Introduction

The origin of allergy may be strongly influenced by a variety of environmental exposures; however, host susceptibility and a variety of genes are also likely to be involved in the etiology and pathogenesis of allergic diseases such as asthma and hay fever [1–3]. Japanese cedar pollinosis (JCP) is a springtime hay fever caused by inhalation of the pollen of Japanese cedar *(Cryptomeria japonica)*, representing a major health problem in Japan because of its high prevalence, severe symptoms, impairment of the patient's quality of life and expenses in controlling the disease [4–6]. Recently, several candidate genes such as the *FCER1B* gene [7], the *IL4RA* gene [8], the *EPO* gene [8, 9] and the *ADAM33* gene [10], have been reported to underlie JCP and its intermediate phenotypes, suggesting a contributory role of genetic factors in the development of this common atopic disorder.

The type 2 cytokine IL-13, which shares signaling pathways and many biological activities with IL-4, plays a pivotal role in the generation of allergic airway inflammation [11–13]. To date, numerous genetic analyses have indicated that the gene encoding human IL-13 (located on chromosome 5q31) is implicated in the development of asthma and atopy [14, 15]. Of the *IL13* gene, Arg110Gln, which is a functional single nucleotide polymorphism (SNP) in the coding region [16, 17], has been comprehensively studied and has been found to be associated with asthma phenotypes in ethnically diverse populations [18–23]. Furthermore, recent genetic association studies in the German population [24] and Chinese samples [25] suggested a potential role of the *IL13* variant Arg110Gln (referred to as Arg130Gln in their reports) for heightened IgE production and atopic sensitization in allergic rhinitis/hay fever. To address whether this coding SNP affects susceptibility to JCP, the most common hay fever in Japan, we performed a case-control study in a Japanese population.

Subjects and Methods

Subjects and Phenotypes

The present study was performed with the approval of the Ethical Committee of the RIKEN Yokohama Institute, and written informed consent was obtained from all participants. 95 unrelated adult individuals with JCP and 95 age-matched unrelated healthy controls were enrolled in the study. All subjects were from the population of the Kinki area (west Japan).

The phenotypic characteristics of recruited subjects have been described in detail elsewhere [10] and are summarized in table 1. Briefly, of 95 patients with JCP, 5 cases (5.3%) were mild, 22 cases (23.2%) were moderate and 68 cases (71.6%) were diagnosed as severe according to the scores of three main nasal symptoms (sneezing, rhinorrhea and nasal obstruction) based on the clinical severity classification for allergic rhinitis (Okuda's method), as previously described [26]. None of the patients had a history of asthma and allergen-specific immunotherapy. The control subjects were all symptom free, had no history of atopic disorders and had negative allergen-specific IgE (<0.7 arbitrary unit/ml) in serum against house dust mites (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), Japanese cedar pollen and three other common pollens in the study area (orchard grass, ragweed and *Artemisia*). The geometric mean of serum total IgE levels was 162.5 (range 5.3–10,000) IU/ml in cases and 30.0 (range 3.2–240) IU/ml in healthy controls. Patients with JCP had higher total IgE levels than control subjects (mean ± SD: 2.21 ± 0.51 vs. 1.48 ± 0.51 log IU/ml; $p < 0.0001$, t test).

Table 1. Phenotypic characteristics of recruited subjects

Category	Cases n = 95	Controls n = 95
Total serum IgE levels (means ± SD)		
log IU/ml	2.21 ± 0.51	1.48 ± 0.51
RAST positive to Japanese cedar pollen	95 (100%)	0 (0.0%)
RAST positive to house dust mites	43 (45.3%)	0 (0.0%)
Eosinophil positive in nasal secretions	82 (86.3%)	NA
Rhinitis severity		
Mild	5 (5.3%)	NA
Moderate	22 (23.2%)	NA
Severe	68 (71.6%)	NA

Genotyping

DNA samples were extracted from whole peripheral blood of study subjects by standard methods. PCR reaction was performed with 5 ng of template genomic DNA, in a 10-μl solution consisting of 13.75 pmol of each primer of 5′-tgacctctttgtcctgcag-3′ for forward and 5′-tgatgctttcgaagtttcagt*a*gat*c*-3′ for reverse (italic nucleotides modified to create a *Bgl*II restriction site), 1.1 μl of 10× Vogelstein buffer (pH 8.8), 0.55 μl of 78 m*M* $MgCl_2$, 0.55 μl of 25 m*M* each dNTPs and 0.55 U of Ex-*Taq* DNA polymerase (TaKaRa Bio, Otsu, Japan). Thermocycling started with an initial denaturation step for 2 min at 95°C, and then 37 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C and extension for 30 s at 72°C, with a final extension step for 7 min at 72°C. A 263-bp PCR fragment including the Arg110Gln polymorphism was then digested by addition of 3 U *Bgl*II (TaKaRa Bio) overnight at 37°C. The digestion products were visualized on a 4% agarose gel stained with ethidium bromide.

Statistics

Statistical analysis was performed using SPSS 10.0J for Windows (SPSS, Chicago, Ill., USA). The Hardy-Weinberg equilibrium was assessed by χ^2 test. The genotype and allele frequencies for the *IL13* variant Arg110Gln in cases and control subjects were compared using Pearson's χ^2 test. If an expected number was less than 5, Fisher's exact test was used. Quantitative traits relating to rhini-

Table 2. Genotype and allele frequencies for Arg110Gln variant

Category	Cases n = 95	Controls n = 94	Odds ratio (95% CI)	p value
Genotype				
Arg/Arg	0.568	0.479	–	
Arg/Gln	0.347	0.426	0.69 (0.38–1.26)	0.226
Gln/Gln	0.084	0.096	0.74 (0.26–2.08)	0.568
Allele				
Arg	0.742	0.691	–	
Gln	0.258	0.309	0.78 (0.50–1.22)	0.275
Major homozygote	0.568	0.479	–	
Minor homozygote + heterozygote	0.432	0.521	0.70 (0.39–1.24)	0.217
Major homozygote + heterozygote	0.916	0.904	–	
Minor homozygote	0.084	0.096	0.87 (0.32–2.36)	0.782

In healthy controls, 94 samples were successfully genotyped. The reference category was assigned an odds ratio of 1.00. CI = Confidence interval.

Table 3. Arg110Gln genotypes and total serum IgE levels

Category	Cases n = 95	Controls n = 94	Total n = 189
Total IgE (means ± SD), log IU/ml			
Arg/Arg	2.196 ± 0.442	1.486 ± 0.490	1.873 ± 0.583
Arg/Gln	2.263 ± 0.585	1.539 ± 0.544	1.866 ± 0.666
Gln/Gln	2.097 ± 0.632	1.213 ± 0.459	1.629 ± 0.697
p value	0.676	0.231	0.188[a]

In healthy controls, 94 samples were successfully genotyped.
[a] Analysis using a general liner model incorporating disease status (case or control) as a covariate.

tis severity and nasal eosinophila in patients with JCP were also analyzed with χ^2 test. Association of the Arg110Gln genotypes with total serum IgE levels (logarithm transformed) and cedar pollen-RAST scores was examined by ANOVA and general liner model. Nonparametric tests were employed to analyze associations between Arg110Gln genotypes and cedar pollen-specific IgE values in patient sera. Two-tailed p values of less than 0.05 were considered statistically significant.

Results

In our study population, the distributions of Arg110Gln genotypes of the *IL13* gene were in Hardy-Weinberg equilibrium, and the overall allele frequencies for Arg110 and Gln110 were 0.717 (271/378) and 0.283 (107/378), respectively. No significant association was detected between the Arg110Gln variant and susceptibility to JCP ($p > 0.05$, table 2). Moreover, this variant was not significantly associated with rhinitis severity and nasal eosinophila in patients with JCP (outlined in table 1). The frequency of the minor allele Gln110 was 0.278 in severe cases compared to 0.250 in mild-to-moderate cases (odds ratio = 1.15, 95% confidence interval = 0.57–2.35; $p > 0.05$), and was 0.308 in patients with eosinophil-positive compared to 0.250 in those with eosinophil-negative nasal secretion (odds ratio = 1.33, 95% confidence interval = 0.54–3.30; $p > 0.05$).

An analysis was also carried out on the relationship between investigated genotypes and IgE measurements. We did not find significant differences in total serum IgE levels among the Arg110Gln genotypes in JCP patients, healthy controls and both groups combined ($p > 0.05$, table 3). There was no correlation between the Arg110Gln genotype and cedar pollen-RAST scores in our study population ($p > 0.05$). The Arg110Gln variant was also not significantly associated with cedar pollen-specific IgE val-

ues in sera from patients ($p > 0.05$). A tendency to lower cedar pollen-RAST scores was observed in those homozygous for Gln110 compared to those homozygous for Arg110 and heterozygous combined, but statistical significance was not reached ($p = 0.067$). In addition, we analyzed the allele and genotype frequencies of the Arg110Gln variant in JCP-affected individuals with or without sensitization to house dust mites and did not observe any significant association ($p > 0.05$).

Discussion

The *IL13* gene encodes a T-lymphocyte-derived cytokine, IL-13, which is produced primarily by activated Th2 cells. IL-13 has been shown to be an important and unique mediator of allergic processes such as IgE production, eosinophilic inflammation, mucus hypersecretion and airway hyperresponsiveness [27]. Recently, numerous SNPs have been identified at the *IL13* locus, and a significant association has been found between these SNPs and asthmatic and/or allergic phenotypes in several populations of distinct ethnic background [18–23, 28–32]. The role of a common coding SNP in the fourth exon that causes a substitution of the amino acid arginine by glutamine at position 110 of the mature protein (Arg110Gln) in the development of asthma and atopy has been widely investigated in ethnically diverse groups; however, less attention was directed to the genetic influence of this functional SNP on the risk of allergic rhinitis/hay fever.

This study represents an evaluation of the Arg110Gln variant in the *IL13* gene as a susceptibility locus for JCP, one of the most common seasonal allergic diseases in the Japanese population. Using a case-control study, we evaluated the Arg110Gln variant for evidence of association to JCP and related phenotypes. Based on the results, we found no evidence to support a significant association between the Arg110Gln variant and the diagnosis of JCP. We also noticed no significant association between this coding SNP and cedar-pollinosis-related traits including serum levels of total and allergen-specific IgE, eosinophil counts in nasal secretion and clinical severity of rhinitis. Our findings might indicate that genetic variation in Arg110Gln at the *IL13* locus is not likely to be involved in the development of JCP.

Of course, the lack of association in our study could reflect a type II error. However, a previous case-control study has shown no significant association of the *IL13* variant Arg110Gln with self-reported hay fever in a large cohort of Germans [24]. Moreover, there was no relationship between this coding SNP and the diagnosis of allergic rhinitis due to *Artemisia* pollen and/or Der p 1 in a Chinese population [25]. For atopy-related phenotypes, Nieters et al. [24] found a marginal significance for the association ($p = 0.046$) of the Arg110Gln variant with in vitro specific IgE responses to common inhalant allergens in their study subjects, being almost completely of Caucasian origin, while Wang et al. [25] showed a borderline effect ($p = 0.039$) of this SNP on serum total IgE levels, but not on specific IgE concentrations against either *Artemisia* pollen or Der p 1, in Chinese patients with allergic rhinitis. Most recently, Miyazawa et al. [33] also reported a negative association of this SNP with JCP susceptibility and anti-Cry j 1 antibody titers in a small Japanese study cohort. Combined with the results of our study, these facts suggest that the Arg110Gln variant in *IL13* is unlikely to represent a major determinant in the development of hay fever and allergic sensitization in ethnically diverse populations.

Hay fever is a typical atopic disease characterized by type I hypersensitivity reactions following induction of IgE-sensitized mast cell release by allergenic pollens. Although T-cell activation is also a characteristic feature of allergic disorders in the upper and lower airways, increased T-cell activation is not consistently found in hay fever [34, 35]. This may explain the lack of association between the Arg110Gln variant of the *IL13* gene (encoding cytokine IL-13 produced principally by activated Th2 cells) and susceptibility to hay fever seen in our study as well as in others [24, 25, 33].

It should also be mentioned that several previous case-control association studies did not reveal any relationship between the *IL13* variant Arg110Gln and asthma per se or asthma-related traits including bronchial hyperresponsiveness, elevated total IgE levels and positive allergen skin tests [28, 36, 37]. However, these data were in contrast to studies showing an association of this SNP with asthma susceptibility [18, 23] or allergy phenotypes [20, 21, 23]. The effects of racial and ethnic differences in environmental and/or genetic risk factors on the development of complex common diseases [38] may account for the conflicting findings. Interestingly, a significant correlation was well demonstrated between the Gln110 variant and high total IgE levels [19, 29, 30], but not specific IgE against common allergens [19, 31], in an unselected population of German children. Consequently, it might be deduced that rather than controlling allergen-specific IgE responses, the Arg110Gln variant at the *IL13* locus may play a potential role in total serum IgE production during the early life.

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 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

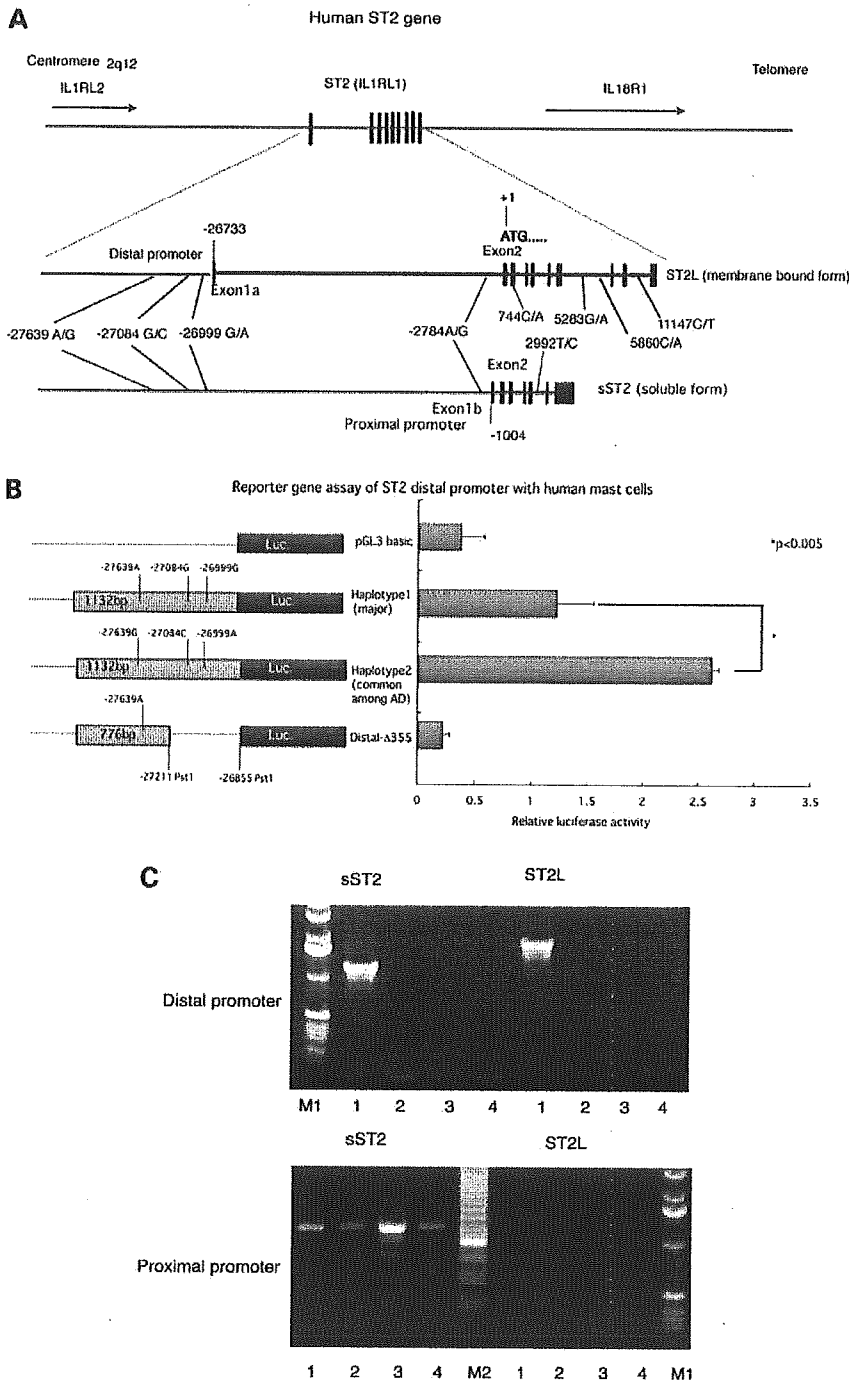

Figure 1. *ST2* gene structures and the roles of promoters in the induction of ST2 transcripts. (**A**) ST2 (IL1RL1) locus SNP map in the genomic region. The complete coding region of ST2, intron/exon boundaries, ~3 kb of 5′-genomic DNA, is shown. The longer variant (ST2L) has 11 exons and the shorter 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 −27639, −26999	Haplotype frequency Case	Control	χ^2	*P*-value	OR
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		−27639	−26999	744	2992	5283	5860	11147
	Case	Control							
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

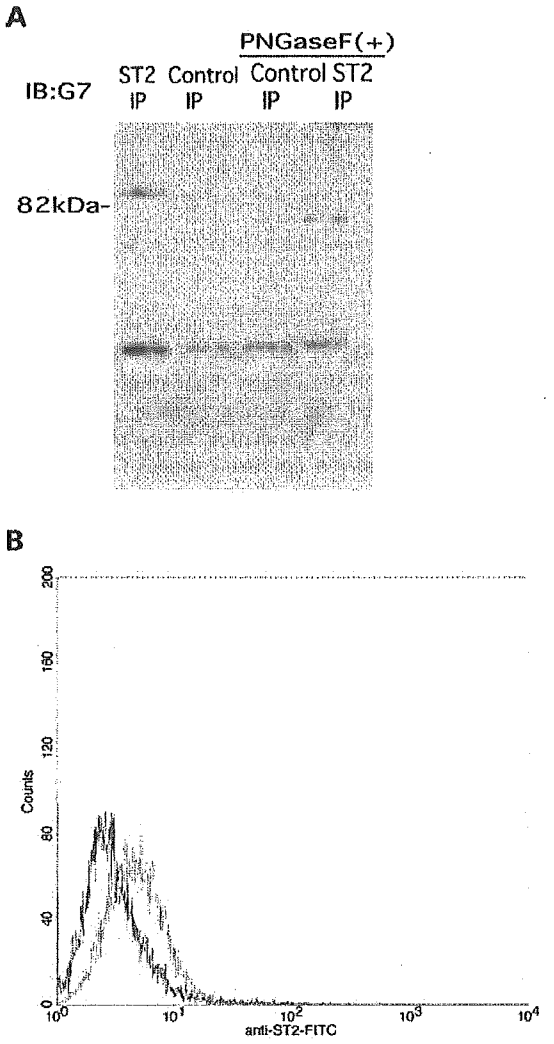

Figure 2. ST2L expression in LAD2 cells. (**A**) LAD2 cell lysate samples were immunoprecipitated with either an anti-ST2 IgG antibody (2A5) or an isotype-matched control antibody. The immunoprecipitated samples were electrophoresed and immunoblotted with an anti-ST2 IgM antibody (clone: G7). Duplicated 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.

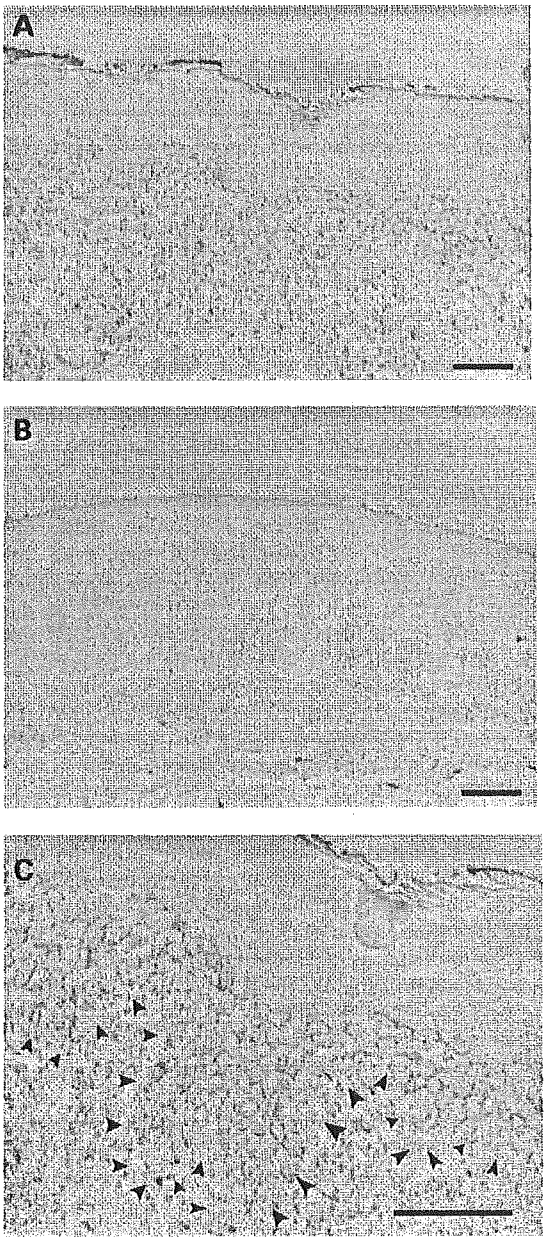

Figure 3. Immunohistochemical staining of human skin samples obtained from AD patients with anti-ST2 monoclonal antibody. Paraffin sections of AD skin biopsies were immunostained with an anti-ST2 antibody (HB12). (**A**) Skin biopsy from acute stage AD. Suprabasal layers of KC show membranous ST2-positive 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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Association of the *IL12RB1* promoter polymorphisms with increased risk of atopic dermatitis and other allergic phenotypes

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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.

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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

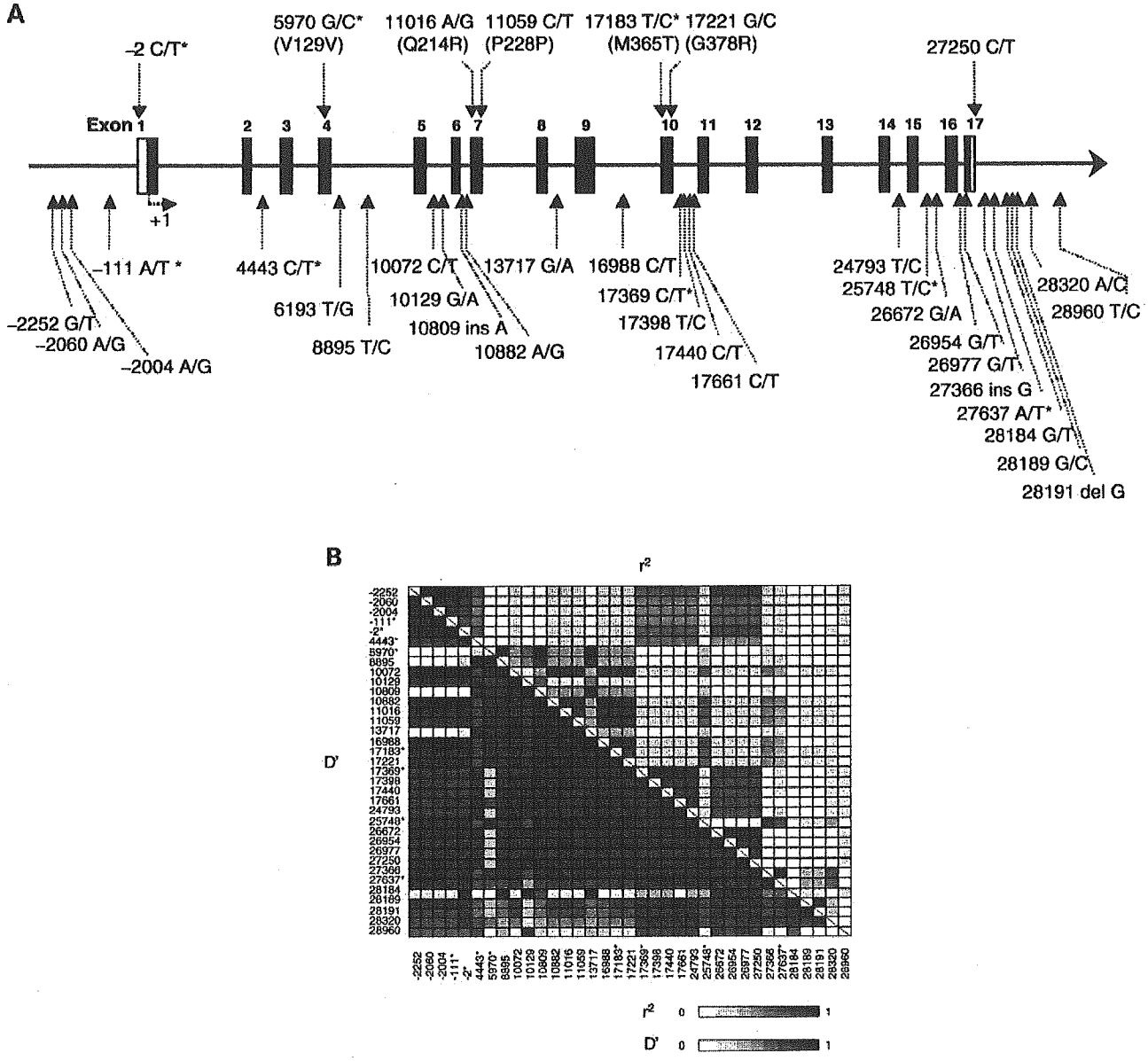

Figure 1. (**A**) A graphical overview of the 36 identified SNPs in relation to the exon/intron structure of the human *IL12RB1* gene. Nucleotide position one (+1) is the first adenine of the initiation codon (ATG) such that SNP positions are given relative to the ATG. The 17 coding exons are shown by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