In summary, we used a candidate gene approach to assess the possible association of the Arg110Gln variant in *IL13* with cedar pollinosis in a Japanese population. Our results indicate that this functional coding SNP does not contribute significantly to JCP susceptibility. It is worthy of note that, to our knowledge, this study is the first detailed investigation showing a genetic association between the *IL13* variant Arg110Gln and hay fever and its related traits, i.e. serum levels of total and specific IgE, nasal eosinophila as well as disease severity. Further studies will be required to determine the relevance of other polymorphisms in the *IL13* gene as well as *IL13* haplotypes to the development of JCP in order to more accurately define the potential causative polymorphism underlying the pathogenesis of the disease.

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Functional haplotypes of *IL-12B* are associated with childhood atopic asthma

Tomomitsu Hirota, DDS,[a,j] Yoichi Suzuki, MD,[b] Koichi Hasegawa, MS,[c] Kazuhiko Obara, MS,[a] Akira Matsuda, MD,[a] Mitsuteru Akahoshi, MD,[a] Kazuko Nakashima, MS,[a,c] Lei Cheng, MD,[c] Naomi Takahashi, BS,[a] Makiko Shimizu, BS,[a] Satoru Doi, MD,[d] Kimie Fujita, MD,[e] Tadao Enomoto, MD,[f] Motohiro Ebisawa, MD,[g] Shigemi Yoshihara, MD,[h] Yusuke Nakamura, MD,[i] Fumio Kishi, MD,[j] Taro Shirakawa, MD,[c] and Mayumi Tamari, MD[a] *Kanagawa, Chiba, Kyoto, Osaka, Shiga, Wakayama, Tochigi, Tokyo, and Kagoshima, Japan*

Background: IL-12 is a heterodimeric proinflammatory cytokine that forms a link between innate and adaptive immunity. Although associations between polymorphisms of *IL-12B* on chromosome 5q31-33 and asthma have been reported, the genetic influences of the polymorphisms and haplotype of *IL-12B* are unclear.

Objective: To examine whether polymorphisms in *IL-12B* are associated with childhood atopic asthma in a Japanese population.

Methods: We identified a total of 13 polymorphisms and characterized the linkage disequilibrium mapping of the gene. Three variants in the promoter and 3′ untranslated region were genotyped, and we conducted case-control and case-only association studies between those variants and asthma-related phenotypes (childhood atopic asthma, n = 297; normal controls, n = 555). Haplotype association analysis and functional analysis of these variants were also performed.

Results: 3′ Untranslated region 10841C>A was significantly associated with the risk of childhood atopic asthma ($P = .00062$). The −6415 promoter variant, in linkage disequilibrium with the 10841C>A ($D' = 0.78$ and $r^2 = 0.61$), was also marginally associated with childhood atopic asthma ($P = .051$). We analyzed the 2-locus haplotype by using these 2 polymorphisms and found a positive association with haplotype CTCTAA-C (−6415 CTCTAA and 10841C; $P = .00078$). Furthermore, 10841C>A affects the stability of transcripts, and promoter variant −6415GC enhances the transcriptional level of *IL-12B*.

Conclusion: Our results imply that functional haplotype CTCTAA-C, which affects the instability of transcripts and the lower transcriptional level of *IL-12B*, has a protective effect in childhood atopic asthma. On the basis of these findings, the *IL-12B* gene might be involved in the development of atopic asthma through functional genetic polymorphisms. (J Allergy Clin Immunol 2005;116:789-95.)

Key words: *Asthma*, IL-12B, *polymorphism, association, linkage disequilibrium, haplotype*

Asthma is defined as a chronic inflammatory lung disease characterized by airway hyperreactivity and mucus hypersecretion that results in intermittent airway obstruction.[1] A growing body of evidence suggests that 2 subsets of T_H cells, T_H1 and T_H2, may play important roles in allergic disorders.[2,3] Although IL-12 is an immunomodulatory cytokine that is the primary inducer of the development of T_H1 cells with downregulation of T_H2 cytokines,[4,5] recent studies have shown that p80 (a p40 homodimer) and the p40 monomer have important roles in T_H2-type immune responses.[6-9] Because of alternative heterodimeric partnering and monomer secretion, IL-12 p40 encoded by *IL-12B* is a component of 4 secreted proteins: IL-12 (a p40 and p35 heterodimer),[5] IL-23 (a p40 and p19 heterodimer),[10] p80 (a p40 homodimer), and p40 (a p40 monomer).[6-9] Each protein has distinct biologic properties. There is strong evidence that IL-12 p40 is crucial for the etiology of asthma and for the induction of T_H2-type allergic inflammation in an animal model.[11-13] Recent studies have indicated that IL-12 p40 may function as an antagonist of IL-12 action.[6-9] Furthermore, increased IL-12 p40 homodimer (p80) levels are associated with enhanced airway macrophage accumulation and overexpression of IL-12 p40 selectivity in airway epithelial cells in subjects with asthma.[13]

IL-12B locates in 5q31.1-33.1, which is linked to asthma susceptibility and asthma-related phenotypes,[14-18] and is also one of the most consistently replicated regions

From [a]the Laboratory for Genetics of Allergic Diseases, Single Nucleotide Polymorphism Research Center, Institute of Physical and Chemical Research (RIKEN), Kanagawa; [b]the Department of Public Health, Graduate School of Medicine, Chiba University; [c]the Department of Health Promotion and Human Behavior, Kyoto University Graduate School of Public Health; [d]the Osaka Prefectural Medical Center for Respiratory and Allergic Diseases; [e]the College of Nursing, University of Shiga; [f]the Department of Otolaryngology, Japanese Red Cross Society, Wakayama Medical Center; [g]the National Sagamihara Hospital, Clinical Research Center for Allergy and Rheumatology, Kanagawa; [h]the Department of Pediatrics, Dokkyo University School of Medicine, Tochigi; [i]the Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, University of Tokyo; and [j]the Department of Microbiology and Immunology, Kagoshima University Dental School.

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Reprint requests: Mayumi Tamari, MD, PhD, Laboratory for Genetics of Allergic Diseases, SNP Research Center, Institute of Physical and Chemical Research (RIKEN), 1-7-22 Suehiro, Tsurumi-ku, Yokohama 230-0045 Kanagawa, Japan. E-mail: tamari@src.riken.jp.

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Abbreviations used
LD: Linkage disequilibrium
OR: Odds ratio
SNP: Single nucleotide polymorphism
tIgE: Total IgE
UTR: Untranslated region

in diverse populations. In the Japanese population, convincing evidence of linkage to atopic asthma susceptibility on chromosome 5q has been observed by sibling pair analysis.[14] Several polymorphisms have been identified in the *IL-12B* gene, and genetic studies have been conducted for these polymorphisms.[19-21] It is important to determine whether the finding is replicable in other ethnic or independent populations and examine the roles of the associated variants in the development of the asthma phenotype.

To test whether variants of *IL-12B* were related to asthma, we first performed linkage disequilibrium mapping of the gene and conducted an association study and haplotype analyses with regard to the linkage disequilibrium (LD) pattern. In addition, we performed functional analyses of the associated polymorphisms.

METHODS

Study subjects

All subjects with asthma were diagnosed according to the criteria of the National Institutes of Health[22] and demonstrated at least 12% improvement in their FEV_1 measurement after β_2-agonist inhalation. The diagnosis of atopic asthma was based on 1 or more positive skin scratch test responses to seven common aeroallergens in the presence of a positive histamine control and a negative vehicle control. The seven aeroallergens were house dust, *Felis domestics* dander (Fel d), *Canis familiaris* dander, *Dactylis glomerata*, *Ambrosia*, *Cryptomeria japonica* and *Alternaria alternata*. Peripheral blood was obtained from each of 297 pediatric atopic outpatients with asthma at the Osaka Prefectural Habikino Hospital and National Sagamihara Hospital (mean age, 9.6 years; range, 3-15 years; male:female ratio, 1.63:1.0; mean serum IgE level, 490 IU/mL; *Dermatophagiodes pteronyssinus* or *Dermatophagiodes farinae* RAST-positive, 80.0%). Specific IgE was considered positive when values exceeded 0.35 UA (arbitrary units)/mL (RAST score ≥ 1) according to an enzyme immunoassay. Serum IgE levels were $\log_{10}$-transformed before analyses. The mean of $\log_{10}$(total IgE [tIgE] [IU/mL]) of patients with childhood atopic asthma was 2.69 (= $\log_{10}$[490 IU/mL]). In this study, high IgE levels were defined as those values in the 75th percentile or higher for total IgE. The 75th percentile value of $\log_{10}$ (tIgE) in patients with childhood atopic asthma was 3.04 (= $\log_{10}$[1106 IU/mL]).[23] The severity of asthma was defined according to the degree of therapy required to control symptoms at the time of entry into the study. The grades were as follows: grade 1, β-agonists only; grade 2, sodium cromoglycate and/or theophylline; grade 3, inhaled beclomethasone 400 μg/d or less; grade 4, inhaled beclomethasone of more than 400 μg/d.[23] A total of 555 healthy individuals who had neither respiratory symptoms nor a history of asthma-related diseases (mean age, 45 years; range, 18-75 years; male:female ratio, 2.44:1.0) were recruited by physicians' interviews about whether they had been diagnosed with asthma and/or atopy. Because there was a large age difference between the cases and controls, we performed linear regression analysis between age and allele frequencies of genotyped single nucleotide polymorphisms (SNPs). R^2 values of all SNPs were less than 0.001, so there was no evidence of association between age and allele frequencies. All individuals were Japanese and gave written informed consent to participate in the study (or, for individuals younger than 16 years, their parents gave consent) according to the rules of the process committee at the SNP Research Center, Institute of Physical and Chemical Research (RIKEN).

Screening for polymorphisms and genotyping

To identify polymorphisms in the human *IL-12B* gene, we sequenced all 8 exons, including a minimum of 100 bases of the flanking intronic sequence, 2.98 kb of the 5′ flanking region, and a 0.2-kb continuous 3′ flanking region of the 8th exon from 24 subjects with asthma and 12 control subjects. Fourteen primer sets were designed on the basis of the *IL-12B* genomic sequence from the GenBank database (accession number AC011418.5; see Table E1 in the Online Repository in the online version of this article at www.jacionline.org). The sequences were analyzed and polymorphisms identified by using the SEQUENCHER program (Gene Codes Corp, Ann Arbor, Mich). The promoter polymorphisms, −6415 CTCTAA>GC and −5741G>C, were genotyped by use of the TaqMan system (Applied Biosystems, Foster City, Calif). For the 10841C>A 3′ untranslated region (UTR) polymorphism, genotyping was performed by PCR-RFLP analysis by using *Taq*I as described.[24]

Luciferase assay

The human embryonic kidney 293 cell line was obtained from the RIKEN cell bank. Three concatenated copies of the 20-bp or 16-bp DNA fragments were cloned into pGL3-basic vector (Promega, Madison, Wis) in the 5′-3′ orientation. The DNA fragments were as follows: for −6415CTCTAA, 5′-AGAGAGGCTCTAATGTGGCC-3′, and −6415GC, 5′-AGAGAGGGCTGTGGCC-3′. We then transfected subconfluent 293 cells (1×10^6) cultured in 12-well plates with 0.5 μg of each construct and 0.01 μg pRL-TK Renilla luciferase vector (Promega), an internal control for transfection efficiency, using 1.5 μL FuGENE 6 transfection reagent (Roche Diagnostics, Basel, Switzerland). After 24 hours, we lysed cells and measured luciferase activities in a luminometer by using the Dual-Luciferase Reporter Assay System. The relative luciferase activity of the *IL-12B* reporter constructs was represented as the ratio of the firefly luciferase activity to that of Renilla. Each experiment was repeated 3 times, and each sample was studied in triplicate as described.[25]

Stability of 2 types of *IL-12B* mRNA

We amplified genes encoding 2 *IL-12B* variants by PCR from cDNAs synthesized by using a first-strand cDNA synthesis kit (Invitrogen, Carlsbad, Calif) with PBMC total RNA. We cloned these amplified genes into the pENTR vector (Invitrogen) and constructed cDNA in pDEST14 (Invitrogen). After digestion with ClaI and *in vitro* expression using RiboMax Large Scale RNA Production System-T7 (Promega), we mixed and incubated 0.5 μg of each synthesized RNA and diluted whole-cell extracts of U937 cells (1:1500) at room temperature as described.[26] We detected RNA by using Northern blot hybridization and measured signal intensities of full-length RNAs.

Statistical analysis

We calculated allele frequencies and tested agreement with Hardy-Weinberg equilibrium by using a χ^2 goodness-of-fit test at each locus. We then compared differences in allele frequencies and genotype distribution of each polymorphism between case and

TABLE I. Locations and allele frequencies of polymorphisms in *IL-12B* screened with 36 Japanese subjects*

Polymorphism	Location	Sequence	Allele frequency (%)†	JSNP (IMS-JST)	NCBI
−6415 CTCTAA/GC	5′g	5′-AGAGG(CTCTAA/GC)TGTGG-3′	53	—	—
−6110 C/T	5′g	5′-CACTG(C/T)GGGAA-3′	50	—	rs2546890
−5741 G/C	5′g	5′-TGGTG(G/C)AGGTG-3′	18	—	—
−5027 G/A	5′g	5′-GGGAG(G/A)AAGTG-3′	2	—	—
−3624 C/T	Intron1	5′-TACCT(C/T)CCTCC-3′	2	—	—
−3312 G/A	Intron1	5′-TAAAG(G/A)ATTGT-3′	2	—	—
1521 A/G	Intron2	5′-TTAGC(A/G)AGCTC-3′	55	132913	rs3181217
1645 A/G	Intron2	5′-ACGAT(A/G)CAGTG-3′	55	132914	rs3181218
3778 G/A	Intron3	5′-ATAAG(G/A)GTTAA-3′	55	040193	rs2288831
6557 G/A	Intron5	5′-TGTGG(G/A)GTAGG-3′	2	—	—
9945 C/T	Intron6	5′-CTTCA(C/T)AGCTT-3′	2	—	rs11574790
10559 WT/delG	Intron7	5′-AGGAA(WT/delG)CCTGG-3′	55	—	rs3213111
10841 C/A	Exon8	5′-TAGTT(C/A)GATGC-3′	55	—	rs3212227

JSNP, Number from the Japanese SNP database (http://snp.ims.u-tokyo.ac.jp/); *IMS-JST*, Institute of Medical Science-Japan Science and Technology Agency; *NCBI*, number from the dbSNP of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/).
*Positions are numbered according to their position relative to the published *IL-12B* gene-containing clone (GenBank AC011418.5). Position 1 is the A of the initiation codon.
†Frequency of right indicated allele.

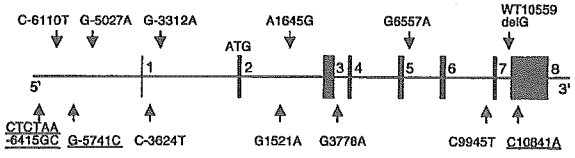

FIG 1. Graphical overview of polymorphisms identified in relation to the exon/intron structure of the human *IL-12B* gene. Eight exons are shown by *black boxes* with their numbers, and positions for polymorphisms are relative to the translation start site (+1). *Underlined* polymorphisms were genotyped in the whole samples.

control subjects by using a 2 × 2 contingency χ^2 test with 1 *df* and calculated odds ratios (ORs) with 95% CIs. Pairwise LD was calculated as |D′| and r^2 by using the SNP Alyze statistical package (Dynacom, Chiba, Japan) as described.[27] To infer the frequencies of haplotypes in control and patient groups and diplotypes in individuals with uncertain phases of genotypes, software programs PHASE version 2.0.2 (http://www.stat.washington.edu/stephens/software.html)[28] (University of Washington, Seattle, Wash) and HAPLOTYPER (http://www.people.fas.harvard.edu/~junliu/Haplo/docMain.htm)[29] (Harvard University, Cambridge, Mass) were used. Haplotype frequencies in cases and controls were evaluated both by the whole distribution with the Fisher exact test and by χ^2 tests of one haplotype against others (haplotype-wise test). We applied Bonferroni corrections, the multiplication of *P* values by the number of variants. Corrected *P* values of less than .05 were judged to be significant. Comparisons in reporter assays as well as mRNA degradation assays were performed with the Student *t* test. A *P* value of less than .05 was considered statistically significant.

RESULTS

We identified 13 biallelic polymorphisms in *IL-12B*: 4 in the 5′ flanking region, 1 in the 3′ UTR, and 8 in the intron (Table I and Fig 1). Seven polymorphisms were contained in the public databases available at Web sites. 10841C>A in exon 8 was reported previously as 1188C>A.[24] Rare SNPs with minor allele frequencies of 2% were not included in the analysis. Pairwise LD among

TABLE II. Pairwise linkage disequilibrium for all possible 2-way comparisons among 8 polymorphisms in *IL-12B* with 36 Japanese subjects

		SNP1 −6415 CTCTAA/GC*	SNP2 −6110 C/T	SNP3 −5741 G/C*	SNP4 1521 A/G	SNP5 1645 A/G	SNP6 3778 G/A	SNP7 10559 WT/delG	SNP8 10841 C/A*
SNP1	D′		1.00	1.00	0.78	0.78	0.78	0.78	0.78
	r^2		0.87	0.20	0.61	0.61	0.61	0.61	0.61
SNP2			D′	1.00	0.74	0.74	0.74	0.74	0.74
			r^2	0.22	0.45	0.45	0.45	0.45	0.45
SNP3				D′	0.35	0.35	0.35	0.35	0.35
				r^2	0.02	0.02	0.02	0.02	0.02
SNP4					D′	1.00	1.00	1.00	1.00
					r^2	1.00	1.00	1.00	1.00
SNP5						D′	1.00	1.00	1.00
						r^2	1.00	1.00	1.00
SNP6							D′	1.00	1.00
							r^2	1.00	1.00
SNP7								D′	1.00
								r^2	1.00

*Polymorphisms were genotyped in this case-control study.

8 SNPs with a frequency >0.15 was measured by different parameters, |D′| and r^2 (Table II). D′ is inversely biased with sample size, and the degree of bias will be greater for SNPs with lower allele frequencies. In contrast, r^2 is highly dependent on allele frequency.[30] There is no single best measure of LD under all possible situations; we used complementary measures, D′ and r^2.[30] The promoter variant, SNP1, was in strong LD with another promoter variant, SNP2 −6110C>T (D′ = 1.00 and r^2 = 0.87). Although SNP3 −5741G>C was in LD with SNP1 when it was evaluated by D′ (= 1.00), the R^2 value of LD between SNP1 and SNP3 was not so high (r^2 = 0.20). The 3′ UTR variant 10841C>A was in complete LD (D′ = 1.00 and r^2 = 1.00) with 1521A>G, 1645A>G, 3778G>A, and 10559WT>delG (Table II). We finally selected 3 polymorphisms with minor allele frequencies of more than

TABLE III. Association between polymorphisms of *IL-12B* and childhood atopic asthma

Locus	Allele1/2	Genotype	Childhood atopic asthma (%)	Control (%)	*P* value	OR (95% CI)
SNP 1	CTCTAA/GC	11	72 (25)	162 (30)	.051*†	1.28 (1.05-1.57)
−6415		12	132 (46)	273 (50)		
		22	81 (28)	112 (20)		
SNP 3	G/C	11	176 (64)	340 (62)	.55	1.08 (0.84-1.40)
−5741		12	88 (32)	194 (35)		
		22	10 (4)	19 (3)		
SNP 8	C/A	11	68 (23)	175 (32)	.00062*†	1.46 (1.20-1.79)
10841		12	144 (49)	273 (50)		
		22	84 (28)	101 (18)		

*P value corrected with Bonferroni correction (raw *P* values were multiplied by 3).
†Allele1 vs allele2.

TABLE IV. Frequencies of haplotypes and ORs in the control group and childhood atopic asthma group

	Haplotypes		Childhood asthma		Control				
	SNP1 CTCTAA/GC	SNP8 C/A	Number of allele	Ratio	Number of allele	Ratio	OR (95% CI)	χ^2 (*df* = 1)	*P* value*
1	CTCTAA	C	243	0.409	559	0.504	0.68 (0.56-0.84)	13.9	.00078
2	GC	A	275	0.463	433	0.390	1.35 (1.10-1.65)	8.46	.015
3	GC	C	37	0.062	70	0.063	0.99 (0.65-1.49)	0.00	>1
4	CTCTAA	A	39	0.066	48	0.043	1.56 (1.01-2.40)	4.01	.18
		Total allele	594		1110				

*P value corrected with Bonferroni correction (raw *P* values were multiplied by 4).

TABLE V. Frequencies of diplotypes and ORs in the control group and in the asthma group

Name	Number	Diplotype frequencies			OR (95% CI)	χ^2 (*df* = 1)	*P* value*
Haplotype 1		Homozygote	Heterozygote	Others			
Childhood asthma	297	0.178	0.461	0.360			
Controls	555	0.256	0.495	0.249	0.59 (0.43-0.80)†	11.8†	.0054†
Haplotype 2		Homozygote	Heterozygote	Others			
Childhood asthma	297	0.222	0.481	0.296			
Controls	555	0.141	0.499	0.360	1.75 (1.22-2.51)‡	9.20‡	.022‡

*P value corrected with Bonferroni correction (raw *P* values were multiplied by 9).
†Haplotype1/haplotype1 + haplotype1/others vs others/others.
‡Haplotype2/haplotype2 vs haplotype2/others + others/others.

15%, SNP1 promoter variant CTCTAA>GC, SNP3 −5741G>C, and SNP8 3′ UTR variant 10841C>A.

All of these loci were in Hardy-Weinberg equilibrium in the control group. The allele frequency of each selected polymorphism was compared between the patients and the normal controls by the χ^2 test using codominant, dominant, and recessive models. After correcting for the number of variants investigated (Bonferroni correction), we found a significant association between the 3′ UTR polymorphism at 10841 and childhood atopic asthma with the codominant model (P = .00021; corrected P=.00062). The promoter polymorphism, −6415 CTCTAA>GC, was marginally associated with childhood atopic asthma (P = .017; corrected P = .051). There was no significant association between SNP3 −5741G>C and childhood atopic asthma (Table III).

We further analyzed the haplotype structure by using 2 SNPs, SNP1 and SNP8, and associations of each of the 4 haplotypes with childhood atopic asthma as described (Table IV).[31] PHASE tests the null hypothesis that the case and control haplotypes are a random sample from a single set of haplotype frequencies, versus the alternative that cases are more similar to other cases than to controls. We obtained a P value of .0011 with 10,000 permutations and found a positive association with haplotype CTCTAA-C (P = .00039; corrected P = .00078; haplotype 1 vs others; Table IV). As shown in Table V, we examined the association of the diplotypes of *IL-12B* with asthma. The results suggested that a homozygote or heterozygote of haplotype 1 showed a lower risk for childhood atopic asthma (OR, 0.59; 95% CI, 0.43-0.80) and a homozygote of haplotype 2 showed a higher risk for

TABLE VI. Association between polymorphisms of *IL-12B* and childhood atopic asthma with related phenotype*

SNP		Samples	Genotype (%) 11	12	22	P†	OR (95% CI)
SNP1	Control	547	162 (30)	273 (50)	112 (20)		
−6415	Severity ≤ grade 2	156	42 (27)	68 (44)	46 (29)		
CTCTAA /GC	Severity ≥ grade 3	112	28 (25)	52 (46)	32 (29)		
	IgE < 1106 IU/mL	146	32 (22)	62 (42)	52 (36)		
	IgE ≥ 1106 IU/mL	116	40 (34)	70 (60)	29 (25)	.020‡	1.54 (1.11-2.15)‡
SNP8	Control	549	175 (32)	273 (50)	101 (18)		
10841	Severity ≤ grade 2	163	41 (25)	77 (47)	45 (28)		
C/A	Severity ≥ grade 3	116	26 (22)	54 (47)	36 (31)	.0042§	1.56 (1.17-2.07)§
	IgE < 1106 IU/mL	221	57 (26)	102 (46)	62 (28)		
	IgE ≥ 1106 IU/mL	75	11 (15)	42 (56)	22 (29)	.0024‖	1.76 (1.25-2.49)‖

*Total IgE ≥ 1106 IU/mL (=75th percentile of adult asthma patient's value).
†*P* value corrected with Bonferroni correction (raw *P* values were multiplied by 2).
‡Childhood atopic asthma with IgE < 1106 IU/mL vs with IgE ≥ 1106 IU/mL (allele1 vs allele2).
§Childhood atopic asthma with severity ≥ grade 3 vs control (allele1 vs allele2).
‖Childhood atopic asthma with IgE ≥ 1106 IU/mL vs control (allele1 vs allele2).

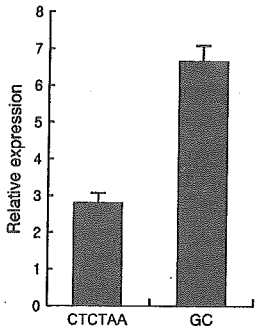

FIG 2. Effect of CTCTAA>GC polymorphism on the transcriptional activity of human *IL-12B* promoter. Relative activity was calculated by taking the relative luciferase activity of the empty vector (pGL3-basic) to be 1.

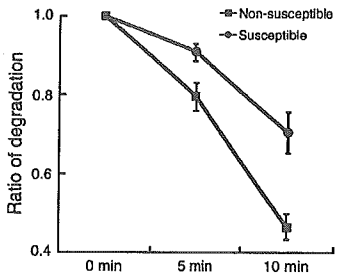

FIG 3. Stability of susceptible (3′ UTR variant 10841A) and non-susceptible (10841C) transcripts of *IL-12B* mRNA measured as degradation rate. Values represent means ± SDs of data from triplicate experiments.

childhood atopic asthma (OR, 1.75; 95% CI, 1.22-2.51) compared with other diplotypes.

We next conducted case-control studies of patients stratified by asthma-related phenotypes, asthma severity, and high total IgE level. Association between SNP8 10841C>A and asthma was evident in childhood atopic asthma with severity ≥ 3 (P = .0021; corrected P = .0042) or with high total IgE ≥ 1106 IU/mL (P = .0012; corrected P = .0024; Table VI). We also investigated associations between asthma-related phenotypes and SNPs within patients with asthma (case-only association study), and Table VI includes the results of the case-only study. SNP1 −6415 CTCTAA>GC was associated with high total IgE (P = .0099; corrected P = .020; Table VI). A recent report showed that patients who were heterozygous for the SNP1 had increased asthma severity. In this study, the proportion of subjects with heterozygous genotypes in severe groups (severity ≥ 3) was not significantly different from those found in the groups with milder asthma (severity ≤ 2).

We examined transient expression of the CTCTAA and GC luciferase reporter constructs. Polymorphism in promoter of *IL-12B* affected relative luciferase activity (P = .00018 for comparison between allele CTCTAA and allele GC by Student t test; 2.82 [SD, 0.27] vs 6.66 [SD, 0.42], respectively; Fig 2). The GC construct had 2.4-fold higher luciferase reporter activity than the CTCTAA construct. These results suggested that the GC allele might affect the increased transcriptional activity of the *IL-12B* gene *in vivo*.

We further examined whether 3′ UTR polymorphism in the coding region affected the stability of *IL-12B* mRNA. RNAs from susceptible alleles were more stable than those of the nonsusceptible alleles, and differences were significant after 5 minutes and 10 minutes of reaction time (P = .0089 and .0024, respectively; Fig 3). The current results suggested that 3′ UTR polymorphism in mRNA contributed to mRNA stability.

DISCUSSION

We showed here a significant association between asthma susceptibility and an SNP in the 3′ UTR region, 10841C>A, and promoter polymorphism −6415 CTCTAA>GC was also associated with asthma. Furthermore, we found a haplotype that affected the stability of transcripts and

enhanced the transcriptional level of *IL-12B*. Although the functional effects of these 2 polymorphisms were analyzed in this study, the polymorphisms that are in linkage disequilibrium with these 2 variants were not examined. −6110C>T, 1521A>G, 1645A>G, 3778G>A, and 10559WT>delG might affect the expression level of the *IL-12B* gene. Functional involvement of these linked polymorphisms in the pathogenesis of asthma should be investigated.

Several studies have surveyed the *IL-12B* gene as a potential candidate gene for asthma.[19-21] Khoo et al[21] reported that *IL-12B* promoter polymorphism was not associated with asthma susceptibility, severity, or atopy at ages 7 and 42 years in an Australian population.[21] However, total serum IgE levels of adult men (age 42 years) with at least 1 CTCTAA allele were higher than those homozygous for the GC allele, whereas no difference was observed for adult women (geometric mean IgE = 133 kU/L vs 80 kU/L; $P = .042$). In this study, we examined childhood atopic asthma, and the mean of $\log_{10}$(tIgE [IU/mL]) of patients was 2.69 (= $\log_{10}$[490 IU/mL]), which was higher than the values in the Australian adult population. In the case-only study, the promoter variant was associated with high total IgE. Heterozygosity for an *IL-12B* promoter polymorphism was associated with asthma severity in a cohort of Australian children.[19] Khoo et al[21] presented no evidence to support the presence of a heterozygote effect of the *IL-12B* promoter variant on the level of asthma in early childhood and adulthood.[21] Although we could not reproduce the finding that patients who were heterozygous for the *IL-12B* promoter polymorphism had increased asthma severity, we confirmed the association between the *IL-12B* promoter variant and asthma susceptibility and elevated serum IgE levels. Polymorphisms in *IL-12B* were found to be associated with children with asthma and atopy phenotypes in whites in the Childhood Asthma Management Program cohort using a family-based association test, and there was a strong association between the *IL-12B* 4237 and *IL-12B* 6402 polymorphisms and the asthma severity phenotype in white subjects.[20] The investigation confirmed positive associations for replication in a case-control study comparing adults with moderate-to-severe asthma with controls. Although we screened all 8 exons, including a minimum of 100 bases of the flanking intronic sequence of the *IL-12B* gene, we could not identify these 2 related variants reported for white subjects, *IL-12B* 4237 in intron 2 and *IL-12B* 6402 in intron 4. Although it is unclear whether these 2 polymorphisms are in LD with SNP8 in 3′ UTR, we have found that variation of the *IL-12B* is associated with susceptibility of childhood atopic asthma, the asthma severity phenotype, and elevated serum IgE levels across ethnic lines.

Failure to replicate genetic associations in complex disease is a common occurrence.[32,33] In the Japanese population, no associations were noted for 3′ UTR polymorphism with asthma and allergic rhinitis using transmission disequilibrium test analyses.[24] They reported that preferential transmission of 10841A/C allele to the asthma was not observed (transmitted:not transmitted = 61:60; $P > .1$). Sample size might effect on the contradictory result. Another possible explanation for the discordance with our study is unrecognized differences in environmental exposures. A recent study showed that genetic variation in *TLR2* is a major determinant of the susceptibility to asthma and allergies in a farming environment.[34] The functional role of genetic polymorphisms of *IL-12B*, involved in immune responses against environmental antigens, might be affected by the proportion of microbes. Furthermore, epistatic interactions may reflect the interethnic contradictory result.

IL-12 p40 encoded by *IL-12B* is a component of 4 secreted proteins that have diverse biologic function: IL-12, IL-23, p80, and the p40 monomer.[5-10] It is possible for functional polymorphisms of *IL-12B* to affect production of these proteins in various tissues. Recently, functions of p80 (a p40 homodimer) and the p40 monomer in T_H2-type immune responses were intensively studied.[6-9] Human airway epithelial cells express programmed immune response genes, and IL-12 p40 overexpression has been observed selectively in airway epithelial cells in subjects with asthma.[13] Our data were consistent with immunological data indicating IL-12 p40 overexpression selectivity in airway epithelial cells in subjects with asthma. Overexpression of IL-12 p40 is also an important contributing factor for the generation of the dominant T_H2-type environment in large intestinal T_H2-type allergic diarrhea in mice.[12] In IL-12 p40 transgenic mice, p40 functions as an IL-12 antagonist *in vivo*, and T_H1 responses in p40 transgenic mice are significantly reduced.[11] These findings suggest that IL-12 p40 plays a crucial role in the T_H2 immune responses as a monomer or a homodimer. We have identified a susceptibility haplotype, which effects a more stable transcript and higher levels of transcription. Although it is unclear whether the related haplotype regulates IL-12 p40 containing protein synthesis in bronchial tissue, the haplotype might contribute to the T_H2-polarized immunity in asthma through higher IL-12 p40 monomer or homodimer production in childhood atopic asthma.

Our data strongly support the important role of *IL-12B* in asthma. Further investigations of the connection between genotypes and the functional role of IL-12 p40 would be helpful to clarify the etiology of asthma.

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Association of the *IL12RB1* promoter polymorphisms with increased risk of atopic dermatitis and other allergic phenotypes

Naomi Takahashi[1,2], Mitsuteru Akahoshi[1,3,*], Akira Matsuda[1], Kouji Ebe[4], Naoko Inomata[5], Kazuhiko Obara[1,6], Tomomitsu Hirota[1], Kazuko Nakashima[1,7], Makiko Shimizu[1], Mayumi Tamari[1], Satoru Doi[8], Akihiko Miyatake[9], Tadao Enomoto[10], Hitoshi Nakashima[3], Zenro Ikezawa[5] and Taro Shirakawa[1,7]

[1]Laboratory for Genetics of Allergic Diseases, SNP Research Center, RIKEN, Kanagawa 230-0045, Japan, [2]Department of Dermatology, School of Medicine, Tokyo Medical and Dental University, Tokyo, Japan, [3]Department of Medicine and Biosystemic Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, [4]Takao Hospital, Kyoto, Japan, [5]Department of Dermatology, Yokohama City University School of Medicine, Kanagawa, Japan, [6]Hitachi Chemical Co., Ltd, Tokyo, Japan, [7]Department of Health Promotion and Human Behavior, Kyoto University Graduate School of Public Health, Kyoto, Japan, [8]Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Osaka, Japan, [9]Miyatake Asthma Clinic, Osaka, Japan and [10]Department of Otolaryngology, Japanese Red Cross Society Wakayama Medical Center, Wakayama, Japan

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Atopic dermatitis (AD) is frequently associated with eosinophilia, highly elevated immunoglobulin E (IgE) levels and increased levels of T-helper 2-type (Th2) cytokines in skin lesions due to infiltrating T cells. Interleukin-12 (IL-12), in combination with interferon-γ (IFN-γ), inhibits IgE synthesis and Th2 cell function. As the IFN-γ-inducing cytokines IL-12 and IL-23 utilize IL-12Rβ1 as part of their receptors, it is possible that polymorphic variants of the IL-12Rβ1 (*IL12RB1*) gene might determine an individual's susceptibility to AD. Here, we carried out a systemic search for genetic variants of the human *IL12RB1* in Japanese subjects and identified 48 genetic variants. In a case–control association study, we found that promoter polymorphisms −111A/T and −2C/T were significantly associated with an increased risk of AD under a recessive model. The −111T-allele frequency in the independent population of child asthmatics was also much higher than that in the control group. In addition, the −111T/T genotype was progressively more common in AD with high total serum IgE levels in an IgE-level-dependent manner. Deletion analysis of the *IL12RB1* promoter suggested that the −265 to −104 region that contained the −111A/T polymorphic site harbored an important regulatory element. Furthermore, we showed that the −111A/T substitution appeared to cause decreased gene transcriptional activity such that cells from −111A/A individuals exhibited higher *IL12RB1* mRNA levels than those from −111T allele carriers. Our results suggested that 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.

*To whom correspondence should be addressed at: Laboratory for Genetics of Allergic Diseases, SNP Research Center, RIKEN Yokohama Institute, RIKEN, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan. Tel: +81 455039616; Fax: +81 455039615; Email: akahoshi@src.riken.jp

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease primarily occurring in infants and children, which is characterized by pruritic and eczematous skin lesions at characteristic locations (1). Although its prevalence has increased 2–3-fold during the past three decades in industrialized countries, wide variations in prevalence rates have been observed within countries inhabited by groups with similar genetic backgrounds, suggesting that environmental factors may be critical in determining AD onset (2,3). Nonetheless, it is widely accepted that AD has a genetic component responsible for its high familial occurrence. Twin studies of AD have shown concordance rates of 72–86% in monozygotic and 21–23% in dizygotic twin pairs (4,5) such that genetic factors also play an important role in AD development. Taken together, it appears that changes in environmental exposure *in utero* and during the early years of life may lead to the manifestation of AD in genetically predisposed individuals (6).

AD is frequently associated with blood eosinophilia and highly elevated immunoglobulin E (IgE) levels. Biopsies from clinically unaffected skin from AD patients demonstrate increased number of T-helper 2-type (Th2) cells that express interleukin-4 (IL-4) and IL-13, but not interferon-γ (IFN-γ), mRNA when compared with normal non-atopic skin (7,8). When compared with normal or uninvolved AD skin, acute AD skin lesions exhibit significantly increased number of IL-4, IL-5 and IL-13 mRNA-expressing cells. Furthermore, transgenic mice genetically engineered to over express IL-4 in their skin and to develop inflammatory pruritic skin lesions similar to AD, which suggests that local skin expression of Th2 cytokines plays a critical role in AD (9).

IL-12, primarily produced by antigen-presenting cells, is a heterodimeric cytokine consists of two disulfide-linked subunits designated as p35 and p40. It plays a central role in regulating Th1 differentiation and in promoting cell-mediated immunity (10). Conversely, IL-12, in combination with IFN-γ, inhibits IgE synthesis and antagonizes Th2 differentiation, including the production of Th2 cytokines such as IL-4 (11). The IL-12 receptor (IL-12R) consists of at least two distinct subunits, β1 and β2, and is primarily expressed on activated T and NK cells (12). Co-expression of human IL-12Rβ1 and IL-12Rβ2 is required for the formation of high-affinity IL-12 binding sites, and analysis of IL-12R-deficient mice showed that both subunits were essential for IL-12R function (13,14). Recently, it was reported that IL-12Rβ1 is also a component of the receptor complex for another IFN-γ-inducing cytokine, IL-23 (15).

To date, case–control association studies have found significant associations between AD and gene polymorphisms in *IL4*, *IL4R*, *IL13*, *RANTES (CCL5)*, *TGFB1*, *GMCSF (CSF2)*, *CARD15*, *FCER1B (MS4A2)*, *SPINK5* and *IL12B* (3,16–18). Although few studies have examined the association between Th1-related genes and the development of AD, there have been recent epidemiological studies showing an inverse relationship between AD and Th1-associated phenotypes. For example, AD was inversely associated with insulin-dependent diabetes mellitus, a Th1-biased autoimmune disorder (19). Moreover, a strong inverse association was also found between positive tuberculin responses and a range of atopic symptoms, including AD (20). Therefore, we hypothesized that functional single nucleotide polymorphisms (SNPs) in Th1-related genes encoding the IL-12 family of cytokines and cytokine receptors might also contribute to AD susceptibility. To test this hypothesis and to assess the role of IL-12/IL-12R and IL-23/IL-23R systems in AD, we examined the influence of IL-12β1 (*IL12RB1*) gene polymorphisms in AD susceptibility in the Japanese population.

RESULTS

Identification of sequence variants in *IL12RB1*

Direct DNA sequencing revealed 48 *IL12RB1* variants, which included previously reported variants (21–24) and 10 novel variants (−3966C/A, −2163C/T, −1973C/T, −355G/A, 3377C/A, 5854G/A, 9354G/T, 10129G/A, 18205C/T and 20228G/A) in Japanese (Table 1). We identified nine variants in the 5′-flanking region, nine in the coding region (seven non-synonymous and two synonymous), one in the 5′-untranslated region (UTR), one in the 3′-UTR, 21 within introns and seven at the 3′ end. Twelve of the 48 variants had estimated minor allele frequencies (MAFs) of <10% (on the basis of the sequencing of 24 DNA samples). Nucleotide position one (+1) was defined as the first adenine of the initiation codon (ATG) and positions for other SNPs were described relative to the ATG on genome contig AC020904. A graphical overview of the structure of the human *IL12RB1* gene with the location of the 36 common polymorphisms (MAF ≥ 10%) identified in this study is shown in Figure 1A.

LD and case–control comparisons

For the successfully genotyped 35 of 36 common variants, we calculated both D' and r^2 as statistical values for pair-wise linkage disequilibrium (LD) analysis between SNPs. The LD block structure defined by the 35 genotyped SNPs is shown in Figure 1B. Strong LD was detected across the *IL12RB1* region, although at least two historical recombination events seem to have occurred, which divided the region into three strongly correlated LD blocks. Next, on the basis of location and LD with other sites ($r^2 < 0.9$), we selected eight representative SNPs (−111A/T, −2C/T, 4443C/T, 5970G/C, 17183T/C, 17369C/T, 25748T/C and 27637A/T) from the 36 common polymorphisms for further genotyping and association studies in our AD population. The distribution of all eight SNPs was in Hardy–Weinberg equilibrium in both AD and control groups ($P > 0.05$).

For the case–control association study, we genotyped the eight selected SNPs in a set of 382 unrelated individuals with AD and 658 population-based controls. The clinical characteristics of our AD patients are summarized in Table 2. Allele and genotype frequencies of each selected SNP were compared between the patients and the normal controls using the χ^2 test under different association models. We found a significant association between two promoter SNPs at −111 and −2 (−111A/T and −2C/T) and AD in our Japanese cohort under a recessive model (−111AA + AT versus TT, $P = 0.00044$; −2CC + CT versus TT, $P = 0.00075$; Table 3).

Table 1. Overview of *IL12RB1* variants identified in Japanese

Number	RefSNP ID	JSNP ID	Position	Variation	Location	Amino acid change	Minor allele frequency[a]	Genotyping method	Primers[b]
1	Novel	—	−3966	C/A	5′g	—	0.021[c]		F1R1
2	rs374326	—	−3633	A/G	5′g	—	0.043[c]		F2R2
3	rs845331	—	−2252	G/T	5′g	—	0.167		F4R4
4	Novel	—	−2163	C/T	5′g	—	0.042[c]		F4R4
5	rs447259	—	−2060	A/G	5′g	—	0.167		F4R4
6	rs845329	—	−2004	A/G	5′g	—	0.167		F4R4
7	Novel	—	−1973	C/T	5′g	—	0.042[c]		F4R4
8	Novel	—	−355	G/A	5′g	—	0.042[c]		F7R7
9	rs393548	063138	−111[d]	A/T	5′g	—	0.167	Invader	F8R8
10	rs436857	063137	−2[d]	C/T	5′-UTR	—	0.146	Invader	F8R8
11	Novel	—	3377	C/A	Exon 2	P37T	0.022[c]		F9R9
12	rs2305743	063136	4443[d]	C/T	Intron 2	—	0.188	Invader	F10R10
13	Novel	—	5854	G/A	Exon 4	A91T	0.024[c]		F11R11
14	rs11086087	—	5970[d]	G/C	Exon 4	V129V	0.188	TaqMan	F11R11
15	rs2305742	063135	6193	T/G	Intron 4	—	0.139		F11R11
16	rs17880761	—	8895	T/C	Intron 4	—	0.229		F12R12
17	Novel	—	9354	G/T	Intron 5	—	0.022[c]		F12R12
18	rs382634	—	10072	C/T	Intron 5	—	0.375		F13R13
19	Novel	—	10129	G/A	Intron 5	—	0.208		F13R13
20	rs17885316	—	10809	Ins A	Intron 6	—	0.217		F13R13
21	rs429774	—	10882	A/G	Intron 6	—	0.375		F13R13
22	rs11575934	—	11016	A/G	Exon 7	Q214R	0.375		F13R13
23	—	—	11059	C/T	Exon 7	P228P	0.375		F13R13
24	rs3761041	114595	13717	G/A	Intron 8	—	0.229		F14R14
25	rs391410	—	16988	C/T	Intron 9	—	0.348		F16R16
26	rs375947	063134	17183[d]	T/C	Exon 10	M365T	0.375	Invader	F16R16
27	rs401502	063133	17221	G/C	Exon 10	G378R	0.375		F16R16
28	rs17882636	063132	17369[d]	C/T	Intron 10	—	0.146	TaqMan	F16R16
29	rs2305740	063131	17398	T/C	Intron 10	—	0.146		F16R16
30	rs2305739	063130	17440	C/T	Intron 10	—	0.146		F16R16
31	rs12150884	—	17661	C/T	Intron 10	—	0.146		F16R16
32	Novel	—	18205	C/T	Intron 10	—	0.021[c]		F17R17
33	Novel	—	20228	G/A	Exon 12	—	0.021[c]		F18R18
34	—	—	22903	G/A	Exon 13	—	0.021[c]		F19R19
35	rs17878533	—	24793	T/C	Intron 14	—	0.146		F20R20
36	rs383483	—	25748[d]	T/C	Intron 15	—	0.438	TaqMan	F21R21
37	rs17882370	—	26460	T/C	Intron 15	—	0.063[c]		F22R22
38	rs1870063	114596	26672	G/A	Intron 15	—	0.125		F22R22
39	—	—	26954	C/T	Intron 15	—	0.125		F22R22
40	—	—	26977	G/T	Intron 15	—	0.125		F22R22
41	rs3746190	097709	27250	C/T	Exon 17	3-UTR	0.125		F23R23
42	rs3833286	097710	27366	Ins G	3′g	—	0.479		F23R23
43	rs404733	097711	27637[d]	T/A	3′g	—	0.458	Invader	F24R24
44	rs11307847	—	28184	G/T	3′g	—	0.167		F25R25
45	rs5014130	—	28189	C/G	3′g	—	0.438		F25R25
46	rs5827394	—	28191	Del G	3′g	—	0.438		F25R25
47	rs445521	—	28320	A/C	3′g	—	0.438		F25R25
48	rs382410	—	28960	T/C	3′g	—	0.354		F25R25

[a]On the basis of the sequencing of 24 DNA samples.
[b]Primer sequences are listed in Supplementary Material, Table S1.
[c]Variations with MAF of <10%.
[d]SNPs genotyped in a larger populaton.

The result for each SNP remained significant after correction for multiple tests (corrected P: $P_c = 0.0035$ for −111A/T and $P_c = 0.006$ for −2C/T). Homozygotes for the −111T or −2T alleles were significantly more common in AD patients when compared with controls. Odds ratios (ORs) of developing AD with respect to positions −111 and −2 were 2.46 (95%CI 1.47–4.13) and 2.60 (95%CI 1.46–4.61), respectively. Genotype frequencies in Japanese AD cases and controls for −111 and −2 SNPs were shown in Table 4.

In further analyses of patient subgroups, we observed strong associations between the presence of high total serum IgE, early age of disease onset (≤3) and peripheral blood eosinophilia (>500/μl), as well as history of childhood asthma and allergic rhinitis (Table 5). No significant differences were observed for the other clinical features tested (data not shown). Notably, the −111T/T genotype was progressively more common in AD with high total serum IgE levels in an IgE-level-dependent manner. Interestingly, when we analyzed

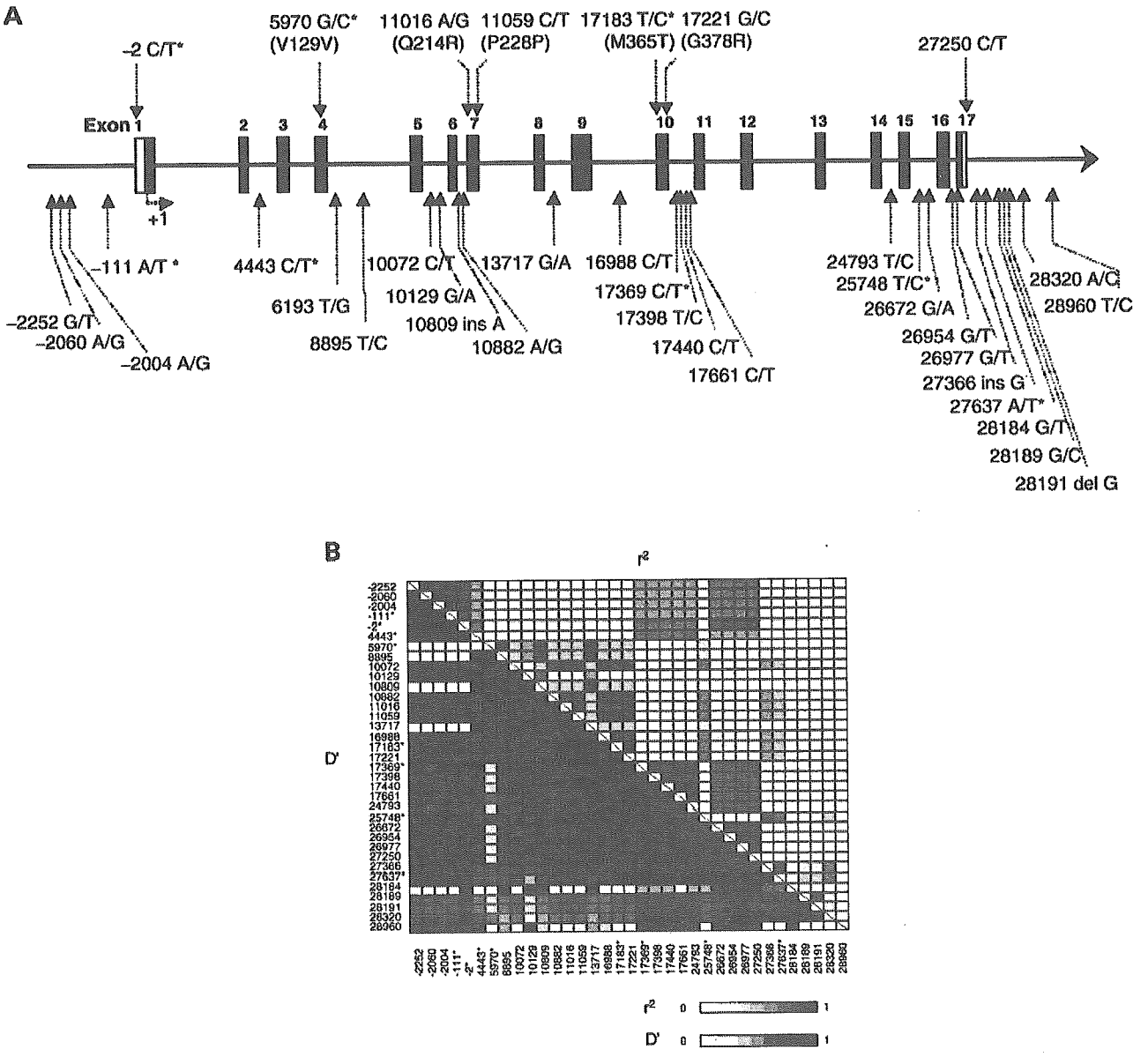

Figure 1. (**A**) A graphical overview of the 36 identified SNPs in relation to the exon/intron structure of the human *IL12RB1* gene. Nucleotide position one (+1) is the first adenine of the initiation codon (ATG) such that SNP positions are given relative to the ATG. The 17 coding exons are shown by black boxes and the 5′- and 3′-UTRs by white blocks. Asterisks indicate SNPs that were genotyped in a larger population. (**B**) Pair-wise LD, as measured by D' and r^2, was calculated for 35 common polymorphisms (MAF $\geq$ 10%) identified in 24 sequenced samples. Two measures of LD are shown: D' in the lower left triangle and r^2 in the upper right triangle. The markers are plotted equidistantly. Scales for both LD measures are provided below. Asterisks indicate SNPs that were genotyped in a larger population.

the −111A/T SNP in the independent population of physician-diagnosed asthma, the −111T/T genotype frequency in 304 child asthmatics aged 4–15 years (9.2%) tended to be much higher than that in the control group (4.2%). Then, to further confirm the influence of the −111 genotype on IgE regulation, we compared total serum IgE levels in AD patients according to genotype. Although the results for AD patients were not statistically significant, we observed the same trend of increasing total serum IgE levels with increasing occurrence of the −111T allele (Fig. 2).

Haplotype analysis

Among eight representative SNPs, SNPs located out of the first LD block were not in strong LD with the −111 and −2 SNPs ($r^2 \leq 0.6$). We analyzed the distribution of

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

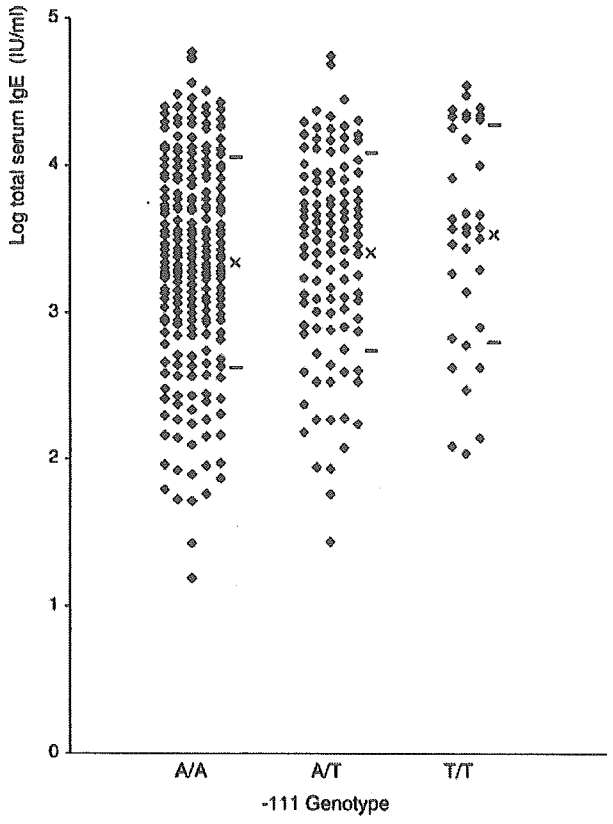

Figure 2. Relationship of *IL12RB1* −111 promoter genotype with total serum IgE levels in AD patients. Log-transformed individual IgE values are plotted, with the mean (×) and SD (bar) shown for each genotype group.

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 $P = 0.005$).

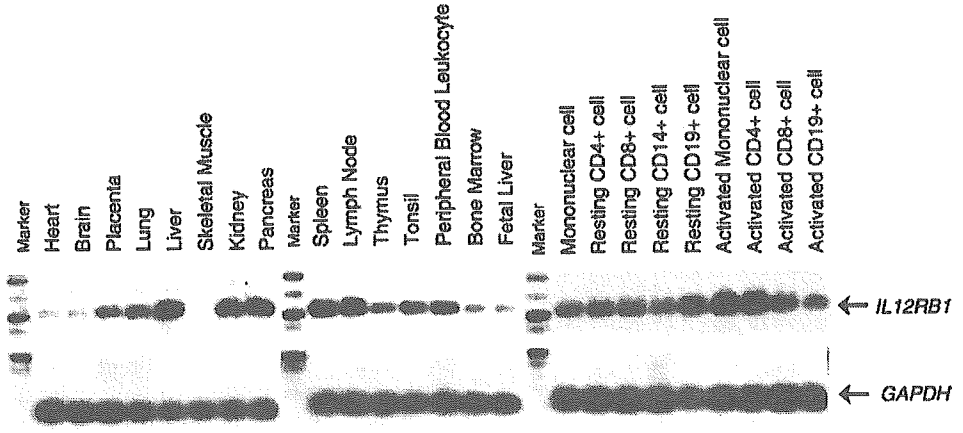

Figure 3. Expression of *IL12RB1* mRNA in multiple tissue cDNA panels. PCR amplification between *IL12RB1* exons 10 and 13 is shown in the upper panel. The *GAPDH* control is shown in the lower panel.

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

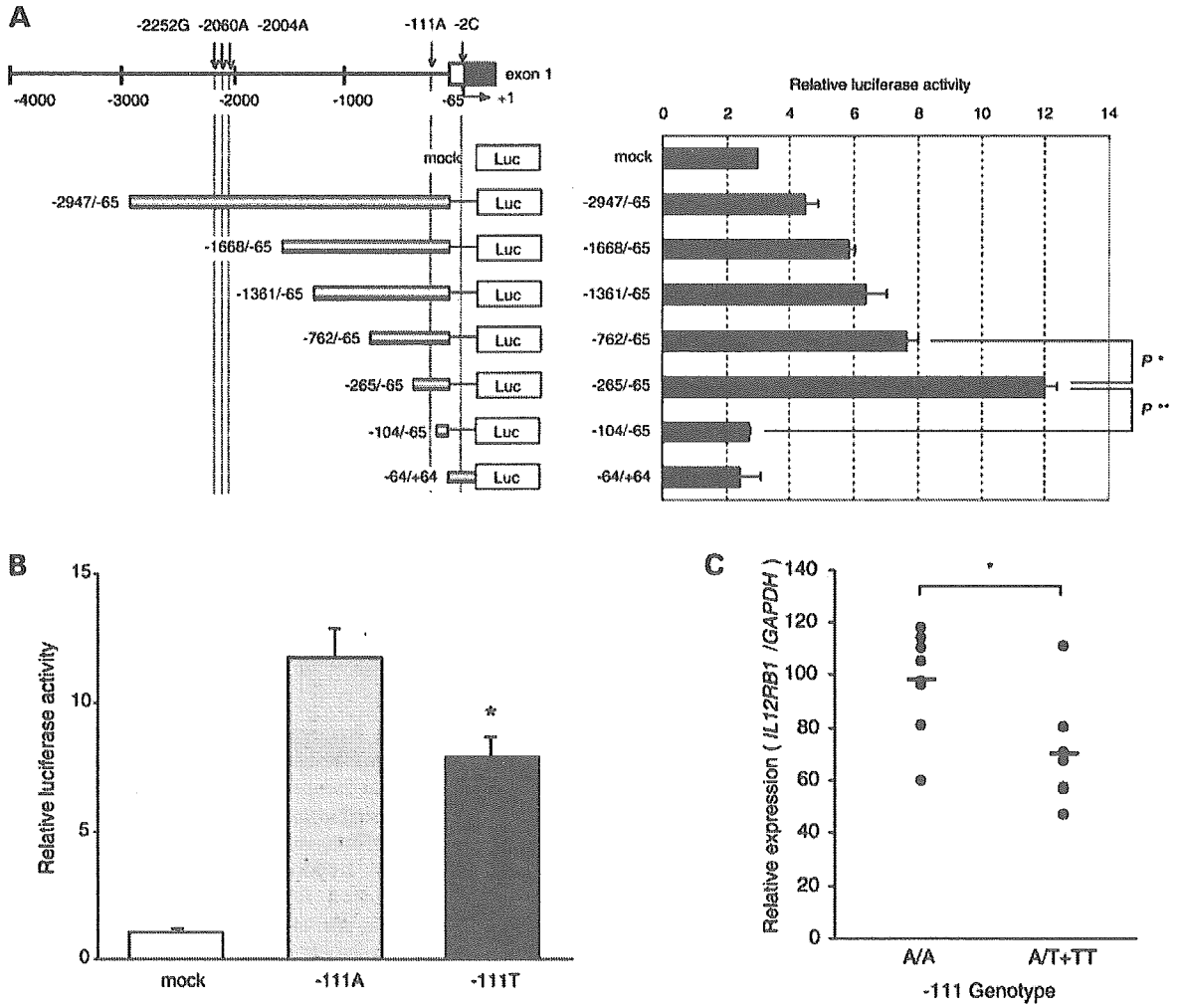

Figure 4. Identification of regulatory elements in the *IL12RB1* promoter, transcriptional effect of the −111A/T SNP and association of the −111 genotype with *IL12RB1* mRNA expression. (**A**) The indicated *IL12RB1* gene promoter fragments derived from wild-type allele sequence (−2252G/−2060A/−2004A/−111A/−2C) 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 pG L3-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.

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Conflict of Interest statement. None declared.

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Functional SNPs in the distal promoter of the *ST2* gene are associated with atopic dermatitis

Makiko Shimizu[1,2], Akira Matsuda[1,*], Ken Yanagisawa[3], Tomomitsu Hirota[1], Mitsuteru Akahoshi[1], Naoko Inomata[2], Kouji Ebe[4], Keiko Tanaka[5], Hisashi Sugiura[5], Kazuko Nakashima[1,6], Mayumi Tamari[1], Naomi Takahashi[1], Kazuhiko Obara[1], Tadao Enomoto[7], Yoshimichi Okayama[8], Pei-Song Gao[9], Shau-Ku Huang[9], Shin-ichi Tominaga[3], Zenro Ikezawa[2] and Taro Shirakawa[1,6]

[1]Laboratory for Genetics of Allergic Diseases, SNP Research Center, RIKEN, Yokohama, Japan, [2]Department of Dermatology, Yokohama City University School of Medicine, Yokohama, Japan, [3]Department of Biochemistry, Jichi Medical School, Tochigi, Japan, [4]Takao Hospital, Kyoto, Japan, [5]Department of Dermatology, Shiga Medical School, Shiga, Japan, [6]Department of Health Promotion and Human Behavior, Kyoto University Graduate School of Public Health, Kyoto, Japan, [7]Department of Otolaryngology, Japanese Red Cross Society, Wakayama Medical Center, Wakayama, Japan, [8]Laboratory of Allergy Transcriptome, Research Center for Allergy and Immunology, RIKEN, Yokohama, Japan and [9]Johns Hopkins Asthma and Allergy Center, Johns Hopkins University School of Medicine, Baltimore, MD, USA

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Atopic dermatitis (AD) is a common inflammatory skin disease associated with the local infiltration of T helper type 2 (Th2) cells. The *ST2* gene encodes both membrane-bound ST2L and soluble ST2 (sST2) proteins by alternative splicing. The orphan receptor ST2L is functionally indispensable for Th2 cells. We found a significant genetic association between AD and the −26999G/A single nucleotide polymorphism (SNP) (χ^2-test, raw *P*-value = 0.000007, odds ratio 1.86) in the distal promoter region of the *ST2* gene (chromosome 2q12) in a study of 452 AD patients and 636 healthy controls. The −26999A allele common among AD patients positively regulates the transcriptional activity of the *ST2* gene. In addition, having at least one −26999A allele correlated with high sST2 concentrations and high total IgE levels in the sera from AD patients. Thus, the −26999A allele is correlated with an increased risk for AD. We also found that the −26999G/A SNP predominantly affected the transcriptional activity of hematopoietic cells. Immunohistochemical staining of a skin biopsy specimen from an AD patient in the acute stage showed ST2 staining in the keratinocytes as well as in the infiltrating cells in the dermal layer. Our data show that functional SNPs in the ST2 distal promoter region regulate ST2 expression which induces preferential activation of the Th2 response. Our findings will contribute to the evaluation of one of the genetic risk factors for AD.

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease associated with the local infiltration of T helper type 2 (Th2) cells that secrete interleukin (IL)-4, IL-5 and IL-13 in the acute stage followed by the infiltration of T helper type 1 (Th1) cells, which is responsible for the chronicity of AD lesions (1). Genetic susceptibility to AD has been suggested by epidemiological and genetic studies (2–4). In one study, monozygotic twin pairs had a concordance rate of 0.72 and dizygotic twin pairs had one of 0.23 for AD (5). The IL-1 receptor (IL1R) gene cluster (2q12–14) has many immunoregulatory genes including IL1R1, IL1R2, IL18R1 and IL18RAP. We and others reported some genetic association studies of atopic diseases investigating this region (6–8). We carried out detailed genetic association studies of the IL1R region and found that genetic polymorphisms within the ST2 (IL1RL1) gene region had a strong association with AD.

*To whom correspondence should be addressed at: Laboratory for Genetics of Allergic Diseases, SNP Research Center, RIKEN, 1-7-22, Suehiro, Tsurumi-KU, Yokohama 230-0045, Japan. Tel: +81 455039616; Fax: +81 455039615; Email: akimatsu@src.riken.go.jp

ST2 was originally reported as a gene induced by serum in mouse fibroblasts (9). There are three alternatively spliced variants in ST2 in mammals, membrane-bound ST2L (10), soluble ST2 (sST2) (9) and ST2V (11). ST2L is an orphan receptor which has a conserved cytosolic domain called as the Toll-IL1R (TIR) domain. The functional role of ST2L is relevant to AD because ST2L is expressed in Th2 cells but not in Th1 cells (12), it has critical roles in Th2 effector functions (13) and is considered as a functional marker for Th2 cells. In addition, ST2L expression was also reported in mast cells (14), effector cells in the acute stage of AD (15). In contrast to other members of the TIR family that activate NF-κB signaling pathways (16), ST2L negatively regulates IL1R1 and toll-like receptor (TLR)-4 signals by sequestrating MyD88 and Mal signals (17). In this study, we found single nucleotide polymorphisms (SNPs) associated with increased risk for AD in the promoter region of the *ST2* gene. The high-risk SNPs showed higher ST2 promoter activity and hence increased serum sST2 as well as total IgE levels in AD patients.

RESULTS

Identification of genetic polymorphisms in ST2 and intragenic LD

We discovered 67 genetic variants in the ST2 region (Supplementary Material, Table S2) by resequencing DNA samples from 24 Japanese individuals (12 AD patients and 12 controls). Among the 67 genetic polymorphisms, 34 variants had estimated minor allele frequencies (MAF) of >10% (based on the sequencing of 24 DNA samples). We calculated r^2 as the statistical value for pairwise linkage disequilibrium (LD) between the SNPs (Supplementary Material, Fig. S1). On the basis of location and LD with other sites, we selected seven haplotype tagging SNPs as representative SNPs (Fig. 1A and Table 1) from the 34 common SNPs. Two SNPs (−27639A/G and −26999G/A) were in the 5′-genomic region for exon 1a, one SNP (744C/A) in exon 3 and four SNPs (2992C/T, 5283G/A, 5860C/A, 11147C/T) in the introns. Positions are numbered according to their positions relative to the published *ST2* gene sequence (GenBank accession no. AC007248), and position 1 is the adenine of the first methionine.

Case–control study

A summary of the case–control association study with representative SNPs is shown in Table 1. All seven SNPs were in Hardy–Weinberg equilibrium in both AD and control groups ($P > 0.05$). One −26999G/A SNP showed a significant association under a dominant model [−26999GG versus G/A + A/A, raw P-value = 0.000007, $P = 0.000049$ after Bonferroni correction, odds ratio (OR) = 1.86] (Table 2). The association became stronger (raw P-value = 0.000000038, corrected P = 0.00000027, OR = 2.55) for the AD patients with very high serum total IgE levels (IgE > 1700 IU/ml, $n = 290$) (Table 2). Weak association was also observed at −27639A/G SNP (−27639AA versus A/G + G/G, raw P-value = 0.0001, corrected P-value = 0.0007). The −27084G/C SNP was also fully genotyped and we found that the −27084G/C and −26999A/G SNPs were in a state of complete LD.

Haplotype analysis

We also tested the distribution of two- and seven-locus haplotypes in AD and control samples. Among the two-locus haplotypes of the promoter region SNPs (−27639A/G and −26999G/A), haplotype −27639G/−26999A showed an increased risk for AD (Table 3, G, A versus others; $P = 0.0004$, OR = 1.41). We also analyzed the haplotypes of the seven representative SNPs and found that haplotype-A was associated with AD (Table 4, haplotype-A versus others; $P = 0.000028$, OR = 1.45). However, none of these associations was stronger than those observed for the single locus (−26999G/A).

Reporter gene analysis

We made a construct for haplotype-1 (the major haplotype: −27639A, −27084G and −26999G) and haplotype-2 (the common haplotype among AD patients: −27639G, −27084C and −26999A) with pGL3 basic vector. The assay was performed in triplicate, and a representative result of three independent experiments is shown as mean ± SD in Fig. 1B, right. The relative strengths of luciferase activity were 1517 ± 41 (mean ± SD) for haplotype-1 and 3226 ± 84 for haplotype-2, 267 ± 7 for distal-Δ355 (−27639A clone). The distal-Δ355 clone with the −27639G allele showed a result similar to that for the −27639A allele (data not shown). Haplotype-2 induced stronger ST2 promoter activity than haplotype-1.

RT–PCR analysis with a panel of hematopoietic cells, keratinocytes and dermal fibroblasts

For analysis of differential promoter usage, we made specific primer sets to distinguish each promoter and subtype of ST2 expression and performed RT–PCR with cDNA from a human mast cell line (LAD2), human keratinocytes (KC) cultured with serum-free medium and dermal fibroblasts cultured with 10% fetal bovine serum (FBS). For some studies, KC were stimulated with 10% FBS for 24 h. The results showed that only mast cells used both distal and proximal promoters. The other cells (skin fibroblasts and KC) used the proximal promoter exclusively (Fig. 1C). LAD2 cells could express sST2 mRNA using both promoters and ST2L mRNA using the distal promoter, whereas skin fibroblasts and KC could only express sST2 using the proximal promoter (Fig. 1C).

Quantification of sST2 protein and total IgE using the sera from AD patients

The concentration of sST2 in the sera of 124 AD patients was measured with ELISA. The sST2 concentration of the serum of patients with the −26999G/G genotype was 0.225 ng/ml (mean). For the −26999G/A + A/A genotype, it was 0.365 ng/ml (Supplementary Material, Fig. S4A). The sera from −26999G/G genotype patients showed a significantly lower ST2 concentration than those from −26999G/A + A/A patients ($P = 0.0000008$ by Mann–Whitney U-test). All measurements were performed in duplicate. We carried out