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

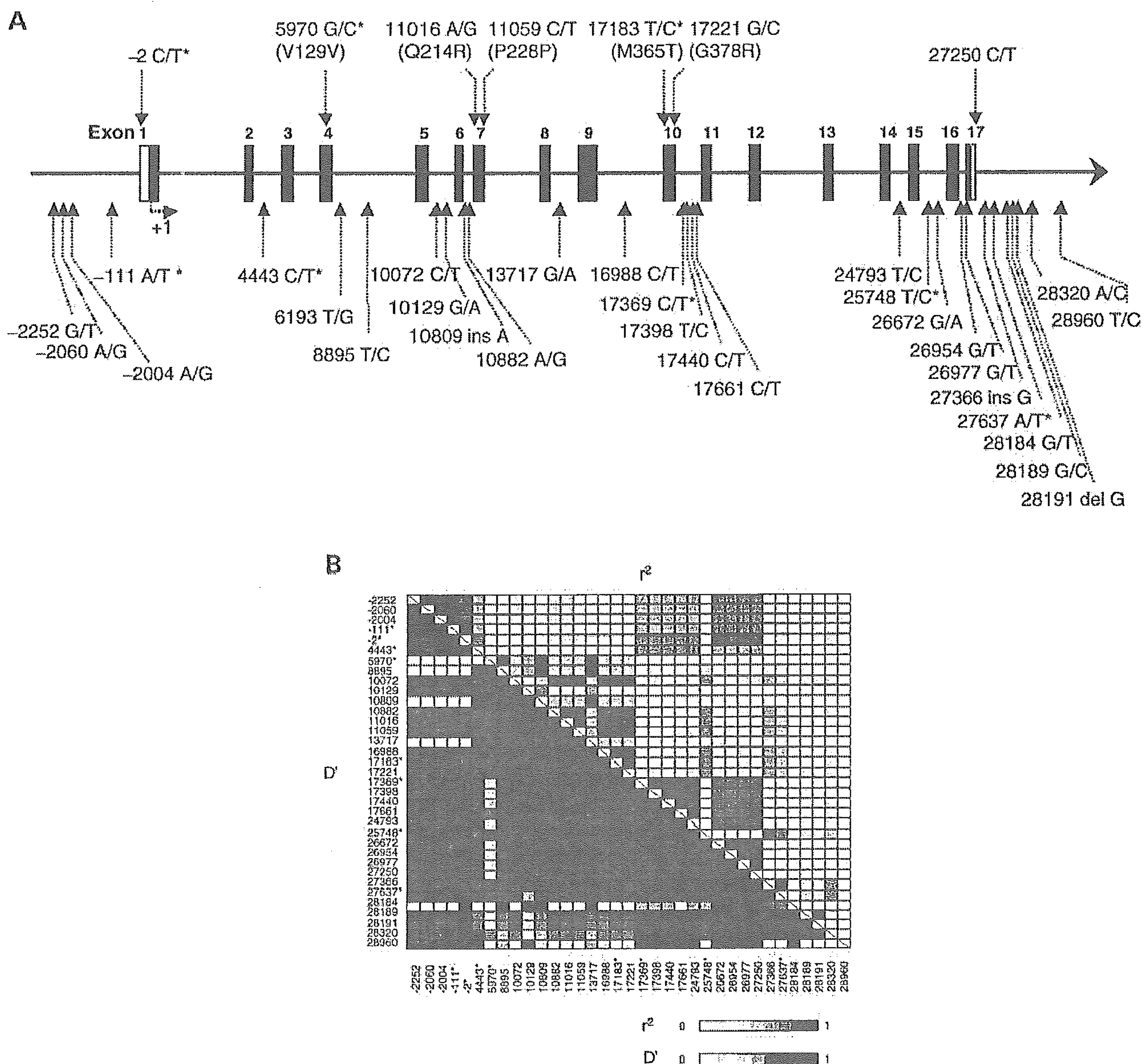

Figure 1. (A) A graphical overview of the 36 identified SNPs in relation to the exon/intron structure of the human *IL12RB1* gene. Nucleotide position one (+1) is the first adenine of the initiation codon (ATG) such that SNP positions are given relative to the ATG. The 17 coding exons are shown by black 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 ≥ 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

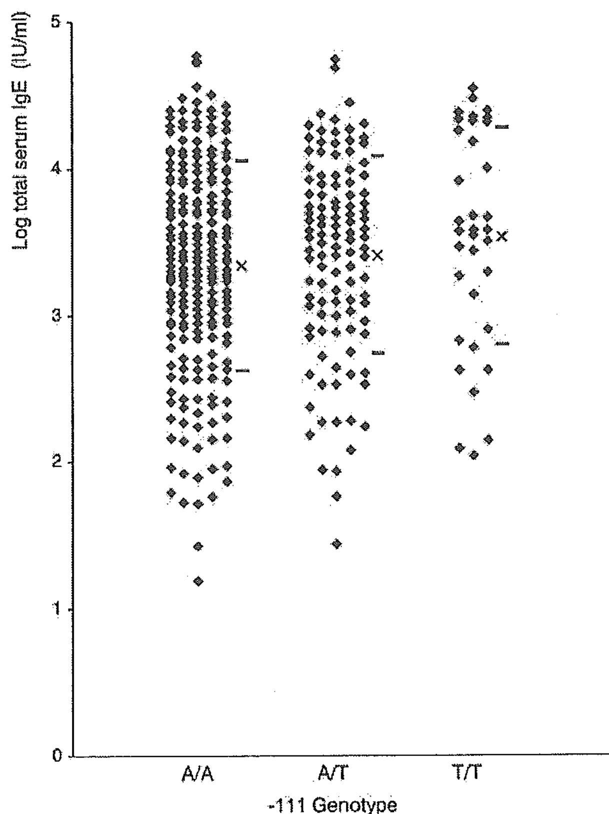

Figure 2. Relationship of *IL12RB1* −111 promoter genotype with total serum IgE levels in AD patients. Log-transformed individual IgE values are plotted, with the mean (×) and SD (bar) shown for each genotype group.

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 $P = 0.005$).

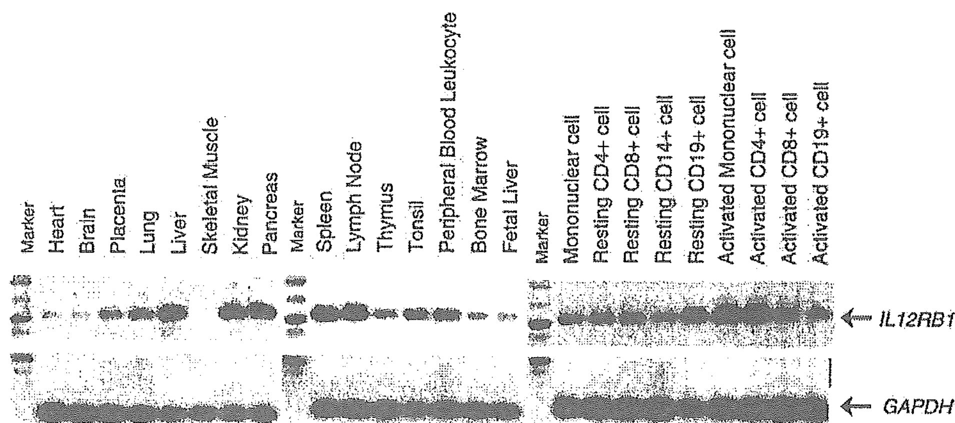

Figure 3. Expression of *IL12RB1* mRNA in multiple tissue cDNA panels. PCR amplification between *IL12RB1* exons 10 and 13 is shown in the upper panel. The *GAPDH* control is shown in the lower panel.

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

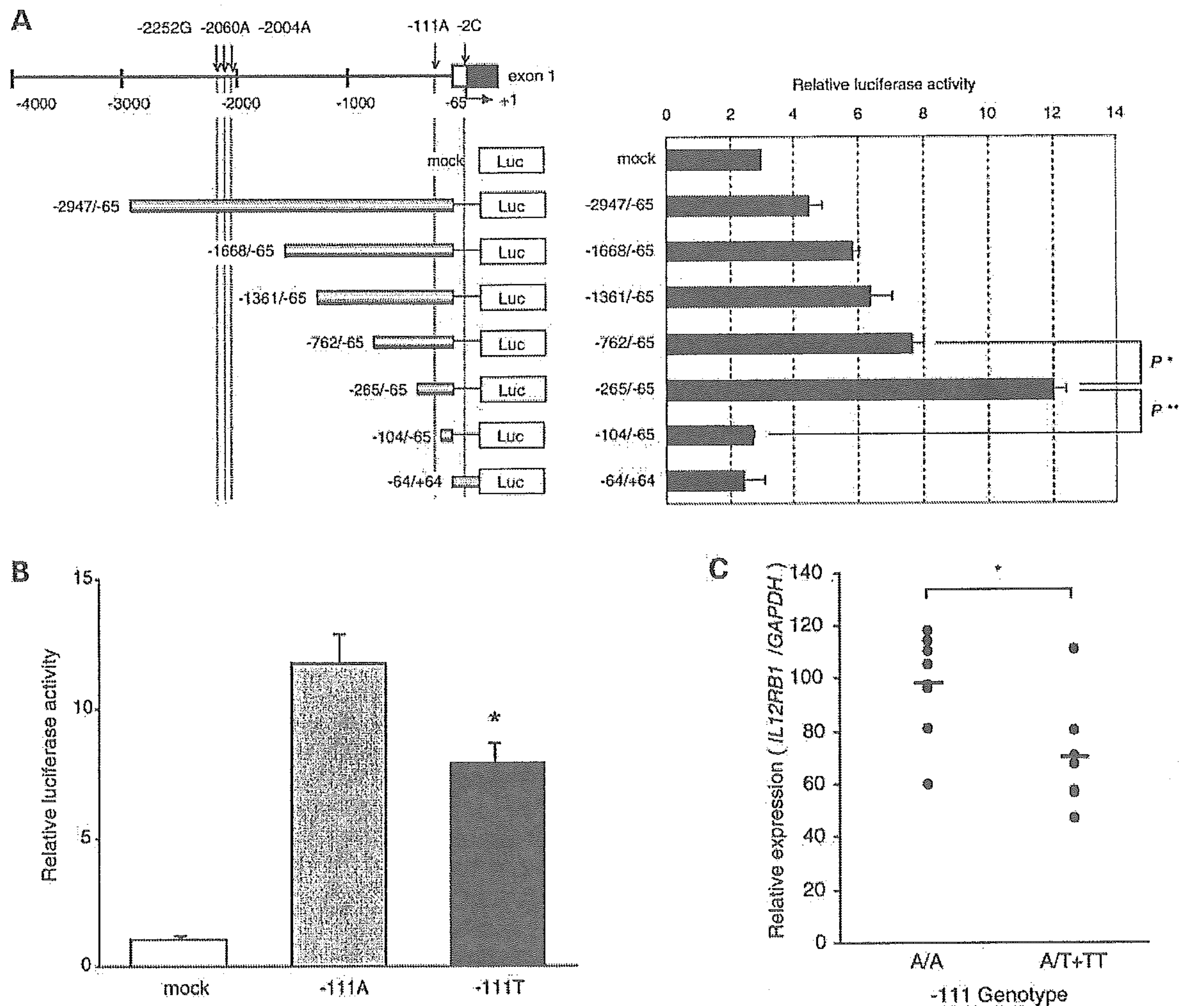

Figure 4. Identification of regulatory elements in the *IL12RB1* promoter, transcriptional effect of the −111A/T SNP and association of the −111 genotype with *IL12RB1* mRNA expression. (**A**) The indicated *IL12RB1* gene promoter fragments derived from wild-type allele sequence (−2252G/−2060A/−2004A/−111A/−2C)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

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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.

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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

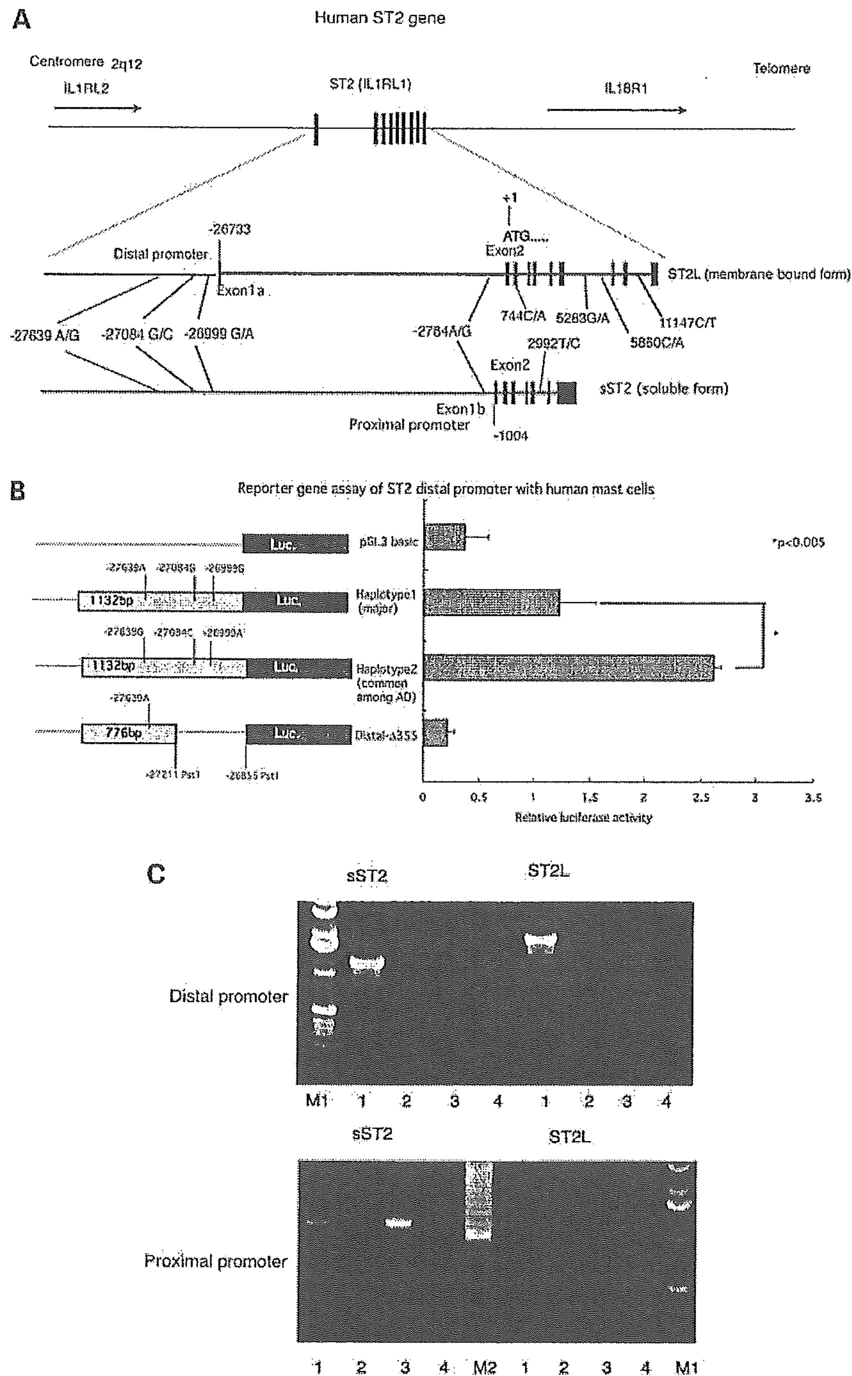

Figure 1. *ST2* gene structures and the roles of promoters in the induction of ST2 transcripts. (A) ST2 (IL1RL1) locus SNP map in the genomic region. The complete coding region of ST2, intron/exon boundaries, ~3 kb of 5′-genomic DNA, is shown. The longer variant (ST2L) has 11 exons and the shorter variant (sST2) has eight exons. These exons are indicated by closed rectangles. (B) Comparison of allelic variants of the ST2 distal promoter region analyzed by luciferase activity. Allelic differences in luciferase activity were examined using human mast (LAD2) cells. The constructs of the reporter plasmids are shown on the left. Five hundred nanograms of each plasmid was transfected with 10 ng of pRL-TK vector. Transcriptional activity was determined by assaying the *firefly* luciferase activity of cellar extracts prepared 24 h after transfection. Data show the mean ± SD relative activity from a representative experiment done in triplicate. *$P = 0.004$ by Student's *t*-test. (C) RT–PCR with cDNA from various cells in skin using specific primer sets for distinguishing each promoter and subtype (ST2L/sST2) expression. (Top left) Forward primer: exon 1a (distal promoter), reverse primer: sST2 specific region. (Top right) Forward: exon 1a, reverse: ST2L-specific region. (Bottom left) Forward: exon 1b (proximal promoter), reverse: sST2. (Bottom right) Forward: exon 1b, Reverse: ST2L. Lane 1: LAD2 (mast cells), lane 2: KC cultured with serum-free medium (SFM), lane 3: KC cultured with SFM + 10% FBS for 24 h, lane 4: dermal fibroblasts. M1: 1 kb molecular marker, M2: 100 bp molecular marker.

Table 1. Genotype frequencies for ST2 SNPs and AD susceptibility

	SNP number	Location	Control ($n = 636$)					AD ($n = 452$)					*P*-value[a]	*P*-value[b]	*P*-value[c]
			1	2	3	Sum	Minor allele frequency	1	2	3	Sum	Minor allele frequency			
1	14	−27639A/G	205	295	124	624	0.44	99	235	115	449	0.52	0.0026[d]	0.0007[d]	NS
2	18	−26999G/A	223	279	112	614	0.41	106	240	106	452	0.50	0.00024[d]	0.000049[d]	NS
3	41	744C/A	415	182	28	625	0.19	313	123	9	445	0.16	NS	NS	NS
4	49	2992C/T	221	286	113	620	0.41	183	205	57	445	0.36	NS	NS	NS
5	51	5283G/A	272	273	79	624	0.35	204	195	48	447	0.33	NS	NS	NS
6	57	5860C/A	225	284	110	619	0.41	187	205	56	448	0.35	NS	NS	NS
7	67	11147C/T	251	280	91	622	0.37	209	189	47	445	0.32	NS	NS	NS

NS, not significant.
[a]Allele1 versus allele2.
[b]Genotype11 versus 12 + 22.
[c]Genotype11 + 12 versus 22.
[d]*P*-value statistically significant after Bonferroni correction (raw *P*-values were multiplied by 7).

Table 2. Association between ST2-26999 G/A SNP and AD

	Controls ($n = 614$)	AD ($n = 452$)	χ^2 (*P*-value)	OR (95% CI)	AD total IgE>1700 ($n = 290$)	χ^2 (*P*-value)	OR (95% CI)
−26999G/A			GG:others			GG:others	
GG	223	106	20.20	1.86	53	30.23	2.55
GA	279	240	(0.0000070)[a]	(1.42–2.45)	166	(0.000000038)[a]	(1.81–3.58)
AA	112	106	(0.000049)[b]		71	(0.00000027)[b]	

[a]Raw *P*-value.
[b]*P*-value after Bonferroni correction.

Table 3. Haplotype structures and frequencies in ST2 distal promoter

Haplotype	Haplotype frequency		χ^2	*P*-value	OR
−27639, −26999	Case	Control			
A, G	0.56	0.48	13.00	0.0012	1.37
G, A	0.41	0.50	15.14	0.0004	1.41
G, G	0.025	0.019	0.85	0.35	1.32

two separate experiments and the results were similar. The total IgE concentration in the sera of 428 AD patients was measured with the fluorescence-enzyme immunoassay (FEIA) (Supplementary Material, Fig. S4B). The total IgE concentrations were 5371.9 IU/ml (mean) for the sera from −26999G/G genotype patients and 7898.7 IU/ml for those from −26999 G/A + A/A genotype patients. The serum concentration of total IgE was significantly lower in the sera of −26999G/G patients ($P = 0.0024$ by Mann–Whitney *U*-test). The correlation between the sST2 and the total IgE concentration was examined among −26999A/A genotype patients (Supplementary Material, Fig. S4C); Pearson's correlation coefficient was 0.28.

ST2L protein expression on the surface of human mast cells

Immunoprecipitation (IP) and subsequent western blotting using LAD2 cell lysate showed a positive band around 90 kDa in the IP samples with an anti-ST2 antibody (clone2A5). Deglycosylation with PNGaseF showed a shift of the band to lower molecular weight, corresponding to the molecular weight of non-glycosylated ST2L protein (Fig. 2A). To further demonstrate the surface expression of ST2L protein, non-stimulated LAD2 cells were stained with the anti-ST2 antibody (with FITC) and analyzed by FACS. The histogram showed a positive shift of the mean FITC intensity of ST2 staining (dotted line, Fig. 2B) compared with that of isotype-matched mouse IgG.

Immunohistochemistry

A paraffin section of the skin biopsy sample from an AD patient in the acute stage was stained with an anti-ST2 monoclonal antibody (clone HB12). Positive staining was observed on the cell surface of KC in the suprabasal layer and infiltrating cells in the dermal layer (Fig. 3A and C). ST2-positive staining was observed only with the infiltrating cells in the dermal layer of the skin of another AD patient in the chronic stage (Fig. 3B). Immunostaining with control mouse IgG_1 did not show positive signals (data not shown).

DISCUSSION

We found an SNP in the distal promoter region of ST2 (−26999G/A) that showed a significant association with AD during our series of genetic association studies within the IL1R gene cluster. This is the first association study for the

Table 4. Haplotype structures and frequencies in ST2

HaplotypeID	Haplotype frequency Case	Control	−27639	−26999	744	2992	5283	5860	11147
Haplotype A	0.41	0.5	G	A	C	T	A	C	C
Haplotype B	0.33	0.32	A	G	C	C	G	A	T
Haplotype C	0.13	0.12	A	G	A	T	A	C	C

Haplotype1/others: $\chi^2 = 17.5$; $P = 0.000028$; OR = 1.45.
Haplotype2/others: $\chi^2 = 0.15$; $P = 0.703$; OR = 1.04.
Haplotype3/others: $\chi^2 = 0.57$; $P = 0.451$; OR = 1.1.

ST2 gene and the results are intriguing, because the SNPs directly affect the expression level of Th2 cell marker ST2. Recent studies have clearly shown essential functional roles of ST2L protein for Th2-mediated immune responses (13,18,19), so it seems reasonable to investigate ST2 genetic polymorphism as a candidate for conferring susceptibility to AD. The result of case–control association studies of seven representative SNPs (Table 1) and haplotype analysis (Tables 3 and 4) showed that the highest association with AD was observed with the −26999G/A SNP as a single locus. There were four other SNPs in the ST2 genomic region that showed tight LD with the −26999G/A SNP. Three SNPs were located distal to the −26999G/A SNP in the distal promoter region. Two SNPs (−28214T/C; 3258 bp distal from the transcription starting site and −29778C/A; 1694 bp distal from the site) were not included in the functional analysis because our series of 5′-deletion promoter assays showed that the critical region for ST2 distal promoter activity was located within 300–500 bp from the transcriptional starting point (20); therefore, these two SNPs seemed to be less functional. Of the remaining two SNPs, one SNP (−27084G/C) was located at 85 bp distal to the −26999G/A SNP (236 bp distal to the transcriptional starting site); therefore, we decided to analyze these two SNPs together by reporter gene assay. The last SNP (−2874A/G) in tight LD with −26999G/A, located in the proximal promoter region of the *ST2* gene, did not affect the proximal promoter activity (Supplementary Material, Fig. S2).

The distal promoter reporter gene assay was performed with two 1132 bp distal promoter constructs, including two major haplotypes −27639A/−27084G/−26999G and −27639G/−27084C/−26999A covering >97% of haplotype frequency (Table 3). In addition to the two SNPs in the state of complete LD, (−27084/−26999), another SNP (−27639 A/G) that also showed a weak association with AD (Table 1) was included for analysis. We have reported that a GATA element commonly observed in both human and mouse *ST2* gene distal promoter region was indispensable for the activation of the promoter activity in Th2 cells (20,21). Therefore, we made another set of promoter assay constructs (Distal-Δ355) deprived of the GATA binding site and two SNP sites, which showed abrogated transcriptional activity (Fig. 1B). From these results, we concluded that this 356 bp region was essential for ST2 transcription and that the two SNPs (−27084G/C and −26999G/A) had major influences on the distal promoter activity among the SNPs with significant associations.

For further analysis of the roles of the genetic polymorphisms, we measured the serum concentrations of sST2 and total IgE and sorted the results by the genotype in the distal promoter. As the association study showed the most significant result under a dominant model (Tables 1 and 2), we compared the results by the genotype −26999G/G (low risk for AD) versus −26999A/G + A/A (high risk for AD). The results matched the results of the reporter gene assay and the association study. Furthermore, the genetic association between the −26999G/A SNP and AD patients for very high serum total IgE (IgE > 1700 IU/ml) became stronger (Table 2). These results suggested that having at least one allele of −26999A was correlated with a high sST2 level and a high total IgE concentration and an increased risk for AD. There is some controversy over the role of IgE in the pathogenesis of AD (22); therefore, it will be useful to genotype intrinsic AD (1) patients in the future.

We found a weak correlation ($r = 0.28$) between the serum sST2 level and the total IgE concentration with the genotype −26999AA patients. This finding was consistent with the recent report that the increase of food-specific IgE is paralleled by elevated sST2 levels, not by serum IL-4, IL-13 and interferon gamma levels (23). These results suggested possible effects of sST2 in IgE production, so further studies seem to be essential.

We reported sST2 concentrations of 200 healthy controls and 56 asthmatic patients previously (24). The sST2 concentration of healthy controls was 0–1.65 ng/ml (mean 0.415 ng/ml) and that of asthmatic patients was 0–2.40 ng/ml (mean 0.493 ng/ml). A differential rise of the serum ST2 level that correlated well with the severity of asthma exacerbation was observed (24). The serum concentration of sST2 in AD patients [0–1.02 ng/ml (mean 0.326 ng/ml)] was not significantly higher or lower than that of healthy controls or asthmatics; nonetheless, there was a correlation between the ST2 genotype and the sST2 concentration. We are now investigating the changes of the sST2 concentration during the clinical stages of AD, and the results might further clarify the role of sST2 in AD.

It has been reported that the usage of two different promoters (distal and proximal) depends on the type of cell for the human *ST2* gene (21). Consistent with this report, we showed that only hematopoietic cells utilized the distal promoter and that ST2 transcription of other skin cells (KC, dermal fibroblasts) was initiated from the proximal promoter (Fig. 1C). These results suggested that the significant association of SNPs −27084 and −26999 in the ST2 distal promoter

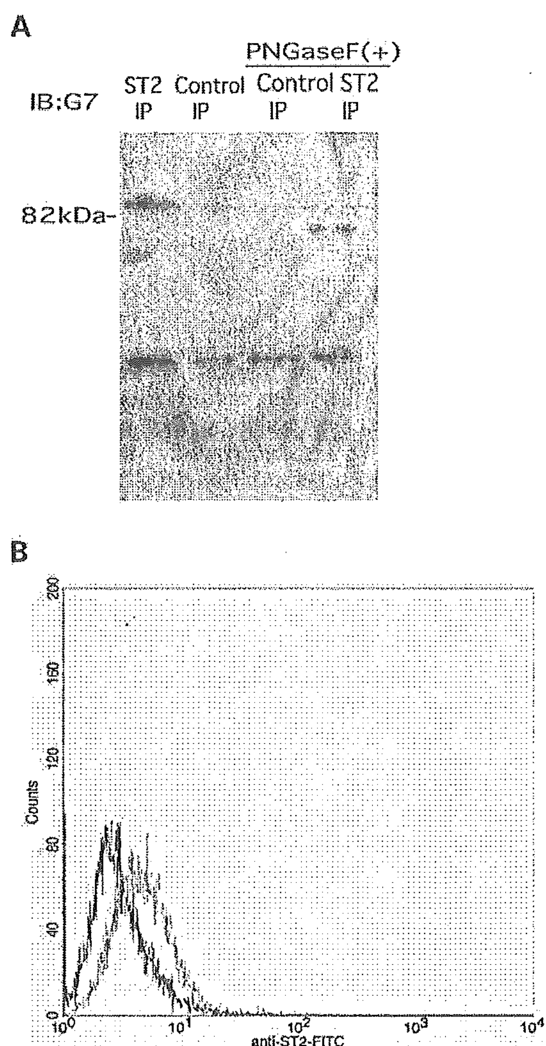

Figure 2. ST2L expression in LAD2 cells. (**A**) LAD2 cell lysate samples were immunoprecipitated with either an anti-ST2 IgG antibody (2A5) or an isotype-matched control antibody. The immunoprecipitated samples were electrophoresed and immunoblotted with an anti-ST2 IgM antibody (clone: G7). Duplicated samples after IP were treated with PNGaseF for 1 h and then immunoblotted simultaneously. Lane 1: anti-ST2-IP, lane 2: control antibody-IP, lane 3: control antibody-IP-PNGaseF-treated, lane 4: anti-ST2-IP-PNGaseF-treated. (**B**) Cell surface ST2L protein expression in LAD2 was analyzed with a FACS Calibur. FcR of LAD2 cells were blocked and then stained with an anti-ST2 antibody (2A5). FITC-goat anti-mouse IgG1 was used as the secondary antibody. Staining with control mouse IgG1 is shown with a black line and the anti-ST2 antibody is shown with the dotted red line.

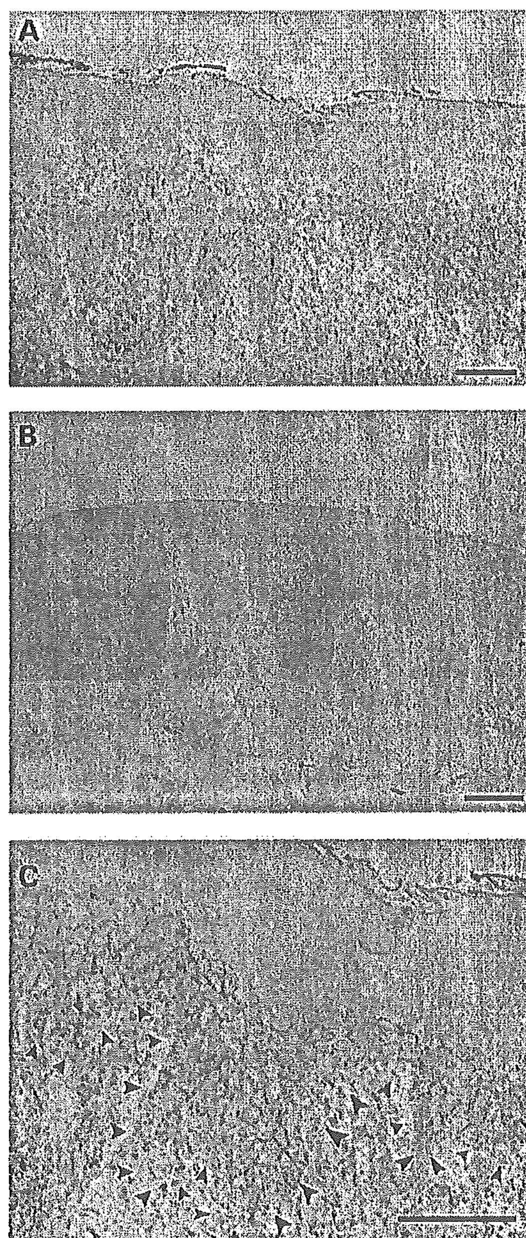

Figure 3. Immunohistochemical staining of human skin samples obtained from AD patients with anti-ST2 monoclonal antibody. Paraffin sections of AD skin biopsies were immunostained with an anti-ST2 antibody (HB12). (**A**) Skin biopsy from acute stage AD. Suprabasal layers of KC show membranous ST2-positive staining. Sporadic positive staining in the dermal region was also observed. (**B**) Skin biopsy from chronic stage AD. Some of the infiltrating cells in the dermal layer show positive ST2 staining. (**C**) High magnification of anti-ST2 immunostaining with acute stage AD. The arrowheads indicate the limit of basement membrane. Bar = 200 μm.

region predominantly affected hematopoietic cells. We found that both ST2L and sST2 mRNAs were expressed most abundantly in mast cells (Supplementary Material, Fig. S3) and confirmed ST2L expression on mast cells at the protein level by western blotting (Fig. 2A) and FACS analysis (Fig. 2B). Moritz *et al.* (14) reported that ST2L was selectively expressed during the development of mast cell lineage, and very recently Chen *et al.* (25) showed that ST2L could be one of the markers for mast cell progenitors in adult mice. These results suggested that abundant ST2L expression might positively affect the number of mature mast cells in the skin, as observed in the AD skin region

(26). The functional roles of ST2L in mast cells will be clarified with an ST2L overexpression system (10), and a study is ongoing.

The positive immunostaining around the cell membrane of suprabasal KC in the acute stage of AD (Fig. 3A and C) reflects the accumulated sST2 in intercellular space because ST2L mRNA expression in KC was not observed in experiments *in vitro*. This is consistent with a previous study that showed intense ST2 protein accumulation in mouse epidermis (27), and we think that serum extravasation during the acute stage of AD may induce sST2 expression in KC as observed in our *in vitro* study (Fig. 1C) (Supplementary Material, Fig. S3). On the other hand, a histological sample from the chronic phase of AD showed slight ST2 staining (Fig. 3B). This might be a reflection of the shift toward the Th1 dominant immunological character observed in the chronic stage of AD (1,28).

Another clinical feature of AD is a reduced skin innate immune response (1). ST2L expression could inhibit the TLR-dependent innate response by sequestering the adaptor molecules Myd88 and Mal (17). Several reports showed that both anti-ST2 antibodies and ST2-immunoglobulin fusion protein could abrogate the Th2 immune response and eosinophilic responses (18,29). Therefore, we consider that sST2/ST2L will be a good therapeutic target of AD and that understanding of the genetic predisposition for high ST2 promoter activity may contribute to the prevention of severe AD.

MATERIALS AND METHODS

Antibodies and cell lines

Anti-ST2 monoclonal antibodies (mouse IgG1; clones 2A5 and HB12) were purchased from MBL (Nagoya, Japan), and an anti-ST2 monoclonal antibody (mouse IgM; clone G7) was generated as previously described (30). Human mast cell line LAD2 was kindly provided by Dr Arnold Kirshenbaum (NIAID, NIH) and maintained as previously described (31). Human neonatal skin fibroblasts were obtained from RIKEN cell bank (Tsukuba, Japan), immortalized human normal keratinocyte cells (PHK16-06b) were obtained from the Japanese Collection of Research Bioresources (JCRB) cell bank (Osaka, Japan).

Subjects

All subjects with AD were diagnosed according to the criteria of Hanifin and Rajka (32). Peripheral blood was obtained from 452 AD patients (mean age 30.0, 11–64 years old at enrollment of the study; mean age 7.1, 0–45 years old at the onset of AD; 236 males and 216 females) from Takao Hospital, Shiga Medical College Hospital and Yokohama City University Hospital. Sera for sST2 ELISA assay were also obtained from some of the patients enrolled in this genetic study. As a control group, we analyzed 636 randomly selected population-based individuals (mean age 42.2, 18–70 years). We excluded the presence of asthma, AD and nasal allergy in the control population via careful interview by physicians. All individuals were Japanese and gave written informed consent to participate in the study (or, for individuals less than 16 years old, their parents gave consent), according to the rules of the process committee at SNP Research Center, RIKEN.

Screening for genetic polymorphisms

The ST2 genomic region targeted for SNP discovery included a 2.5 kb continuous region 5′ to exon 1a (distal promoter region) and a 2.5 kb continuous region 5′ to exon 1b (proximal promoter region) and 11 exons, each with a minimum of 200 bases of flanking intronic sequences (Fig. 1A). Primer sets (Supplementary Material, Table S1) were designed on the basis of the ST2 genomic sequence (GenBank accession no. AC007248). Each polymerase chain reaction (PCR) was carried out with 5 ng of genomic DNA from 24 individuals. Sequence reaction was performed with Big Dye Terminator v3.1 using an ABI 3700 DNA analyzer.

Genotyping

We genotyped a total of seven representative SNPs in the *ST2* gene selected on the basis of the allele frequency (MAF > 10%) and LD (Table 1) (Supplementary Material, Table S2 and Fig. S1). Additional typing was carried out for some SNPs, in relation to the functional assay for *ST2* genes. The SNP typing was carried out either with the invader assay (33) or with the Taqman genotyping assay using an ABI PRISM 7700 sequence detection system. Invader assay was performed with multiplex PCR products as the template. Taqman genotyping assay was carried out according to the manufacturer's protocol.

Statistical analysis

Allele frequencies in AD cases and controls were compared by the contingency χ^2-test. A *P*-value of less than 0.01, also in the case of multiple comparisons after Bonferroni adjustment, was considered to be statistically significant. ORs and 95% confidence intervals (95% CI) were calculated. Pairwise LD coefficients were calculated and expressed as r^2. Intragenic LD and haplotype analyses were performed using SNPAlyze v2.0 (DYNACOM, Chiba, Japan) as recommended by the manufacturer. We estimated haplotype frequencies using the expectation–maximization algorithm. Comparison in reporter gene assay was performed with Student's *t*-test. The association between the serum sST2 level or total IgE concentration and the genotype was evaluated by the Mann–Whitney *U*-test. A *P*-value of less than 0.05 was considered to be statistically significant.

Reporter gene assay

We subcloned 1131 bp distal promoter sequences continuous to exon 1a into pGL3 basic vector (Promega Corporation, Madison, WI, USA). Two SNPs in this region (−27084G/C and −26999G/A) were in the state of complete LD. We made two haplotype clones 1 (−27639A, −27084G, −26999G) and 2 (−27639G, −27084C, −26999A). Another set of constructs was made by deleting a 355 bp long promoter sequence

between two PstI sites, which contained the −27084G/C and −26999G/A SNPs as well as two putative GATA binding sequences (named distal-Δ355). All subcloned plasmids were verified by direct sequencing. We transfected the pGL3-ST2 promoter plasmid and pRL-TK renilla luciferase vector (Promega) as an internal control for transfection efficiency into human cell line LAD2 with DMRIE-C (Invitrogen, Carlsbad, CA, USA). After 24 h, luciferase activity was measured with a Dual Luciferase Reporter Assay Kit (Promega).

Measurement of sST2 protein and total IgE

The protein level of sST2 in the sera of AD patients was measured using human ST2 ELISA kits (MBL) following the manufacturer's protocol. The total IgE concentration in serum was measured by the FEIA method in a commercial laboratory.

RT–PCR analysis for differential promoter usage

mRNA was isolated from cultured cells (LAD2, KC and human dermal fibroblasts) with a Quick Prep micro-mRNA purification kit (Amersham Bioscience, Little Chalfont, UK). cDNA was made with the Super Script III First-Strand Synthesis System (Invitrogen) using oligo(dT)$_{20}$ primer. To distinguish promoter usage for specific cell types and subtypes (sST2/ST2L) of mRNA, we made sets of specific primers and performed RT–PCR as previously described (21).

IP and western blotting analysis

First, 1×10^7 LAD2 cells were solublized with lysis buffer [1% Triton X-100 in 20 mM Tris–HCl, pH 7.6, 150 mM NaCl with Complete Mini protease inhibitor cocktail tablets (Roche, Penzberg, Germany)]. The cell lysate was centrifuged at 20 000*g* for 15 min at 4°C. The supernatant was taken and pre-cleared with Protein-A Sepharose (Amersham) for 30 min. The sample was reacted with 2 μg of the anti-ST2 antibody (2A5) or control mouse IgG1 for 1 h and then Protein-A Sepharose was added. After 3 h rotation at 4°C, the Sepharose was washed with the lysis buffer and finally suspended with SDS sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 20% glycerol, 0.4% bromophenol blue, 50 mM DTT). To check the glycosylation status of ST2L protein, aliquots of the IP samples were treated with PNGaseF (New England Biolaboratory, Bevery, MA, USA). SDS–PAGE and subsequent immunoblotting were essentially performed as previously described (34). In brief, samples were subjected to SDS–PAGE using 4–20% Tris–glycine polyacrylamide gels and then electrophoretically transferred onto a PVDF membrane (Millipore). The membrane was incubated with the mouse anti-human ST2 IgM antibody (G7) overnight at 4°C. After washing with PBS, the membrane was reacted with a horseradish peroxidase (HRP)-conjugated anti-mouse IgM antibody for 30 min. The membrane was developed onto X-ray film with ECL plus (Amersham).

Flow cytometric analysis

Flow cytometric analysis was carried out using the anti-ST2 monoclonal antibody (2A5). LAD2 cells were washed with PBS, and Fc receptors (FcR) were blocked with FcR blocking reagent (Miltenyi Biotec, Gladbach, Germany). Cells were reacted with 4 μg of the anti-ST2 IgG monoclonal antibody in a volume of 40 μl for 15 min at room temperature. As a control, an isotype-matched mouse IgG1 antibody was used. After washing with PBS, the cells were reacted with an FITC-conjugated anti-mouse IgG antibody (Dako Japan, Kyoto). The stained cells were analyzed with a FACS Caliber (BD Japan).

Immunohistochemistry

ST2 immunohistochemistry was performed essentially as described previously (35). In brief, formaldehyde-fixed paraffin sections of the skin biopsies from AD patients were deparaffinized, then the endogenous peroxidase activity was quenched with 0.3% H_2O_2 in methanol for 20 min. Non-specific staining was blocked with blocking buffer (10% normal goat serum, 1% BSA in PBS) for 30 min. The anti-ST2 monoclonal antibody (clone HB12) was applied and reacted overnight at 4°C. After washing with PBS, slides were incubated with HRP-conjugated anti-mouse IgG for 30 min. The slides were developed with DAB (Dojindo, Kumamoto, Japan).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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Coding SNP in tenascin-C Fn-III-D domain associates with adult asthma

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The extracellular matrix glycoprotein tenascin-C (TNC) has been accepted as a valuable histopathological subepithelial marker for evaluating the severity of asthmatic disease and the therapeutic response to drugs. We found an association between an adult asthma and an SNP encoding TNC fibronectin type III-D (Fn-III-D) domain in a case–control study between a Japanese population including 446 adult asthmatic patients and 658 normal healthy controls. The SNP (44513A/T in exon 17) strongly associates with adult bronchial asthma (χ^2 test, $P = 0.00019$, Odds ratio = 1.76, 95% confidence interval = 1.31–2.36). This coding SNP induces an amino acid substitution (Leu1677Ile) within the Fn-III-D domain of the alternative splicing region. Computer-assisted protein structure modeling suggests that the substituted amino acid locates at the outer edge of the beta-sheet in Fn-III-D domain and causes instability of this beta-sheet. As the TNC fibronectin-III domain has molecular elasticity, the structural change may affect the integrity and stiffness of asthmatic airways. In addition, TNC expression in lung fibroblasts increases with Th2 immune cytokine stimulation. Thus, Leu1677Ile may be valuable marker for evaluating the risk for developing asthma and plays a role in its pathogenesis.

INTRODUCTION

Asthma is a chronic inflammatory disease characterized by smooth muscle hypertrophy, excess mucus secretion and increased deposition of extracellular matrix (ECM) around the basement membrane (1–3). Many asthmatic patients also have an atopic tendency characterized by a Th2 dominant cytokine profile including interleukin (IL)-4 and IL-13 (4). Several studies showed genetic associations between asthma and proteinases like ADAM33 (5) or Th2 cytokine receptors (4,6), but to the best of our knowledge, there is no report of an association between asthma and ECM genes. The hexametric

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