

Polymorphisms in *ADAM33* are associated with allergic rhinitis due to Japanese cedar pollen

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Summary

Background A recent report provided evidence that a disintegrin and metalloprotease domain 33 (*ADAM33*), a member of the ADAM family, is a novel susceptibility gene in asthma linked to bronchial hyper-responsiveness. However, there has been no investigation of the genetic role of *ADAM33* variants in nasal allergy.

Objective The purpose of this study was to test the association between *ADAM33* polymorphisms and Japanese cedar pollinosis (JCPsis), a most common seasonal allergic rhinitis in Japan.

Methods We conducted a case-control association study among a Japanese population, involving 95 adult individuals with JCPsis and 95 normal healthy controls. A total of 22 single-nucleotide polymorphisms (SNPs) in *ADAM33* were genotyped using PCR-based molecular methods.

Results Six SNPs of *ADAM33* gene, three in introns (7575G/A, 9073G/A and 12540C/T) and three in the coding region (10918G/C, 12433T/C and 12462C/T), were strongly associated with JCPsis ($P = 0.0002 - 0.022$ for absolute allele frequencies) and most of the SNPs were in linkage disequilibrium with each other. A higher frequency of the common alleles of these SNPs was noted for the subjects with JCPsis in comparison with healthy controls. We also identified a haplotype associated with the disease susceptibility. In addition, associations were found between *ADAM33* polymorphisms and various cedar pollinosis phenotypes including clinical severity, eosinophil counts in nasal secretion and allergen-specific IgE levels in sera, but not total serum IgE levels.

Conclusion These results indicate that polymorphisms in the *ADAM33* gene are associated with susceptibility to allergic rhinitis due to Japanese cedar pollen, but the functional relationship still needs clarification.

Keywords *ADAM33*, allergic rhinitis, Japanese cedar pollinosis, single-nucleotide polymorphism, susceptibility gene

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Introduction

Japanese cedar pollinosis (JCPsis) is an IgE-mediated type I allergy affecting the nasal mucosa caused by exposure to Japanese cedar (*Cryptomeria japonica*) pollen (JCP). It is one of the most common allergic diseases in Japan and represents a public health issue affecting 5–20% of the Japanese population, with increasing prevalence over the past two to three decades [1–3]. JCPsis is known to be a complex disease with genetic predisposition [4], although the underlying genes

have not been pinpointed. Several previous studies, focused on the relationship between HLA class II molecules and JCPsis, showed antigen-specific T cell responsiveness to the major allergens of JCP was strikingly associated with certain specific *HLA-D* alleles [5–7]. Recently, a significant association has been found between a coding variant (Glu237Gly) of the β chain of the high-affinity IgE receptor (Fc ϵ RI β) and JCPsis, as well as serum total and specific IgE levels [8], suggesting a strong genetic component to the onset and disease phenotype of JCPsis.

Allergic rhinitis (AR) and asthma may both be manifestations of the atopic syndrome, affecting different anatomical parts of the respiratory tract, which share a common genetic background [9], and are characterized by a similar inflammatory process [10, 11]. Significant progress has been made in

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the study of genetics of atopy during the past decade since Cookson et al. [12], in 1989, first reported a linkage between generalized IgE responsiveness and a variable number of tandem repeats polymorphism on chromosome 11q13 in British families. Nevertheless, intensive research on the complex genetics of allergic disease has focused primarily on asthma and related traits, with very little attention given to rhinitis [9, 13]. Recent information regarding asthma genetics comes from the work of Van Eerdewegh et al. [14]. These investigators performed a genetic study by combining linkage and association approaches in Caucasian affected sib-pair families from the UK and the US, and identified a disintegrin and metalloprotease domain 33 (*ADAM33*) as a major susceptibility gene for asthma linked to bronchial hyper-responsiveness (BHR). Most recently, the association of *ADAM33* polymorphisms with asthma and related phenotypes has been replicated in four unique asthma populations (African American, US white, US Hispanic and Dutch white) [15] and in the German samples [16], but not in the Latino populations (Puerto Rican and Mexican) [17].

We hypothesized that association of the *ADAM33* gene with asthma may not be specific for asthma *per se* but rather may reflect an overall genetic predisposition for allergic airway diseases. To clarify whether the *ADAM33* polymorphisms relate to JCPsis, a most important springtime AR in Japan, we genotyped and analysed a total of 22 single-nucleotide polymorphisms (SNPs) within the *ADAM33* locus in a case-control association study.

Materials and methods

Subjects

The study included 95 unrelated adult individuals with JCPsis and 95 age-matched unrelated healthy controls. All subjects were from the population of Kinki area, west Japan. The diagnosis of JCPsis was based on a positive history of rhinitis symptoms during the cedar pollen season, and positive allergen-specific IgE reactivity to JCP in serum. Exclusion criteria were a present or past history of asthma and asthma-like symptoms. Among a total of 95 patients with JCPsis, 45.3% were also allergic to house dust mites (HDM), and 18.9% of patients had perennial symptoms with seasonal exacerbations. None of the patients had received specific immunotherapy for allergic conditions. The control subjects were all symptom free, had no history of atopic disorders, and had negative allergen-specific IgE against JCP as well as HDM. All participants gave written informed consent to participate in the study, according to the process approved by the Ethical Committee in RIKEN Yokohama Institute.

Clinical parameters

Detailed characteristics of recruited patients are described in Table 1. Among a total of 95 patients with JCPsis, five cases (5.3%) were mild, 22 cases (23.2%) were moderate and 68 cases (71.6%) were diagnosed as severe according to the scores of three main nasal symptoms (sneezing, rhinorrhoea and nasal obstruction), on the basis of the clinical severity

Table 1. Phenotypic characteristics of recruited patients

Number of subjects	95
Clinical severity	
Mild	5 (5.3%)
Moderate	22 (23.2%)
Severe	68 (71.6%)
Total serum IgE (IU/mL)	
Geometric mean	162.5
Ranges	5.3–10 000
RAST scores for Japanese cedar pollen	
Score 2	9 (9.5%)
Score 3	42 (44.2%)
Score 4	25 (26.3%)
Score 5	16 (16.8%)
Score 6	3 (3.2%)
RAST scores for house dust mites	
Score ≥ 2	43 (45.3%)
Eosinophil counts in nasal secretions	
None	13 (13.7%)
1+	36 (37.9%)
2+	16 (16.8%)
3+	30 (31.6%)

classification for AR (Okuda's method) described in detail elsewhere [18, 19].

Serum total and allergen-specific IgE levels were measured for all participants with an AutoCAP System (Pharmacia Diagnostics AB, Uppsala, Sweden). The geometric mean (range) of total IgE levels in patients and control subjects was 162.5 (5.3–10 000) IU/mL and 30.0 (3.2–240) IU/mL, respectively. Subjects with JCPsis had higher total IgE levels than healthy controls (2.21 ± 0.51 (mean $\pm$ SD) log IU/mL vs. 1.48 ± 0.51 , $P < 0.001$, *t*-test). Allergen-specific IgE levels were determined by RAST. The grading of RAST scores 0–6 was made according to the manufacturer's instructions, with a specific IgE level of 0.7 UA/mL (RAST score 2 or more) being positive. In addition, to evaluate the nasal eosinophilia in patients with JCPsis, eosinophils in nasal secretion (nEOS) obtained from nose blowing were stained by Hansel staining and semi-quantitated ranging from 0 to 3+ (0: none; 1+: scattered; 2+: between 1+ and 3+; and 3+: clustered) [18, 19].

SNPs

We screened a total of 22 SNPs at the human *ADAM33* locus as the target polymorphisms for a case-control association study. The SNPs studied were based on the database of SNPs in Japanese population (JSNP, data are available through website at <http://snp.ims.u-tokyo.ac.jp/>) [20, 21], and our own data for SNP discovery (data not shown). The 22 SNPs are shown in Table 2, five SNPs (–3989T/C, –3982C/T, –3835C/T, –3705C/A and –85T/C) are in the 5' genomic region, 10 SNPs (6716G/C, 7575G/A, 7667A/G, 9073G/A, 11188A/T, 11434C/A, 12540C/T, 12601T/G, 12946G/A and 12954C/T) are in introns, four SNPs (7441A/G, 10918G/C, 12433T/C and 12462C/T) are in the coding region and the remaining three SNPs (13236T/C, 13506C/G and 13527A/G) belong to the 3' untranslated region (3'UTR) of exon 22.

Table 2. Overview of the 22 SNPs of *ADAM33* gene on chromosome 20p13 that were genotyped in our case-control association study

SNP No.	Variation*	Location	SNP name in ref. [14]	Forward (f) and reverse (r) primers for PCR†	Annealing temp. (°C)	Genotyping methods
01	– 3989 T/C	5' genomic		5'-CCTCTAAATGCACCAGGCTG-3' (f) 5'-AGTGTGGATGTCAGGTACAG-3' (r)	60	Direct sequencing
02	– 3982 C/T	5' genomic		5'-CCTCTAAATGCACCAGGCTG-3' (f) 5'-AGTGTGGATGTCAGGTACAG-3' (r)	60	Direct sequencing
03	– 3835 C/T	5' genomic		5'-CCTCTAAATGCACCAGGCTG-3' (f) 5'-AGTGTGGATGTCAGGTACAG-3' (r)	60	Direct sequencing
04	– 3705 C/A	5' genomic		5'-CCTCTAAATGCACCAGGCTG-3' (f) 5'-AGTGTGGATGTCAGGTACAG-3' (r)	60	Direct sequencing
05	– 85 T/C	5' genomic		5'-CCAGGAGACCACGGAAGATC-3' (f) 5'-AAGCTGGTTGTGAGAGCGAG-3' (r)	60	Direct sequencing
06	6716 G/C	Intron 3	D-1	5'-TTTTCTCCACAATAAGCCATCACCA TG-3' (f) 5'-TTCCTCCCTAAATGCTAATGG-3' (r)	58	RFLP, <i>Eco</i> T14 I
07	7441 A/G (Thr178Ala)	Exon 6	F1	5'-TTCTGATCTGAGCAAGTCACC-3' (f) 5'-TTGGAGGGCTATAGATACAGC-3' (r)	60	Direct sequencing
08	7575 G/A	Intron 6	F+1	5'-GGGGAGCCCTCCAAATCAGAAGAGCC-3' (f) 5'-AGTGGAAAGCTGCTGGGCTT-3' (r)	58	RFLP, <i>Msp</i> I
09	7667 A/G	Intron 6	G-1	5'-GCTGTATCTATAGCCCTCCA-3' (f) 5'-AAGGGCTTGAGCCCTGACCA TTAA-3' (r)	58	RFLP, <i>Psh</i> B I
10	9073 G/A	Intron 11	L-1	5'-CAAGGGTGCTCGTGTCTC-3' (f) 5'-GCTCAGGCAAAGTGATTCTC-3' (r)	60	Invader assay
11	10918 G/C (Gly717Gly)	Exon 19	S2	5'-GGAACCGCAGGAGTAGGCTC-3' (f) 5'-GGAAGAATGCCTTCCAGGAG-3' (r)	60	Invader assay
12	11188 A/T	Intron 19	S+1	5'-CTTCTCAGTGGCCTCCAGTCAA TCG-3' (f) 5'-AGGGTGAGGCAGCCAGCT-3' (r)	58	RFLP, <i>Taq</i> I
13	11434 C/A	Intron 19	ST+4	5'-TCGCCCTGTTTTCCCTGG-3' (f) 5'-AAGGCCCAGCATTTGGGAAC TCA TG-3' (r)	55	RFLP, <i>Eco</i> T14 I
14	12433 T/C (Met764Thr)	Exon 20	T1	5'-TCAAGTTCCTGGAGTGGCTG-3' (f) 5'-GGTCTTTGGAAGCTGAGCG-3' (r)	60	Invader assay
15	12462 C/T (Pro774Ser)	Exon 20	T2	5'-TGGACTCTTATCACGTTGCTC-3' (f) 5'-GGGAAGAACTTCCAAGCTGC-3' (r)	60	Direct sequencing
16	12540 C/T	Intron 20	T+1	5'-TCAAGTTCCTGGAGTGGCTG-3' (f) 5'-GGTCTTTGGAAGCTGAGCG-3' (r)	60	Invader assay
17	12601 T/G	Intron 20	T+2	5'-TGGGTGAGTGAGGCACCA-3' (f) 5'-TTTACTTGTAATATGGTCAGCAGGCG-3' (r)	58	RFLP, <i>Hha</i> I
18	12946 G/A	Intron 21	V-3	5'-TGGACTCTTATCACGTTGCTC-3' (f) 5'-GGGAAGAACTTCCAAGCTGC-3' (r)	60	Direct sequencing
19	12954 C/T	Intron 21	V-2	5'-TGGACTCTTATCACGTTGCTC-3' (f) 5'-GGGAAGAACTTCCAAGCTGC-3' (r)	60	Direct sequencing
20	13236 T/C	3' UTR	V3	5'-GCTCCAGAACCTCAGGTGTAGC-3' (f) 5'-CTGACAGCCACTCCAGGAAC-3' (r)	60	Invader assay
21	13506 C/G	3' UTR	V4	5'-GCTCCAGAACCTCAGGTGTAGC-3' (f) 5'-CTGACAGCCACTCCAGGAAC-3' (r)	60	Invader assay
22	13527 A/G	3' UTR	V5	5'-GCTCCAGAACCTCAGGTGTAGC-3' (f) 5'-CTGACAGCCACTCCAGGAAC-3' (r)	60	Invader assay

*The position of each variation is given in base pairs according to the GenBank accession numbers AF466287 for *ADAM33* cDNA, and AP002898 for *ADAM33* gene containing clone. Nucleotide numbering starts from the 'A' of the initiation codon. †Italic nucleotide was exchanged to incorporate the polymorphic site, and underlined sequence was added as an elongation of PCR products.

UTR, untranslated region; *ADAM33*, a disintegrin and metalloprotease domain 33; SNPs, single-nucleotide polymorphisms.

Using genomic DNA extracted from whole peripheral blood of study subjects by standard methods, we genotyped all 22 SNPs with one of the following three PCR-based methods: Invader assay [22, 23], restriction fragment length polymorphism (RFLP), or direct sequencing.

Invader assay

To simplify the SNP-typing procedure, a panel of seven SNPs (see Table 2) was genotyped with multiplex PCR-Invader

assay, as described in detail elsewhere [23]. In brief, we amplified 5 ng of genomic DNA with PCR primers designed to include one or more SNPs (sequences of the primers are listed in Table 2). Invader assay was then performed through use of multiplex PCR products as template. Third Wave Technologies (Madison, WI, USA) designed probe sets for each locus. Genotyping was performed on an ABI PRISM 7700 Sequence Detection System and attached analysis software SDS 2.0 (Applied Biosystems, Foster City, CA, USA).

PCR-RFLP

PCR-RFLP analysis was used to genotype six SNPs (see Table 2). Briefly, genomic DNA was amplified with PCR primers (for primer sequences see Table 2). PCR fragment generated was then digested overnight with a site-specific restriction endonuclease (see Table 2) according to the manufacturer's recommendation. The digestion products were visualized on a 4% agarose gel stained with ethidium bromide.

Direct sequencing

Remaining nine SNPs, as shown in Table 2, were subsequently determined directly on a capillary-based sequencer, using BigDye-terminator cycle sequencing according to standard procedures. In brief, sequencing reaction was accomplished with PCR products, BigDye Terminator v.2 (Applied Biosystems), and one unlabelled primer either reverse (5'-CGAAGTCTCTGAGAAAGGGT-3' for four SNPs of -3989T/C, -3982C/T, -3835C/T and -3705C/A, and 5'-ATGCCCTCTGCTTTGCCAGC-3' for -85T/C) or forward (5'-TGGGATGAGGTGAGCTCT-3' for 7441A/G, 5'-TGGACTCTTATCACGTTGCTC-3' for 12462C/T, and 5'-CCTGAGAACTCTCATGAG-3' for both 12946G/A and 12954C/T). Following refinement of the reaction products by ethanol, the purified products were sequenced on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems). Genotypes were identified by comparing the peak levels of signals within the expected allele range for all lanes through use of a computer programme SEQUENCHER 3.1.1 (Gene Codes Corporation, Ann Arbor, MI, USA).

Statistics

Statistical analyses were performed using a SPSS 10.0J for Windows (SPSS Inc., Chicago, IL, USA). The Hardy-Weinberg equilibrium of genotype distribution for each of 22 SNPs in *ADAM33* gene was assessed by χ^2 test. Genotype and allele frequencies in cases and control subjects were compared using Pearson's χ^2 test. If an expected number was less than 5, Fisher's exact test was then used. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated as measures of the association of *ADAM33* polymorphisms with JCPsis. Quantitative traits relating to clinical severity (severe vs. mild-to-moderate) and nEOS counts (positive vs. negative) were also analysed with χ^2 test. When genotypic frequencies were compared between different groups, a 2×2 table was performed. Association between total serum IgE levels and *ADAM33* genotypes was tested with ANOVA, *t*-test and general liner model (GLM). For these analyses, total IgE values were logarithm transformed to normalize the distribution. Non-parametric tests (Kruskal-Wallis test and Mann-Whitney test) were employed for an association analysis of *ADAM33* genotypes with actual values of specific IgE to JCP.

Pairwise linkage disequilibrium (LD) and haplotypes for *ADAM33* polymorphisms were evaluated using the programme SNPalyze 3.1 (DYNACOM Co. Ltd, Chiba, Japan), by the method described elsewhere [24]. Haplotype frequencies were estimated by the expectation-maximization algorithm. Significance levels were corrected for multiple comparison of the number of correlations by Bonferroni

adjustment. Two-tailed *P*-value of less than 0.05 was considered statistically significant.

Results

Association of *ADAM33* polymorphisms with JCPsis

Overall success rate for the SNP genotyping in the present study was 99.4%. All 22 SNPs investigated were in Hardy-Weinberg equilibrium ($P > 0.05$). Therefore, genotyping errors can most probably be excluded.

Among typed 22 SNPs of *ADAM33* gene, six of them had significant associations with JCPsis (Table 3). The statistical *P*-values were 0.0002–0.022 (OR = 4.81–1.66) for absolute allele frequencies. These SNPs were 7575G/A (SNP F+1 in ref. [14]) in intron 6, 9073G/A (SNP L-1) in intron 11, 10918G/C (SNP S2, Gly717Gly) in exon 19, 12433T/C (SNP T1, Met764Thr) and 12462C/T (SNP T2, Pro774Ser) in exon 20 and 12540C/T (SNP T+1) in intron 20. Furthermore, a comparison of allelic frequencies in these SNPs indicated that the frequencies of common alleles (7575G, 9073G, 10918G, 12433T, 12462C and 12540C) were significantly higher for the JCPsis-affected individuals than for the healthy control subjects ($P < 0.05$).

As shown in Table 3, we also evaluated the frequencies of diploid genotypes in cases and controls, and observed that five of the SNPs (9073G/A, 10918G/C, 12433T/C, 12462C/T and 12540C/T) were significantly associated with JCPsis ($P = 0.00004$ – 0.004 , OR = 6.71–2.39).

No significant association was detected ($P > 0.05$) in the analyses for other variants – a coding SNP (7441A/G, Thr178Ala), seven intronic SNPs (6716G/C, 7667A/G, 11188A/T, 11434C/A, 12601T/G, 12946G/A and 12954C/T), five 5' genomic SNPs (-3989T/C, -3982C/T, -3835C/T, -3705C/A and -85T/C) and three 3'UTR SNPs (13236T/C, 13506C/G and 13527A/G).

In addition, to clarify whether sensitization to HDM would affect the association between *ADAM33* polymorphisms and JCPsis, we analysed genotype and allele frequencies for each of the 22 SNPs in patients with or without positive allergen-specific IgE against HDM, and did not identify any significant correlation ($P > 0.05$). Namely, association of the *ADAM33* gene with JCPsis was independent of allergic sensitization to HDM in our study population.

Intragenic LD and haplotype frequencies in *ADAM33* gene

To investigate the pattern of LD in the *ADAM33* locus, pairwise LD was measured by $|D'|$ and r^2 among the 22 SNPs genotyped. These 22 SNPs were located in one extended block of LD and some were in tight LD (Fig. 1). However we found that six of the SNPs had significant associations with JCPsis, when pairwise LD was examined between these six SNPs, most of the SNPs were in LD with each other (Table 4). We further analysed the haplotype structure using four SNPs (7575G/A [SNP F+1], 9073G/A [SNP L-1], 10918G/C [SNP S2] and 12540C/T [SNP T+1]) and identified six common haplotypes covering more than 98% of the population in both case and control groups (Table 5). Estimated frequencies of the four-locus haplotypes were compared between cases and control subjects, and found the most common haplotype was 7575G, 9073G, 10918G and

Table 3. Association between *ADAM33* polymorphisms and Japanese cedar pollinosis

			Genotype frequency				Allele frequency			
SNP*	Location	n†	Genotype			P-value‡	OR (95% CI)¶	Allele	P-value§	OR (95% CI)¶
7575 G/A F+1	Intron 6		GG	GA	AA			A		
	Cases	95	0.516	0.432	0.053	0.072	1.70 (0.95–3.05)	0.268	0.022	1.66 (1.07–2.58)
	Controls	91	0.385	0.473	0.143			0.379		
9073 G/A L-1	Intron 11		GG	GA	AA			A		
	Cases	94	0.947	0.043	0.011	0.00004	6.71 (2.45–18.37)	0.032	0.0002	4.81 (1.93–11.98)
	Controls	95	0.726	0.274	0.000			0.137		
10918 G/C S2	Exon 19		GG	GC	CC			C		
	Cases	94	0.691	0.266	0.043	0.004	2.39 (1.32–4.33)	0.176	0.006	1.96 (1.21–3.20)
	Controls	95	0.484	0.442	0.074			0.295		
12433 T/C T1	Exon 20		TT	TC	CC			C		
	Cases	95	0.905	0.084	0.011	0.0003	4.20 (1.86–9.47)	0.053	0.001	3.24 (1.53–6.86)
	Controls	95	0.695	0.305	0.000			0.153		
12462 C/T T2	Exon 20		CC	CT	TT			T		
	Cases	95	0.895	0.095	0.011	0.00007	4.52 (2.08–9.87)	0.058	0.0003	3.55 (1.74–7.24)
	Controls	95	0.653	0.337	0.011			0.179		
12540 C/T T+1	Intron 20		CC	CT	TT			T		
	Cases	95	0.905	0.084	0.011	0.0005	3.99 (1.77–9.03)	0.053	0.002	3.11 (1.47–6.60)
	Controls	95	0.705	0.295	0.000			0.147		

*SNP with *P*-value for genotype or allele frequencies between groups less than 0.05 listed. SNP name in ref. [14] indicated in bold. †Number of valid subjects who were successfully genotyped for each of SNP. ‡Analysis performed by a 2×2 table for each SNP using major homozygotes vs. others in cases and controls. §Analysis performed by a 2×2 table for the number of each allele in cases and controls. ¶Reference group (controls) designated with an OR of 1.00. SNP, single-nucleotide polymorphism; *ADAM33*, a disintegrin and metalloprotease domain 33; CI, confidence interval; OR, odds ratio.

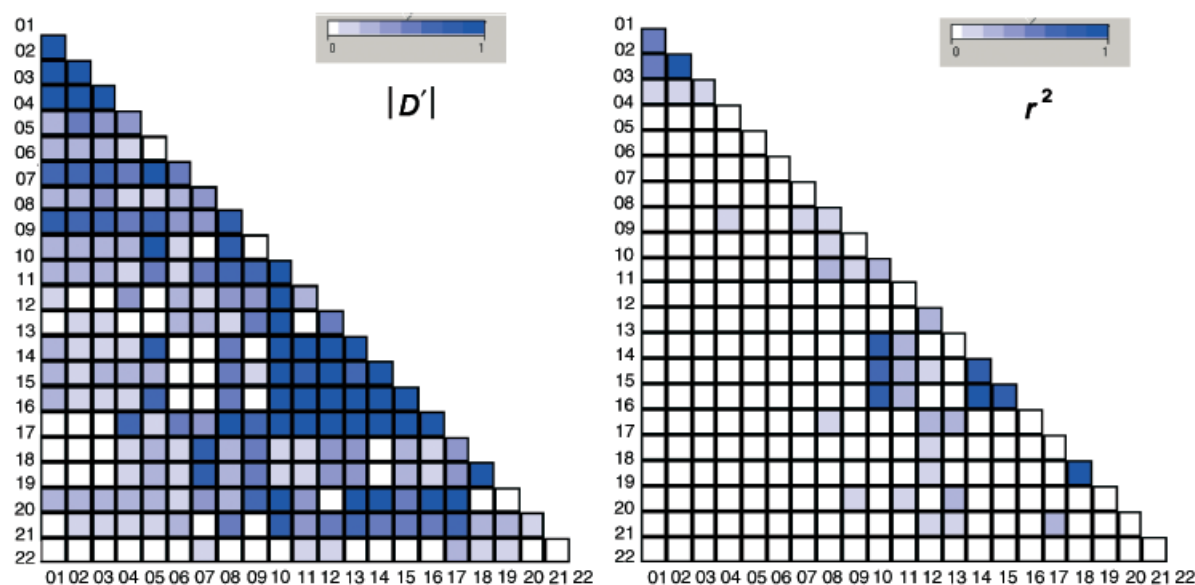


Fig. 1. Pairwise linkage disequilibrium in a disintegrin and metalloprotease domain 33 (*ADAM33*), as measured by D' -value (left panel) and r^2 (right panel) between all pairs of single-nucleotide polymorphisms (SNPs) examined. The physical locations of 22 SNPs refer to the SNP number (from 01 to 22) shown in Table 2.

12540C (GGGC haplotype) in both groups. This haplotype was more frequently observed in patients than in healthy controls (68.9% vs. 57.8%, $P = 0.027$, OR = 1.62; see Table 5); however, when Bonferroni's correction was applied to the result we found no significant difference between the groups ($P = 0.188$). In contrast, a tight association with JCPsis was

observed with AACT haplotype ($P = 0.0013$, OR = 4.19; see Table 5), and the association was also statistically significant after correcting for multiple testing ($P = 0.009$). In the control subjects, this haplotype had a frequency of 11.8%, whereas the frequency in the JCPsis-affected individuals was substantially lower, 3.1%.

Table 4. Pairwise linkage disequilibrium coefficients between the six SNPs of *ADAM33* gene

SNP*	7575 G/A, F+1	9073 G/A, L-1	10918 G/C, S2	12433 T/C, T1	12462 C/T, T2	12540 C/T, T+1
7575 G/A, F+1	–	0.126	0.348	0.083	0.102	0.078
9073 G/A, L-1	0.812	–	0.300	0.804	0.684	0.828
10918 G/C, S2	0.743	1.000	–	0.345	0.256	0.334
12433 T/C, T1	0.589	1.000	0.961	–	0.851	0.972
12462 C/T, T2	0.602	1.000	0.763	1.000	–	0.827
12540 C/T, T+1	0.579	1.000	0.960	1.000	1.000	–

*The disease-associated polymorphisms in this study are shown. SNP name in ref. [14] indicated in bold.
 D' -value (abs) is listed in lower left triangle, and r^2 in upper right.

SNP, single-nucleotide polymorphism; *ADAM33*, a disintegrin and metalloprotease domain 33.

Table 5. *ADAM33* haplotype profiles in our study population

Haplotype*	SNP position†				Haplotype frequency		<i>P</i> -value‡	OR (95% CI)§
	7575, F+1	9073, L-1	10918, S2	12540, T+1	Cases (<i>n</i> = 94)	Controls (<i>n</i> = 91)		
Haplotype 1	G	G	G	C	0.689	0.578	0.027	1.62 (1.06–2.48)
Haplotype 2	A	G	G	C	0.130	0.131	0.985	1.01 (0.55–1.84)
Haplotype 3	A	G	C	C	0.110	0.130	0.554	1.21 (0.64–2.26)
Haplotype 4	A	A	C	T	0.031	0.118	0.0013	4.19 (1.64–10.74)
Haplotype 5	G	G	C	C	0.018	0.013	0.698	1.40 (0.26–7.61)
Haplotype 6	G	G	C	T	0.016	0.011	0.687	1.45 (0.24–8.79)
Others					0.006	0.019	0.272	3.03 (0.38–24.28)

*Haplotype structure using four SNPs analysed. †SNP name in ref. [14] indicated in bold. ‡Analysis using a 2×2 table for each haplotype vs. all others combined in cases and controls. §Reference group designated with an OR of 1.00.

SNP, single-nucleotide polymorphism; *ADAM33*, a disintegrin and metalloprotease domain 33; OR, odds ratio; CI, confidence interval.

Association of *ADAM33* polymorphisms with cedar pollinosis-related phenotypes

Of the 22 SNPs examined, five of them showed statistically significant associations with cedar pollinosis closely related phenotypes including disease severity, nEOS counts and specific IgE to JCP (summarized in Table 6); however, no SNP was associated with two of these three phenotypes.

Ninety-five affected individuals were further stratified into two subgroups: those with mild-to-moderate JCPsis (*n* = 27) and those with severe JCPsis (*n* = 68). As shown in Table 6, the difference between two subgroups was significant in the analyses for genotype distribution of two intronic SNPs (P = 0.027, both). These two SNPs were 12946G/A (SNP V-3) and 12954C/T (SNP V-2) in intron 21, which were in complete LD with each other (see Fig. 1). A trend was observed for an association between the allele frequencies and severe JCPsis relative to mild-to-moderate JCPsis, but not at a statistically significant level (47.1% vs. 31.5%, P = 0.050). Analysis of other polymorphisms in *ADAM33* did not identify any significant correlation between clinical severity of JCPsis and genotype distribution, as well as allele frequencies (P > 0.05).

No significant difference was detected in total serum IgE levels (log IU/mL) between the genotypes for each of the 22 SNPs (using codominant, dominant and recessive models), either in patients with JCPsis or in healthy control subjects (P > 0.05). Moreover, we examined the genetic association between *ADAM33* polymorphisms and total IgE levels using a whole study population, while incorporating disease status (case or control) as a covariate in a GLM analysis, and found no evidence of significant association as well (P > 0.05).

However, we found that the genotype distributions for 11188A/T (SNP S+1) in intron 19 and 12601T/G (SNP T+2) in intron 20 were associated with the actual levels of allergen-specific IgE to JCP in sera from patients with JCPsis, but the significance was marginal (P = 0.027 and 0.045, respectively; see Table 6). No significant effects were demonstrated from analysis to determine the associations between any other SNPs of *ADAM33* and specific IgE values for JCP (P > 0.05).

We next investigated the impact of *ADAM33* variants on eosinophil counts in nasal secretion from JCPsis-affected individuals. A 5' genomic polymorphism in *ADAM33*, –3989T/C, showed a significant difference in either genotype or allele frequencies between nEOS-positive and -negative subjects (see Table 6). Using the TT homozygotes as a reference, we observed a significant association between nEOS counts (positive vs. negative) and genotype frequencies (P = 0.015). Comparison of allele frequencies showed that the C allele frequency was significantly higher in the subjects with positive nEOS than in those with negative nEOS (60.4% vs. 38.5%, P = 0.036). No significant association was obtained in the analyses regarding whether other polymorphisms of *ADAM33* were related to nEOS counts (P > 0.05).

Discussion

ADAM33, which maps to human chromosome 20p13 and consists of 22 exons [25], was recently identified to be a novel asthma susceptibility gene [14]. To our knowledge, this study represents the first evaluation of the *ADAM33* gene as a

Table 6. Association between *ADAM33* polymorphisms and cedar pollinosis related phenotypes

SNP*	Location	Clinical severity	Nasal eosinophilia	Specific IgE levels against Japanese cedar pollen
		Severe vs. mild-to-moderate	nEOS positive vs. negative	
– 3989 T/C	5' genomic		$P = 0.015^{\dagger}$; $P = 0.036^{\ddagger}$	
11188 A/T, S+1	Intron 19			$P = 0.027^{\S}$
12601 T/G, T+2	Intron 20			$P = 0.045^{\S}$; $P = 0.048^{\P}$
12946 G/A, V-3	Intron 21	$P = 0.027^{\dagger}$		
12954 C/T, V-2	Intron 21	$P = 0.027^{\dagger}$		

*SNP with *P*-value less than 0.05 listed. SNP name in ref. [14] indicated in bold. † Analysis for genotype frequencies by a 2×2 table using major homozygotes vs. others. ‡ Analysis for allele frequencies by a 2×2 table. § Comparison made by a Kruskal–Wallis test using codominant model. ¶ Comparison made by a Mann–Whitney test using major homozygotes vs. others.

nEOS positive, eosinophil counts in nasal secretion ranging from 1+ to 3+. nEOS negative, none of eosinophils counted in nasal secretion. *ADAM33*, a disintegrin and metalloprotease domain 33; SNP, single-nucleotide polymorphism.

susceptibility locus for nasal allergy. Using a case–control study, we have typed a total of 22 SNPs in *ADAM33* among a Japanese population living in the same area by using PCR-based molecular methods, and evaluated the genetic variants for evidence of association to JCPsis and related traits. The results showed that three intronic and three coding SNPs at the *ADAM33* locus were significantly associated with JCPsis ($P = 0.0002 - 0.022$, OR = 4.81 – 1.66 for allele frequencies). These SNPs were 7575G/A (SNP F+1) in intron 6, 9073G/A (SNP L–1) in intron 11, 10918G/C (SNP S2, Gly717Gly) in exon 19 encoding the transmembrane domain, 12433T/C (SNP T1, Met764Thr) and 12462C/T (SNP T2, Pro774Ser) in exon 20 encoding the cytoplasmic domain and 12540C/T (SNP T+1) in intron 20. Unexpectedly, we found significantly increased frequencies of the common alleles (7575G, 9073G, 10918G, 12433T, 12462C and 12540C) in patients with JCPsis compared with healthy control subjects.

Six of these JCPsis-associated SNPs (F+1, L–1, S2, T1, T2 and T+1) have been previously reported to be associated with asthma and BHR in Caucasian populations from the UK (SNPs F+1 and S2) or the US (SNPs L–1, T1, T2 and T+1), but not significant in both samples combined [14]. Associations with asthma were then replicated in the African American (SNP S2), US white (SNPs T1 and T2) and US Hispanic (SNPs S2 and T2) populations [15], and in a German family-based study sample (SNP F+1) [16]; however, no SNP was consistently associated across ethnically diverse groups. Additionally, we found no association between JCPsis and each of the two SNPs (11434C/A [SNP ST+4] in intron 19 and 13506C/G [SNP V4] in 3'UTR), which was described to be implicated in asthma susceptibility among the UK population and combined UK and US samples [14], the Dutch population (SNP V4 only) [15] and the German families (SNP ST+4 only) [16]. There were pronounced differences in allele frequencies for SNPs S2, ST+4, T1, T2 and V4 (which were tested in ethnically diverse groups) between our population and others [14–16]. The variability of genetic background (including LD pattern) and environmental exposure among the populations may account for the observed differences [26]. There is of course also the possibility that different SNPs of the *ADAM33* gene contribute to asthma or rhinitis susceptibility in specific ethnic populations.

Subsequent assessment of the pattern of LD between all pairs of SNPs showed that most of the disease-associated polymorphisms in this study were in LD with each other. We

further analysed the *ADAM33* haplotype structure and frequencies using four SNPs (7575G/A [SNP F+1], 9073G/A [SNP L–1], 10918G/C [SNP S2] and 12540C/T [SNP T+1]), and identified a haplotype (AACT haplotype) that accounted for JCPsis susceptibility risk. Because this haplotype is more frequently present in the healthy controls, it could be interpreted as a protective or resistant allele.

In addition to targeting disease status, as stated above, we observed weak correlations between five of non-coding SNPs in the *ADAM33* gene and JCPsis-related traits that included clinical severity, nasal eosinophilia, and specific IgE levels; however, no SNP was associated with two or more phenotypes. These five SNPs were – 3989T/C in 5' genomic region, 11188 A/T (SNP S+1) in intron 19, 12601 T/G (SNP T+2) in intron 20 and 12946 G/A (SNP V–3) and 12954 C/T (SNP V–2) in intron 21. None of the SNPs was overlapped with those found to be associated with susceptibility to JCPsis. Interestingly, five of these SNPs had not been identified as the risk for asthma and BHR in the original report of Van Eerdewegh et al. [14]. It is likely that different SNPs of *ADAM33* may be important in specific phenotypes.

In the present study, we found no evidence for association of SNPs in *ADAM33* with total serum IgE levels. Lack of association of *ADAM33* polymorphisms with total IgE was also reported in the Latino populations [17] but contradicted by the data in others [15, 16], suggesting an existence of race and ethnic difference.

One of the main finding from Van Eerdewegh et al. [14]; original analysis showed that the linkage of *ADAM33* gene to asthma was strongly correlated with BHR. This raises the possibility that asymptomatic BHR might be influencing the results of our study, because there is evidence that a proportion of subjects with pollinosis, even in the absence of asthma, also exhibit non-specific BHR during and out of the pollen seasons [27, 28]. However, some characteristics of aeroallergens, such as inhaled particles' size and allergenic properties, are likely to determine or influence the degree of BHR and the level of bronchial airways inflammation in subjects with AR [28, 29]. One carefully conducted study in Japanese young adult population revealed negative BHR (based on PC₂₀ values to methacholine) in non-asthmatic subjects with JCPsis [29]. Furthermore, among these subjects the airway inflammation markers (percentages of eosinophils, and levels of ECP and TNF- α) in induced sputum were below detection limits [29], indicating less inflammatory process in the lower airways in non-asthmatics with JCPsis. It therefore

seems less likely that the finding of our study concerning the association of *ADAM33* with JCPsis is attributable to the impact of BHR, although we did not test it in our subjects. In fact, recent study by Werner et al. [16] has shown that BHR was even absent in many asthmatics, and demonstrated that the *ADAM33* gene was associated with asthma susceptibility in the absence of BHR.

Taken together, despite the relatively small sample size in this study, our findings provided evidence for the *ADAM33* gene as an important determinant of JCPsis susceptibility, and the 3' portion of the gene that encodes the transmembrane and cytoplasmic domains may be the key region. However, without further functional analysis it is difficult to ascribe primacy to any individual SNP, since the extent of LD exists in this region [14].

ADAM33 is a newly discovered member of the ADAM protein family of zinc-dependent metalloproteinase superfamily and contains multiple domains including signal sequence, pro-domain, followed by catalytic, disintegrin, cysteine-rich, epidermal growth factor (EGF)-like, transmembrane and cytoplasmic domains [25]. ADAMs were originally identified as membrane-anchored proteins on the cell surface that mediate adhesion and proteolysis, and play pivotal roles in cell-to-cell interactions, cell signalling and the remodelling of extracellular matrix components [30]. The potential importance of ADAM33 in asthma pathogenesis is supported by the cellular-expression profile of *ADAM33* mRNA [14, 31], as well as protein [32], in human bronchial smooth muscle cells and lung fibroblasts. *ADAM33* polymorphisms may thus accelerate the proliferation of smooth muscle cells, fibroblasts and myofibroblasts and subsequent structural changes leading to airway remodelling in the asthmatic lung [14]. Also, *ADAM33* might affect asthma through playing a role in the alteration of growth factor expression, the regulation of cytokine shedding or a shift toward an immune response mediated by Th2 cells [33], although its function is at present unknown. This raises an intriguing question regarding similar potential mechanisms being implicated in nasal allergy, because the upper and lower airways show similar epithelial features and inflammatory reactions to irritants and allergens [10, 11].

Viewing the airways as a whole, from the nostrils to the alveoli, anatomic differences exist between the two ends of the respiratory tract, plentiful smooth muscle in the lower airways, but venous sinusoids and prominent glands in the nasal airways. Some studies show less tissue remodelling in the upper airways than in the lower airways of allergic individuals [34, 35]. However, allergic nasal mucosa also displays a variety of characteristic histopathological features including thickening and hyperplasia with polypoid degeneration of the epithelium, infiltration with eosinophilic, mononuclear and lymphoid cells; oedema and proliferation of the fibrous tissue with dilatation as well as thickening and compression of blood vessels in the lamina propria [36]. This points towards a potential underlying role for epithelium damage in contributing to nasal airways inflammation and remodelling responses by virtue of interaction of nasal mucosa with inhaled allergen in AR [37–39]. Interestingly, it is quite likely that the nasal mucosa has enhanced mechanisms for epithelial regeneration and repair through EGF receptor (EGFR) and its ligands, such as EGF, heparin-

binding EGF-like growth factor (HB-EGF) and TGF- α [40, 41].

Also, it is plausible that the epithelium damage and remodelling processes in AR might link to an imbalance between matrix metalloproteinases (MMPs) and their inhibitors, the specific tissue inhibitors of metalloproteinases (TIMPs) [42]. Several studies have shown that MMPs, especially MMP-9, may contribute to the pathogenesis of asthma via their influence on the function and migration of inflammatory cells, as well as matrix deposition and degradation [43]. In contrast, there are few data available on the role of MMPs in AR [42, 44] and other upper airway diseases characterized by chronic inflammation and tissue remodelling, such as nasal polyposis [45]. These limited studies have, however, shown the presence of MMPs and consequently the potential for epithelial damage and remodelling in the nasal airways. On the other hand, evidence reveals that TIMPs, a group of endogenous inhibitors of MMPs and ADAMs [46], are present in large amounts in the nasal mucosa and that they might suppress MMP-mediated damage in AR [42], suggesting the potential influence of MMP-TIMP interactions on the development of nasal allergy.

A recent study has demonstrated proteolytic activity of human ADAM33 catalytic protein, and found that the activity could be inhibited by TIMPs [47]. Importantly, ADAM33 belongs to a subfamily [25] where members are shown to be catalytically active. For example, ADAM12 sheds HB-EGF [48]; ADAM19 participates in the proteolytic processing of membrane-anchored neuregulin- β 1, a member of the EGFR family [49]; ADAM8 and ADAM15 catalyse ectodomain shedding of CD23, the low affinity IgE receptor [50]. It has been speculated that alteration in ADAM33 activity might underlie abnormalities in the regulation function for shedding of growth factors, cytokines and their receptors [33]. This raises the possibility that polymorphic variation in *ADAM33* might contribute to AR through influencing the course of epithelium inflammation, damage, repair and consequent tissue remodelling in the nasal airways.

In summary, we conclude that polymorphisms in the *ADAM33* gene are associated with susceptibility to AR due to JCP, but the functional relationship still needs clarification. Our case-control study has shown that some of the associated SNPs were splice variants located in non-coding sequences of introns, while, there were other disease-related SNPs that cause amino acid changes in the coding sequence; this highlights the potential for alterations in gene function and ADAM33 activity. Thus, further genetic and functional studies are needed to elucidate the role of *ADAM33* polymorphisms in the molecular mechanisms underlying JCPsis and nasal allergy.

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