. **Results:** We found 8 polymorphisms, Ile40Met, Gln122His, Arg202Arg (A660G), Asn303Asn (C909T), Arg326Pro, Arg326His, Pro358Leu, and Asn572Ty, in the EPO gene. As a result of the transmission disequilibrium test, we recognized significant transmissions of 202Arg (660G) in

exon 6 in addition to 358Leu of exon 7 in the EPO gene of affected children. **Conclusions:** Our results might indicate that polymorphisms of the EPO gene are associated with Japanese cedar pollinosis.

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Japanese cedar pollinosis is the most common hay fever in spring and is a very serious medical problem because its prevalence has increased in recent years [1, 2]. Cross-sectional and ecological studies suggest that environmental factors such as NO_x or suspended particular matter and lifestyle including smoking are associated with the development of the disease [3–5]. However, it is generally accepted that the most established risk factor for Japanese cedar pollinosis is a family history, indicating that genetic factors strongly influence cedar pollinosis [6, 7]. Using an association study we have previously demonstrated that a polymorphism at amino acid 358 in exon 7 of the eosinophil peroxidase (EPO) gene as substitutions of Pro into Leu (Pro358Leu) as well as the interleukin-4

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receptor α chain (IL4RA) gene are associated with Japanese cedar pollinosis [8]. Although the involvement of polymorphisms of the IL4RA gene in allergic diseases such as atopic dermatitis and asthma is well known, that of EPO had not been pointed out until our study. A problem accompanying an association study used in our previous method might include a structured population [9]. Furthermore, as we have not examined the association between cedar pollinosis and markers other than exon 7 in the EPO gene, neighbor loci of the exon 7 in EPO might be responsible for the development of cedar pollinosis. Therefore, the association between EPO and cedar pollinosis must be confirmed by an analysis of the whole gene in a different population with the methods compensating for the defect of the association study, e.g., a transmission disequilibrium test (TDT) [10].

We recruited 432 subjects in Makioka-cho, a countryside area of Yamanashi Prefecture, west of Metropolitan Tokyo, who voluntarily underwent an examination for the diagnosis of the cedar pollinosis and to the study its etiology. All subjects received a self-administered questionnaire regarding their history of allergic diseases, i.e., cedar pollinosis, asthma, and atopic dermatitis as well as nasal and conjunctival symptoms, sneezing, nasal discharge, itching of nasal mucosa or conjunctiva, 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 a history of cedar pollinosis. The criteria for a diagnosis of cedar pollinosis were as follows: (1) under drug therapy or immunotherapy for cedar pollinosis, and (2) at least one positive IDT or NPT in subjects with symptoms. Sixty children under 16 years of age, who were diagnosed as having cedar pollinosis according to the criteria, and their 80 parents, including 48 patients and 32 healthy subjects, constituted the subjects of this study. As 40 of the 60 affected children were siblings, our subjects included 40 families. The average age $\pm$ standard deviation for 60 children and their 80 patients were 10.4 ± 3.20 and 38.8 ± 3.42 years, respectively. All affected children and patients had 2 CAP radioallergosorbent test scores of 0.70 (U/ml) or higher as an indicator of the specific IgE antibody level to pollens of Japanese cedar. All subjects were fully informed of the protocol and had given their informed consent before the experiment. This study was approved by the Ethics Committee on Experimentation of Kanazawa University, Takara-machi Campus.

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, Kyoto, 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 Ex Taq, 2 μ l MgCl₂ and 1 μ l specific primer pairs. The specific primer pairs used were as follows: EPO exon 1–3, forward 5'-CTG GAG GAA GTG AGA GGT CGG-3' and reverse 5'-TCT CCT CCC AGC ACC CCA A-3'; EPO exon 4–5, forward 5'-TTG GGG TGC TGG GAG GAG A-3' and reverse 5'-TGC GTG ACT CTG GGC TGT G-3'; EPO exon 6, forward 5'-GGA ATG AGA GCA GGA GGT GGC-3' and reverse 5'-ACC TCT CAA ACA CAC ACG GCG-3'; EPO exon 7, forward 5'-CTG GGT GAG TCA AGG AGG GC-3' and reverse 5'-CAC ATC CCA CCA TTG CTT CCC-3'; EPO exon 8–9, forward 5'-GGA GGG TGG CGA TGA GGA G-3' and reverse 5'-CTG GGG AGA TAT TAG CAG TGG C-3'; EPO exon 10–11, forward 5'-GGG TAC TGT CTT ACT TGT GTC T-3' and reverse 5'-CCT GGG TCA GTG TCT CTC TTC C-3'. To detect polymorphisms, direct DNA sequencing of PCR products for 50 unrelated patients with cedar pollinosis was performed on an ABI Prism 377 Genetic Analyzer (Perkin Elmer, Wellesley, Mass., USA) using the ABI Prism Big Dye Terminator DNA sequencing kit (Perkin Elmer). We found 8 polymorphisms of Ile40Met in exon 2, Gln122His in exon 4, Arg202Arg (A660G) in exon 6, Asn303Asn (C909T), Arg326Pro, Arg326His, and Pro358Leu in exon 7, and Asn572Ty in exon 11. The PCR-restriction fragment length polymorphism method was used to determine polymorphisms of Ile40Met, Gln122His, and Arg202Arg (A660G). The specific primer pairs and restriction enzymes used were as follows: Ile40Met, forward 5'-C CTC GGT CCT GCG AGA CTC CAT-3' and reverse 5'-GAC CCA AGT CCA CCT CTT CTG-3', Nco I; Gln122His, forward 5'-TTG GGG TGC TGG GAG GAG A-3' and reverse 5'-TGC GTG ACT CTG GGC TGT G-3', Pvu II; Arg202Arg (A660G), forward 5'-GGA ATG AGA GCA GGA GGT GGC-3' and reverse 5'-ACC TCT CAA ACA CAC ACG GCG-3', Apa I. For polymorphisms of exon 7–11, direct DNA sequencing was performed in the manner described above. The PCR protocol consisted of a pre-PCR heat activation step followed by denaturation, and ethidium bromide staining. PCR products were purified by use of Microcon (Millipore, Bedford, Mass., USA). TDT was analyzed using Genehunter (version 2.1). The calculations of linkage disequilibrium and Hardy-Weinberg equilibrium were computed by employing the Arlequin software package (version 2.0), which implements the EM algorithm to estimate maximum likelihood haplotype frequencies.

The allele frequencies for 140 individuals for 40Met in exon 2, 122His in exon 4, 202Arg (660G) in exon 6, 303Asn (909T), 326Pro, 326His, and 358Leu in exon 7, and 572Ty in exon 11 were 5.00, 6.79, 22.5, 0.714, 0.714, 5.36, and 4.29%, respectively. As a result of the TDT for Ile40Met, Gln122His, Arg202Arg (A660G), Asn303Asn (C909T), Arg326Pro, Arg326His, Pro358Leu, and Asn572Ty, we found significant transmissions of 202Arg (660G) in exon 6 in addition to 358Leu in exon 7 of the EPO gene to affected children (table 1). A significant linkage disequilibrium was observed between Arg202Arg (A660G) and Pro358Leu polymorphisms ($D = 0.011$, $D' = 0.29$, $p = 0.013$). Allele frequencies of the Arg202Arg (A660G) or Pro358Leu did not deviate from expected Hardy-Weinberg equilibrium ($p = 0.14$ for Arg202Arg (A660G); $p = 0.56$ for Pro358Leu).

We do not know candidate genes of cedar pollinosis excluding a coding variant of the FcεRIβ gene Glu237Gly [11] as well as EPO and IL4RA, the involvement of which we have previously demonstrated [8].

Eosinophil granulocytes are important effector cells in allergic inflammatory disorders. During the allergic response eosinophils are present at the site of inflammation, where they produce a variety of inflammatory mediators and reactive oxidant species, generate free radicals and release toxic granules including EPO upon activation [12–14]. 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 [15, 16]. We have previously demonstrated a strong association between cedar pollinosis and Pro358Leu in EPO, the region encompassing which is well conserved among other peroxidases such as neutrophil myeloperoxidase, lactoperoxidase and thyroid peroxidase [15, 16] in humans as well as in porcine. This study confirmed the asso-

Table 1. Transmission disequilibrium test for EPO gene polymorphisms in 40 families including 60 affected children with Japanese cedar pollinosis

	Allele	Transmitted	Not transmitted	p value
Exon 2	40Met	5	6	1
Exon 4	122His	8	4	0.39
Exon 6	202Arg (A660G)	24	9	0.015
Exon 7	303Asn (C909T)	1	0	1
Exon 7	326Pro	1	0	1
Exon 7	326His	1	0	1
Exon 7	358Leu	8	1	0.046
Exon 11	572Tyr	5	2	0.45

ciation between cedar pollinosis and the exons 6–7 region including Arg202Arg (A660G) and Pro358Leu in EPO. As a pathogenesis of asthma, EPO is released into the bronchial space after the allergen challenge, resulting in increased permeability and damage of bronchial epithelial cells [17]. The missense mutation Pro358Leu polymorphism in EPO is assumed to be involved in the hyperactivity of EPO, leading to increased epithelial permeability. Our result showing an association between silent mutation Arg202Arg (A660G) and cedar pollinosis may be due to linkage disequilibrium between exons 6 and 7 in EPO. These assumptions should be confirmed by further function analysis of variant of EPO gene.

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