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) (χ²-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 common 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.
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 r² 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 −26999A/G) 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 = 0.00000038, 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 (−27693A clone). The distal-Δ355 clone with the −27693G allele showed a result similar to that for the −27693A 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 in 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 concentration of sST2 protein was significantly lower in the sera of AD patients than those from −26999G/A + A/A patients (P = 0.000008 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 (s*ST2*) has 8 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 cellular 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*/s*ST2*) expression. (Top left) Forward primer: exon 1a (distal promoter), reverse primer: s*ST2* specific region. (Top right) Forward: exon 1a, reverse: *ST2L*-specific region. (Bottom left) Forward: exon 1b (proximal promoter), reverse: s*ST2*. (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.
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 = 0.0026 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.

**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 1. Genotype frequencies for ST2 SNPs and AD susceptibility

<table>
<thead>
<tr>
<th>SNP number</th>
<th>Location</th>
<th>Control (n = 636)</th>
<th>AD (n = 452)</th>
<th>P-valuea</th>
<th>P-valueb</th>
<th>P-valued</th>
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<td></td>
<td></td>
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<td>2</td>
<td>3</td>
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<td>205</td>
<td>295</td>
<td>124</td>
<td>624</td>
<td>0.44</td>
</tr>
<tr>
<td>2</td>
<td>−26999</td>
<td>223</td>
<td>279</td>
<td>112</td>
<td>614</td>
<td>0.41</td>
</tr>
<tr>
<td>3</td>
<td>744C/A</td>
<td>415</td>
<td>182</td>
<td>28</td>
<td>625</td>
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</tr>
<tr>
<td>4</td>
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<td>286</td>
<td>113</td>
<td>620</td>
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<tr>
<td>5</td>
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<td>79</td>
<td>624</td>
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<tr>
<td>6</td>
<td>5860C/A</td>
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<td>284</td>
<td>110</td>
<td>619</td>
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<td>7</td>
<td>11147C/T</td>
<td>251</td>
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<td>91</td>
<td>622</td>
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</table>

NS, not significant.
aAllele1 versus allele2.
bGenotype11 versus 12 + 22.
cGenotype11 + 12 versus 22.
dP-value statistically significant after Bonferroni correction (raw P-values were multiplied by 7).

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

<table>
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<tr>
<th></th>
<th>Controls (n = 614)</th>
<th>AD (n = 452)</th>
<th>χ² (P-value)</th>
<th>OR (95% CI)</th>
<th>AD total IgE&gt;1700 (n = 290)</th>
<th>χ² (P-value)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
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<td>−26999G/A</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>223</td>
<td>106</td>
<td>20.20</td>
<td>1.86</td>
<td>53</td>
<td>30.23</td>
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<tr>
<td>GA</td>
<td>279</td>
<td>240</td>
<td>(0.00000070)a</td>
<td>(1.42–2.45)</td>
<td>166</td>
<td>(0.000000038)a</td>
<td>(1.81–3.58)</td>
</tr>
<tr>
<td>AA</td>
<td>112</td>
<td>106</td>
<td>(0.0000049)b</td>
<td>71</td>
<td></td>
<td>(0.00000027)b</td>
<td></td>
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</tbody>
</table>

aRaw P-value.
bP-value after Bonferroni correction.

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 IgG1 did not show positive signals (data not shown).
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 (−27639A/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 dispensable 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 −26999G/A + 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
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).

Allele frequencies in AD cases and controls were compared by the contingency $\chi^2$-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 SNPAllyze 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.

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 $\chi^2$-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 SNPAllyze 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 Pst1 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 Kit (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, $1 \times 10^7$ LAD2 cells were solubilized 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 Biolabs, Beverly, 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 horse-radish 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$_2$O$_2$ 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.

REFERENCES


