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

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Received August 4, 2005; Revised and Accepted September 9, 2005

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 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-12RB1 and IL-12RB2 is required for the formation of high-affinity IL-12 binding sites, and analysis of IL-12-deficient mice showed that both subunits were essential for IL-12R function (13,14). Recently, it was reported that IL-12RB1 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), TGFβ1, G M C S F (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-12B1 (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 nonsynonymous 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 r² 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 (r² < 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 χ² 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).
The result for each SNP remained significant after correction for multiple tests (corrected \( P_c = 0.0035 \) 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
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
Expression analysis of IL12RB1 in human tissue panels

Previous reports have shown that IL-12 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 lympho-hematopoietic 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 (−111A versus AT, 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 2. Clinical characteristics of patients with AD

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>382</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>30.2 (16–65)</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>189/193</td>
</tr>
<tr>
<td>Log serum total IgE (mean ± SD)</td>
<td>3.36 ± 0.74</td>
</tr>
<tr>
<td>IgE &gt; 250 IU/ml (%)</td>
<td>11.4</td>
</tr>
<tr>
<td>IgE &gt; 400 IU/ml (%)</td>
<td>84.1</td>
</tr>
<tr>
<td>IgE &gt; 1800 IU/ml (%)</td>
<td>60.5</td>
</tr>
<tr>
<td>Blood eosinophil count &gt;500/μl (%)</td>
<td>42.1</td>
</tr>
<tr>
<td>Early age of disease onset ≤3 years (%)</td>
<td>54.5</td>
</tr>
<tr>
<td>History or coexisting condition (%)</td>
<td>25.9</td>
</tr>
<tr>
<td>Childhood asthma (%)</td>
<td>51.8</td>
</tr>
</tbody>
</table>
 Polymorphism Genotype AD (n = 382) (%) Controls (n = 658) (%) OR (95%CI) χ² 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 IL12RB1 gene expression-dependent manner. The promoter SNPs were shown to be in strong LD with each other (D' = 0.99; r² = 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 IL12R expression and allow the development of AD. To the best of 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 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.
in the majority of affected patients, giving rise to the so-called 'atopic march', which suggests that AD is an initial step or entry point for subsequent allergic diseases (3,28). Therefore, early intervention in AD-susceptible individuals may be an effective strategy in preventing the atopic march. For this reason, IL12RB1 genotype may be an important genetic marker.

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

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

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

<table>
<thead>
<tr>
<th>−111 genotype</th>
<th>Genotype AA + AT versus TT</th>
<th>Genotype AA (%) AT (%) TT (%) OR (95%CI)</th>
<th>x²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD (Total)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA (%)</td>
<td>AT (%)</td>
<td>TT (%)</td>
<td>2.46 (1.47–4.13)</td>
<td>12.4</td>
</tr>
<tr>
<td>IgE ≤ 250 IU/ml</td>
<td></td>
<td></td>
<td>2.41 (0.80–7.23)</td>
<td>2.6</td>
</tr>
<tr>
<td>IgE &gt; 250 IU/ml</td>
<td></td>
<td></td>
<td>2.47 (1.45–4.20)</td>
<td>11.8</td>
</tr>
<tr>
<td>IgE &gt; 400 IU/ml</td>
<td></td>
<td></td>
<td>2.53 (1.48–4.32)</td>
<td>12.3</td>
</tr>
<tr>
<td>IgE &gt; 1800 IU/ml</td>
<td></td>
<td></td>
<td>2.87 (1.63–5.06)</td>
<td>14.3</td>
</tr>
<tr>
<td>Blood eosinophil count &gt;500/µl</td>
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<td>3.24 (1.77–5.94)</td>
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<td>Early age of disease onset ≤3 year</td>
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<td></td>
<td>3.23 (1.83–5.70)</td>
<td>17.9</td>
</tr>
<tr>
<td>History of asthma in childhood</td>
<td></td>
<td></td>
<td>3.54 (1.76–7.12)</td>
<td>14.0</td>
</tr>
<tr>
<td>History of allergic rhinitis</td>
<td></td>
<td></td>
<td>2.52 (1.37–4.65)</td>
<td>9.4</td>
</tr>
<tr>
<td>Childhood asthma (n = 304)</td>
<td></td>
<td></td>
<td>2.32 (1.34–4.01)</td>
<td>9.5</td>
</tr>
</tbody>
</table>

Figure 2. Relationship of IL12RB1 −111 promoter genotype with total serum IgE levels in AD patients. Log-transformed individual IgE values are plotted, with the mean (×) and SD (bar) shown for each genotype group.
The human IL12RB1 gene is located on chromosome 19p13.1. Although this region has not been previously implicated by AD linkage studies, a recent study suggested that chromosome 19p13 might harbor a genetic determinant of IgE-related traits (36). Another report showed modest evidence of linkage for atopic phenotypes on chromosome 19p13.3 in an Italian population (37), and a genome-wide scan of a large cohort of German families revealed significant evidence for a psoriasis-susceptibility locus on 19p13 (38). Thus, chromosome 19p13 may indeed contain immunoregulatory genes that influence inflammatory skin diseases such as AD and/or atopy-related phenotypes. In addition, the IL12RB1 gene is a novel and attractive candidate susceptibility gene for skin inflammatory diseases such as AD and/or atopy-related phenotypes. In addition, the IL12RB1 gene is a novel and attractive candidate susceptibility gene for skin inflammatory diseases such as AD, on the basis of its location as well as its function. However, further genetic analyses and biological studies will be required to address whether other atopic disease-related genes or SNPs are also present on 19p13.

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

MATERIALS AND METHODS

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

Screening for polymorphisms and genotyping

To identify genetic variants of the human \( \text{IL12RB1} \) gene, we sequenced all 17 exons, adjacent intronic sequence, 4.0 kb 5' flanking region and 1.5 kb 3' flanking region in 24 individuals from our study cohort. On the basis of \( \text{IL12RB1} \) genomic and mRNA sequences from the GenBank database (accession nos AC020904 and U03187, respectively), 25 primer sets were designed (Supplementary Material). All PCR products were sequenced using BigDye terminator v3.1 and an ABI Prism 3700 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA). The sequences were analyzed and polymorphisms identified using the SEQUENCHER program (Gene Codes Corporation, Ann Arbor, MI, USA). On the basis of information available from the public JSNP database (http://snp.ims.u-tokyo.ac.jp), eight selected SNPs were genotyped by one of two methods: the Invader assay (41) (for \( -111A/T: \text{IMS-JST063138}, -2C/T: \text{IMS-JST063137}, 4443C/T: \text{IMS-JST063136}, 17183T/C: \text{IMS-JST063134} \) and \( \text{IL12RB1}^{+/-} \)) and \( \text{IL12RB1}^{-/-} \) individuals following anti-CD3 stimulation are shown. Relative mRNA levels were defined as the net intensity of \( \text{IL12RB1} \) and GAPDH (\( \text{IL12RB1}/\text{GAPDH} \)). * \( P \) (A/A versus A/T + T/T) = 0.019.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Identification of regulatory elements in the \( \text{IL12RB1} \) promoter, transcriptional effect of the \( -111A/T \) SNP and association of the \( -111 \) genotype with \( \text{IL12RB1} \) mRNA expression. (A) The indicated \( \text{IL12RB1} \) gene promoter fragments derived from wild-type allele sequence (–2252G/–2060A/–2004A/–111A/–2C) were cloned into the pGL3-basic vector and transiently cotransfected with pRL-TK vector as internal control. * \( P < 0.01; ** P < 0.001. \) (B) Relative luciferase activities of constructs containing the human \( \text{IL12RB1} \) gene fragments (from –265 to –65 bp), with –111A or –111T, were compared in transient transfection assays using Jurkat cells. The relative luciferase activity of the \( \text{IL12RB1} \) reporter constructs is represented as the ratio of firefly luciferase activity to that of \( \text{Renilla} \). Data are expressed as mean ± SD of three independent experiments performed in triplicate. * \( P < 0.01. \) (C) Quantitative \( \text{IL12RB1} \) mRNA expression in stimulated PBMCs from healthy volunteers (A/A, \( n = 8 \); A/T, \( n = 6 \); T/T, \( n = 1 \)). \( \text{IL12RB1} \) mRNA levels in cells from \( \text{IL12RB1}^{+/-} \), \( \text{IL12RB1}^{-/-} \) and \( \text{IL12RB1}^{+/-} \) individuals following anti-CD3 stimulation are shown. Relative mRNA levels were defined as the net intensity of \( \text{IL12RB1} \) and GAPDH (\( \text{IL12RB1}/\text{GAPDH} \)). * \( P \) (A/A versus A/T + T/T) = 0.019.
Detect density gradient centrifugation, after which 1 × 10^6 cells/ml PBMCs were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 1% HEPES buffer, 1% L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids and 1% penicillin–streptomycin solution. PBMCs were stimulated with 100 µg/ml PHA or 100 µg/ml anti-CD3 antibody for 72 h at 37°C in a 5% CO₂ atmosphere.

Tissue expression

We assessed IL12RB1 expression in a panel of cDNA tissue samples (Human Multiple Tissue, Human Immune System and Human Blood Fractions Multiple Tissues cDNA Panels, Clontech) by PCR amplification of target sequences and Southern blotting. The primer sets were 5’-CAGCTTC TGATATCACGC-3’ and 5’-TGCACTGTAGGCTACTACCC-3’ for IL12RB1 and 5’-CCCATGTTCGTTCATGGGT-3’ and 5’-TGATGGCTGAGTACCTGG-3’ for GAPDH. Southern blotting was performed with a non-radioactive nucleic acid labeling and detection kit (Roche Diagnostic, Basel, Switzerland), according to the manufacturer’s instructions. The probes for IL12RB1 and GAPDH were 5’-TGCAAAAC TACAGCTGGAGT-3’ and 5’-CCATGAGAAGTATGACAA CAG-3’, respectively.

Luciferase assay

After restriction enzyme digestion with KpnI and XhoI, luciferase reporter constructs were generated by cloning the different promoter fragments of the IL12RB1 gene into the pGL3-basic vector (Promega, Madison, WI, USA) between unique KpnI and XhoI sites. Forward primers used were: −2947/−65, 5’-CCACTTGGGCCTCAGTTTCC-3’; −1668/ −65, 5’-CTGACATTTAGAGGCTTTGCC-3’; −1361/−65, 5’-CAAACCTCTGACCTGTGATC-3’; −762/−65, 5’-CCG TGATGGACCACTGCAC-3’; −265/−65, 5’-ACCTGAC TTGCTTCAAAAGTC-3’; −104/−65, 5’-TCTCTTGTCTC AGCTTC-3’, with 5’-CTGTCCTCCCCACTCGAAC-3’ used as a common reverse primer. Using plasmid DNA as template, −111T constructs were created using the QuickChange Site-Directed Mutagenesis kit (Stratagene, USA), forward primer (5’-CTTTTTTTTTTTTTTTTTTTTTCTTTGTC ATCAGCTTC-3’), complementary reverse primer and the various pGL3-basic clones. The orientation and integrity of the inserts for each construct were confirmed by DNA sequencing. Jurkat cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO₂. Subconfluent cells cultured in 12-well plates were transiently cotransfected with 2 µg pGL3-basic vector DNA or each reporter construct and 40 ng pRL-TK vector DNA (Promega) as an internal control for transfection efficiency, using DMRIE-C transfection reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer’s instructions. After 24 h, cells were harvested, and firefly and Renilla luciferase activities measured as previously described (40). Data are presented as relative luciferase activity of firefly/Renilla luciferase activity.

PBMC preparation

To analyze IL12RB1 mRNA expression, PBMCs from 16 randomly selected healthy individuals were isolated by Ficoll density gradient centrifugation, after which 1 × 10^6 cells/ml PBMCs were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 1% HEPES buffer, 1% L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids and 1% penicillin–streptomycin solution. PBMCs were stimulated with 100 µg/ml PHA or 100 µg/ml anti-CD3 antibody for 72 h at 37°C in a 5% CO₂ atmosphere.

Real-time quantitative PCR

Total RNA was isolated from cultured PBMC using the NucleoSpin 96 RNA kit (MACHEREY-NAGEL, Düren, Germany), according to the manufacturer’s instructions. cDNA was then synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen). Real-time quantitative PCR was performed on the ABI PRISM 7900 (Applied Biosystems) using an Assay-on-Demand TaqMan probe and primers (Hs00234651_ml for IL12RB1), according to the manufacturer’s instructions. Relative expression levels of IL12RB1 mRNA were normalized according to GAPDH expression, using a standard curve method as described by the manufacturer.

Statistical analysis

We calculated allele frequencies and tested agreement with Hardy–Weinberg equilibrium using a χ² goodness-of-fit test at each locus. We also compared differences in allele frequencies and genotype distribution of each polymorphism between case and control subjects, using a 2 × 2 contingency χ² test with one degree of freedom or Fisher’s exact test. All P-values are unadjusted for multiple comparisons unless specifically indicated. We calculated LD coefficients (D’ and r²) using the SNP Alyze statistical package (Dynacom, Chiba, Japan), as described elsewhere (42). We estimated haplotype frequencies using the expectation–maximization algorithm. We calculated ODs with 95% confidence intervals (95%CI) using logistic regression. Comparisons in reporter assays as well as quantitative PCR experiments were performed using Student’s t-test. Association between log-transformed total serum IgE levels in AD patients and individual genotypes was calculated by the Mann–Whitney U-test or the Kruskal–Wallis test. P-value of less than 0.05 was considered to indicate statistical significance.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

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

Conflict of Interest statement. None declared.
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