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′-TTTTTCTCCACAACTAAGCCATCACCA*T*G-3′ (f) 5′-TTCCTCCCTAAATGCTAATGG-3′ (r)	58	RFLP, *EcoT14* 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′-GGGGAGCCCTCCAAATCAGAAGAG*C*C-3′ (f) 5′-AGTGGAAGCTGCTGGGCTT-3′ (r)	58	RFLP, *Msp* I
09	7667 A/G	Intron 6	G-1	5′-GCTGTATCTATAGCCCTCCA-3′ (f) 5′-AAGGGCTTGAGCCCTGACCA*TT*AA-3′ (r)	58	RFLP, *PshB* I
10	9073 G/A	Intron 11	L-1	5′-CAAGGGTGCTCGTGTCCTC-3′ (f) 5′-GCTCAGGCAAAGTGGATTCTC-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′-CTTCCTCAGTGGCCTCCCAGTCAA*T*CG-3′ (f) 5′-AGGGTGAGGCAGCCAGCT-3′ (r)	58	RFLP, *Taq* I
13	11434 C/A	Intron 19	ST+4	5′-TCGCCCTGTTTTTCCCTGG-3′ (f) 5′-AAGGCCCAGCATTTGGGAACT*CC*A*T*G-3′ (r)	55	RFLP, *EcoT14* I
14	12433 T/C (Met764Thr)	Exon 20	T1	5′-TCAAGTTCCTGGAGTGGCTG-3′ (f) 5′-GGTTCTTTGGAAGCTGAGCG-3′ (r)	60	Invader assay
15	12462 C/T (Pro774Ser)	Exon 20	T2	5′-TGGACTCTTATCACGTTGCTC-3′ (f) 5′-GGGAAGAAACTTCCAAGCTGC-3′ (r)	60	Direct sequencing
16	12540 C/T	Intron 20	T+1	5′-TCAAGTTCCTGGAGTGGCTG-3′ (f) 5′-GGTTCTTTGGAAGCTGAGCG-3′ (r)	60	Invader assay
17	12601 T/G	Intron 20	T+2	5′-TGGGTGAGTGAGGCACCA-3′ (f) 5′-TTTACTTGTGAATATGGTCAGCAGG*C*G-3′ (r)	58	RFLP, *Hha* I
18	12946 G/A	Intron 21	V-3	5′-TGGACTCTTATCACGTTGCTC-3′ (f) 5′-GGGAAGAAACTTCCAAGCTGC-3′ (r)	60	Direct sequencing
19	12954 C/T	Intron 21	V-2	5′-TGGACTCTTATCACGTTGCTC-3′ (f) 5′-GGGAAGAAACTTCCAAGCTGC-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

SNP*	Location	n†	Genotype frequency					Allele frequency		
			Genotype			P-value‡	OR (95% CI)¶	Allele	P-value§	OR (95% CI)¶
7575 G/A	Intron 6		GG	GA	AA			A		
F+1	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	Intron 11		GG	GA	AA			A		
L-1	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	Exon 19		GG	GC	CC			C		
S2	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	Exon 20		TT	TC	CC			C		
T1	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	Exon 20		CC	CT	TT			T		
T2	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	Intron 20		CC	CT	TT			T		
T+1	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.

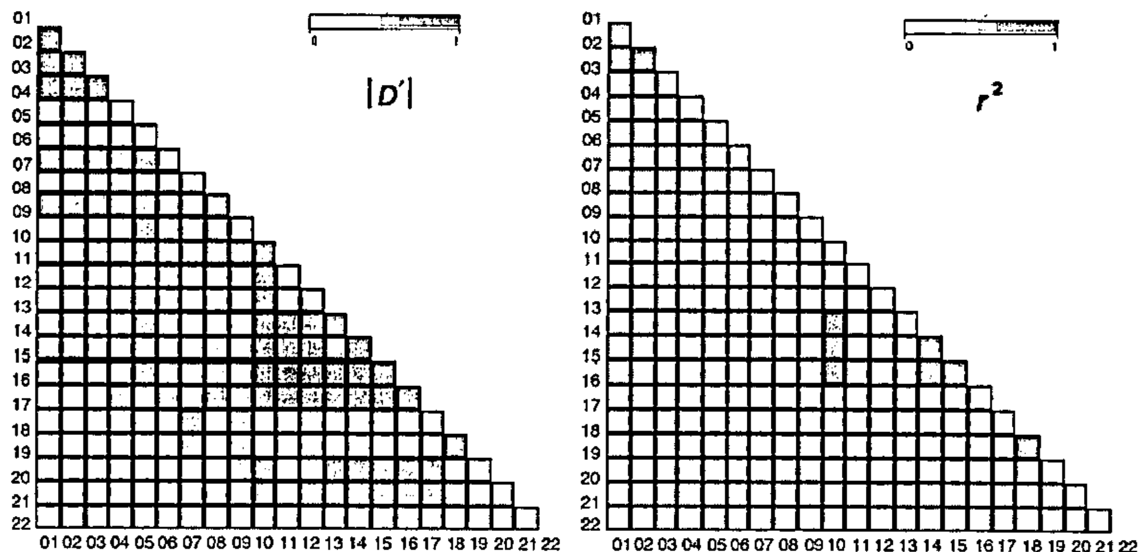

Fig. 1. Pairwise linkage disequilibrium in a disintegrin and metalloprotease domain 33 (*ADAM33*), as measured by D'-value (left panel) and r^2 (right panel) between all pairs of single-nucleotide polymorphisms (SNPs) examined. The physical locations of 22 SNPs refer to the SNP number (from 01 to 22)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

	SNP position†				Haplotype frequency			
Haplotype*	7575, **F+1**	9073, **L-1**	10918, **S2**	12540, **T+1**	Cases (n = 94)	Controls (n = 91)	*P*-value ‡	OR (95% CI)§
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 eosinophila	
		Severe vs. mild-to-moderate	nEOS positive vs. negative	Specific IgE levels against Japanese cedar pollen
− 3989 T/C	5′ genomic		$P = 0.015$†; $P = 0.036$‡	
11188 A/T, **S+1**	Intron 19			$P = 0.027$§
12601 T/G, **T+2**	Intron 20			$P = 0.045$§; $P = 0.048$¶
12946 G/A, **V-3**	Intron 21	$P = 0.027$†		
12954 C/T, **V-2**	Intron 21	$P = 0.027$†		

*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 eosinophila, 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 PC_{20} 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-β1, 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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ORIGINAL ARTICLE

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Association between genetic variation in the gene for death-associated protein-3 (DAP3) and adult asthma

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Abstract Lung epithelium plays a central role in modulation of the lung inflammatory response, and lung repair and airway epithelial cells are targets in asthma and viral infection. Activated T lymphocytes release cytokines such as interferon-gamma (IFN-γ) that induce apoptosis, or programmed cell death, of damaged epithelial cells. Death-associated protein-3 (DAP3) is involved in mediating IFN-γ-induced cell death. To assess the possible involvement of genetic variants of *DAP3* with asthma, we searched for single-nucleotide polymorphisms (SNPs) in the gene and conducted a case-control study with 1,341 subjects. We found a strong association between bronchial asthma (BA) in adults ($P=0.0051$, odds ratio = 1.87, 95% CI = 1.20–2.92), whereas no association was found with childhood asthma. The tendency was more prominent in patients with higher serum total immunoglobulin E (IgE) (>250 IU/ml) ($P=0.00061$, odds ratio = 2.40, 95% CI = 1.44–4.00). *DAP3* was expressed in normal bronchial epithelial cells, and the expression was induced by IFN-γ. These results indicated that specific variants of the *DAP3* gene might be associated with the mechanisms responsible for adult BA and contribute to airway inflammation and remodeling.

Keywords Bronchial asthma · Single-nucleotide polymorphism · DAP3 · Association study · Immunoglobulin E

Introduction

Bronchial asthma (BA) is a complex disorder caused by a combination of genetic and environmental factors (Busse and Lemanske 2001). In recent years, a growing body of clinical and experimental evidence has

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highlighted the importance of respiratory infections in acute asthma exacerbation (Gern and Busse 2002; Message and Johnston 2001). Most infections induce asthma exacerbation and a T helper (Th) 1 inflammatory response. Some studies have shown that some Th1 cells are present in asthmatic patients, which could be related to bronchial hyperreactivity (Krug et al. 1996; Magnan et al. 2000; Cho et al. 2002). Interferon-γ (IFN-γ), produced by Th1 cells, exerts an inhibitory effect on Th2 cells and has extensive and diverse immunoregulatory effects on various cells (Chung and Barnes 1999). A recent study showed that IFN-γ alone and in combination with activation of the Fas pathway induced apoptosis, or programmed cell death, in A549 lung epithelial cells (Wen et al. 1997). Disruption of the bronchial epithelium is frequently observed in mucosal biopsies obtained from asthmatic airways (Laitinen et al. 1985; Jeffery et al. 1989; Montefort et al. 1992).

Apoptosis plays crucial roles in numerous biological processes ranging from growth and development to combating viral infections. Human *DAP3* was originally isolated as a novel mediator of IFN-γ-induced cell death by performing functional selection by gene cloning. The gene codes for a 46 kDa protein with a potential nucleotide-binding motif (Kissil et al. 1995). Functional analyses of the protein indicate that the intact full-length protein is required for its ability to induce apoptosis when overexpressed, and DAP3 is implicated as a positive mediator of tumor necrosis factor α (TNF-α) and Fas (Kissil et al. 1999). Furthermore, genome screens for asthma and related phenotypes have been completed in 11 study populations. The human *DAP3* gene lies on chromosome 1q21–q22 (Kissil and Kimchi 1997), a locus linked to atopy susceptibility (Ober et al. 1999). To investigate the possible involvement of variants of *DAP3* with asthma in humans, we searched for single-nucleotide polymorphisms (SNPs) in the gene and conducted a genetic association study in Japanese.

Materials and methods

Subjects

We recruited 305 patients with child atopic asthma [mean age 9.6, range 1–15 years; male/female ratio = 1.54:1.0; mean serum immunoglobulin E (IgE) level, 1,083 U/ml], 322 with adult atopic asthma (mean age 49, range 20–91 years; male/female ratio = 1.0:1.18; mean serum IgE level, 762.8 U/ml), and 95 with adult nonatopic asthma (mean age 60, range 42–80 years; male/female ratio = 1.0:1.71; mean serum IgE level, 152.5 U/ml) from Osaka Prefectural Habikino Hospital and the Miyatake Asthma Clinic.. All subjects with asthma were diagnosed according to the criteria of the National Institutes of Health, with minor modifications (National Heart, Lung, and Blood Institute, National Institutes of Health, 1997). The diagnosis of atopic asthma was based on a positive immunoassay test to one or more allergens or total serum IgE level of ≥400 kU/l. The criteria for a diagnosis of nonatopic asthma were a total serum IgE level of <400kU/l and all allergen-specific IgE level of ≤ 0.35 kU/l (Mao et al. 2001). As controls, we analyzed 571 randomly selected population-based individuals who did not have atopy-related diseases (mean age 36, range 18–83 years; male/female ratio = 2.42:1.0). All individuals were Japanese and gave written informed consent to participate in the study (or, for individuals younger than 16 years old, their parents gave consent) in accord with the rules of the process committee at the SNP Research Center, The Institute of Physical and Chemical Research (RIKEN).

Screening for polymorphisms and genotyping

The *DAP3* genomic region targeted for SNP discovery included a 0.5 kb continuous region 5′ to the gene and 13 exons, each with a minimum of 200 bases of a flanking intronic sequence. Twelve primer sets (Table 1) were designed on the basis of the *DAP3* genomic sequence available from GenBank (accession number NT_079484). Each polymerase chain reaction (PCR) was performed with 5 ng of genomic DNA from 24 individuals. The PCR product was reacted with BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA). Sequences were assembled, and polymorphisms were identified using the Sequencher program (Gene Codes Corporation, Ann Arbor, MI, USA).

Genotyping

For the −20632 G/T polymorphism, genotyping was performed by PCR-restriction fragment-length

Table 1 Primers used in screening for single-nucleotide polymorphisms (SNPs)

F1	5′-AAGCCTTCTCCACCTCCTCT	R1	5′-CCATCACCAGGCTGTCTAAC
F2	5′-TGTCATGCCAGATGTAATAGC	R2	5′-AAGTAAACCAGCAGAATGGTC
F3	5′-CATGGCTCACCTTCTCAGTG	R3	5′-ATGGGAACTGATACAGACCG
F4	5′-GTCACTTTGTCACCATTATACC	R4	5′-CTATTTAGCTCAGGAGTGTAC
F5	5′-AGTGGCCCTAAGTGGTATAC	R5	5′-ACTGGAAGGATCACTTGTATG
F6	5′-CTCATGAACCAATGCTTTCTC	R6	5′-AGGATAACTAAACCTAACAGAC
F7	5′-TGACTTACCTTTCAAACCTGC	R7	5′-TCAAGTTATGAGTCCACTGTG
F8	5′-GAGGTCTCTTACAGAACCTG	R8	5′-ATATTGCATGGTCTACAAGGC
F9	5′-TTCTAGGGCCAGAGGCTACG	R9	5′-TCTTTTCCAAAGACGGTAAGG
F10	5′-GTATCTCCTTACCGTCTTTGG	R10	5′-GACCTACAAAATAGCTGGCATG
F11	5′-TGTGGAGTCCATATGTGGAG	R11	5′-CTCAAATGTGAGGTCAAGGG
F12	5′-TGGGGTGAGGGTCTAGAAG	R12	5′-GCCTCAACAGAACCACTGG

polymorphism (PCR-RFLP) analysis using *EcoT14*I (Takara, Shiga, Japan). The primers for the SNP were 5′TCAGGACGGGCGCTTTGTG and 5′GGTCTACCGGCCTCACT.

Statistical analysis

Allele frequencies for each SNP were calculated. A χ^2goodness-of-fit test was used to assess deviation from Hardy–Weinberg equilibrium for genotype frequencies at each locus. Associations of genotypes or alleles with patient groups versus nonatopic control subjects were determined by using χ^2 analysis with appropriate df.

Quantitative real-time PCR

Human bronchial epithelial cells (NHBE) were purchased from BioWhittaker, and human recombinant IFN-γ was purchased from Genzyme/Techne. Total RNA (5 μg) extracted from each sample was treated with DNaseI (Roche), and cDNA was synthesized with Thermoscript reverse transcriptase (Life Technologies) by dT_{15} priming. TaqMan probes and primers for *DAP3* and *GAPDH* were Assay-on-Demand gene expression products (Applied Biosystems). TaqMan PCR was done with an ABI PRISM 7900 Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. The relative expression of *DAP3* mRNA was normalized to the amount of *GAPDH* in the same cDNA by using the standard curve method described by the manufacturer.

Tissue expression

We used human multiple tissue, human immune system, and human blood fractions multiple tissues cDNA panels from CLONTECH for expression analysis by PCR amplification of target sequences. The primers for *DAP3* were 5′GCACTTGTTCACAACTTGAG and 5′TCTGTAGGAGCTTTCTCATG. The primers for *GAPDH* were 5′CCCCATGTTCGTCATGGGT and 5′GTGATGGCATGGACTGTGG. Southern blotting was done with DIG reagents and kits for nonradioactive nucleic acid labeling and detection (Roche) according to the manufacturer's instructions. The probes for *DAP3* and *GAPDH* were 5′AAATGATTGGCATGGAGGCG and 5′CCATGAGAAGTATGACAACAG, respectively.

Results

An extensive search identified four SNPs by PCR-directed sequencing using genomic DNA from 24 individuals on the basis of the *DAP3* genomic sequence. Position one was the adenine of the initiation codon.

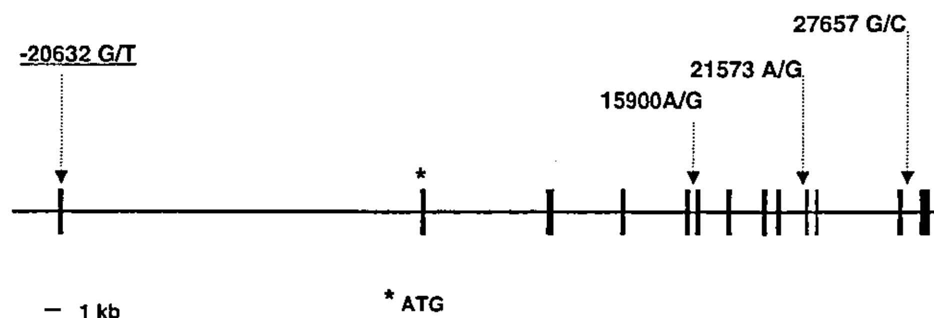

Fig. 1 Genomic structure and polymorphism map of *DAP3*. Thirteen exons are indicated by *closed squares*. ATG represents the translational start codon. The *underlined* single-nucleotide polymorphism (SNP), −20632 G/T, was genotyped in this study

Table 2 Locations and allele frequencies of single-nucleotide polymorphisms (SNPs). *JSNP* Japanese Single Nucleotide Polymorphisms Database, *NCBI* National Center for Biotechnology Information

SNP[a]	Location	Gene sequence	Allele frequency (%)[b]	Primers	JSNP ID[c]	NCBI dbSNP[d]
−20632 G/T	Exon 1	GGTCGCCTAG(G/T)CTGGAGAACT	27	F1R1	–	rs1058207
15900 A/G	Intron 5	CCTGGCCAAC(A/G)TGGCGAAACC	2	F6R6	IMS-JST084352	–
21573 A/G	Intron 9	TGTCCGATAT(G/A)CAGATTGCCC	4	F9R9	IMS-JST070763	rs2274787
27657 G/C	Intron 12	GTGCAAGGGC(T/C)ACTCCAGCAC	2	F11R11	–	–

[a]Positions are numbered according to their position relative to the published DAP3 gene (GenBank NT_079484). Position 1 is the A of the initiation codon
[b]Frequency of minor allele
[c]JSNP ID, number from the Japanese SNP database (http://snp.ims.u-tokyo.ac.jp/index_ja.html)
[d]dbSNP ID, Number from the dbSNP database of NCBI (http://www.ncbi.nlm.nih.gov/SNP/)

These were −20632 G/T, 15900 A/G, 21573 A/G, and 27657 G/C (Fig. 1, Table 2). The 15900 A/G (IMS-JST084352) and 21573 A/G (IMS-JST070763) are contained in the J-SNPs that are available from the Web site (http://snp.ims.u-tokyo.ac.jp). Since three of the SNPs were quite rare, further case-control analysis focused on −20632 G/T, which was located in the 5′ noncoding region of *DAP3*. We found a strong association between −20632 G/T and BA in adults (P=0.0051, odds ratio = 1.87, 95% CI = 1.20–2.92), whereas no association was found with childhood asthma (Table 3). The tendency was more prominent in patients with higher serum total IgE (>250 IU/ml) (P=0.00061, odds ratio = 2.40, 95% CI = 1.44–4.00) (Table 4).

We investigated *DAP3*expression of cultured NHBE by RT-PCR, and *DAP3* was expressed in NHBE (data not shown). Next we compared relative expression of *DAP3* in NHBE that were either unstimulated or stimulated with 10 ng/ml IFN-γ for 3 h. Levels of *DAP3* mRNA were increased 10.6 fold by stimulation with IFN-γ (Fig. 2).

We performed RT-PCR using multiple tissue cDNA panels. Transcripts were expressed in lung and tissues and cells associated with immune function. The bands were present in both cDNAs from activated and inactivated lymphocytes (Fig. 3).

Discussion

Asthma is an inflammatory airway disease associated with infiltration of T cells and eosinophils, increased levels of proinflammatory cytokines, and shedding of bronchial cells (Busse and Lemanske 2001), which is an important histologic feature observed in bronchial biopsy specimens from asthmatic patients (Laitinen et al. 1985; Jeffery et al. 1989; Montefort et al. 1992). A recent study demonstrated that both T cells and eosinophils contribute to the induction of bronchial epithelial cell apoptosis by secretion of IFN-γ and TNF-α (Trautmann et al. 2002). Although we could not show functional data on this SNP in this study, we found that *DAP3* was expressed in NHBE, and the transcript was induced by IFN-γ. DAP3, a positive mediator of cell death induced by IFN-γ, may play an important role in the epithelial cell shedding frequently observed in asthma.

In our study, adult BA showed a stronger association with variant −20632 G/T than did childhood BA, especially in patients with high levels of serum IgE. Asthma in adults is often a slowly progressive, irreversible disease (Reed 1999). The majority of older

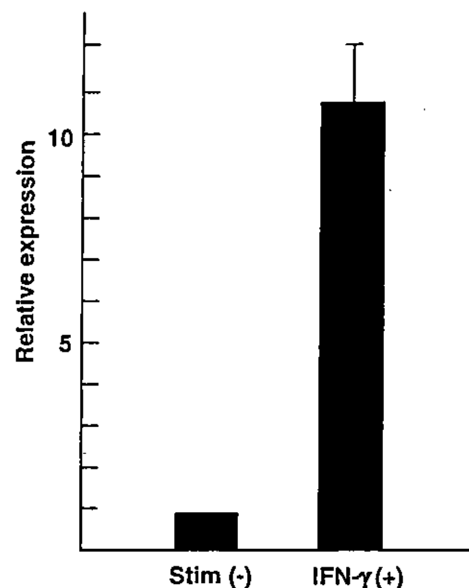

Fig. 2 Expression pattern of *DAP3* in NHBE cells. Results displayed are the averages of these measurements normalized to 1.0 for the nonstimulated control. Data are from three separate experiments

Table 3 Association between asthma and single-nucleotide polymorphisms (SNPs) in *DAP3*. *BA* bronchial asthma

−20632 G/T	Controls (n=571)	Childhood BA (n=305)	Adult BA (n=465)	TT vs GG+GT			
				χ^2 (P-value)		Odds ratio (95% CI)	
				vs childhood BA	vs adult BA	vs childhood BA	vs adult BA
GG	301 (53%)	170 (56%)	238 (51%)	0.48	7.85	1.21	1.87
GT	234 (41%)	112 (37%)	175 (38%)	(0.49)	(0.0051)	(0.70–2.09)	(1.20–2.92)
TT	36 (6%)	23 (7%)	52 (11%)				

Table 4 Association between −20632 G/T substitution of *DAP3* and adult asthmatics with high serum total immunoglobulin E (IgE) (>250 IU/ml). *BA* bronchial asthma

Genotype −20632 G/T	Control (%) n=571	Adult BA with total IgE >250 IU/ml (%) n=216	OR (95% CI)	P
GG and GT	535 (94%)	186 (86%)	2.40	0.00061
TT	36 (6%)	30 (14%)	(1.44–4.00)	

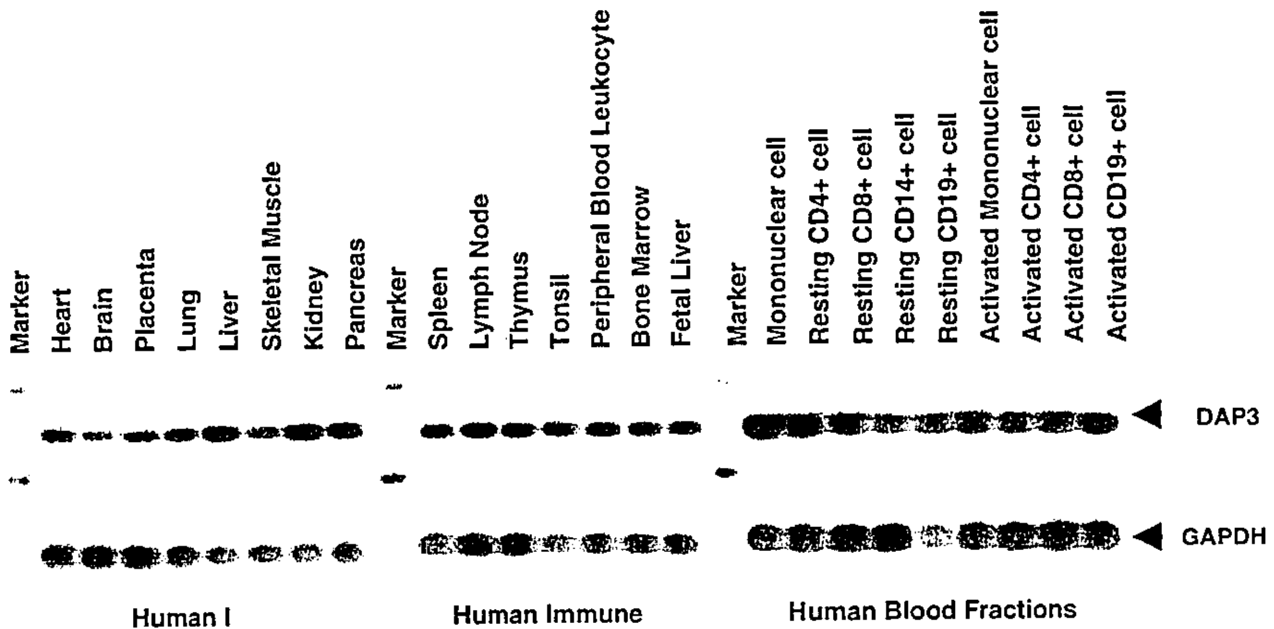

Fig. 3 Expression pattern of *DAP3* in multiple tissue cDNA panels

patients have a substantial degree of irreversible impairment of lung function that results from a combination of pathologic changes. These changes include airway remodeling from chronic lymphocytic-eosinophilic inflammation and bronchiectasis from repeated infections. DAP3 mediating IFN-γ-induced cell death might be involved in the development of chronic progressive irreversible inflammation, which is more frequently observed in adult asthma than childhood asthma. A recent report showed that influenza A viral infection, which induces a large amount of intrapulmonary IFN-γ production, enhanced later allergen-specific asthma and promoted dual allergen-specific Th1 and Th2 responses (Dahl et al. 2004). Several studies reported that some Th1 cells were present in asthmatic patients, which could be related to bronchial hyperreactivity (Krug et al. 1996; Magnan et al. 2000; Cho et al. 2002). The epithelial injuries might induce inflammatory reactions and change the mucosal permeability to allow allergens easier access to dendritic cells and enhance subsequent allergen sensitization.

Apoptosis provides a mechanism for removal of antigen-activated T cells and eosinophils, thereby leading to the resolution of the inflammatory response (Haslett 1992; Lenardo et al. 1995; Woolley et al. 1996). The accumulation of eosinophils in the asthmatic airway significantly contributes to the persistence of airway inflammation in these patients (Kroegel et al. 1994). Decreased numbers of eosinophils undergoing apoptosis have been observed in asthmatic subjects when compared with a nonasthmatic control group (Vignola et al. 1999). Although we found that *DAP3* was expressed in lung, tissue, and cells associated with immune function and NHBE, the expression profile of *DAP3* in eosinophils and antigen-activated T cells remains unclear. Thus, further study is required to clarify the physiological role of DAP3 in these cells.

Apoptosis is an essential process for functions such as the immune response, and glucocorticoids, the most effective treatments for asthma, are one of the important regulators of the cellular functions underlying these events (Walsh et al. 2003). It has been suggested that DAP3 directly interacts with the glucocorticoid receptor (GR), and DAP3 is needed to efficiently increase the GR level and enhance the transcriptional activity of GR (Hulkko et al. 2000; Hulkko and Zilliacus 2002). Dexamethasone has been reported to inhibit lung epithelial cell apoptosis induced by IFN-γ (Wen et al. 1997). Further analyses of genetic predisposition for expression of *DAP3*contributing to the pathogenesis of asthma might lead to improved diagnosis, treatment, and prevention.

Acknowledgement This work was supported by a grant from the Japanese Millennium Project.

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ORIGINAL ARTICLE

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Linkage and association of childhood asthma with the chromosome 12 genes

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Abstract Several studies have shown linkage of chromosome region 12q13–24 to bronchial asthma and related phenotypes in ethnically diverse populations. In the Japanese population, a genome-wide study failed to show strong evidence of linkage of this region. Chromosome 12 genes that showed association with the disease in at least one report include: the signal transducer and activator of transcription 6 gene (*STAT6)*, the nitrogen oxide synthetase 1 gene (*NOS1*), the interferon γ gene (*IFNG*), and the activation-induced cytidine deaminase gene (*AICDA*). To evaluate the linkage between chromosome 12 and childhood asthma in the Japanese population, we performed sib-pair linkage analysis on childhood asthma families using 18 microsatellite markers on chromosome 12. To investigate association between chromosome 12 candidate genes and asthma, distributions of alleles and genotypes of repeat polymorphisms of *STAT6*, *NOS1*, and *IFNG* were compared between controls and patients. Single nucleotide polymorphism of *AICDA* was also investigated. Chromosome region 12q24.23–q24.33 showed suggestive linkage to asthma. The *NOS1* intron 2 GT repeat and *STAT6* exon 1 GT repeat were associated with asthma. Neither the *IFNG* intron 1 CA repeat nor 465C/T of *AICDA* showed any association with asthma. Our results suggest that *NOS1* and *STAT6* are asthma-susceptibility genes and that chromosome region 12q24.23–q24.33 contains other susceptibility gene(s).

Keywords Childhood asthma · Linkage · Association · Polymorphism · NOS1 · STAT6 · IFNG · AICDA

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Introduction

Bronchial asthma is an inflammatory disease of the airways characterized by airway obstruction and increased airway responsiveness. Asthma is an etiologically complex disease and develops by the interaction of multiple genes and environmental factors. Genome-wide

linkage studies have identified a number of autosomal regions providing evidence of linkage to asthma, atopy, eosinophilia, and/or other associated phenotypes (CSGA 1997; Daniels et al. 1996; Dizier et al. 2000; Haagerup et al. 2002; Hakonarson et al. 2002; Laitinen et al. 2001; Ober et al. 2000; Wjst et al. 1999; Xu et al. 2001a; Xu et al. 2000; Xu et al. 2001b; Yokouchi et al. 2000). Some of these studies (CSGA 1997; Dizier et al. 2000; Haagerup et al. 2002; Wjst et al. 1999; Xu et al. 2001a; Xu et al. 2000; Yokouchi et al. 2000) and those focused on a single chromosome (Barnes et al. 1999; Barnes et al. 1996; Kruglyak et al. 1996; Malerba et al. 2000; Nickel et al. 1997; Wilkinson et al. 1998; Raby et al. 2003) suggested linkage of chromosome 12q regions to asthma or related phenotypes in diverse populations.

In a genome-wide linkage analysis of mite-sensitive Japanese childhood asthma, the 110–145 cM region from the pter (the telomere of the short arm) showed maximum logarithm of odds score (MLS) more than 1.0 with the highest MLS of 1.92 at 111.9–125.3 cM (Yokouchi et al. 2000). The highest MLS did not reach the value of "significant" (MLS = 3.6) or "suggestive" (MLS = 2.2) linkage to the disease (Lander and Kruglyak 1995). The region in which MLS exceeded 1.0 was roughly overlapped by those of studies on Afro-Caribbean, French, and British populations (Barnes et al. 1996; Dizier et al. 2000; Wilkinson et al. 1998). To establish the linkage between asthma and chromosome 12 region, evidence of the suggestive linkage must be replicated using a different set of samples from the same population (Lander and Kruglyak 1995).

Candidate genes of chromosome 12q15–q24 include the signal transducer and activator of transcription 6 gene (*STAT6*), interferon-γ (*IFNG*), stem cell factor (*SFC*), leukotriene A4 hydrolase (*LTA4H*), insulin-like growth factor (*IGFI*), β-subunit of nuclear factor-Y (*NFYB*), B-cell translocation gene 1 (*BTG1*), and nitrogen oxide synthetase 1 (*NOS1*) (Barnes et al. 1996; Dizier et al. 2000; Wjst et al. 1999). Of these, *STAT6*, *NOS1*, and *IFNG* were investigated with case-control studies and showed positive association with asthma in at least one study. Gao et al. (2000b) demonstrated the association of the single nucleotide polymorphism (SNP) 2964G/A of *STAT6* with adult asthma in Japanese populations. However, this association was not replicated in later studies on German/Swedish (Duetsch et al. 2002) or Japanese populations (Tamura et al. 2001). Instead, a GT repeat polymorphism in exon 1 was associated with eosinophil count in the German/Swedish study and with allergic diseases in the Japanese study. A dinucleotide repeat marker in *NOS1* was also reported to be associated with the disease in the British population (Gao et al. 2000a). An association between a SNP in *NOS1* and eosinophil count was also shown in German/Swedish patients (Immervoll et al. 2001). Hyden et al. (1997) reported that no polymorphism in the *IFNG* was associated with atopic asthma, whereas an association between the GT repeat in intron 1 of *IFNG* and childhood asthma was suggested in the Japanese population (Nakao et al. 2001). Heinzmann et al. (2000a) screened polymorphisms in *SCF*, *STAT6*, *TR2* (thyroid receptor 2), and *LTA4H* and found two polymorphisms in *SCF* and one in *TR2* in the German population. They found no evidence of linkage or association of these genes with atopy.

All of the above-mentioned studies were based on case-control design. On the other hand, using the transmission disequilibrium test (TDT), Noguchi et al. (2001) reported that the activation-induced cytidine deaminase gene (*AICDA*) was associated with childhood asthma in the Japanese population. The *AICDA* gene is located in the short arm of chromosome 12, where linkage has never been suggested. They selected this gene as a candidate gene for asthma because deficiency of *AICDA* resulted in low IgE production, thereby the variations of the gene might be responsive to atopy. More recently, Isidoro-García et al. (2003) reported a case-control study on the same SNP of *AICDA* gene in the Spanish population. They failed to show the association of this SNP with the disease.

Among candidate genes of chromosome region 12q15–q24, *STAT6*, *IFNG*, and *NOS1* have been suggested to be associated with asthma in at least one study. Although the locus was not a suspected linkage to asthma, the *AICDA* gene showed a positive result in one study. As often seen in genetic analyses of a complex disease (Ioannidis et al. 2001), inconsistencies were noticed between the studies of chromosome 12 candidate genes for asthma. Studying other sets of samples in the same population is necessary to conclude whether a particular gene is truly associated with this complex disease.

In the present study, we investigated linkage of markers on chromosome 12 to childhood asthma in the Japanese population. We also investigated association of four candidate genes, *AIDCA*, *STAT6*, *NOS1*, and *IFNG*, with Japanese childhood asthma.

Materials and methods

Families and individuals

For linkage analysis, 18 families with affected sib pairs and one family with an affected sib trio were recruited. For the association study, 184 controls and 115 patients were genotyped. One hundred control subjects were selected in the Osaka area, Japan, as previously described (Heinzmann et al. 2000b; Mao et al. 1996), and 84 controls were selected from adult staff and student volunteers from Tohoku University School of Medicine in Sendai, Japan. Individuals with a history of treatment for asthma or eczema were excluded from controls. Forty-two patients were diagnosed at hospitals in the Sendai area, which included patients from 19 families for linkage analysis. For an association study, one patient per family was selected. Other patients were recruited as described (Heinzmann et al. 2000b; Mao et al. 1996). None of the samples were previously analyzed for chromosome 12 linkage markers, *NOS1*, *IFNG*, *STAT6*, or *AICDA*. Diagnosis of asthma of probands was made by pediatricians specializing in allergic diseases.

The criteria of asthma were two or more episodes of wheezing and shortness of breath and reversibility of the wheezing and dyspnea, either spontaneously or by bronchodilator treatment. The definition of "childhood" asthma was asthma with onset before age 15 years. Diagnosis of other family members was based on the modified ATS-DLD questionnaire (Ferris 1978). Total serum IgE was regarded as high when the level was 250 IU/ml or higher. The specific IgE against house dust mite [*Dermatophagoides pteronyssinus* (Dp)] was judged positive when the RAST score against Dp was 2 (0.70 U_A/ml) or higher. "Atopy" was defined as either having high total IgE and/or positive Dp-specific IgE. Eighteen families had an affected sibpair and one family had an affected sib trio. All patients with childhood asthma were atopic. All affected sibs and their parents were genotyped.

This study was approved by the ethics committee of Tohoku University School of Medicine.

Genotyping

DNA was extracted from peripheral blood leukocytes using the Genomic DNA purification kit (Promega, Madison, WI, USA). Chromosome 12 microsatellite markers of the Human GenePairs Primers version 9 (Invitrogen, Carlsland, CA, USA) were used for the linkage analysis. The 18 markers genotyped in this study are shown in Table 1. Information on marker order and position was obtained from LDB2000: Sequence-based Integrated Maps of the Human Genome (http://cedar.genetics.soton.ac.uk/public_html/LDB2000.html) (Wilkinson et al. 1998). The location of *AICDA* was not cited in this database and was estimated using the NCBI Human Map Viewer.

In chromosome 12 microsatellite marker analyses, PCR mixtures contained 10 mM Tris/HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 µM each of deoxynucleotide triphosphate (dNTPs), 0.25 U of rTaq DNA polymerase (TAKARA, Tokyo, Japan), 5 µM of each primer, and 10 ng of template DNA in a total volume of 10 µl. The cycle conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 1 min, with a final extension of 72°C for 10 min. The size of the PCR products was estimated using a 373XL DNA sequencer (Applied Biosystems, Foster City, CA, USA). GeneScan 500XL TAMRA labeled standard (Applied Biosystems) was used for estimation of fragment lengths.

Primers for the *NOS1* intron 2 GT repeat were as described previously (Gao et al. 2000a). One of the primers, 5'-ATAGAGCCTGTGCTGAGCCTTC, was 6-FAM labeled. The PCR mixture contained 10 mM Tris/HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 250 µM each of dNTPs, 0.5 U of rTaq DNA polymerase, 200 µM of each primer, and 10 ng of template DNA in a total volume of 15 µl. The cycle conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 40 s, with a final extension of 72°C for 10 min.

The primers for the *STAT6* exon 1 GT repeat were 5'-GGAGAAGCCGGAAACAGCGG and 5'-GTTCAAGGCTGGCCCTGCTAGC (6-FAM labeled). The PCR mixture was the same as for of *NOS1*. The cycle conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 1 min, with a final extension of 72°C for 10 min.

Primers for the *IFNG* intron 2 CA repeat were as previously described (Nakao et al. 2001). The PCR mixture was the same as for *NOS1*, except that 0.25 U of rTaq and 600 µM of each primer were used. The cycle conditions were 95°C for 5 min, followed by 25–34 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min, with a final extension of 72°C for 5 min.

The 467C/T (His155His) polymorphism of *AICDA* (GeneBank AB040430) was the same polymorphism reported by Noguchi et al. (2001), where they designated this polymorphism as 7888C/T. This was genotyped using a modified TaqMan PCR method employing allele-specific amplification (Fujii et al. 2000). The common forward primer was 5'-GGCCCCGAGGAAATGAGAAAAT. The reverse primers were 5'-TCCCAGGCTTTGAAAGTTCTTTAG for the C allele and 5'-TCCCAGGCTTTGAAAGTTCTTTGA for the T allele. The TaqMan probe was 5'-FAM-AGAAGACAGTTCAGGTTCCAAATCGAGG- TAMRA -3'. The PCR mixture contained 7.5 µl of 2X TaqMan Universal PCR Master Mix (Applied Biosystems), 400 µM of each PCR primer, 0.12 µM of TaqMan probe, and 5 ng of template DNA in a final volume of 15 µl. The cycle conditions were 50°C for 2 min, 95°C for 3 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min.

Table 1 Map locations for chromosome 12 markers and genes

Locus	Kb from pter	Band	Male cM	Female cM	Averaged cM
ptr	0	p13.33	0.0	0.0	0.0
D12S372	3761	p13.33	7.5	2.5	5.0
AICDA	8468	p13.31	20.0	9.3	14.7
GATA49D12	8513	p13.31	20.1	9.4	14.7
D12S391	13246	p13.2	26.1	21.4	23.8
D12S373	18347	p12.3	32.8	35.5	34.1
D12S1042	28440	p11.23	36.9	56.2	46.5
cen	39000	q11	38.7	65.0	51.9
D12S1301	46378	q12	39.7	71.3	55.5
D12S398	56808	q13.13	44.4	87.0	65.7
STAT6	61349	q13.13	45.9	92.5	69.2
D12S1294	73218	q14.2	50.4	97.7	74.0
IFNG	73860	q14.2	51.7	100.3	76.0
D12S375	74485	q14.3	52.7	103.0	77.8
D12S1052	80438	q15	57.1	106.9	82.0
D12S1064	96885	q21.33	67.2	120.5	93.8
D12S1300	105918	q23.1	69.8	133.1	101.4
PAH	111421	q23.3	73.5	143.5	108.5
D12S2070	125845	q24.22	81.8	167.6	124.7
NOS1	127541	q24.22	82.8	174.6	128.7
D12S395	130349	q24.23	84.1	184.3	134.2
D12S392	138666	q24.32	91.9	200.3	146.1
D12S2078	140437	q24.33	98.1	201.8	149.9
D12S1045	143552	q24.33	110.7	212.9	161.8
qtr	146025	qtr	119.2	218.4	168.8

Statistical analysis

Allele frequencies of microsatellite markers were estimated from the parental chromosomes. All chromosome 12 markers were assessed by PEDCHECK (version 1.0) (O'Connell et al. 1998) for pedigree inconsistencies. There was no genotype inconsistency at any loci in all the families. Multipoint linkage analysis was conducted using GENEHUNTER 2 (Kruglyak et al. 1996). For score calculations of the sib trio, we used "all independent pairs of affected/phenotyped sibs" option of the GENEHUNTER 2 program where one sib trio yielded two pairs. In all allelic and genotypic distribution analyses, Fisher's exact *P* values were calculated using SPSS for Windows version 11.0 J (SPSS Japan, Japan). For multiallelic markers, the *P* value of association with the disease of each allele or genotype was multiplied by the number of the alleles or genotypes to compensate for multiple testing and expressed as *Pc*. The level of significance for the association studies was set at $P(Pc) = 0.05$.

Results

Linkage analysis of chromosome 12

Thirty-nine sibs with childhood asthma used for the linkage analysis consisted of 20 males and 19 females. Ages of the patients ranged from 1 to 14 years with the average age of 7.3 years. All affected sibs were positive

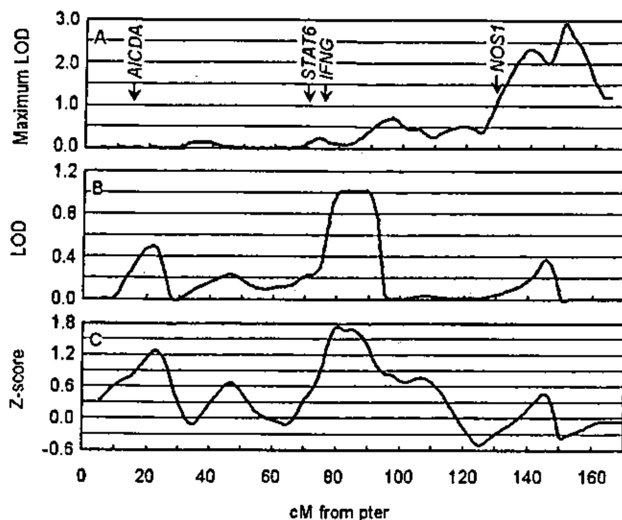

Fig. 1A–C Linkage results for asthma and total IgE level. All calculations were performed by GENEHUNTER 2. **A** Multipoint maximum likelihood (ML) estimate of identity by decent sharing for asthma affection status. **B** ML quantitative locus (QTL) variance estimation for $\log_{10}$ (total IgE level). ML QTL variance estimation (Kruglyak and Lander 1995) is performed instead of traditional Haseman-Elston (HE) QTL analysis because the values calculated with HE QTL analysis were affected by selection of sib pairs from the sib trio. **C** Nonparametric QTL analysis (Kruglyak and Lander 1995) for $\log_{10}$ (total IgE level)

for Dp-specific IgE. Thirty also showed atopic dermatitis. Results of the linkage analysis of the 18 microsatellite markers of chromosome 12 are shown in Fig. 1. A broad region from 135 cM to 160 cM exceeded 1.5 in MLS (Fig. 1A). Two peaks at 140 and 150 cM were observed, and the latter peak showed a MLS of 2.92, greater than the suggestive linkage level (Lander and Kruglyak 1995). Serum total IgE levels were $\log_{10}$ transformed and subjected to sib-pair quantitative locus (QTL) analysis. The result of maximum likelihood (ML) QTL variance estimation is shown in Fig. 1B. ML QTL variance estimation (Kruglyak and Lander 1995) was performed instead of the traditional Haseman-Elston (HE) QTL analysis, because the values with HE QTL analysis were affected greatly by the selection method of pairs from the sib trio. The highest peak of logarithm of odds (LOD) score (max = 1.02) was observed at 83 cM. No significant peaks were observed where linkage to asthma was observed. Nonparametric QTL analysis (Kruglyak and Lander 1995) showed the highest peak to be at 80 cM with a Z-score of 1.74 ($P = 0.082$) (Fig. 1C). The results of QTL analyses did not suggest evidence of QTL for $\log_{10}$ (total IgE level) on chromosome 12.

Association studies of genes on chromosome 12

Results of the case-control studies of chromosome 12 candidate genes are shown in Table 2. There were four types of alleles for the GT repeat of exon 1 of *STAT6* with the repeat number varying from 13 to 16 in our entire series. Comparison of allele frequencies between patients and controls showed a significant difference in the whole-allele distribution ($P = 0.0054$). This difference was primarily derived from the difference in the frequency of allele 3 (15 repeats). The frequency of this allele was significantly lower in the patients ($Pc = 0.0044$, odds ratio (OR) = 0.71, 95% confidence interval (95%CI) = 0.58–0.87). Other alleles did not show difference in frequency between controls and patients. Seven genotypes of this GT repeat were observed. The genotypic distribution differed between patients and controls ($P = 0.0054$). Homozygotes for allele 3 had a lower risk of asthma ($Pc = 0.0035$, OR = 0.718, 95%CI = 0.60–0.86).

We identified six alleles in the GT repeat polymorphism of intron 2 of *NOS1* (Table 2). The whole-allele distribution of this polymorphism of patients was significantly different from that of controls ($P = 0.0082$). This difference was primarily due to the difference in frequency of allele 3 (16 repeats) ($Pc = 0.049$, OR = 0.75, 95%CI = 0.60–0.93). Eleven genotypes of this GT repeat were observed. The whole genotypic distribution differed between patients and controls ($P = 0.0019$). Homozygotes for allele 3 had a lower risk for asthma than the other genotypes ($Pc = 0.030$, OR = 0.53, 95%CI = 0.33–0.84). There was a significant difference in the frequency of heterozygote for allele 4/5 between controls and patients ($Pc = 0.028$). The numbers of this genotype is small (zero in controls and six in patients), and its biological meaning is difficult to interpret.

Association of asthma was also tested for the CA repeat of the interferon γ gene (*IFNG*). We identified seven allele types and 16 genotypes of this polymorphism in the Japanese population (Table 2). There was no evidence of association between this polymorphism and asthma in our series.

We also investigated the SNP 465C/T of *AICDA*. Frequencies of the major allele were almost the same between controls and patients. Genotype distribution of controls was in Hardy-Weinberg equilibrium, whereas that of patients was not ($\chi^2 = 7.17$, $P = 0.027$). We regenotyped the patients and found no evidence of typing errors. Patients showed lower CT and higher TT genotype frequencies when compared to those of controls. However, these changes did not reach statistical significance in the association study (Table 2).

Discussion

We have found evidence of a suggestive linkage between 12q24.32–33 markers (144–155 cM) and childhood asthma in the Japanese population. The region identified was very close to the region reported by Wilkinson et al. (1998), who investigated linkage to their unique asthma score in the English population. In the genome-wide analysis of Japanese mite-sensitive childhood asthma, the highest MLS (1.92) was observed between D12S78 and D12S86 (110–132 cM) (Yokouchi et al. 2000). This

Table 2 Association study of the genes on chromosome 12

	Controls (frequency %)	Asthma (frequency %)	Significance level*
STAT6 exon 1 GT repeat			
Allelic distribution			
Allele 1 (13 repeat)	77 (22.4)	72 (31.6)	Pc = 0.061
Allele 2 (14 repeat)	1 (0.3)	4 (1.4)	Pc = 0.34
Allele 3 (15 repeat)	233 (67.7)	123 (53.8)	Pc = 0.0044
Allele 4 (16 repeat)	33 (9.6)	29 (12.7)	Pc > 1.0
Overall	344 (100.0)	228 (100.0)	P = 0.0032
Genotypic distribution			
Allele 1/allele 1	14 (8.1)	12 (10.5)	Pc > 1.0
Allele 1/allele 3	46 (26.7)	41 (36.5)	Pc = 0.81
Allele 1/allele 4	3 (1.7)	7 (6.1)	Pc = 0.67
Allele 2/allele 3	1 (0.6)	4 (3.5)	Pc = 0.59
Allele 3/allele 3	81 (47.1)	30 (26.3)	Pc = 0.0035
Allele 3/allele 4	24 (14.0)	18 (15.8)	Pc > 1.0
Allele 4/allele 4	3 (1.7)	2 (1.8)	Pc > 1.0
Overall	172 (100.0)	114 (100.0)	P = 0.0054
NOS1 intron 2 GT repeat			
Allelic distribution			
Allele 1 (14 repeat)	2 (0.5)	1 (0.5)	Pc > 1.0
Allele 2 (15 repeat)	11 (3.0)	2 (0.9)	Pc = 0.88
Allele 3 (16 repeat)	189 (51.4)	88 (40.0)	Pc = 0.049
Allele 4 (17 repeat)	3 (0.8)	7 (3.2)	Pc = 0.27
Allele 5 (18 repeat)	126 (34.2)	97 (44.1)	Pc = 0.11
Allele 6 (19 repeat)	37 (10.1)	25 (11.4)	Pc > 1.0
Overall	368 (100.0)	220 (100.0)	P = 0.0082
Genotypic distribution			
Allele 1/allele 3	2 (1.1)	1 (0.9)	Pc > 1.0
Allele 2/allele 3	10 (5.4)	2 (1.8)	Pc > 1.0
Allele 2/allele 5	1 (0.5)	0 (0.0)	Pc > 1.0
Allele 3/allele 3	53 (28.8)	15 (13.6)	Pc = 0.030
Allele 3/allele 4	3 (1.6)	1 (0.9)	Pc > 1.0
Allele 3/allele 5	50 (27.2)	43 (39.1)	Pc = 0.42
Allele 3/allele 6	18 (9.8)	11 (10.0)	Pc > 1.0
Allele 4/allele 5	0 (0.0)	6 (5.5)	Pc = 0.028
Allele 5/allele 5	28 (15.2)	18 (16.4)	Pc > 1.0
Allele 5/allele 6	19 (10.3)	12 (10.9)	Pc > 1.0
Allele 6/allele 6	0 (0.0)	1 (0.9)	Pc > 1.0
Overall	184 (100.0)	110 (100.0)	P = 0.0019
IFNG intron 1 CA repeat			
Allelic distribution			
Allele 1 (12 repeat)	39 (11.0)	24 (10.6)	Pc > 1.0
Allele 2 (13 repeat)	209 (58.7)	110 (48.7)	P = 0.12
Allele 3 (14 repeat)	6 (1.7)	7 (3.1)	Pc > 1.0
Allele 4 (15 repeat)	91 (25.6)	78 (34.5)	P = 0.17
Allele 5 (16 repeat)	7 (2.0)	3 (1.3)	Pc > 1.0
Allele 6 (17 repeat)	0 (0.0)	1 (0.4)	Pc > 1.0
Allele 7 (18 repeat)	4 (1.1)	3 (1.3)	Pc > 1.0
Overall	356 (100.0)	226 (100.0)	P = 0.12
Genotypic distribution			
Allele 1/allele 1	6 (3.4)	3 (2.7)	Pc > 1.0
Allele 1/allele 2	19 (10.7)	8 (7.1)	Pc > 1.0
Allele 1/allele 3	0 (0.0)	1 (0.9)	Pc > 1.0
Allele 1/allele 4	8 (4.5)	9 (8.0)	Pc > 1.0
Allele 2/allele 2	62 (34.8)	32 (28.3)	Pc > 1.0
Allele 2/allele 3	3 (1.7)	0 (0.0)	Pc > 1.0
Allele 2/allele 4	54 (30.3)	35 (31.0)	Pc > 1.0
Allele 2/allele 5	6 (3.4)	2 (1.8)	Pc > 1.0
Allele 2/allele 7	3 (1.7)	1 (0.9)	Pc > 1.0
Allele 3/allele 3	0 (0.0)	2 (1.8)	Pc > 1.0
Allele 3/allele 4	2 (1.1)	1 (0.9)	Pc > 1.0
Allele 3/allele 7	1 (0.6)	1 (0.9)	Pc > 1.0
Allele 4/allele 4	13 (7.3)	15 (13.3)	Pc > 1.0
Allele 4/allele 5	1 (0.6)	1 (0.9)	Pc > 1.0
Allele 4/allele 6	0 (0.0)	1 (0.9)	Pc > 1.0
Allele 4/allele 7	0 (0.0)	1 (0.9)	Pc > 1.0
Overall	178 (100.0)	113 (100.0)	P = 0.289
AICDA 465C/T			
Allelic distribution			
C	214 (58.8)	119 (57.8)	
T	150 (41.2)	87 (42.2)	
Overall	364 (100.0)	206 (100.0)	P = 0.860
Genotypic distribution			
C/C	63 (34.6)	41 (39.8)	
C/T	88 (48.4)	37 (35.9)	
T/T	31 (17.0)	25 (24.3)	
Overall	182 (100.0)	103 (100.0)	P = 0.099

* All significance levels were calculated by Fisher's exact methods. In allele-by-allele or genotype-by-genotype comparison, the *P* value was corrected by multiplying the number of alleles or genotypes and expressed as *Pc*

region is a little different from Wilkinson's and our peak regions. However, if we compare the region with MLS exceeding 1.0, the three regions overlap each other—our data 130–160 cM, Yokouchi et al. 110–150 cM, and Wilkinson et al. 130–160 cM. In an analysis of the Danish population, linkage with an MLS of 1.58 to atopic asthma at D12S392 (146 cM) was reported (Haagerup et al. 2002). More recently, linkage to airway responsiveness was also suggested at 147 cM by the Childhood Asthma Management Program (CAMP) study (Raby et al. 2003). Thus, all these studies shared a region from 140 to 150 cM. Several studies have reported linkage between asthma and the different regions of chromosome 12. The Collaborative Study on the Genetics of Asthma reported evidence of linkage to asthma of the 12q22 region. This study analyzed Caucasians, African Americans, and Hispanics and found a linkage peak at D12S2070 (125 cM) in Hispanics (CSGA 1997; Xu et al. 2001a). When the condition of loci of chromosome 14 was considered, this linkage peak became broader and greater and the region with an LOD score greater than 1.0 extended from 120 cM to the telomere (Xu et al. 2001a). The region detected in Barbados families was located at 12q21.1 (90 cM), which is clearly different from those found in the Japanese and English (Barnes et al. 1999; Barnes et al. 1996). Linkage to asthma of this region has also been suggested in German and Swedish populations (Wjst et al. 1999). A suggestive linkage (MLS = 2.81) for asthma was reported at a region around D12S390 (65 cM) in the Italian population (Malerba et al. 2000). The region was close to the region suggested by a CAMP study (Raby et al. 2003). In a founder population, Hutterites, Ober et al. (1998) suggested linkage of D12S375 (80 cM) to asthma in an earlier study, a finding not replicated in a more recent study (Ober et al. 2000). Chromosome 12 showed no evidence of linkage to asthma in Finnish families (Laitinen et al. 2001). A genome-wide study of the French population failed to detect a linkage to asthma but detected linkage to eosinophilia around the 130 cM region (Dizier et al. 2000). There was no evidence of

linkage to asthma-related phenotypes of chromosome 12 markers in the Chinese population, which is ethnically close to the Japanese population (Xu et al. 2001b).

In combination, the results of our and previous studies suggest that several genes located in 12q24–q33 are likely to be responsible for susceptibility to asthma. Some susceptibility genes of this region may be shared by several populations, and others may not. The MLS at the position of *NOS1* (128 cM), an association with which was detected in this study, was less than 1.0. The *NOS1* locus was 20 cM apart from the peak of the MLS. Thus, the region we detected in the present study most likely contains other asthma-susceptibility gene(s). In the 12q24 region, more than a hundred genes are listed in the Human Genome Map. Narrowing down the linked region by dense mapping and an intensive survey of SNPs in this region will be required to identify new susceptibility genes.

We investigated the existence of QTL for total IgE on chromosome 12 using asthma sib pairs. No region on chromosome 12 showed significant linkage to total IgE. Although type-2 error (false negative) could not be excluded due to the relatively small sample size, we speculate that loci in 12q22–q23 are asthma-susceptibility loci rather than QTLs that affect the total IgE level in the Japanese population. Using HE sib-pair QTL approaches, Barnes et al. (1996) reported that markers from 107 to 135 cM showed evidence of linkage to log (total IgE) in Barbados and Amish families. Xu et al. (2000) suggested linkage (LOD score = 2.73) to log(total IgE) of the region from *PAH* (108 cM) and D12S2070 (125 cM) in the Danish population using variance-component linkage analysis. Analysis of total IgE as a dichotomous trait (high versus normal IgE) was also performed by Nickel et al. (1997). They performed TDT in German children and presented evidence of association between markers located from 75 to 108 cM and high total IgE. On the other hand, many studies have shown negative linkage for total IgE. These include studies of the Australian population by Daniels et al. (1996), the Germans by Heinzmann et al. (2000a), the Germans and Swedish by Wjst et al. (1999), the Hutterites by Ober et al. (2000), the Chinese by Xu et al. (2001b), the French by Dizier et al. (2000), the Finnish by Leitinen et al. (2001), the Italians by Malerba et al. (2000), and the Danish by Haagerup et al. (2002). This inconstancy suggests that the relative importance of chromosome 12 loci in controlling total IgE varies from population to population.

We detected association of *STAT6* exon 1 GT repeat with Japanese childhood asthma. Our results clearly show association of the 15-repeat allele (allele 3) and asthma by allelic and genotypic distribution analyses. In Tamura's study using fewer samples than the present study, the number of heterozygote of allele 1 and allele 3 was significantly higher in patients with "allergic diseases" and that of homozygotes of allele 3 was lower in the patients with marginal statistical significance (Tamura et al. 2001). This observation was further confirmed by our present results: a change in the frequency of allele 3 homozygotes is more essential than that in the frequency of allele 1/allele 3 heterozygotes. Allele 3 appears to be a protective allele against the development of asthma in Japanese childhood asthma.

We also investigated the 2964G/A polymorphism in the 3'-UTR that was reported to be associated with adult asthma by Gao et al. (2000b) but failed to detect association in our samples (data not shown). Similar results were reported by Deutsch et al. (2002). These observations suggest that the *STAT-6* exon 1 GT repeat, but not 2964 G/A, is an asthma-susceptibility polymorphism. Further study is necessary to establish whether the polymorphism is functionally relevant to disease development or only a marker for the true functional polymorphism(s).

Association of the *NOS1* intron 2 GT repeat with asthma was also demonstrated in this study. Another study also describing the association of this polymorphism with asthma was carried out in the British population (Gao et al. 2000a). The 16-repeat allele of *NOS1* is a protective allele against asthma development in both British and Japanese populations, suggesting that this allele represents an old protective haplotype that evoked before the division of races. It is therefore possible that this polymorphism is associated with the disease in many ethnic groups. Moreover, Grasemann et al. (2000) have shown that *NOS1* exon 29 CA repeat in the 3'-UTR was associated with asthma in the US population. This is additional evidence that *NOS1* is a susceptibility gene for asthma in diverse populations. Again, further study is required to conclude whether these known polymorphisms are functionally relevant to the disease development or only markers for the true functional polymorphism(s).

We were unable to detect any association between *IFNG* and asthma in our series. Nakao et al. (2001) reported that allele frequency distribution differed between their 218 controls and 158 patients with atopic asthma in the Japanese population. Deviation of the distribution was primarily derived from the difference in frequency of allele 5 ($P = 0.0069$, $Pc = 0.048$). Frequencies in controls and patients of this allele were 4.8% and 1.3% respectively. In our study, the corresponding frequencies were 2.0% and 1.3% respectively. If we combine Nakao's and our data, P values become 0.012, which is not statistically significant after correction for the number of alleles ($Pc = 0.083$). More subjects are required to evaluate the significance of the association. Even if the association was confirmed, allele 5 would affect less than 5% of the population, i.e., the attributable fraction of this polymorphism would be low.

Although association of *AICDA* 465C/T (His155His) and asthma was suggested by TDT (Noguchi et al. 2001), our case-control study failed to prove the association of this polymorphism with asthma in the same population. In allelic distribution, allele frequency was essentially the same between the patient and control groups. Genotype distribution of the patients was not in