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Positional Identification of an Asthma Susceptibility Gene on Human Chromosome 5q33

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Rationale: Asthma is a common respiratory disease with complex genetic components. We previously reported strong evidence for linkage between mite-sensitive asthma and markers on chromosome 5q33. This area of linkage includes a region homologous to a mouse area that contains a locus involved in regulation of airway hyperreactivity. *Objective:* The aim of the present study is to identify asthma susceptibility genes on chromosome 5q33. *Methods and Results:* We performed mutation screening and association analyses of genes in the 9.4-Mb human linkage region. Transmission disequilibrium test analysis of 105 polymorphisms in 155 families with asthma revealed that six polymorphisms in cytoplasmic fragile X mental retardation protein (FMRP)–interacting protein 2 gene were associated significantly with the development of asthma ($p = 0.000075$; odds ratio, 5.9). These six polymorphisms were in complete linkage disequilibrium. In real-time quantitative polymerase chain reaction analysis, subjects homozygous for the haplotype overtransmitted to asthma-affected offspring showed significantly increased level of cytoplasmic FMRP interacting protein 2 gene expression in lymphocytes compared with ones heterozygous for the haplotype ($p = 0.038$). *Conclusions:* Our data suggest that cytoplasmic FMRP interacting protein 2 are associated with the development of atopic asthma in humans, and that targeting cytoplasmic FMRP interacting protein 2 could be a novel strategy for treating atopic asthma.

Keywords: inducible tyrosine kinase; transmission disequilibrium test; polymorphism

Atopic diseases, such as asthma, atopic dermatitis, and allergic rhinitis, are major causes of morbidity in developed countries, and they have been increasing in frequency (1, 2). Asthma affects nearly 155 million individuals worldwide (3). It is a complex disorder involving genetic and environmental factors, and several asthma susceptibility loci have been identified through genomewide screens (4–10). A region of human chromosome 5q has been linked to asthma and asthma-associated phenotypes in several genomewide studies (4, 8, 10, 11). In our genomewide screen for loci associated with mite-sensitive atopic asthma, we found strong evidence for linkage of marker *D5S820* to atopic asthma (10).

Our linkage region on chromosome 5q includes the mouse homologous region that contains an airway hyperreactivity regulatory locus, which contains Epsin 4, a disintegrin and metalloproteinase domain 19, Sry-box 30, cytoplasmic fragile X mental retardation protein (FMRP) interacting proteins 2 (*CYFIP2*), cofactor required for sp1 transcriptional activation, subunit 9, interleukin 2 (IL-2)–inducible tyrosine kinase (*ITK*), hepatitis virus cellular receptor 1 (*HAVCR1*), and *HAVCR2* (12). It was reported that *HAVCR1* and *HAVCR2* are associated with differentiation of T-helper type 1 (Th1) and Th2 cells and airway hyperresponsiveness in mice and suggested that HAVCRs play an important role in the regulation of asthma and allergic diseases (12). Also, it was recently reported that HAV seropositivity protects against atopy only in individuals carrying an insertion/deletion coding polymorphism in *HAVCR1* (13). We previously screened for polymorphisms in *HAVCR1* and *HAVCR2* and identified seven, including two insertion/deletion coding polymorphisms, in *HAVCR1* and two in *HAVCR2*. However, we did not detect any association between these polymorphisms and development of asthma (14).

Our linkage region also includes the *IL-12B* gene. IL-12 is a macrophage-derived cytokine that modulates T-lymphocyte responses and can suppress allergic inflammation. We performed a mutation screen of *IL12B* and identified four variants in *IL12B*; however, none of these polymorphisms was associated with development of atopic asthma (15).

In the present study, we screened for mutations in 26 genes located in the 5q33 linkage region, and we describe herein the results of transmission disequilibrium tests of the identified polymorphisms. We identified functional polymorphisms associated with asthma in our Japanese study population.

METHODS

Probands were children with mite-sensitive asthma who visited the Pediatric Allergy Clinic of the University Hospital of Tsukuba. A full verbal and written explanation of the study was given to all family members interviewed, and 155 families (538 members), including 47 families used for our genomewide screening (10), gave informed consent and participated in this study. Criteria used for the diagnosis of asthma were described previously (16).

We constructed a saturation map of our linkage region on chromosome 5q33 with 27 microsatellite markers between *D5S2013* and *D5S211*. The 95% confidence interval was calculated based on a method described previously (17). There were 26 refseq genes in the 95% confidence interval, and we performed mutation screens of these 26 genes. All exons, exon–intron junctions, and 5′ flanking regions of the 26 genes were amplified from genomic DNAs of 16 unrelated subjects with asthma. Ninety polymorphisms with minor allele frequencies of greater than 0.05 were identified. Because we found strong association between asthma and polymorphisms in *CYFIP2*, we screened for

(*Received in original form September 16, 2004; accepted in final form April 17, 2005*)

Supported by scientific research grants (12204001 and 12024201) from the Ministry of Education, Science, and Culture of Japan.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 172. pp 183–188, 2005
Originally Published in Press as DOI: 10.1164/rccm.200409-1223OC on May 5, 2005
Internet address: www.atsjournals.org

mutations in the region that was 2 kb upstream of exon 1 and across all of intron 1 of *CYFIP2*. Eighteen additional polymorphisms, including four polymorphisms in complete linkage disequilibrium with c.2061C/T, were identified in intron 1 of *CYFIP2*. Genotyping of all 105 polymorphisms with minor allele frequencies greater than 0.05 was done by fluorescence correlation spectroscopy (18), TaqMan Assay-on-Demand single nucleotide polymorphism typing (Applied Biosystems, Foster City, CA), or direct sequencing.

We used human multiple-tissue, human immune system, and human blood fraction cDNA panels (Clontech, Palo Alto, CA) to analyze expression of *CYFIP2* in various tissues. Primers used for polymerase chain reaction (PCR) were 5′-CATTGTCCTCGCCATAGAGG and 5′-ACGGTGGATACGGAATGATG, and the expected product size was 467 bp.

Peripheral blood lymphocytes from 18 adult donors without allergic symptoms were purified by Ficoll-Paque gradient (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Naive T cells were sorted from cord blood cells, and Th1- and Th2-skewed cells were then developed in culture medium. Detailed methods are available in the online supplement. Total RNA was extracted from lymphocytes with RNeasy (Qiagen, Valencia, CA). Real-time PCR was performed with the TaqMan Universal Master Mix and Assay-on-Demand gene expression kit (Applied Biosystems) per the manufacturer's instructions. All samples were tested in triplicate, and quantification of mRNA in each sample was performed with serial-diluted reference cDNA using SDS 2.1 software (Applied Biosystems). *GAPDH* was analyzed as an internal control. Relative gene expression was calculated as the ratio of the target gene (*CYFIP2*) to the internal control (*GAPDH*). The difference in quantities of mRNA between genotypes was analyzed by Student's *t* test.

Detailed methods for electrophoretic mobility shift assays are given in the online supplement.

Statistics

Multipoint linkage analysis on chromosome 5q33 was done with the GeneHunter program (19). A family-based association test was performed with a transmission disequilibrium test as implemented in the ASPEX program (20). A haplotype association test was performed with Haploview software (21). Linkage disequilibrium was calculated and visualized with graphical overview of linkage disequilibrium, or GOLD, software (22). The p values for multiple comparisons were adjusted by Bonferroni correction, and a p value less than 0.00033 was considered statistically significant.

RESULTS

To identify asthma susceptibility genes present in the area of human chromosome 5q33, we constructed a saturation map of 27 microsatellite markers that span 23.6 Mb between *D5S2013* and *D5S211* (Figure 1A). A portion of the microsatellite genotype data included in constructing the saturation map was generated as part of our previous study (10). We found strong evidence (maximum lod score, 5.28) for linkage between asthma and a region between *D5S487* and *D5S422*. The 95% confidence interval for the location of the asthma susceptibility gene was defined by markers *D5S2077* and *D5S1955*, which are separated by 9.4 Mb according to the annotated human genome sequences. We screened for mutations in 26 genes located in this 95% confidence interval region and genotyped 90 polymorphisms with minor allele frequencies of greater than 0.05 in 538 members of 155 families with asthma. The locations of the polymorphisms and reference single nucleotide polymorphism numbers are listed in Table E2 in the online supplement. Allele frequencies of three polymorphisms found in the parents were not in Hardy–Weinberg equilibrium (rs6870491 in *GLRA1*, p = 0.02, rs2289852 in *CYFIP2*, p = 0.041, and rs2277040 in *FLJ25267*, p = 0.008). Pairwise linkage disequilibrium between polymorphisms in the 9.4-Mb region is presented in Figure E1. Results of the transmission disequilibrium test for these 155 families with asthma are shown in Figure 1B and Figure E2. Two polymorphisms in *CYFIP2* (IVS3+20G/A and c.2061C/T) showed the strongest association with the development of asthma (transmitted, 28, vs. not transmitted, 5; p = 0.000075; odds ratio, 5.9), and these two polymorphisms were in complete linkage disequilibrium (D' and $r^2 = 1$; Table 1). The IVS3+20G and c.2061C alleles were transmitted preferentially to asthma-affected offspring. Linkage disequilibrium studies of polymorphisms around *CYFIP2* showed that linkage disequilibrium was restricted to a region containing *ITK* and *CYFIP2* (Figure 1C). Polymorphisms in *ITK* are in linkage disequilibrium with those in *CYFIP2*, and the A allele of ITK-IVS14-588A/G tended to be transmitted preferentially to asthma-affected offspring (transmitted, 13; not transmitted, 3; p = 0.041), although the statistical significance was not significant after correction for multiple comparisons.

Reverse transcription PCR was performed to examine whether the IVS3+20G/A and c.2061C/T polymorphisms affect splicing of *CYFIP2*. We designed primer pairs specific for exons 2 and 4 and for exons 16 and 20 because the IVS3+20G/A and c.2061C/T polymorphisms were located in intron 3 and exon 17, respectively. We performed reverse transcription PCR using RNAs extracted from lymphocytes of subjects homozygous or heterozygous for these alleles, and no splice variants were observed (data not shown).

To identify a causal polymorphism in the genomic region of *CYFIP2*, we extended our mutation screen to a region 2 kb upstream of the transcription initiation site and to intron 1. We identified 18 polymorphisms in intron 1, and four (CY-In1-4A/T, CY-In1-8T/C, CY-In1-9G/A, and CY-In1-10A/G) were in complete linkage disequilibrium with IVS3+20G/A and c.2061C/T. The results of transmission disequilibrium tests with *CYFIP2* polymorphisms are shown in Table 1. Six polymorphisms in *CYFIP2* were in complete linkage disequilibrium and showed strong association with asthma (p = 0.000075; Table 1 and Figure E2). We then performed haplotype association tests with the family data. The region was divided into 16 linkage disequilibrium blocks by the methods of Gabriel and colleagues (23), and a haplotype association test was performed for each linkage disequilibrium block. A total of 47 association tests were performed; however, none of the haplotypes showed stronger associations than the one observed in the single-polymorphism association test.

We next performed electrophoretic mobility shift assays with fragments containing CY-In1-8T/C, CY-In1-9G/A, or CY-In1-10A/G to assess the functional significance of the variants. CY-In1-4A/T was not evaluated because the single base-pair change AAAAAATTTTTTT to AAAAATTTTTTTT is unlikely to cause a functional difference. In electrophoretic mobility shift assays with K562 and Jurkat cell nuclear extracts, bands with retarded mobility were detected for the CY-In1-8T allele (Figure 2A, *lane 1, bands a* and *b*) but not for the CY-In1-8C allele (Figure 2A, *lane 3*). Binding specificity was confirmed by cross-competition with unlabeled CY-In1-8T or CY-In1-8C. Competitive binding of CY-In1-8T, but not CY-In1-8C, eliminated DNA/nuclear protein bindings of bands a and b (Figure 2B, *lanes 13–15*). Computer prediction of a potential binding protein (Match; http://www.gene-regulation.com/) to the CY-In1-8T/C polymorphic site suggested that GATA binding proteins might have more binding affinity to CY-In1-8T than to CY-In1-8C. The competition experiment revealed that DNA/nuclear protein bindings represented by bands a and b were eliminated by the additional oligonucleotide specific to GATA binding proteins (Figure 2B, *lane 16*), suggesting that GATA binding proteins may differently bind to CY-In1-8T/C polymorphic site. A band with retarded mobility was observed for the CY-In1-9A allele but not for the CY-In1-9G allele (Figure 2A, *band c, lanes 5* and *6*); however, this was not competed with a 100-mol/L excess of cold oligonucleotide, suggesting that band c is nonspecific. A

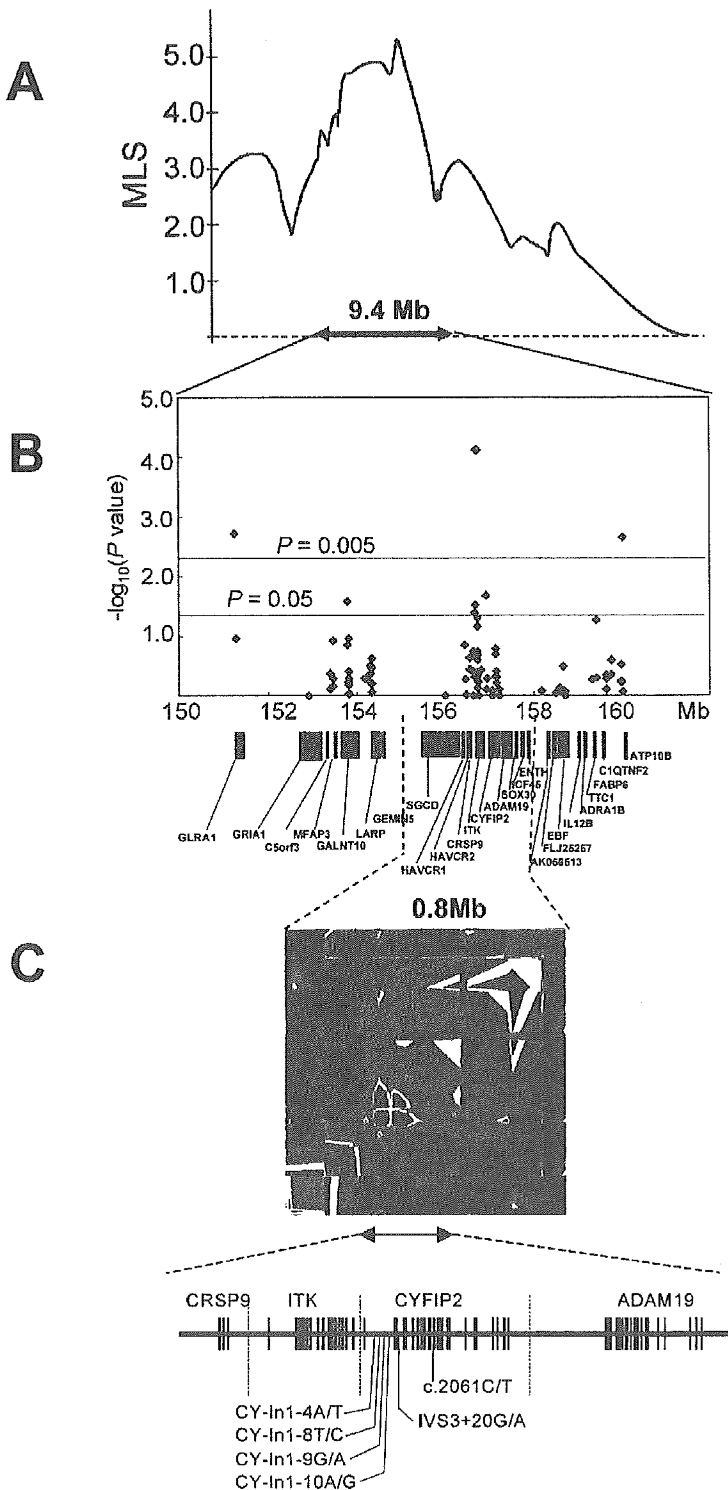

Figure 1. (*A*) Maximum logarithm of odds score (MLS) plot for asthma on human chromosome 5q33 in 47 families with asthma identified through children with mite-sensitive asthma. (*B*) Results of transmission disequilibrium test in the 95% confidence interval for the location of the asthma susceptibility gene for 155 families with asthma. *Y axis:* $-\log_{10}$ (p value); *X axis:* location in Mb. (*C*) Pairwise linkage disequilibrium between polymorphisms in a 0.8-Mb region as measured by *D′* in 155 families with asthma. Areas indicated in *red* or *yellow* show strong linkage disequilibrium.

protein-DNA complex was also observed with CY-In1-10, and the intensity for the band with G allele was much stronger than that with the A allele (Figure 2A, *band d, lanes 9* and *11*). Because no difference of DNA/nuclear protein bindings was observed in CY-In1-9, and the only one strong binding was observed in CY-In1-10, we have not determined the band c in CY-In1-9 and the band d in the CY-In1-10 in the competitive electrophoretic mobility shift assay (Figure 2A). Tissue expression of *CYFIP2* was analyzed by RT-PCR (Figure 3). We observed strong expression in brain, kidney, lymph nodes, lymphocytes, and thymus, and weak expression in skeletal muscle. In the human blood fraction panel (Figure 3C), *CYFIP2* expres-

TABLE 1. RESULTS OF TRANSMISSION DISEQUILIBRIUM TESTS OF *CYFIP2* IN FAMILIES WITH ASTHMA

	Families with Asthma (*n* = *538*)			
Polymorphisms	Allele	T/NT	p Values	Position*
c.−122C/G (rs767007)	G (0.52)	114/102	0.44	156629073
CY-In1-4A/T	A (0.05)	28/5	0.000075	156634537
CY-In1-8T/C (rs12654973)	T (0.05)	28/5	0.000075	156640526
CY-In1-9G/A	G (0.05)	28/5	0.000075	156641892
CY-In1-10A/G (rs10040318)	A (0.05)	28/5	0.000075	156642604
IVS1-152C/T (rs2288069)	T (0.24)	73/64	0.48	156644775
IVS3+20G/A (rs2288068)	G (0.05)	28/5	0.000075	156646715
IVS10-132G/A (rs2289852)	A (0.21)	74/53	0.05	156671118
IVS11+41G/C (rs393178)	C (0.76)	82/71	0.46	156671408
IVS12+112A/G	G (0.22)	81/64	0.18	156674161
IVS12+203T/A	A (0.22)	80/64	0.21	156674252
IVS12+272C/T	T (0.23)	80/65	0.25	156674321
IVS14-31C/G (rs6555939)	G (0.22)	72/64	0.56	156680192
IVS14-49G/T (rs2863198)	T (0.22)	72/67	0.73	156680210
c.1530A/G	G (0.22)	73/64	0.4	156680247
c.2061C/T	C (0.05)	28/5	0.000075	156685835
IVS18+9T/C(rs2289850)	C (0.21)	77/55	0.058	156685862
IVS22+9G/A(rs3734028)	G (0.74)	83/75	0.59	156698851
IVS24+12C/A(rs2289851)	C (0.75)	78/77	1	156718746

* Positions are based on build 35, version 1, of the annotated human genome sequences (http://www.ncbi.nlm.nih.gov). Numbers in parentheses in polymorphisms are reference single nucleotide polymorphism numbers (http://www.ncbi.nlm.nih.gov/SNP/). CY-In1-4A/T, CY-In1-8T/C, CY-In1-9G/A, CY-In1-10A/G, IVS3+20G/A, and c.2061C/T were in complete linkage disequilibrium (r^2 = 1), and the haplotype frequency of CY-In1-4T/CY-In1-8C/CY-In1-9A/CY-In1-10G/IVS3+20A/c.2061T was 0.05. T/NT = transmitted/not transmitted alleles.

sion was stronger in resting cells than in activated cells. We then performed real-time PCR using RNA extracted from naive T cells, Th1-skewed cells, and Th2-skewed cells. Naive T cells derived from umbilical cord blood were cultured in an environment suitable for Th1 development (IL-12 and anti–IL-4) or in a Th2-skewed environment (IL-4 and anti–IL-12) for 9 days. On flow cytometric analysis, all naive T cells showed high expression of CD45RA. Among Th1-skewed cells, 50% were positive for IFN-γ and 1.9% were positive for IL-13. Among Th2-skewed cells, 1.7% were positive for IFN-γ and 30% were positive for IL-13. Real-time quantitative analysis revealed that *CYFIP2* was expressed more in undifferentiated cells than in differentiated cells (Th0/Th1 ratio = 7.1, Th0/Th2 ratio = 2.9, and Th2/Th1 ratio = 2.4).

Levels of expression for different haplotypes were quantified by real-time PCR. As shown in Table 1, six polymorphisms, CY-In1-4A/T, CY-In1-8T/C, CY-In1-9G/A, CY-In1-10A/G, IVS3+20G/A, and c.2061C/T, were in complete linkage disequilibrium (r^2 = 1). The mean level of *CYFIP2* expression in lymphocytes from subjects homozygous for the ATGAGC haplotype (n = 9) was significantly higher than that in lymphocytes from subjects heterozygous for the ATGAGC haplotype (ATGAGC/TCAGAT, n = 9; 16.1 for homozygotes and 12.0 for heterozygotes, p = 0.038). Neither *ITK* nor *CRSP9* expression in lymphocytes was associated with *CYFIP2* haplotypes by real-time quantitative analysis (p > 0.1). The expression level of *ADAM19* was too low to be detected by real-time quantitative analysis.

DISCUSSION

Our present data show that polymorphisms in the *CYFIP2* gene on human chromosome 5q33 are associated with childhood atopic asthma. CYFIP2 was originally identified as a protein induced by p53 and p53 mutant protein 121F (24). The CYFIP family includes two proteins, CYFIP1 and CYFIP2, that share 88% amino acid sequence identity. The sequences of these proteins are highly conserved among species (24). *CYFIP2* is expressed in various tissues, such as brain, liver, kidney, lymph nodes, and lymphocytes. Interestingly, *CYFIP2* is expressed in resting cells more than in activated cells, and real-time quantitative analysis revealed that expression is stronger in undifferentiated cells than in differentiated cells. Thus, *CYFIP2* may be involved in differentiation of T cells. Schenck and colleagues (25) reported that CYFIP2 interacts with FMRP and that CYFIP is involved in controlling synaptogenesis and axonogenesis and affects axonal path-finding, growth, and branching. The role of CYFIP2 in the immune system is less clear; however, Mayne and coworkers (26) showed that CYFIP2 is involved in Rac-1-mediated T-cell adhesion and that overabundance of CYFIP2 protein facilitates increased adhesion of T cells obtained from patients with multiple sclerosis. Our real-time quantitative PCR analysis revealed that subjects homozygous for the ATGAGC haplotype, which was overtransmitted to asthma-affected offspring, showed a significantly increased level of *CYFIP2* expression in lymphocytes compared with the expression level in subjects heterozygous for the ATGAGC haplotype. These data suggest involvement of CYFIP2 in the development of both Th2-mediated asthma and Th1-mediated multiple sclerosis. CYFIP2 may be involved in a Th1/Th2 imbalance.

Transcriptional factor binding sites in intron 1 play critical roles in enhancing expression of some genes (27, 28), and polymorphisms in intron 1 in *RANTES* (28) and lymphotoxin α (29) are shown to bind nuclear proteins differently and are associated with HIV-1 infection and myocardial infarction, respectively. The *CYFIP2* intron 1 polymorphism, CY-In1-8T/C, binds nuclear proteins differently *in vitro*, and the competitive experiment showed that GATA binding proteins might have more binding affinity to CY-In1-8T than to CY-In1-8C (Figures 2A and 2B, *bands a* and *b*). These findings combined with results of our real-time PCR analysis indicate that the intronic polymorphisms are important for *CYFIP2* expression.

Allele frequencies of three polymorphisms of parents were not in Hardy-Weinberg equilibrium (rs6870491 in *GLRA1*, rs2289852 in *CYFIP2*, and rs2277040 in *FLJ25267*). We set our significance level at 0.05. In other words, deviation from Hardy-Weinberg equilibrium would be expected to occur at a frequency of 5% or less under Hardy-Weinberg equilibrium. Because we genotyped 90 polymorphisms, four polymorphisms would be expected to have p values less than 0.05 under Hardy-Weinberg equilibrium; therefore, it is possible that they occur by chance. Other reasons for the deviation include nonrandom mating and genotyping errors. Because the other 87 polymorphisms were in Hardy-Weinberg equilibrium, nonrandom mating is unlikely. Concerning genotyping, these three polymorphisms were analyzed by fluorescence correlation spectroscopy, and the accuracy of sequencing was confirmed by the sequence of at least 16 unrelated individuals.

There are a number of limitations to our study. First, we examined a 9.4-Mb region to identify asthma susceptibility genes. Although we calculated the 95% confidence interval region with the method of Glidden and others (17), the possibility remains that the actual susceptibility gene may be located further away from the linkage peak. Therefore, we cannot exclude the possibility that there may be other asthma susceptibility genes outside of this 9.4-Mb region on chromosome 5q33. Second, our polymorphism screening did not cover the introns and intergenic regions that may contain causal variants for asthma. Regulatory regions of genes are sometimes located in introns and intergenic sequences (28, 30). We screened for mutations in exons, exon–intron junctions, and promoter regions because polymorphisms

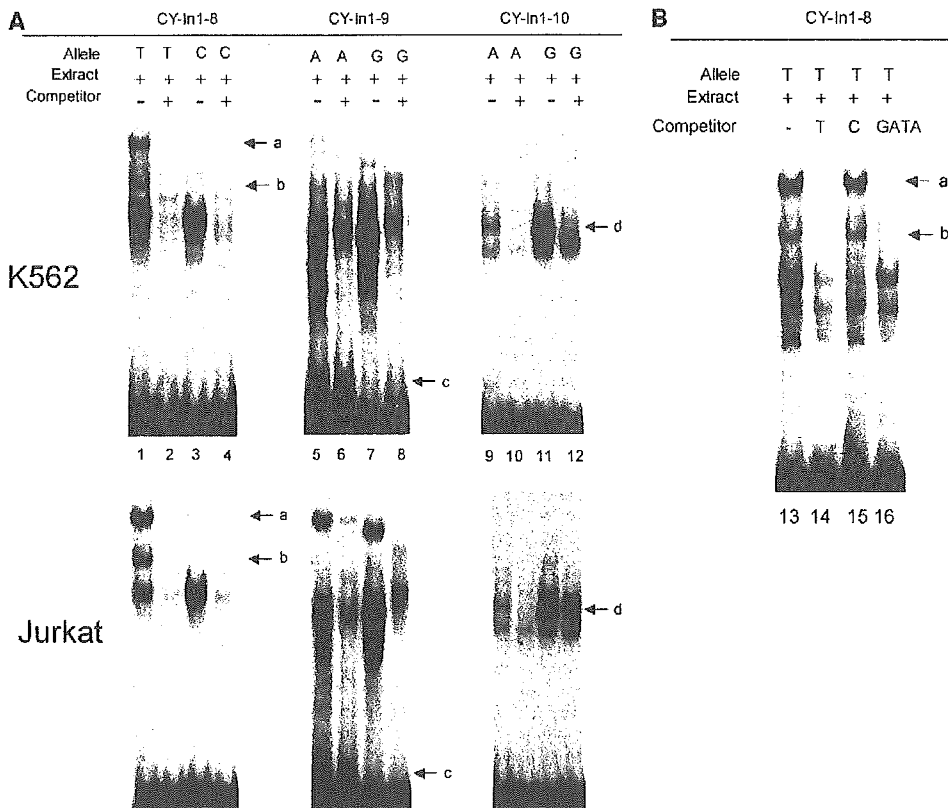

Figure 2. (*A*) Electrophoretic mobility shift assays of fragments of intron 1 of *CYFIP2* with nuclear extracts from K562 (*top*) and Jurkat (*bottom*) cells. Fragments containing either CY-In1-8C/T, CY-In1-9G/A, or CY-In1–10A/G were synthesized and used as DNA probes. One-hundred-fold molar excess cold oligonucleotides were added in the even-numbered *lanes* (*2, 4, 6, 8, 10,* and *12*). Experiments were replicated four times, and the same results were obtained in each replicate. (*B*) Competition experiment of the CY-In1-8T/C polymorphic site. *Lane 13*: CY-In1–8T without cold competitor; *lane 14*: CY-In1-8T with a 100-fold molar excess of cold CY-In1-8T; *lane 15*: CY-In1-8T with a 100-fold molar excess of cold CY-In1-8C; *lane 16*: CY-In1-8T with a 100-fold molar excess of cold oligonucleotide specific to GATA binding proteins. Nuclear extracts from Jurkat cells were used for competition experiments. Experiments were replicated three times, and the same results were obtained in each replicate.

in these regions are more likely to have functional effects than those in introns and intergenic sequences. However, causal variants in introns and intergenic sequences were overlooked in our present approach.

Because we performed multiple tests for the association analysis, appropriate corrections are necessary to avoid spurious associations. We performed 105 single-polymorphism association tests and 47 haplotype tests. We applied Bonferroni correction, one of the most stringent corrections, to this dataset, and $0.05/(105 + 47) = 0.00033$ was set as the p value for the α level of 0.05. The p values for six polymorphisms in *CYFIP2* were 0.000075, which is statistically significant even after Bonferroni correction.

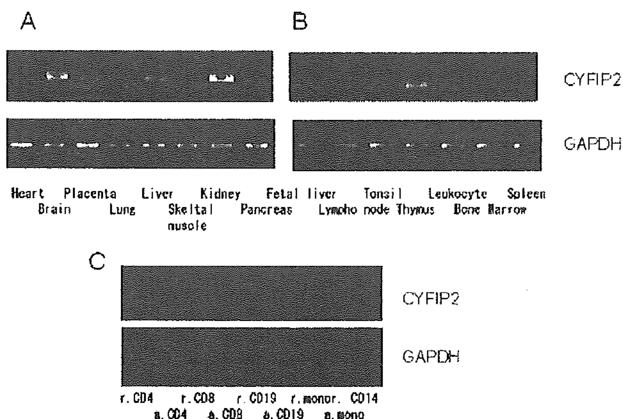

Figure 3. Expression of *CYFIP2* in human multiple-tissue panels. Polymerase chain reaction (PCR) amplification of cDNA from body organs (*A*), immune system (*B*), and blood fractions (*C*) are shown. *GAPDH* was used as a control. a = activated; r = resting. The region between exons 16 and 20 was amplified. PCR products were detected in most of the tissues examined. The experiments were repeated three times, and the pattern of PCR bands was the same in each experiment.

McIntire and coworkers (12) examined congenic mice that differed only at a segment homologous to human 5q23-35, and they identified a region related to the development of bronchial hyperresponsiveness and T-cell production of IL-4 and IL-13. The region includes several candidate genes for asthma, such as *ITK*, *HAVCR1*, and *HAVCR2*. The A polymorphisms in *ITK* are in linkage disequilibrium with those in *CYFIP2*, and the A allele of ITK-IVS14-588A/G tended to be transmitted preferentially to asthma-affected offspring (transmitted, 13; not transmitted, 3; p = 0.041). It has been shown that the genomic regions harboring regulatory elements can stretch as much as 1 Mb in either direction from the transcription unit, and that some elements may reside within the introns of neighboring genes (31, 32). ITK is a member of the tec family of kinases and is critical for both development and activation of T cells. Mice lacking ITK have drastically reduced lung inflammation, eosinophil infiltration, and mucosal production after induction of allergic asthma (33), and a recent study showed that selective ITK inhibitors block T-cell activation and lung inflammation in ovalbumin-induced mice (34). In the present study, the strongest association was observed between polymorphisms in *CYFIP2* and atopic asthma. *CYFIP2* is located adjacent to *ITK* and in the chromosome region related to mouse bronchial hyperresponsiveness. Therefore, it is possible that *CYFIP2* is an evolutionary-conserved locus that affects bronchial hyperresponsiveness in

both humans and mice. However, involvement of ITK in the development of asthma in the Japanese population cannot be excluded.

In summary, we identified *CYFIP2* as a susceptibility gene for childhood-onset atopic asthma by means of a family-based association test. Also, the *CYFIP2* haplotypes are associated with its expression levels, suggesting *CYFIP2* expression is controlled genetically to some extent. CYFIP2 plays a role in adhesion of T cells, and further investigation of *CYFIP2* could clarify the mechanisms underlying the development of asthma.

Conflict of Interest Statement: None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors thank Drs. Satoko Nakahara, Tetsuo Nogami, and Michiharu Inudou for collecting samples, and all family members who participated in the study.

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

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Background: IL-12 is a heterodimeric proinflammatory cytokine that forms a link between innate and adaptive immunity. Although associations between polymorphisms of *IL-12B* on chromosome 5q31-33 and asthma have been reported, the genetic influences of the polymorphisms and haplotype of *IL-12B* are unclear.
Objective: To examine whether polymorphisms in *IL-12B* are associated with childhood atopic asthma in a Japanese population.
Methods: We identified a total of 13 polymorphisms and characterized the linkage disequilibrium mapping of the gene. Three variants in the promoter and 3′ untranslated region were genotyped, and we conducted case-control and case-only association studies between those variants and asthma-related phenotypes (childhood atopic asthma, n = 297; normal controls, n = 555). Haplotype association analysis and functional analysis of these variants were also performed.
Results: 3′ Untranslated region 10841C>A was significantly associated with the risk of childhood atopic asthma (P = .00062). The −6415 promoter variant, in linkage disequilibrium with the 10841C>A (D′ = 0.78 and r^2 = 0.61), was also marginally associated with childhood atopic asthma (P = .051). We analyzed the 2-locus haplotype by using these 2 polymorphisms and found a positive association with haplotype CTCTAA-C (−6415 CTCTAA and 10841C; P = .00078). Furthermore, 10841C>A affects the stability of transcripts, and promoter variant −6415GC enhances the transcriptional level of *IL-12B*.
Conclusion: Our results imply that functional haplotype CTCTAA-C, which affects the instability of transcripts and the lower transcriptional level of *IL-12B*, has a protective effect in childhood atopic asthma. On the basis of these findings, the *IL-12B* gene might be involved in the development of atopic asthma through functional genetic polymorphisms. (J Allergy Clin Immunol 2005;116:789-95.)

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

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Supported by grants-in-aid from the Ministry of Health, Labor and Welfare; Japan Science and Technology Corp; and the Japanese Millennium project.
Received for publication January 7, 2005; revised June 9, 2005; accepted for publication June 13, 2005.
Available online August 8, 2005.
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0091-6749/$30.00
© 2005 American Academy of Allergy, Asthma and Immunology
doi:10.1016/j.jaci.2005.06.010

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

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

Mechanisms of asthma and allergic inflammation

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

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

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

METHODS

Study subjects

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

Screening for polymorphisms and genotyping

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

Luciferase assay

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

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

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

Statistical analysis

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

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

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

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

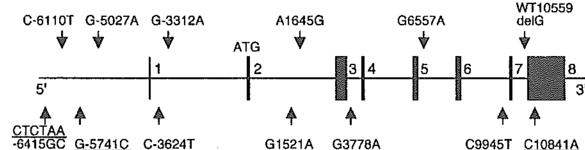

FIG 1. Graphical overview of polymorphisms identified in relation to the exon/intron structure of the human *IL-12B* gene. Eight exons are shown by *black boxes* with their numbers, and positions for polymorphisms are relative to the translation start site (+1). *Underlined* polymorphisms were genotyped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

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

RESULTS

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

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

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

*Polymorphisms were genotyped in this case-control study.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

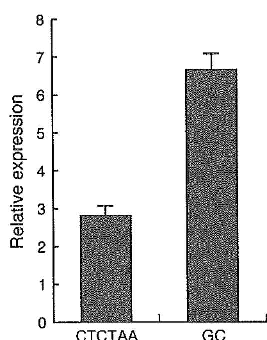

FIG 2. Effect of CTCTAA>GC polymorphism on the transcriptional activity of human *IL-12B* promoter. Relative activity was calculated by taking the relative luciferase activity of the empty vector (pGL3-basic) to be 1.

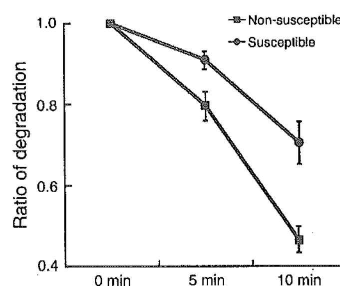

FIG 3. Stability of susceptible (3′ UTR variant 10841A) and non-susceptible (10841C) transcripts of *IL-12B* mRNA measured as degradation rate. Values represent means ± SDs of data from triplicate experiments.

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

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

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

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

DISCUSSION

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

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

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

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

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

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

We thank all participants in the study. We are grateful to members of the Rotary Club of Osaka-Midosuji District 2660 Rotary International in Japan for supporting our study. We also thank Hiroshi Sekiguchi and Miki Kokubo for technical assistance and Chinatsu Fukushima for providing patients' data.

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Original Paper

International Archives of Allergy and Immunology

Int Arch Allergy Immunol 2006;139:25–30
DOI: 10.1159/000089519

Received: May 23, 2005
Accepted after revision: August 18, 2005
Published online: November 3, 2005

Lack of Association between the *IL13* Variant Arg110Gln and Susceptibility to Cedar Pollinosis in a Japanese Population

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Key Words
Allergic rhinitis · Candidate gene · Hay fever · Interleukin-13 · Japanese cedar pollinosis · Single nucleotide polymorphism

Abstract
Background: Interleukin (IL)-13 has come to be appreciated as a molecule critically involved in allergic inflammatory responses. Recent studies revealed that a common variant in the coding region of the *IL13* gene, Arg110Gln, has been implicated in the development of asthma and atopy. ***Methods:*** To assess whether the *IL13* variant Arg110Gln is associated with cedar pollinosis, one of the most common atopic diseases in the Japanese population, we examined the Arg110Gln variant using PCR-RFLP to compare the genotype and allele frequencies between 95 patients with cedar pollinosis and 95 healthy control subjects. Relationships between the Arg110Gln variant and the pollinosis-related traits, e.g. rhinitis severity, eosinophil counts in nasal secretion and serum total and allergen-specific IgE levels, were also investigated. ***Results:*** The frequencies of the minor allele Gln110 were 25.8% in patients with cedar pollinosis and 30.9% in healthy control subjects ($p > 0.05$). There was also no significant difference in the genotype frequencies between cases and controls ($p > 0.05$). In addition, we found no significant association of the Arg110Gln variant with any of the pollinosis-related phenotypes ($p > 0.05$). ***Conclusions:*** Our data suggest lack of evidence for identifying the variant Arg110Gln at the *IL13* locus as a genetic risk factor involved in the development of Japanese cedar pollinosis.

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1018–2438/06/1391–0025$23.50/0

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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*aga*tc-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 Arg110-Gln 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.

Acknowledgment

We wish to acknowledge Drs. Y. Dake and S. Doi for collecting samples, and H. Sekiguchi and M. Kokubo for assistance in the molecular laboratory. This study was supported by a research grant from the Millennium Genome Project of the Japanese Government and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan. L.C. has been a visiting fellow at the University of Wales Swansea, UK.

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Human Molecular Genetics, 2005, Vol. 14, No. 21 3149–3159
doi:10.1093/hmg/ddi347
Advance Access published on September 13, 2005

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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