Table 2. Clinical characteristics of patients with AD

Number of subjects	382
Mean age (years)	30.2 (16–65)
Gender (M/F)	189/193
Log serum total IgE (mean ± SD)	3.36 ± 0.74
IgE ≤ 250 IU/ml (%)	11.4
IgE > 250 IU/ml (%)	88.6
IgE > 400 IU/ml (%)	84.1
IgE > 1800 IU/ml (%)	60.5
Blood eosinophil count >500/μl (%)	42.1
Early age of disease onset ≤3 years (%)	54.5
History or coexisting condition (%)	
Childhood asthma	25.9
Allergic rhinitis	51.8

two-locus haplotype in AD, childhood asthma and control samples. Of the two promoter *IL12RB1* haplotypes that constituted the −111A/T and −2C/T SNPs, the −111A/−2C haplotype was the most common, followed by −111T/−2T and −111T/−2C. Association studies for each haplotype with AD identified a borderline-significant association of both the −111A/−2C and −111T/−2T haplotypes with AD (Table 6). For child asthmatics, results of the haplotype analysis showed a significant association (global $P = 0.005$). These associations were not stronger than those observed for the single loci. However, these results cannot exclude the possibility that any other variants lying on the −111T/−2T haplotype may be involved in AD and asthma. We also analyzed the global distribution of *IL12RB1* haplotypes comprising the eight SNPs between the patient and control groups. Ten haplotypes, each of which consisted of eight SNPs that had a calculated frequency >1%, were observed in the control group (data not shown). These 10 haplotypes accounted for more than 95% of all haplotypes estimated in the study population.

Expression analysis of *IL12RB1* in human tissue panels

Previous reports have shown that IL-12R is detected mostly on activated T cells and NK cells (25) and that dendritic cells express a single class of high-affinity IL-12R (26). IL-12Rβ1 has also been detected on human B cell lines and activated tonsillar B lymphocytes (27). In this study, to confirm the expression of *IL12RB1* mRNA transcripts in target cells, we carried out RT–PCR analysis of multiple tissue cDNA panels. We observed *IL12RB1* expression in various tissues including spleen and lymph nodes, as well as in activated mononuclear and $CD4^+$ cells (Fig. 3). Furthermore, as shown previously (27), we also detected transcripts in lymphohematopoietic cell lines (Jurkat, Daudi, MOLT3, MOLT4, THP-1 and U937), but not in HL60, Hela and HEK293 cell lines (data not shown).

Identification of the regulatory elements in the *IL12RB1* promoter, transcriptional effect of −111A/T SNP and association of the −111 genotype with *IL12RB1* mRNA expression

We constructed a deletion panel of the *IL12RB1* upstream region encompassing nucleotides −2947 to −65 (Fig. 4A). Transcriptional activity of the promoter constructs derived from the wild-type allele (−2252G/−2060A/−2004A/−111A/−2C) was analyzed in transiently transfected Jurkat, HEK293 and THP-1 cells by measuring firefly luciferase activity. The relative changes in transcriptional activity among the *IL12RB1* promoter constructs were generally similar between the cell lines (data not shown). Deletion of 5′ sequence between −762 and −265 significantly increased activity (1.6-fold in Jurkat cells, $P < 0.01$), which suggested the presence of a silencer in the −762/−265 region. Of the promoter constructs tested, the −265/−65 fragment showed the highest activity. Further, 5′ deletion of this fragment to −104/−65 caused a dramatic reduction of promoter activity to 23% of the −265/−65 fragment ($P < 0.001$), which suggested that the −265/−104 region contained an enhancer element.

To determine the effect of the A/T polymorphism at position −111 on promoter activity, we transiently expressed −111A and −111T luciferase reporter constructs (pGL3/−111A and pGL3/−111T, respectively) in Jurkat cells. Luciferase activity in cell extracts was analyzed 24 h after transfection and was standardized against internal control *Renilla* activity. Results indicated that the −111T construct that consisted of the −265/−65 fragment showed a significant decrease in luciferase reporter activity when compared with the −111A construct (33%, $P < 0.01$; Fig. 4B). Similarly, the −111T construct had only 40–70% of the −111A luciferase activity in THP-1 cells and HEK293 cell lines (data not shown), which suggested that the −111A/T substitution impaired a functional promoter element. Thus, it appeared that the −111T allele was associated with decreased transcriptional activity of the *IL12RB1* gene. We also tested whether the C/T SNP at −2 affected *IL12RB1* promoter activity, using the −64/+64 fragment. Results indicated that the −64/+64 region that contained the −2C/T SNP had only slight activity in Jurkat cells (Fig. 4A). Moreover, no significant differences in expression levels were detected between −2C and −2T promoter constructs in transiently transfected Jurkat cells (data not shown). During this study, we had identified three SNPs in the *IL12RB1* promoter, which also showed a high degree of LD (−2252G/T, −2060A/G and −2004A/G). Deletion of 5′ sequence between −2947 and −1668 that contained these SNPs caused no obvious change in transcriptional activity. We also observed almost identical relative luciferase activity between −2252G/−2060A/−2004A and −2252T/−2060G/−2004G promoter constructs (data not shown).

Quantitative real-time PCR was performed to assess *IL12RB1* mRNA expression in peripheral blood mononuclear cells (PBMC) from healthy subjects with different −111 *IL12RB1* promoter genotypes (haplotypes). Relative values for *IL12RB1* mRNA expression were obtained by dividing the *IL12RB1* mRNA abundance by the *GAPDH* mRNA abundance. Results indicated that the *IL12RB1* mRNA expression was significantly higher in anti-CD3-stimulated cells carrying the −111A/A genotype (−111A/−2C homozygotes) when compared with those carrying the −111T allele (−111A/T or −111T/T genotypes) (−111AA versus AT + TT, $P = 0.019$; −111AA versus AT, $P = 0.021$; Fig. 4C). There was a similar tendency observed for unstimulated and PHA-stimulated cells, which was borderline significant ($P = 0.04$–0.06).

Table 3. Association of eight selected polymorphisms in *IL12RB1* with AD

RefSNP ID	JSNP ID	Position	SNP	Location	Amino acid change	Minor allele frequency		P^a	P^b	P^c
						AD ($n = 382$)	Controls ($n = 658$)			
rs393548	IMS-JST063138	−111	A/T	5′g	—	0.250	0.214	0.059	0.58	0.00044[d]
rs436857	IMS-JST063137	−2	C/T	5′-UTR	—	0.225	0.189	0.057	0.47	0.00075[d]
rs2305743	IMS-JST063136	4443	C/T	Intron 2	—	0.240	0.208	0.095	0.38	0.019
rs11086087	—	5970	G/C	Exon 4	V129V	0.200	0.209	0.64	0.37	0.43
rs375947	IMS-JST063134	17183	T/C	Exon 10	M365T	0.427	0.400	0.23	0.72	0.077
rs17882636	IMS-JST063132	17369	C/T	Intron 10	—	0.238	0.216	0.23	0.67	0.024
rs383483	—	25748	T/C	Intron 15	—	0.427	0.417	0.64	0.46	0.078
rs404733	IMS-JST097711	27637	A/T	3′g	—	0.446	0.440	0.26	0.96	0.057

[a]*P*-values for comparisons of allele frequencies between cases and controls.
[b]*P*-values for comparisons of genotype 11 versus 12 + 22 between cases and controls.
[c]*P*-values for comparisons of genotype 11 + 12 versus 22 between cases and controls.
[d]*P*-values statistically significant after correction for multiple comparisons.

Table 4. Genotype frequencies in Japanese AD cases and controls for *IL12RB1* SNPs at nucleotide positions −111 and −2

Polymorphism	Genotype	AD ($n = 382$) (%)	Controls ($n = 658$) (%)	OR (95%CI)	χ^2	*P*
−111 A/T	AA	221 (59.7)	396 (61.5)	1.00	—	—
	AT	113 (30.5)	221 (34.3)	0.92 (0.69–1.21)	0.38	0.54
	TT	36 (9.7)	27 (4.2)	2.39 (1.41–4.04)	11.1	0.00088
−2 C/T	CC	232 (63.2)	415 (65.5)	1.00	—	—
	CT	105 (28.6)	198 (31.2)	0.95 (0.71–1.26)	0.13	0.72
	TT	30 (8.2)	21 (3.3)	2.55 (1.43–4.57)	10.6	0.0011

Values are the number (%) of successfully genotyped chromosomes.

DISCUSSION

In this study, we identified 48 *IL12RB1* gene variants, including 10 novel variants, in a Japanese population. We selected eight representative SNPs from 36 common SNPs (MAF ≥ 10%) for further genotyping and association studies on an AD population. Our results showed that the *IL12RB1* promoter SNPs −111A/T and −2C/T were significantly associated with risk of AD under a recessive model ($P < 0.001$). Moreover, we observed a positive association between −111T/T genotype and total serum IgE levels in an IgE-level-dependent manner. The promoter SNPs were shown to be in strong LD with each other ($D' = 0.99$; $r^2 = 0.85$), and the percentage of −111T/T and −2T/T genotypes was much higher in AD patients than that in normal controls. Deletion analysis of the *IL12RB1* promoter indicated that the −265 to −104 region that contained the −111A/T polymorphic site harbored an important regulatory element. In addition, our data revealed that a single base substitution at the IL12RB1 −111 polymorphic site altered the transcriptional activity of the *IL12RB1* gene such that the wild-type *IL12RB1* (−111A) reporter construct was transcriptionally more active than the −111T construct in Jurkat cells. These results suggested that the A/T SNP at position −111 within the *IL12RB1* promoter affects the *IL12RB1* gene expression and contributes to increased risk of AD as well as raised total serum IgE levels. This is supported by our data showing the effects of the −111 genotype on *IL12RB1* mRNA levels in stimulated PBMC from healthy volunteers. We also found a trend for an association between total IgE level and *IL12RB1* genotype among our Japanese AD patients. Thus, presence of a particular *IL12RB1* allele may lower IL-12Rβ1 expression and allow the development of AD. To the best our knowledge, this is the first report to indicate that the *IL12RB1* gene may be involved in AD onset and IgE regulation.

When we analyzed *IL12RB1* gene haplotypes, the haplotypic findings of the promoter SNPs were weaker than that of individual SNP associations. These and our functional experiments suggested that these SNPs did not act in combination and that the −2 SNP located within the Kozak consensus sequence had little or no effect on translation efficiency. However, these findings could not exclude the possibility that polymorphisms elsewhere, in LD with the −111 and −2 SNPs, within or around the *IL12RB1* gene might also influence *IL12RB1* expression. Our AD patient subgroups analysis also revealed strong associations with the presence of high total serum IgE, early age of disease onset, peripheral blood eosinophilia and history of childhood asthma or allergic rhinitis. Moreover, the percentage of the −111T/T genotype was much higher in child patients with asthma than that in controls as well as in adult asthmatics. Thus, specific *IL12RB1* genotypes may predispose not only toward the development of AD but also toward other atopic conditions such as asthma and allergic rhinitis. In general, the clinical signs of AD predate the development of asthma and allergic rhinitis

Table 5. Genotype frequencies and case-control analysis of the *IL12RB1* −111 SNP in AD patient subgroups and childhood asthma

	−111 genotype			Genotype AA + AT versus TT		
	AA (%)	AT (%)	TT (%)	OR (95%CI)	χ^2	*P*
AD (Total)	221 (59.7)	113 (30.5)	36 (9.7)	2.46 (1.47–4.13)	12.4	0.00044
IgE ≤ 250 IU/ml	27 (64.3)	11 (26.2)	4 (9.5)	2.41 (0.80–7.23)	2.6	0.11
IgE > 250 IU/ml	194 (59.1)	102 (31.1)	32 (9.8)	2.47 (1.45–4.20)	11.8	0.00059
IgE > 400 IU/ml	183 (58.8)	97 (31.2)	31 (10.0)	2.53 (1.48–4.32)	12.3	0.00046
IgE > 1800 IU/ml	128 (57.1)	71 (31.7)	25 (11.2)	2.87 (1.63–5.06)	14.3	0.00015
Blood eosinophil count >500/μl	85 (52.8)	56 (34.8)	20 (12.4)	3.24 (1.77–5.94)	15.9	0.000068
Early age of disease onset ≤3year	115 (56.9)	62 (30.7)	25 (12.4)	3.23 (1.83–5.70)	17.9	0.000024
History of asthma in childhood	53 (54.6)	31 (32.0)	13 (13.4)	3.54 (1.76–7.12)	14.0	0.00018
History of allergic rhinitis	123 (64.4)	49 (25.7)	19 (9.9)	2.52 (1.37–4.65)	9.4	0.0022
Childhood asthma ($n = 304$)	162 (53.3)	114 (37.5)	28 (9.2)	2.32 (1.34–4.01)	9.5	0.0020

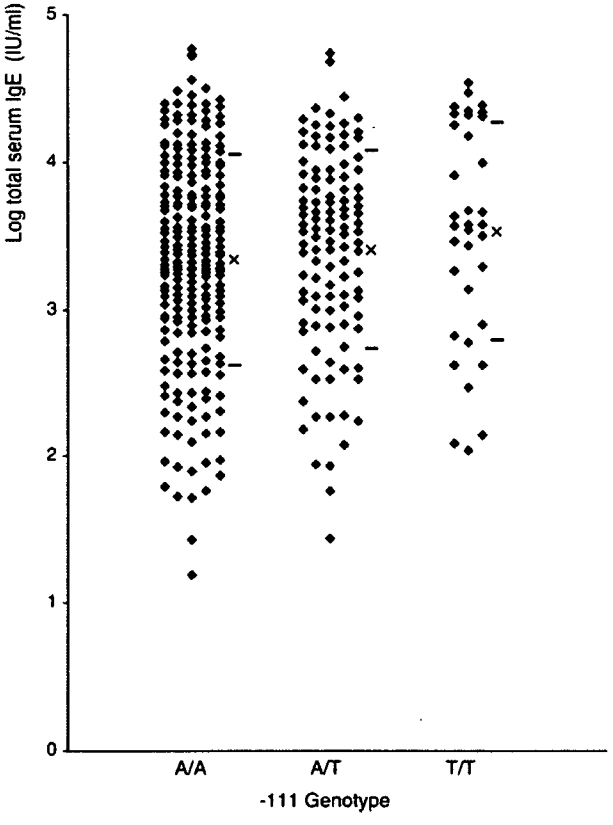

Figure 2. Relationship of *IL12RB1* −111 promoter genotype with total serum IgE levels in AD patients. Log-transformed individual IgE values are plotted, with the mean (×) and SD (bar) shown for each genotype group.

in the majority of affected patients, giving rise to the so-called 'atopic march', which suggests that AD is an initial step or entry point for subsequent allergic diseases (3,28). Therefore, early intervention in AD-susceptible individuals may be an effective strategy in preventing the atopic march. For this reason, *IL12RB1* genotype may be an important genetic marker.

The lymphocytes infiltrating unaffected skin or acute skin lesions in AD patients tend to be Th2-type T cells that produce IL-4, IL-5 and IL-13, whereas expression of IL-12 and the Th1 cytokine IFN-γ are increased in chronic eczematous AD skin lesions (8,29). In addition, AD is known to be associated with a high prevalence of skin infections, particularly involving *Staphylococcus aureus*. Recent studies have shown that at both the mRNA and protein levels, antibacterial peptides such as β-defensins and cathelicidin are decreased or deficient in skin lesions from AD patients when compared with those from psoriasis patients and that the combination of IL-4 and IL-13 inhibited the production of these antimicrobial peptides from keratinocytes (30,31). Although the exact mechanisms by which IL-12Rβ1 regulates these pathophysiological disease features remain unknown, one possibility is that excess Th2 cytokines block a. pathway of innate immune activation, leading to an increased susceptibility to skin infections. This in turn facilitates the continued activation of the adaptive immune system, including the recruitment and activation of atopic Th2 cells and perpetuation of the lesions (32). Therefore, our findings suggest that *IL12RB1* SNPs or haplotypes, which appear to affect protein expression or function, may predispose an individual toward the initiation or development of Th2-mediated immune responses in the skin. Further biological and population studies will be required to confirm the role of *IL12RB1* SNPs.

In contrast to AD, the immune response in psoriasis is Th1-mediated and is associated with local neutrophil infiltration. Recent data have indicated that expression of IL-23 and the IL-23-promoting cytokine IL-17 is increased in lesional skin samples of patients with psoriasis vulgaris. As IL-23 utilizes IL-12Rβ1 as part of its receptor, it is reasonable to speculate that the same promoter SNPs in *IL12RB1* that cause susceptibility to AD might be involved in psoriasis vulgaris as well. Our findings were in accordance with a recent study in Morocco that found an association of pulmonary tuberculosis (TB) with two promoter *IL12RB1* SNPs at −111 and −2 (24). Moreover, an association between heterozygous mutations of the *IL12RB2* gene and reduced IFN-γ production by PBMC following stimulation by IL-12 in some Japanese atopic subjects has also been reported (33) and *IL12B* has been identified as a susceptibility gene in patients with AD (18), asthma (34) and type 1 diabetes (35). These studies along with our present data suggest that functionally relevant SNPs in the IL-12/IL-12R and IL-23/IL-23R systems may be associated with the genetic susceptibility to a variety of diseases, including AD and TB.

Table 6. Structure and frequencies of two-locus haplotype in *IL12RB1*

Haplotypes (−111; −2)	−111	−2	AD ($n = 382$)	Childhood asthma ($n = 304$)	Controls ($n = 658$)	P^a	P^b
1	A	C	0.742	0.717	0.787	0.021	0.00081
2	T	T	0.227	0.238	0.188	0.035	0.011
3	T	C	0.027	0.043	0.025	0.741	0.028
4	A	T	0.004	0.002	0.001	0.095	0.57

[a]AD versus controls (global $P = 0.06$).
[b]Childhood asthma versus controls (global $P = 0.005$).

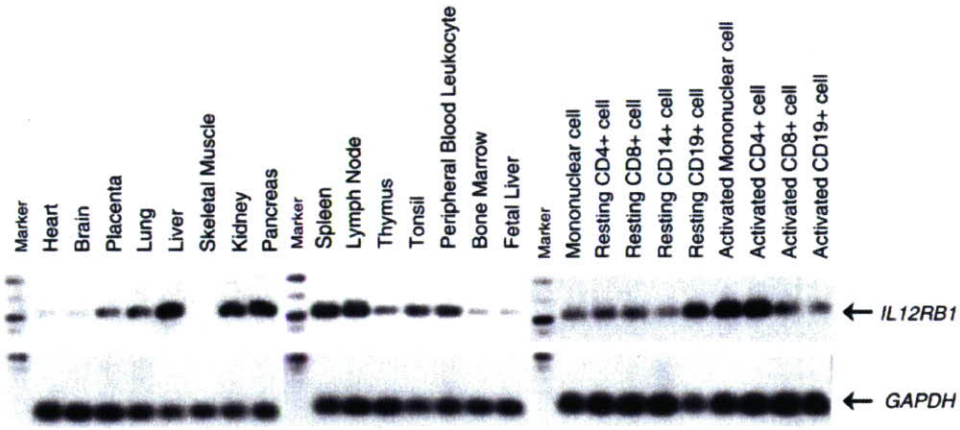

Figure 3. Expression of *IL12RB1* mRNA in multiple tissue cDNA panels. PCR amplification between *IL12RB1* exons 10 and 13 is shown in the upper panel. The *GAPDH* control is shown in the lower panel.

The human *IL12RB1* gene is located on chromosome 19p13.1. Although this region has not been previously implicated by AD linkage studies, a recent study suggested that chromosome 19p13 might harbor a genetic determinant of IgE-related traits (36). Another report showed modest evidence of linkage for atopic phenotypes on chromosome 19p13.3 in an Italian population (37), and a genome-wide scan of a large cohort of German families revealed significant evidence for a psoriasis-susceptibility locus on 19p13 (38). Thus, chromosome 19p13 may indeed contain immunoregulatory genes that influence inflammatory skin diseases such as AD and/or atopy-related phenotypes. In addition, the *IL12RB1* gene is a novel and attractive candidate susceptibility gene for skin inflammatory diseases such as AD, on the basis of its location as well as its function. However, further genetic analyses and biological studies will be required to address whether other atopic disease-related genes or SNPs are also present on 19p13.

In conclusion, we have identified 48 variants (10 novel) of the human *IL12RB1* gene. Our studies demonstrated that the −111T/T *IL12RB1* genotype was associated with high total serum IgE levels and AD susceptibility in a Japanese population. Furthermore, we showed that the −111A/T polymorphism affected the *IL12RB1* gene transcriptional activity and may contribute to low IL-12Rβ1 expression levels. In individuals with the −111T/T genotype, reduced IL-12Rβ1 expression may lead to increased Th2 cytokine production in the skin and contribute to the development of AD and other subsequent allergic diseases. Our findings also highlighted the importance of the IL-12–IFN-γ and/or IL-23–IL-17 pathway in the pathogenesis of AD and regulation of IgE. Although the exact functional role of IL-12Rβ1 in AD remains to be elucidated, the identification of SNPs in *IL12RB1* as a risk factor for AD may provide a strategy to prevent disease onset in susceptible individuals and to represent an attractive target for future therapies for this disorder.

MATERIALS AND METHODS

Study subjects

A total of 382 AD patients (mean age 30.2 years, range 16–65 years; 193 females and 189 males; mean total serum IgE level 2815 IU/ml) were recruited from Yokohama City University Hospital and Kyoto Takao Hospital. All patients with AD were diagnosed by dermatologists according to the criteria of Hanifin and Rajka (39). Controls for the SNP association study were 658 unrelated healthy individuals with an age range of 18–83 years, were from the same geographical areas as the AD patients and had no symptoms or personal and family histories of AD, asthma or allergic rhinitis. We also recruited 304 patients with childhood asthma with an age range of 4–15 years, who have been included in a previous study (40). All subjects in this study were ethnically

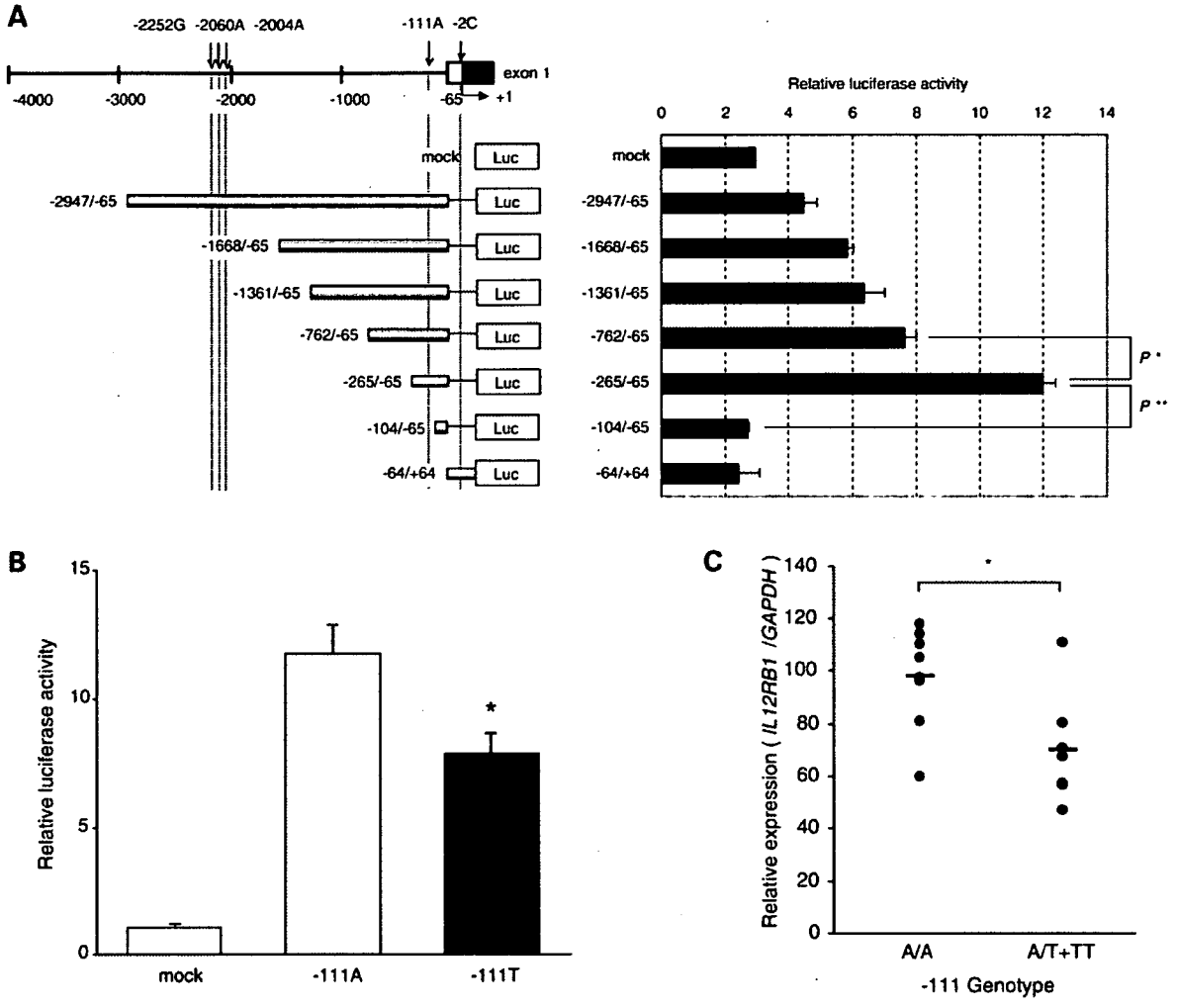

Figure 4. Identification of regulatory elements in the *IL12RB1* promoter, transcriptional effect of the −111A/T SNP and association of the −111 genotype with *IL12RB1* mRNA expression. (**A**) The indicated *IL12RB1* gene promoter fragments derived from wild-type allele sequence (−2252G/−2060A/−2004A/−111A/−2C) were cloned into the pGL3-basic vector and transiently cotransfected with pRL-TK vector as internal control. $^{*}P < 0.01$; $^{**}P < 0.001$. (**B**) Relative luciferase activities of constructs containing the human *IL12RB1* gene fragments (from −265 to −65 bp), with −111A or −111T, were compared in transient transfection assays using Jurkat cells. The relative luciferase activity of the *IL12RB1* reporter constructs is represented as the ratio of firefly luciferase activity to that of *Renilla*. Data are expressed as mean ± SD of three independent experiments performed in triplicate. $^{*}P < 0.01$. (**C**) Quantitative *IL12RB1* mRNA expression in stimulated PBMCs from healthy volunteers (A/A, $n = 8$; A/T, $n = 6$; T/T, $n = 1$). *IL12RB1* mRNA levels in cells from $IL12RB1^{-111A/A}$, $IL12RB1^{-111A/T}$ and $IL12RB1^{-111T/T}$ individuals following anti-CD3 stimulation are shown. Relative mRNA levels were defined as the net intensity of *IL12RB1* and *GAPDH* (*IL12RB1/GAPDH*). $^{*}P$ (A/A versus A/T+ T/T) = 0.019.

Japanese and gave written informed consent to participate in the study, according to the process approved by the Ethics Committee at the SNP Research Center, Institute of Physical and Chemical Research (RIKEN).

Screening for polymorphisms and genotyping

To identify genetic variants of the human *IL12RB1* gene, we sequenced all 17 exons, adjacent intronic sequence, 4.0 kb 5′ flanking region and 1.5 kb 3′-flanking region in 24 individuals from our study cohort. On the basis of *IL12RB1* genomic and mRNA sequences from the GenBank database (accession nos AC020904 and U03187, respectively), 25 primer sets were designed (Supplementary Material). All PCR products were sequenced using BigDye terminator v3.1 and an ABI Prism 3700 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA). The sequences were analyzed and polymorphisms identified using the SEQUENCHER program (Gene Codes Corporation, Ann Arbor, MI, USA). On the basis of information available from the public JSNP database (http://snp.ims.u-tokyo.ac.jp), eight selected SNPs were genotyped by one of two methods: the Invader assay (41) (for −111A/T:IMS-JST063138, −2C/T:IMS-JST063137, 4443C/T:IMS-JST063136, 17183T/C:IMS-JST063134 and 27637T/A:IMS-JST097711) and the TaqMan assay (for 5970G/C, 17369C/T and 25748T/C) on an ABI PRISM 7700 Sequence

Detector Systems (Applied Biosystems), according to the manufacturer's instructions. Probe sets for the Invader assay were designed and synthesized by Third Wave Technologies, and those for the TaqMan assay were obtained from Applied Biosystems.

Tissue expression

We assessed *IL12RB1* expression in a panel of cDNA tissue samples (Human Multiple Tissue, Human Immune System and Human Blood Fractions Multiple Tissues cDNA Panels, Clontech) by PCR amplification of target sequences and Southern blotting. The primer sets were 5′-CAGTGGCTC TGAATATCAGC-3′ and 5′-TGCACCGTGTAGGCTACACC-3′ for *IL12RB1* and 5′-CCCCATGTTCGTCATGGGT-3′ and 5′-GTGATGGCATGGACTGTGG-3′ for *GAPDH*. Southern blotting was performed with a non-radioactive nucleic acid labeling and detection kit (Roche Diagnostic, Basal, Switzerland), according to the manufacturer's instructions. The probes for *IL12RB1* and *GAPDH* were 5′-TGGCAACC TACAGCTGGAGT-3′ and 5′-CCATGAGAAGTATGACAA CAG-3′, respectively.

Luciferase assay

After restriction enzyme digestion with *KpnI* and *XhoI*, luciferase reporter constructs were generated by cloning the different promoter fragments of the *IL12RB1* gene into the pGL3-basic vector (Promega, Madison, WI, USA) between unique *KpnI* and *XhoI* sites. Forward primers used were: −2947/−65, 5′-CCACTTGGGCCTCAGTTTCC-3′; −1668/−65, 5′-CTGACATTTAGAGGCTTTGCC-3′; −1361/−65, 5′-CAAACTCCTGACCTCGTGATC-3′; −762/−65, 5′-CCG TGATTGCACCACTGCAC-3′; −265/−65, 5′-ACCCTGA CTTGCTCCAAAGTC-3′; −104/−65, 5′-TCTCCTTGCTC AGCTTC-3′, with 5′-CCGTCCCCACTCCGGAACAC-3′ used as a common reverse primer. Using plasmid DNA as template, −111T constructs were created using the QuickChange Site-Directed Mutagenesis kit (Stratagene, USA), forward primer (5′-CTTTTTTCTTTTTTTTCTGTCTTTTCTCCTTG CTCAGCTTC-3′), complementary reverse primer and the various pGL3-basic clones. The orientation and integrity of the inserts for each construct were confirmed by DNA sequencing. Jurkat cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO_2. Subconfluent cells cultured in 12-well plates were transiently cotransfected with 2 μg pGL3-basic vector DNA or each reporter construct and 40 ng pRL-TK vector DNA (Promega) as an internal control for transfection efficiency, using DMRIE-C transfection reagent (Invitrogen, San Diego, CA, USA) according to the manufacture's instructions. After 24 h, cells were harvested, and firefly and *Renilla* luciferase activities measured as previously described (40). Data are presented as relative luciferase activity of firefly/*Renilla* luciferase activity.

PBMC preparation

To analyze *IL12RB1* mRNA expression, PBMCs from 16 randomly selected healthy individuals were isolated by Ficoll density gradient centrifugation, after which 1×10^6 cells/ml PBMC were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 1% HEPES buffer, 1% L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids and 1% penicillin–streptomycin solution. PBMCs were stimulated with 100 μg/ml PHA or 100 μg/ml anti-CD3 antibody for 72 h at 37°C in a 5% CO_2 atmosphere.

Real-time quantitative PCR

Total RNA was isolated from cultured PBMC using the NucleoSpin 96 RNA kit (MACHEREY-NAGEL, Düren, Germany), according to the manufacturer's instructions. cDNA was then synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen). Real-time quantitative PCR was performed on the ABI PRISM 7900 (Applied Biosystems) using an Assay-on-Demand TaqMan probe and primers (Hs00234651_m1 for *IL12RB1*), according to the manufacturer's instructions. Relative expression levels of *IL12RB1* mRNA were normalized according to *GAPDH* expression, using a standard curve method as described by the manufacturer.

Statistical analysis

We calculated allele frequencies and tested agreement with Hardy–Weinberg equilibrium using a χ^2 goodness-of-fit test at each locus. We also compared differences in allele frequencies and genotype distribution of each polymorphism between case and control subjects, using a 2×2 contingency χ^2 test with one degree of freedom or Fisher's exact test. All *P*-values are unadjusted for multiple comparisons unless specifically indicated. We calculated LD coefficients (D' and r^2) using the SNP Alyze statistical package (Dynacom, Chiba, Japan), as described elsewhere (42). We estimated haplotype frequencies using the expectation–maximization algorithm. We calculated ODs with 95% confidence intervals (95%CI) using logistic regression. Comparisons in reporter assays as well as quantitative PCR experiments were performed using Student's *t*-test. Association between log-transformed total serum IgE levels in AD patients and individual genotypes was calculated by the Mann–Whitney *U*-test or the Kruskal–Wallis test. A *P*-value of less than 0.05 was considered to indicate statistical significance.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We thank Miki Kokubo, Aya Jodo and Hiroshi Sekiguchi for their technical assistance. This work was supported by grants-in-aid from the Ministry of Health, Labor and Welfare of Japan, the Japan Science and Technology Corporation and the Japanese Millennium Project.

Conflict of Interest statement. None declared.

REFERENCES

1. Rudikoff, D. and Lebwohl, M. (1998) Atopic dermatitis. *Lancet*, **351**, 1715–1721.
2. Williams, H., Robertson, C., Stewart, A., Ait-Khaled, N., Anabwani, G., Anderson, R., Asher, I., Beasley, R., Bjorksten, B., Burr, M. *et al.* (1999) Worldwide variations in the prevalence of symptoms of atopic eczema in the International Study of Asthma and Allergies in Childhood. *J. Allergy Clin. Immunol.*, **103**, 125–138.
3. Leung, D.Y., Boguniewicz, M., Howell, M.D., Nomura, I. and Hamid, Q.A. (2004) New insights into atopic dermatitis. *J. Clin. Invest.*, **113**, 651–657.
4. Larsen, F.S., Holm, N.V. and Henningsen, K. (1986) Atopic dermatitis. A genetic–epidemiologic study in a population-based twin sample. *J. Am. Acad. Dermatol.*, **15**, 487–494.
5. Schultz Larsen, F. (1993) Atopic dermatitis: a genetic–epidemiologic study in a population-based twin sample. *J. Am. Acad. Dermatol.*, **28**, 719–723.
6. Novak, N., Bieber, T. and Leung, D.Y. (2003) Immune mechanisms leading to atopic dermatitis. *J. Allergy Clin. Immunol.*, **112**, S128–S139.
7. Hamid, Q., Boguniewicz, M. and Leung, D.Y. (1994) Differential *in situ* cytokine gene expression in acute versus chronic atopic dermatitis. *J. Clin. Invest.*, **94**, 870–876.
8. Hamid, Q., Naseer, T., Minshall, E.M., Song, Y.L., Boguniewicz, M. and Leung, D.Y. (1996) *In vivo* expression of IL-12 and IL-13 in atopic dermatitis. *J. Allergy Clin. Immunol.*, **98**, 225–231.
9. Chan, L.S., Robinson, N. and Xu, L. (2001) Expression of interleukin-4 in the epidermis of transgenic mice results in a pruritic inflammatory skin disease: an experimental animal model to study atopic dermatitis. *J. Invest. Dermatol.*, **117**, 977–983.
10. Watford, W.T., Moriguchi, M., Morinobu, A. and O'Shea, J.J. (2003) The biology of IL-12: coordinating innate and adaptive immune responses. *Cytokine Growth Factor Rev.*, **14**, 361–368.
11. Abbas, A.K., Murphy, K.M. and Sher, A. (1996) Functional diversity of helper T lymphocytes. *Nature*, **383**, 787–793.
12. Presky, D.H., Yang, H., Minetti, L.J., Chua, A.O., Nabavi, N., Wu, C.Y., Gately, M.K. and Gubler, U. (1996) A functional interleukin 12 receptor complex is composed of two β-type cytokine receptor subunits. *Proc. Natl Acad. Sci. USA*, **93**, 14002–14007.
13. Wu, C., Ferrante, J., Gately, M.K. and Magram, J. (1997) Characterization of IL-12 receptor β1 chain (IL-12Rβ1)-deficient mice: IL-12Rβ1 is an essential component of the functional mouse IL-12 receptor. *J. Immunol.*, **159**, 1658–1665.
14. Wu, C., Wang, X., Gadina, M., O'Shea, J.J., Presky, D.H. and Magram, J. (2000) IL-12 receptor β2 (IL-12Rβ2)-deficient mice are defective in IL-12-mediated signaling despite the presence of high affinity IL-12 binding sites. *J. Immunol.*, **165**, 6221–6228.
15. Oppmann, B., Lesley, R., Blom, B., Timans, J.C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K. *et al.* (2000) Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity*, **13**, 715–725.
16. Leung, D.Y. and Bieber, T. (2003) Atopic dermatitis. *Lancet*, **361**, 151–160.
17. Rafatpanah, H., Bennett, E., Pravica, V., McCoy, M.J., David, T.J., Hutchinson, I.V. and Arkwright, P.D. (2003) Association between novel GM-CSF gene polymorphisms and the frequency and severity of atopic dermatitis. *J. Allergy Clin. Immunol.*, **112**, 593–598.
18. Tsunemi, Y., Saeki, H., Nakamura, K., Sekiya, T., Hirai, K., Fujita, H., Asano, N., Kishimoto, M., Tanida, Y., Kakinuma, T. *et al.* (2002) Interleukin-12 p40 gene (IL12B) 3′-untranslated region polymorphism is associated with susceptibility to atopic dermatitis and psoriasis vulgaris. *J. Dermatol. Sci.*, **30**, 161–166.
19. Olesen, A.B., Juul, S., Birkebaek, N. and Thestrup-Pedersen, K. (2001) Association between atopic dermatitis and insulin-dependent diabetes mellitus: a case–control study. *Lancet*, **357**, 1749–1752.
20. Shirakawa, T., Enomoto, T., Shimazu, S. and Hopkin, J.M. (1997) The inverse association between tuberculin responses and atopic disorder. *Science*, **275**, 77–79.
21. Sakai, T., Matsuoka, M., Aoki, M., Nosaka, K. and Mitsuya, H. (2001) Missense mutation of the interleukin-12 receptor β1 chain-encoding gene is associated with impaired immunity against *Mycobacterium avium* complex infection. *Blood*, **97**, 2688–2694.
22. Akahoshi, M., Nakashima, H., Miyake, K., Inoue, Y., Shimizu, S., Tanaka, Y., Okada, K., Otsuka, T. and Harada, M. (2003) Influence of interleukin-12 receptor β1 polymorphisms on tuberculosis. *Hum. Genet.*, **112**, 237–243.
23. van de Vosse, E., Lichtenauer-Kaligis, E.G., van Dissel, J.T. and Ottenhoff, T.H. (2003) Genetic variations in the interleukin-12/interleukin-23 receptor (β1) chain, and implications for IL-12 and IL-23 receptor structure and function. *Immunogenetics*, **54**, 817–829.
24. Remus, N., El Baghdadi, J., Fieschi, C., Feinberg, J., Quintin, T., Chentoufi, M., Schurr, E., Benslimane, A., Casanova, J.L. and Abel, L. (2004) Association of *IL12RB1* polymorphisms with pulmonary tuberculosis in adults in Morocco. *J. Infect. Dis.*, **190**, 580–587.
25. Desai, B.B., Quinn, P.M., Wolitzky, A.G., Mongini, P.K., Chizzonite, R. and Gately, M.K. (1992) IL-12 receptor. II. Distribution and regulation of receptor expression. *J. Immunol.*, **148**, 3125–3132.
26. Grohmann, U., Belladonna, M.L., Bianchi, R., Orabona, C., Ayroldi, E., Fioretti, M.C. and Puccetti, P. (1998) IL-12 acts directly on DC to promote nuclear localization of NF-κB and primes DC for IL-12 production. *Immunity*, **9**, 315–323.
27. Wu, C.Y., Warrier, R.R., Carvajal, D.M., Chua, A.O., Minetti, L.J., Chizzonite, R., Mongini, P.K., Stern, A.S., Gubler, U., Presky, D.H. *et al.* (1996) Biological function and distribution of human interleukin-12 receptor β chain. *Eur. J. Immunol.*, **26**, 345–350.
28. Spergel, J.M. and Paller, A.S. (2003) Atopic dermatitis and the atopic march. *J. Allergy Clin. Immunol.*, **112**, S118–S127.
29. Grewe, M., Gyufko, K., Schopf, E. and Krutmann, J. (1994) Lesional expression of interferon-γ in atopic eczema. *Lancet*, **343**, 25–26.
30. Nomura, I., Goleva, E., Howell, M.D., Hamid, Q.A., Ong, P.Y., Hall, C.F., Darst, M.A., Gao, B., Boguniewicz, M., Travers, J.B. *et al.* (2003) Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. *J. Immunol.*, **171**, 3262–3269.
31. Ong, P.Y., Ohtake, T., Brandt, C., Strickland, I., Boguniewicz, M., Ganz, T., Gallo, R.L. and Leung, D.Y. (2002) Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N. Engl. J. Med.*, **347**, 1151–1160.
32. Kupper, T.S. and Fuhlbrigge, R.C. (2004) Immune surveillance in the skin: mechanisms and clinical consequences. *Nat. Rev. Immunol.*, **4**, 211–222.
33. Matsui, E., Kaneko, H., Fukao, T., Teramoto, T., Inoue, R., Watanabe, M., Kasahara, K. and Kondo, N. (1999) Mutations of the IL-12 receptor β2 chain gene in atopic subjects. *Biochem. Biophys. Res. Commun.*, **266**, 551–555.
34. Morahan, G., Huang, D., Wu, M., Holt, B.J., White, G.P., Kendall, G.E., Sly, P.D. and Holt, P.G. (2002) Association of *IL12B* promoter polymorphism with severity of atopic and non-atopic asthma in children. *Lancet*, **360**, 455–459.
35. Morahan, G., Huang, D., Ymer, S.I., Cancilla, M.R., Stephen, K., Dabadghao, P., Werther, G., Tait, B.D., Harrison, L.C. and Colman, P.G. (2001) Linkage disequilibrium of a type 1 diabetes susceptibility locus with a regulatory *IL12B* allele. *Nat. Genet.*, **27**, 218–221.
36. Laitinen, T., Ollikainen, V., Lazaro, C., Kauppi, P., de Cid, R., Anto, J.M., Estivill, X., Lokki, H., Mannila, H., Laitinen, L.A. *et al.* (2000) Association study of the chromosomal region containing the *FCER2* gene suggests it has a regulatory role in atopic disorders. *Am. J. Respir. Crit. Care Med.*, **161**, 700–706.
37. Venanzi, S., Malerba, G., Galavotti, R., Lauciello, M.C., Trabetti, E., Zanoni, G., Pescollderungg, L., Martinati, L.C., Boner, A.L. and Pignatti, P.F. (2001) Linkage to atopy on chromosome 19 in north-eastern Italian families with allergic asthma. *Clin. Exp. Allergy*, **31**, 1220–1224.
38. Lee, Y.A., Ruschendorf, F., Windemuth, C., Schmitt-Egenolf, M., Stadelmann, A., Nurnberg, G., Stander, M., Wienker, T.F., Reis, A. and Traupe, H. (2000) Genomewide scan in German families reveals evidence for a novel psoriasis-susceptibility locus on chromosome 19p13. *Am. J. Hum. Genet.*, **67**, 1020–1024.
39. Hanifin, J.M. and Rajka, G. (1980) Diagnostic features of atopic dermatitis. *Acta Derm. Venereol.*, **92**(suppl.), 44–47.
40. Akahoshi, M., Obara, K., Hirota, T., Matsuda, A., Hasegawa, K., Takahashi, N., Shimizu, M., Nakashima, K., Cheng, L., Doi, S. *et al.* (2005) Functional promoter polymorphism in the *TBX21* gene associated with aspirin-induced asthma. *Hum. Genet.*, **117**, 16–26.
41. Ohnishi, Y., Tanaka, T., Ozaki, K., Yamada, R., Suzuki, H. and Nakamura, Y. (2001) A high-throughput SNP typing system for genome-wide association studies. *J. Hum. Genet.*, **46**, 471–477.
42. Nakajima, T., Jorde, L.B., Ishigami, T., Umemura, S., Emi, M., Lalouel, J.M. and Inoue, I. (2002) Nucleotide diversity and haplotype structure of the human angiotensinogen gene in two populations. *Am. J. Hum. Genet.*, **70**, 108–123.

Association of a haplotype block spanning *SDAD1* gene and *CXC* chemokine genes with allergic rhinitis

Jian Zhang, MD,[a] Emiko Noguchi, MD, PhD,[a,b] Ohsuke Migita, MD,[a,b] Yukako Yokouchi, MD, PhD,[a,b] Junko Nakayama, MD, PhD,[a,b] Masanao Shibasaki, MD, PhD,[c] and Tadao Arinami, MD, PhD[a] *Tsukuba City, Japan*

Background: Seasonal allergic rhinitis (SAR) is a common allergic disorder characterized by episodes of sneezing, rhinorrhea, and swelling of the nasal mucosa. Although the pathogenesis of SAR remains unclear, there does appear to be a genetic predisposition to development of SAR. We previously identified regions of chromosomes 1p, 4q, and 9q linked to SAR in 48 families (188 members) identified through children with SAR against orchard grass pollens.

Objective: The aim of the current study was to identify susceptibility genes for SAR on 4q.

Methods: We screened for markers associated with SAR on 4q with 17 microsatellite markers and then for mutations in 11 genes. We genotyped 44 single nucleotide polymorphisms (SNPs) in 48 SAR families and performed haplotype-based haplotype relative risk statistics implemented in the UNPHASED program. We also examined expression of genes with human multiple tissue and immune system cDNA panels.

Results: We found that 1 microsatellite marker, *D4S3042*, was associated with SAR ($P = .034$). The haplotype-based haplotype relative risk approach revealed that SNPs in SDA1 domain containing 1; chemokine, CXC motif, ligand *(CXCL)–9*; *CXCL10*; and *CXCL11* were associated with SAR ($P = .001$-$.04$). These SNPs made up a haplotype block, and the most common haplotype of this block was transmitted preferentially to affected offspring ($P = .002$).

Conclusion: Our results suggests that genetic variations in a haplotype block spanning the SDA1 domain containing 1 and *CXC* chemokine genes on 4q21 may contribute to development of SAR in the Japanese population. (J Allergy Clin Immunol 2005;115:548-54.)

Key words: *Seasonal allergic rhinitis, linkage, HHRR, CXC chemokine, SDAD1*

Abbreviations used

CXCL: Chemokine, CXC motif, ligand
HHRR: Haplotype-based haplotype relative risk
LD: Linkage disequilibrium
LOD: Logarithm of the odds
Mb: Megabase
OG: Orchard grass
SAR: Seasonal allergic rhinitis
SDAD1: SDA1 domain containing 1
SNP: Single nucleotide polymorphism

Atopic diseases such as asthma, allergic rhinitis, and atopic eczema are major causes of morbidity in developed countries and have been increasing in frequency.[1,2] The prevalence of rhinitis in various countries ranges from 3% to 19%.[3,4] Allergic rhinitis is characterized by mucosal inflammation in response to allergen exposure and is a common disease of complex inheritance. Twin and family studies have confirmed the existence of a genetic predisposition to the development of allergic diseases,[5-7] although a clear Mendelian pattern of inheritance has not been established. Several genome-wide screens for genes associated with allergic diseases have been conducted. Ten genome-wide screens for asthma,[8-17] 3 for atopic dermatitis,[18-20] and 2 for allergic rhinitis[21,22] have been reported. Haagerup et al[21] performed a genome-wide screen of 33 Danish families with sibling pairs affected by rhinitis caused by common environmental allergens; they found 1 major candidate region on chromosome 4q24-q27 (logarithm of the odds [LOD] = 2.83) and 8 minor candidate regions on 2q12-q33, 3q13, 4p15-q12, 5q13-q15, 6p24-p23, 12p13, 22q13, and Xp21 (LOD = 1.04-1.63) that were associated with allergic rhinitis. In our genome-wide screen for seasonal allergic rhinitis (SAR) triggered by orchard grass (OG),[22] we found evidence for linkage of marker *D4S392* at 4q13 to SAR (maximum LOD score = 2.01; P = .001), indicating that the chromosome 4q is one of the regions that contain susceptibility genes for SAR.

In the current study, we screened for mutations in 11 genes located in the 4q linkage region to identify genetic variations associated with SAR by haplotype-based haplotype relative risk (HHRR) statistics.

From [a]the Department of Medical Genetics and [b]the Department of Pediatrics, Majors of Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba; and [c]the Department of Pediatrics, Tsukuba College of Technology.

Supported by scientific research grants (12204001 and 12024201) from the Ministry of Education, Science and Culture of Japan.

Received for publication September 8, 2004; revised November 11, 2004; accepted for publication November 12, 2004.

Available online January 31, 2005.

Reprint requests: Emiko Noguchi, MD, PhD, Department of Medical Genetics, Majors of Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Laboratory of Advanced Research D, 1-1-1 Tennodai, Tsukuba, Ibaraki, 305-8577, Japan. E-mail: enoguchi@md.tsukuba.ac.jp.

0091-6749/$30.00

doi:10.1016/j.jaci.2004.11.034

METHODS

Subjects

Probands of the allergic rhinitis families were children with allergic rhinitis who were recruited from 3 elementary schools and a junior high school in Matsukawa, a farming community in central Japan where OG pollens are thought to be a major cause of SAR. Children with rhinitis were identified by means of a questionnaire, and diagnosis was confirmed by physicians. Other family members were contacted, and a total of 48 families (188 individuals) who gave informed consent participated in this study. The diagnostic criteria for SAR induced by exposure to OG included hay fever or persistent nasal symptoms, associated itching of the eyes or nose, and/or watering or redness of the eyes; regular occurrence of any symptom only between April and June; and positive position prick test or radioallergosorbent test reactivity to OG antigen (Torii, Tokyo, Japan).[22] OG-specific IgE levels and total serum IgE level were determined for all participants with the Pharmacia CAP system (Pfizer, New York, NY). To achieve homogeneity of the patients with SAR, only subjects with OG-specific IgE levels higher than 3.5 U_A/mL were included as affected subjects. Characteristics of the families with SAR are shown in Table I. This study was approved by the Committee of Ethics of the University of Tsukuba.

Microsatellite marker screening, single nucleotide polymorphism discovery, and genotyping

We genotyped 17 microsatellite markers between *D4S2638* and *D4S3042* spanning 18.8 megabase (Mb) to screen for association with SAR. The map location and distances between markers were obtained from the Marshfield Human Genetic Map (http://marshmed.org/genetics/). Because we observed a possible association of *D4S3042* with SAR, 11 genes around *D4S3042* and spanning 586 kb were selected for mutation screening. All exons, exon-intron junctions, and the 5′-flanking region of each gene were amplified by PCR from genomic DNAs isolated from 24 unrelated patients, and the products were subjected to direct sequencing with a Big-Dye Terminator Kit (Applied Biosystems, Foster City, Calif). Genotyping of single nucleotide polymorphisms (SNPs) was performed by RFLP analysis, Taqman Assay-on-Demand SNP typing (Applied Biosystems), or direct sequencing.

PCR screening of multiple tissue cDNA panels

We used human multiple tissue and human immune system cDNA panels (BD Biosciences/Clontech, Palo Alto, Calif) to analyze expression of SDA1 domain containing 1 *(SDAD1)*; chemokine, CXC motif, ligand *(CXCL)-9*; *CXCL10*; and *CXCL11* in various tissues by PCR. Primers were 5′-TGGCCCAAATGAGAAAAGAA-3′ and 5′-TCTTTTTCAAAAGTGCATCTCG-3′ for *SDAD1*; 5′-TTTTCCTCTTGGGCATCATC-3′ and 5′-GCTTTTTCTTTTGGCTGACC-3′ for *CXCL9*; 5′-ACCGTACGCTGTACCTGCAT-3′ and 5′-GCTCCCCTCTGGTTTTAAGG-3′ for *CXCL10*; and 5′-GCCTTGGCTGTGATATTGTG-3′ and 5′-AGATGCCCTTTTCCAGGACT-3′ for *CXCL11*. Amplification conditions were an initial denaturation step at 94°C for 2 minutes followed by denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. Samples were removed after a total of 26, 30, 34, and 38 cycles, and products were checked by electrophoresing in 2% agarose gel. Expected product sizes were 295 bp, 276 bp, 233 bp, and 299 bp for *SDAD1*, *CXCL9*, *CXCL10*, and *CXCL11*, respectively.

TABLE I. Characteristics of families with SAR*

Children (n)	123
Mean age (y)	14.1 ± 4.4
Sex ratio (M:F)	66:57
Log (total IgE [U_A/mL])	2.6 ± 0.55 (398.11)
Log (OG-RAST IgE [U_A/mL])	1.3 ± 0.54 (20.51)
Affected children (n)	104
Affected sibling pairs (n)	67

*Log (total IgE) and log (OG specific IgE) were means ± SDs. The values in parentheses were geometric means.

Statistical analysis

Deviation from predicted Hardy-Weinberg frequencies was examined by χ^2 test. Family-based association study by HHRR statistics was performed with the TDTPHASE command implemented in UNPHASED program version 2.43 (available at http://www.hgmp.mrc.ac.uk/~fdudbrid/software/unphased/).[23] EM and droprare options were used. Linkage disequilibrium between polymorphisms and haplotype block structures was evaluated by Haploview software version 2.05 (available at http://www.broad.mit.edu/mpg/haploview/index.php).[24] Haplotype blocks were generated by the default algorithm of Gabriel et al.[25]

RESULTS

In our genome-wide screen, we reported that a region of 4q was linked to SAR triggered by OG with a maximum LOD score of 2.01 at *D4S392*.[22] We genotyped 17 microsatellite markers between 4q12 *(D4S2638)* and 4q21 *(D4S3042)*. HHRR analysis revealed that *D4S3042* is associated with SAR ($P = .034$ for global test).

We then screened for mutations in 11 genes around *D4S3042* that span approximately 583 kb (Fig 1). Forty-seven SNPs were identified and genotyped in DNAs from 48 unrelated patients to evaluate allele frequencies and linkage disequilibrium (LD). Forty-four SNPs with minor allele frequencies greater than 0.05 were genotyped in 188 members of 48 families with SAR. The LD map constructed from 25 SNPs with minor allele frequencies greater than 0.2 is shown in Fig 2. LD was divided into 3 blocks. *D4S3042* was located in block 1, spanning 81.7 kb. There are 4 genes in the LD region: *SDAD1*, *CXCL9*, *CXCL10*, and *CXCL11*.

Haplotype-based haplotype relative risk revealed that SNPs in *SDAD1*, *CXCL9*, *CXCL10*, and *CXCL11* were associated with SAR (Table II); however, none was a nonsynonymous polymorphism. Because these polymorphisms were in 1 LD block, we evaluated haplotype associations with SAR. Only 2 common haplotypes had frequencies greater than 0.1, and the most common haplotypes were transmitted preferentially to the affected offspring (Table III). Selected haplotype tag SNPs (rs271528C/T and rs3733239T/G in *SDAD1*) also showed this association.

Expression of *SDAD1*, *CXCL9*, *CXCL10*, and *CXCL11* in various tissues was analyzed by PCR-based methods (Fig 3). *CXCL9* was expressed strongly in tonsils, spleen, lymph nodes, and thymus. *CXCL10* was expressed in

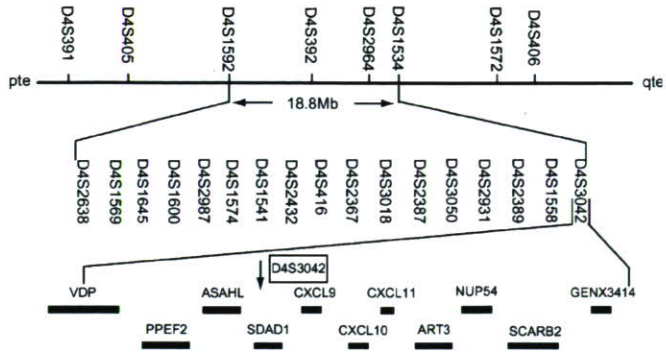

FIG 1. Genetic map of linkage region for SAR on chromosome 4q. *Top,* Microsatellite markers used in linkage study. *Middle,* Microsatellite markers used for dense mapping around *D4S392,* in which a maximum LOD score of 2.01 for SAR was observed in our genome-wide screen. *pte,* Petit telomere; *qte,* queue telomere. *Bottom,* location of genes around *D4S3042. Arrow* indicates location of *D4S3042. VDP,* Vesicle docking protein p 115; *PEF2,* protein phosphatase, EF hand calcium-binding domain 2; *ASAHL,* N-acylsphingosine amidohydrolase (acid ceramidase)-like; *ART,* ADP-ribosyltransferase 3; *NUP54,* nucleoporin 54kDa; *SCARB2,* scavenger receptor class B, member 2; *GENX-3414,* genethonin 1.

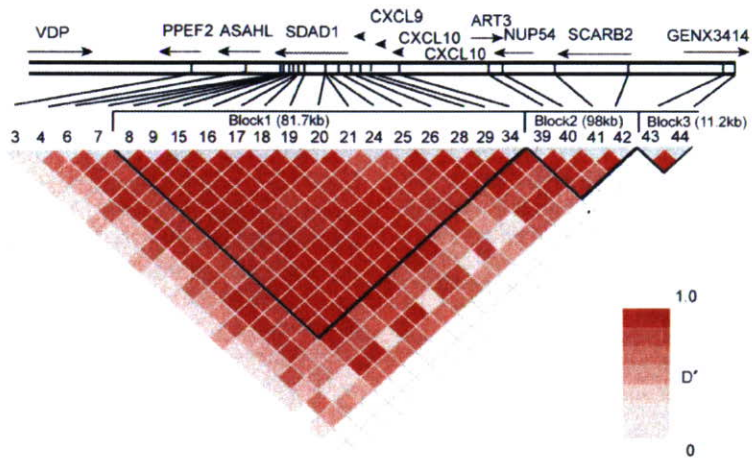

FIG 2. Pairwise linkage disequilibrium between polymorphisms in a 538-kb region as measured by *D'* in 188 subjects. LD is divided into 3 blocks. *D4S3042* is located in block 1. The *D'* values for LD are color-coded, and the extent of *red* indicates the strength of LD.

various organs and immune cells, including placenta, lung, liver, kidney, pancreas, spleen, lymph nodes, and thymus (Fig 3, *A and B*). In the blood fraction panel, *CXCL9* and *CXCL10* were expressed at higher levels in activated cells than in resting cells (Fig 3, *C*). *CXCL11* expression was observed in pancreas, spleen, and lymph nodes, but expression was very low in samples of the blood fraction panel. The expression level of *SDAD1* was very low. It was not observed in samples after 34 cycles of amplification, but it was detected in most of the tissues examined after 38 cycles of amplification (Fig 3, *A-C*).

DISCUSSION

Our data showed that haplotypes of the *SDAD1*, *CXCL9*, *CXCL10*, and *CXCL11* genes, which are located consecutively on chromosome 4q21, were associated with OG-specific SAR. LD is a nonrandom association between alleles at different loci, and it creates opportunities as well as difficulties in gene mapping. In our LD analysis with SNPs, 1 LD block was a 81.7-kb region where polymorphisms in 4 genes showed associations with SAR. These genes are in strong LD with each other, making it difficult to determine which SNP in which gene is associated with susceptibility to SAR. To our knowledge, the current study is the first to show an association between genetic variations in *CXCL9*, *CXCL10*, and *CXCL11* and an allergic disease; *CXCL9*, *CXCL10*, and *CXCL11* are likely to contribute to susceptibility to SAR.

Chemokine, CXC motif, ligand 9 (also called MIG [monokine induced by IFN-γ]); CXCL10 (also called IP-10 [IFN-γ–inducible protein of 10 kDa]); and CXCL11 (also called IP-9/I-TAC [IFN-γ–inducible

TABLE II. Results of HHRR in SAR families on chromosome 4q21

Gene		SNPs*	Location(NCBI Biuld 34)	Major allele	T/NT†	Minor allele	Minor allele freqactency	*P* value
VDP	1	rs3853184	77128575	C	118/120	T	0.024	.47
	2	rs905954	77194606	T	105/102	C	0.122	.56
PPEF2	3	rs2280100	77267964	C	67/69	T	0.495	.80
ASAHL	4	rs3733233	77305546	T	71/65	A	0.446	.42
	5	rs11732759	77314835	T	109/111	C	0.086	.58
SDAD1	6	rs3796482	77330701	A	62/74	G	0.463	.13
	7	rs3796483	77331079	T	68/72	C	0.43	.61
	8	rs3796484	77332909	A	75/58	T	0.423	.03
	9	rs2242470	77337083	C	75/58	T	0.423	.03
	10	rs2242471	77337111	C	114/115	G	0.077	.78
	11	rs2242742	77337122	C	113/112	G	0.048	.82
	12	IVS18+44C/T	77337325	C	114/115	T	0.074	.78
	13	rs2242473	77337351	A	114/115	G	0.074	.78
	14	1415C/A	77339749	C	106/108	A	0.165	.69
	15	IVS16-47A/G	77339787	G	61/78	A	0.455	.03
	16	rs4859577	77339796	A	75/56	G	0.423	.01
	17	rs1857821	77340374	T	75/58	C	0.423	.03
	18	rs2271527	77343818	A	61/78	G	0.455	.03
	19	rs2271528	77347231	C	77/56	T	0.426	.007
	20	rs3733238	77361134	T	77/56	C	0.426	.006
	21	rs3733239	77361170	T	56/73	G	0.452	.03
	22	rs3733240	77361370	A	120/107	G	0.048	.001
	23	rs10008757	77361431	C	115/116	T	0.079	.79
	24	rs10021434	77361690	C	44/65	G	0.421	.006
	25	rs6838162	77370623	C	58/72	G	0.484	.07
CXCL9	26	rs2869460	77380209	T	60/44	C	0.447	.04
	27	rs10336	77381383	G	114/115	A	0.08	.78
	28	rs2276886	77386823	C	75/56	T	0.423	.014
CXCL10	29	rs2869462	77393114	C	75/58	G	0.432	.03
	30	rs4859588	77402072	A	114/115	G	0.08	.78
	31	rs4241578	77402886	C	115/116	T	0.079	.79
CXCL11	32	rs13130221	77414018	A	115/116	G	0.079	.79
	33	rs6532111	77414309	T	115/116	C	0.079	.79
	34	rs12649185	77414574	G	75/58	G	0.423	.03
	35	rs4241580	77414583	C	115/116	T	0.079	.79
	36	rs4859415	77414923	A	115/116	G	0.079	.79
	37	IVS1+25T/C	77415572	T	115/116	C	0.079	.79
ART3	38	rs10017484	77443331	T	107/109	G	0.152	.68
	39	rs6829592	77477967	A	56/66	G	0.481	.18
NUP54	40	HCV158575	77487361	G	76/69	A	0.414	.36
SCARB22	41	HCV7919722	77522909	T	94/103	C	0.239	.14
	42	rs999361	77575969	G	66/53	A	0.487	.09
GENX3414	43	rs736634	77648664	T	58/74	C	0.457	.03
	44	rs1036788	77659882	T	75/66	C	0.417	.23

*Name of SNPs are according to reference SNP numbers (http://www.ncbi.nlm.nih.gov/SNP/), Celera discovery system (http://www.celeradiscoverysystem.com/), or gene nomenclature systems.
†Number of transmitted/nontransmitted alleles.

T cell α-chemoattractant]) are IFN-γ–inducible chemokines that preferentially attract T_H1 lymphocytes through the CXC chemokine receptor, which is a G protein–coupled receptor expressed at high levels on T_H1 lymphocytes.[26-29] Although these chemokines activate the same receptor and are functionally and structurally related, CXCL9 and CXCL10 differ in their patterns of expression in response to inducers such as *Plasmodium yoelii, Toxoplasma gondii*, and vaccinia virus, suggesting specific roles *in vivo*.[30] In human inflammatory skin diseases, *CXCL10* and *CXCL11* were expressed mainly by basal keratinocytes, whereas *CXCL9* mRNA was expressed predominantly in dermal infiltrates.[31] Monoclonal antibody neutralization revealed that CXCL10 is required for survival of mice after infection with *Toxoplasma gondii* and can not be compensated for by other CXCR3 ligands.[32] These data support the idea that these chemokines have different functional roles.

Allergic rhinitis is considered a T_H2-type disease like asthma or atopic dermatitis. The proportion of IL-4–producing lymphocytes increases significantly at the peak of the birch pollen season in comparison with the proportion outside the pollen season in patients with allergic rhinitis, whereas this trend is not observed in nonallergic

TABLE III. Haplotype association*

Haplotype	Freq	T	Freq-T	NT	Freq-NT	OR	χ^2	Individual	Global
ACAATGCTGGGTCCG	0.397	54	0.5	32	0.2963	4.125	9.432	0.002132	0.02
TTGGCATCTCCCTGA	0.389	35	0.3241	48	0.4444	1.782	3.317	0.06856	
ACGATACTTGCCCCG	0.078	10	0.0926	6	0.05556	4.074	1.091	0.2963	
Haplotype tag SNP (rs2271528C/T-rs3733239T/G) *(SDAD1)*									
-----C-G-----	0.4438	60.99	0.5258	41.97	0.3618	1	6.353	0.01172	0.007
-----T-T-----	0.4265	39.99	0.3447	58.97	0.5083	0.467	6.384	0.01152	
-----C-T-----	0.1123	14.01	0.1121	12.03	0.1124	0.686	0.17	0.6802	

Freq, Frequency of haplotypes; *freq-T(NT)*, frequency of transmitted (nontransmitted) haplotypes; *T (NT)*, number of transmitted (nontransmitted) haplotypes; *OR*, odds ratio.

*The order of the SNPs in haplotype is as follows: rs3796484, rs224270, IVS16-47A/G, rs4859577, rs1857821, rs2271527, rs2271528, rs3733238, rs3733239, rs10021434, rs6838162 *(SDAD1)*/rs2869460, rs2276886 *(CXCL9)*/rs2869462 *(CXCL10)*/rs12649185 *(CXCL11)*.

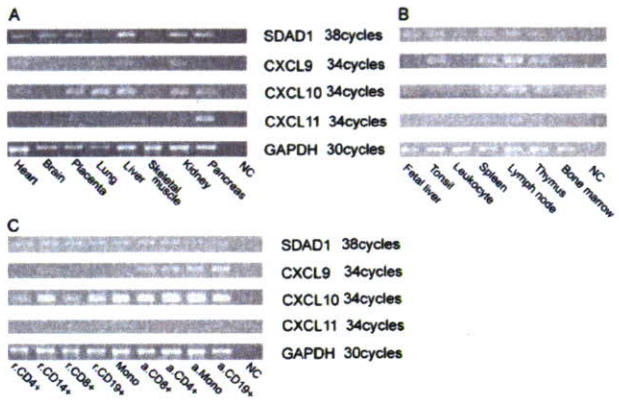

FIG 3. Expression of *SDAD1*, *CXCL9*, *CXCL10*, and *CXCL11* in human multiple tissue panels. PCR amplifications of cDNA from **(A)** body organs, **(B)** immune system, and **(C)** blood fractions are shown. *G3PDH* was used as internal control. *a*, Activated; *GADPH*, glyceraldehyde-3-phosphate dehydrogenase; *mono*, monocyte; *NC*, nontemplate control; *r*, resting.

controls.[33] CXCL9, CXCL10, and CXCL11 are T_H1-type chemokines induced by IFN-γ stimulation and are expressed in various T_H1-type diseases such as psoriasis[34] and multiple sclerosis.[35] There are conflicting data suggesting that T_H1 inflammation can both augment[36,37] and attenuate[38,39] allergic inflammation.

Expression of *CXCL9*, *CXCL10*, and *CXCL11* increases during the inflammatory phase of allergic contact dermatitis.[40] On the contrary, CXCL9 levels in the plasma of subjects with allergy decrease from the preseason to the grass pollen season.[41] Airway neutralization of CXCL9 at the time of allergen challenge significantly increases airway hyperreactivity, airway eosinophil accumulation, and IL-4 levels in bronchoalveolar lavage fluid and significantly decreases airway levels of IL-12. In contrast, introduction of exogenous CXCL9 into the airway at the time of allergen challenge dramatically reduces airway hyperreactivity and eosinophil accumulation.[42] CXCL9 is expressed at high levels in vernal keratoconjunctivitis.[43]

The increased level of CXCL10 in the plasma of patients with atopic dermatitis could be associated with increased activity of skin fibroblasts.[44] Production of *CXCL10* is increased in allergic pulmonary inflammation in a mouse model of asthma.[45] Keratinocytes contribute to the vigorous immigration by sequential expression of monocyte chemoattractant protein 1, RANTES, and CXCL10 induced by CXCL9, indicating that differential and sequential expression of multiple chemokines occurs during allergic contact hypersensitivity.[46] CXCL10 and CXCL9 are expressed in allergic patch test reactions.[47]

Compared with the *CXCL*s, less is known about the function of SDAD1. SDAD1 was initially identified in fetal liver at 22 weeks of gestation.[48] Relatively constant expression of *SDAD1* is observed in organs and immune systems; however, the expression levels are weaker than those of *CXCL*s. However, in the current study, the strongest genetic association with SAR was observed in SNPs in *SDAD1*. Therefore, the relation between the function of *SDAD1* and allergic diseases warrants further study.

We screened 17 microsatellite markers in the region spanning 18.8 Mb from 4q12 to 4q21 for association with SAR. We used all of the microsatellite markers listed in the Marshfield database for this region, and the distances between markers ranged from 17 kb to 3.2 Mb. Therefore, it is possible that genetic variations in regions of 4q12-q21 other than the *D4S3042* region examined in the current study are associated with SAR.

In conclusion, our data showed that genes in the haplotype block containing *CXCL9*, *CXCL10*, *CXCL11*, and *SDAD1* may be associated with SAR. Although the exact relations between *CXCL*s and *SDAD1* and SAR remain to be elucidated, our data may contribute to a better understanding of the pathophysiology underlying allergic rhinitis.

We thank Mrs Shirota, who helped collect samples, and all family members who participated in this study.

REFERENCES

1. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. Lancet 1998;351:1225-32.
2. Gergen PJ, Weiss KB. The increasing problem of asthma in the United States. Am Rev Respir Dis 1992;146:823-4.
3. Nakamura A, Asai T, Yoshida K, Baba K, Nakae K. [Allergic rhinitis epidemiology in Japan]. Nippon Jibiinkoka Gakkai Kaiho 2002;105: 215-24.
4. Skoner DP. Allergic rhinitis: definition, epidemiology, pathophysiology, detection, and diagnosis. J Allergy Clin Immunol 2001;108:S2-8.
5. Genes for asthma? an analysis of the European Community Respiratory Health Survey. Am J Respir Crit Care Med 1997;156:1773-80.
6. Hopp RJ, Bewtra AK, Watt GD, Nair NM, Townley RG. Genetic analysis of allergic disease in twins. J Allergy Clin Immunol 1984;73:265-70.
7. Hopper JL, Hannah MC, Macaskill GT, Mathews JD. Twin concordance for a binary trait, III: a bivariate analysis of hay fever and asthma. Genet Epidemiol 1990;7:277-89.
8. A genome-wide search for asthma susceptibility loci in ethnically diverse populations. The Collaborative Study on the Genetics of Asthma (CSGA). Nat Genet 1997;15:389-92.
9. Daniels SE, Bhattacharrya S, James A, Leaves NI, Young A, Hill MR, et al. A genome-wide search for quantitative trait loci underlying asthma. Nature 1996;383:247-50.
10. Dizier MH, Besse-Schmittler C, Guilloud-Bataille M, Annesi-Maesano I, Boussaha M, Bousquet J, et al. Genome screen for asthma and related phenotypes in the French EGEA study. Am J Respir Crit Care Med 2000; 162:1812-8.
11. Haagerup A, Bjerke T, Schiotz PO, Binderup HG, Dahl R, Kruse TA. Asthma and atopy—a total genome scan for susceptibility genes. Allergy 2002;57:680-6.
12. Laitinen T, Daly MJ, Rioux JD, Kauppi P, Laprise C, Petays T, et al. A susceptibility locus for asthma-related traits on chromosome 7 revealed by genome-wide scan in a founder population. Nat Genet 2001;28: 87-91.
13. Ober C, Tsalenko A, Parry R, Cox NJ. A second-generation genomewide screen for asthma-susceptibility alleles in a founder population. Am J Hum Genet 2000;67:1154-62.
14. Wjst M, Fischer G, Immervoll T, Jung M, Saar K, Rueschendorf F, et al. A genome-wide search for linkage to asthma. German Asthma Genetics Group. Genomics 1999;58:1-8.
15. Xu J, Meyers DA, Ober C, Blumenthal MN, Mellen B, Barnes KC, et al. Genomewide screen and identification of gene-gene interactions for asthma-susceptibility loci in three U.S. populations: collaborative study on the genetics of asthma. Am J Hum Genet 2001;68:1437-46.
16. Yokouchi Y, Nukaga Y, Shibasaki M, Noguchi E, Kimura K, Ito S, et al. Significant evidence for linkage of mite-sensitive childhood asthma to chromosome 5q31-q33 near the interleukin 12 B locus by a genome-wide search in Japanese families. Genomics 2000;66:152-60.
17. Hakonarson H, Bjornsdottir US, Halapi E, Palsson S, Adalsteinsdottir E, Gislason D, et al. A major susceptibility gene for asthma maps to chromosome 14q24. Am J Hum Genet 2002;71:483-91.
18. Bradley M, Soderhall C, Luthman H, Wahlgren CF, Kockum I, Nordenskjold M. Susceptibility loci for atopic dermatitis on chromosomes 3, 13, 15, 17 and 18 in a Swedish population. Hum Mol Genet 2002;11:1539-48.
19. Cookson WO, Ubhi B, Lawrence R, Abecasis GR, Walley AJ, Cox HE, et al. Genetic linkage of childhood atopic dermatitis to psoriasis susceptibility loci. Nat Genet 2001;27:372-3.
20. Lee YA, Wahn U, Kehrt R, Tarani L, Businco L, Gustafsson D, et al. A major susceptibility locus for atopic dermatitis maps to chromosome 3q21. Nat Genet 2000;26:470-3.
21. Haagerup A, Bjerke T, Schoitz PO, Binderup HG, Dahl R, Kruse TA. Allergic rhinitis—a total genome-scan for susceptibility genes suggests a locus on chromosome 4q24-q27. Eur J Hum Genet 2001;9:945-52.
22. Yokouchi Y, Shibasaki M, Noguchi E, Nakayama J, Ohtsuki T, Kamioka M, et al. A genome-wide linkage analysis of orchard grass-sensitive childhood seasonal allergic rhinitis in Japanese families. Genes Immun 2002;3:9-13.
23. Dudbridge F. Pedigree disequilibrium tests for multilocus haplotypes. Genet Epidemiol 2003;25:115-21.
24. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. Epub 2004 Aug 5.
25. Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, et al. The structure of haplotype blocks in the human genome. Science 2002;296:2225-9.
26. Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, et al. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. J Exp Med 1998;187:129-34.
27. Siveke JT, Hamann A. T helper 1 and T helper 2 cells respond differentially to chemokines. J Immunol 1998;160:550-4.
28. Loetscher M, Gerber B, Loetscher P, Jones SA, Piali L, Clark-Lewis I, et al. Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes. J Exp Med 1996;184:963-9.
29. Sallusto F, Lenig D, Mackay CR, Lanzavecchia A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. J Exp Med 1998;187:875-83.
30. Amichay D, Gazzinelli RT, Karupiah G, Moench TR, Sher A, Farber JM. Genes for chemokines MuMig and Crg-2 are induced in protozoan and viral infections in response to IFN-gamma with patterns of tissue expression that suggest nonredundant roles in vivo. J Immunol 1996; 157:4511-20.
31. Flier J, Boorsma DM, van Beek PJ, Nieboer C, Stoof TJ, Willemze R, et al. Differential expression of CXCR3 targeting chemokines CXCL10, CXCL9, and CXCL11 in different types of skin inflammation. J Pathol 2001;194:398-405.
32. Khan IA, MacLean JA, Lee FS, Casciotti L, DeHaan E, Schwartzman JD, et al. IP-10 is critical for effector T cell trafficking and host survival in Toxoplasma gondii infection. Immunity 2000;12:483-94.
33. Wosinska-Becler K, Plewako H, Hakansson L, Rak S. Cytokine production in peripheral blood cells during and outside the pollen season in birch-allergic patients and non-allergic controls. Clin Exp Allergy 2004;34:123-30.
34. Giustizieri ML, Mascia F, Frezzolini A, De Pita O, Chinni LM, Giannetti A, et al. Keratinocytes from patients with atopic dermatitis and psoriasis show a distinct chemokine production profile in response to T cell-derived cytokines. J Allergy Clin Immunol 2001;107:871-7.
35. Sorensen TL, Trebst C, Kivisakk P, Klaege KL, Majmudar A, Ravid R, et al. Multiple sclerosis: a study of CXCL10 and CXCR3 co-localization in the inflamed central nervous system. J Neuroimmunol 2002;127: 59-68.
36. Lack G, Bradley KL, Hamelmann E, Renz H, Loader J, Leung DY, et al. Nebulized IFN-gamma inhibits the development of secondary allergic responses in mice. J Immunol 1996;157:1432-9.
37. Cohn L, Homer RJ, Niu N, Bottomly K. T helper 1 cells and interferon gamma regulate allergic airway inflammation and mucus production. J Exp Med 1999;190:1309-18.
38. Li L, Xia Y, Nguyen A, Feng L, Lo D. Th2-induced eotaxin expression and eosinophilia coexist with Th1 responses at the effector stage of lung inflammation. J Immunol 1998;161:3128-35.
39. Hansen G, Berry G, DeKruyff RH, Umetsu DT. Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. J Clin Invest 1999;103:175-83.
40. Moed H, Boorsma DM, Tensen CP, Flier J, Jonker MJ, Stoof TJ, et al. Increased CCL27-CCR10 expression in allergic contact dermatitis: implications for local skin memory. J Pathol 2004;204:39-46.

41. Campbell JD, Stinson MJ, Simons FE, HayGlass KT. Systemic chemokine and chemokine receptor responses are divergent in allergic versus non-allergic humans. Int Immunol 2002;14:1255-62.
42. Thomas MS, Kunkel SL, Lukacs NW. Regulation of cockroach antigen-induced allergic airway hyperreactivity by the CXCR3 ligand CXCL9. J Immunol 2004;173:615-23.
43. Abu El-Asrar AM, Struyf S, Al-Kharashi SA, Missotten L, Van Damme J, Geboes K. The T-lymphocyte chemoattractant Mig is highly expressed in vernal keratoconjunctivitis. Am J Ophthalmol 2003;136:853-60.
44. Villagomez MT, Bae SJ, Ogawa I, Takenaka M, Katayama I. Tumour necrosis factor-alpha but not interferon-gamma is the main inducer of inducible protein-10 in skin fibroblasts from patients with atopic dermatitis. Br J Dermatol 2004;150:910-6.
45. Medoff BD, Sauty A, Tager AM, Maclean JA, Smith RN, Mathew A, et al. IFN-gamma-inducible protein 10 (CXCL10) contributes to airway hyperreactivity and airway inflammation in a mouse model of asthma. J Immunol 2002;168:5278-86.
46. Goebeler M, Trautmann A, Voss A, Brocker EV, Toksoy A, Gillitzer R. Differential and sequential expression of multiple chemokines during elicitation of allergic contact hypersensitivity. Am J Pathol 2001;158:431-40.
47. Flier J, Boorsma DM, Bruynzeel DP, Van Beek PJ, Stoof TJ, Scheper RJ, et al. The CXCR3 activating chemokines IP-10, Mig, and IP-9 are expressed in allergic but not in irritant patch test reactions. J Invest Dermatol 1999;113:574-8.
48. Yu Y, Zhang C, Zhou G, Wu S, Qu X, Wei H, et al. Gene expression profiling in human fetal liver and identification of tissue- and developmental-stage-specific genes through compiled expression profiles and efficient cloning of full-length cDNAs. Genome Res 2001;11:1392-403.