mouse in an attempt to address the role of HTm4 in both hematopoietic and CNS development. In addition, further analysis of the protein interactions and cell-cycle regulatory capability of HTm4 will identify its importance in both contexts.

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Note

Molecular Monitoring of the Developmental Bacterial Community in the Gastrointestinal Tract of Japanese Infants

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The dynamics of the developmental bacterial community in the Japanese neonatal gastrointestinal tract were examined by monitoring 16S ribosomal RNA gene (rDNA) diversity in fecal samples by PCR and denaturing gradient gel electrophoresis (DGGE). The results showed a certain pattern common in infants without antibiotic treatment, in which aerobes, *e.g.*, *Pseudomonas*, appeared first and were then immediately replaced by facultative anaerobe, *Enterococcus*, *Streptococcus*, and *Enterobacteriaceae* through the first month, and finally strictly anaerobic *Bifidobactrerium* appeared.

Key words: denaturing gradient gel electrophoresis (DGGE); gastrointestinal tract; infant; 16S rRNA; intestinal microbiota

Soon after birth, bacterial colonization starts in the previously germfree gastrointestinal tract and commensal host-microbial relationships begin.[1,2] The colonizing bacteria contribute to maintenance of the mucosal barrier, facilitate carbohydrate assimilation, and modulate the mucosal immune system. Thus, the initial development of intestinal microbiota is considered to have great influence on the health of the infant. In this study, the succession of the gastrointestinal bacterial community was examined for the first two months in nine Japanese infants by monitoring 16S ribosomal RNA gene (rDNA) diversity in fecal samples.

All infant subjects (infants nos. 1, 2, 5, 6, 10, 20, 24, 25, and 33) participated in this study were vaginally delivered. Infant no. 5 was fed formula milk and the other infants were fed both breast and formula milk during the sampling period. Infants no. 1 and no. 33 were subjected to antibiotic therapy, receiving cefalex (50 mg/kg, 4 times a day) the first four days, whereas infant no. 5 was treated on day 0 only. Fecal samples were collected on day 0/1, day 3, day 5, month 1, and month 2 (there was no month-2 sample from infant no. 5). All the parents of our subjects gave written informed consent and the Ethics Committee of the Faculty of Medicine of Kyoto University approved this study protocol.

DNA was isolated from each fecal sample using a bead beating method essentially as previously described,[3] except for 2–3 times washing of the fecal sample before the bead beating step. In order to construct 16S rDNA libraries, a V1–V3 region of 16S rDNA was amplified from each sample by PCR with 8UA (5′-AGAGTTTGATCCTGGCTCAG-3′)[4] and 519B (5′-ATTACCGCSGCTGCTG-3′)[5] primers, and cloned into a pGEM-T vector (Promega, Madison, WI), and transformed in *E. coli* JM109. About ten clones from each library were sequenced. In total, 357 clones were sequenced and the ribotypes found are summarized with the result of the database search in Table 1.

PCR-denaturing gradient gel electrophoresis (PCR-DGGE), which allowed rapid and efficient molecular fingerprinting of gut microbiota,[3,6] was performed in order to monitor the succession of the infant fecal bacterial community. The variable region V2–V3 of 16S rDNA was amplified by PCR using primers HDA1-GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG

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Table 1. List of Ribotypes Obtained in This Study

Ribotype	Closest sequence relative[a] (species)	% identity	Numbers			Accession no.
			clones	subjects	DGGE[b]	
Gram-positive facultative anaerobes						
Ef	*Enterococcus faecalis*	99.4	25	4	7	AY635946
Ss	*Streptococcus salivarius*	99.8	14	6	3	AY635947
Sp	*Streptococcus parasanguis*	96.4	12	3	7	AY635948
Se	*Staphylococcus epidermidis*	100.0	11	4	8	AY635949
Pa	*Propionibacterium avidum*	99.0	4	3	0	AY635950
Em	*Enterococcus faecium*	99.8	4	2	6	AY635951
Sa	*Streptococcus anginosus*	99.8	4	1	1	AY635952
Lg	*Lactobacillus gasseri*	99.8	2	2	0	AY635953
St	*Streptococcus thermophilus*	99.8	2	1	5	AY635954
Sm'	*Streptococcus mitis*	96.6	2	1	0	AY635955
Sm	*Streptococcus mitis*	99.0	1	1	4	AY635956
Sc	*Streptococcus cremoris*	99.1	1	1	3	AY635957
Si	*Streptococcus infantarius*	99.6	1	1	1	AY635958
Gram-positive strict anaerobes						
Cb	*Clostridium butyricum*	100.0	25	4	4	AY635959
Bd	*Bifidobacterium dentium*	99.0	11	5	5	AY635960
Bp	*Bifidobacterium pseudocatanulatum*	100.0	11	2	5	AY635961
Fm	*Finegoldia magna*	98.8	2	2	0	AY635962
Rm	*Ruminococcus* sp.	99.0	2	1	1	AY635963
Ch	*Clostridium hathewayi*	98.6	3	1	1	AY635964
Gram-positive aerobes						
Mm	*Micrococcus mucilaginosus*	98.8	5	2	1	AY635965
Ar	*Acinetobacter rhizospaerae*	98.7	5	1	0	AY635966
Gram-negative facultative anaerobes						
Es	*Enterobacter* sp. B901-2	99.8	32	5	6	AY635967
Kp	*Klebsiella pneumoniae*	99.2	31	6	2	AY635968
Km	*Klebsiella milletis*	99.0	19	8	6	AY635969
Ec	*Escherichia coli*	99.2	15	2	3	AY635970
Ko	*Klebsiella oxytoca*	99.6	12	2	2	AY635971
Cd	*Citrobacter diversus*	98.5	5	2	0	AY635972
Kr	*Klebsiella rennanqify*	99.0	9	3	4	AY635973
Cg	*Calymmatobacterium granulomatis*	99.0	7	4	1	AY635974
Ea	*Enterobacter aerogenes*	97.8	5	2	0	AY635975
Km'	*Klebsiella milletis*	99.0	3	3	1	AY635976
Sf	*Escherichia coli*	99.4	3	2	0	AY635977
Ko'	*Klebsiella oxytoca*	98.3	2	1	1	AY635978
Kr'	*Klebsiella rennanqify*	98.9	2	1	0	AY635979
Gram-negative strict anaerobes						
Bu	*Bacteroides uniformis*	99.4	8	3	1	AY635980
Vp	*Veillonella parvula*	98.9	1	1	1	AY635981
Vp'	*Veillonella parvula*	98.4	1	1	1	AY635982
Gram-negative aerobes						
Pm	*Pseudomonas marginalis/reactans/veronii*	99.4	28	5	5	AY635983
Fh	*Flavobacterium heparinum*	98.1	8	3	3	AY635984
Pt	*Pseudomonas tolaasii*	99.6	7	4	2	AY635985
Ad	*Acidovorax deflvii*	99.4	6	1	1	AY635986
Ph	*Phyllobacterium myrsinacearum*	99.6	4	1	0	AY635987
Pr	*Pseudomonas trivialis/poae*	99.6	2	2	2	AY635988

Ribotypes found with more than two clones in the 16S rDNA libraries or found only once but also detected in DGGE are listed.
[a]Cultured bacteria in Genbank showing highest identity as a result of Blast search.
[b]The number of bands corresponding to the indicated species in the DGGE (Fig. 1).

GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-3′) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA C-3′).[7] The PCR condition was as follows: 94 °C for 5 min, 30 cycles consisting of 94 °C for 40 s, 58°C for 20 s, and 72 °C for 1 min, and finally 72 °C for 5 min. DGGE analysis was performed as described by Muyzer *et al.*[8] and Heilig *et al.*[9] using a Dcode System apparatus (Bio-Rad, CA). Each band in the DGGE gel was assigned one of the ribotypes in Table 1 either by sequencing of DNA fragments excised from the DGGE gel or by comparing band positions with those of reference clones derived from the 16S rDNA clone library.

Figure 1 shows the DGGE profile of nine subjects. Although each subject showed individual banding patterns, a stepwise development from aerobic to

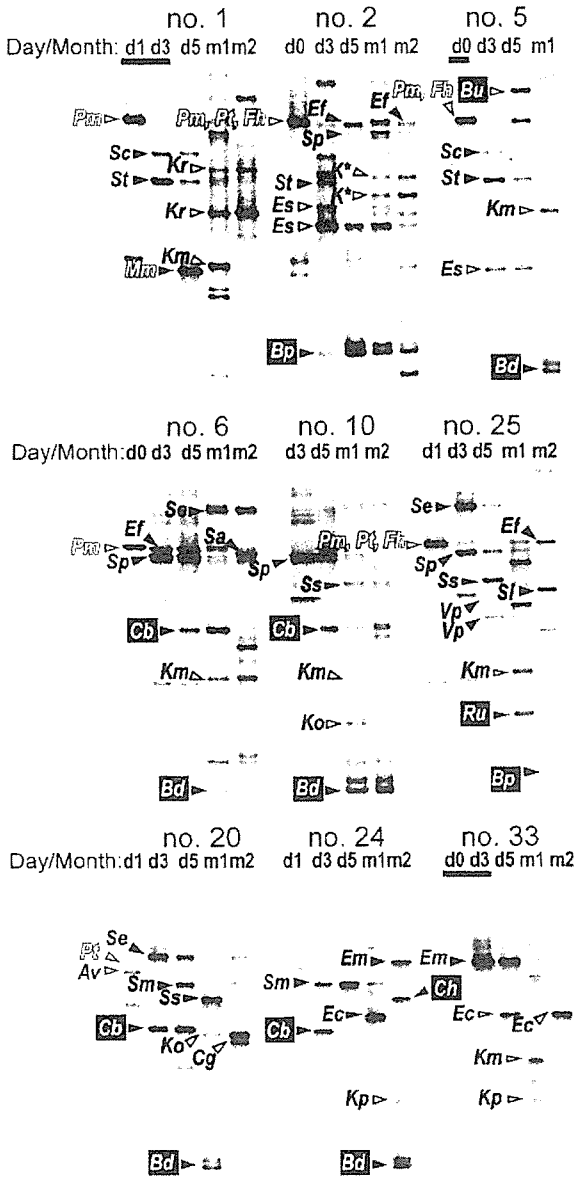

Fig. 1. PCR-DGGE Profiles Representing the Fecal Bacterial Community in the First Two Months of Nine Infants.

The bands identified with ribotypes in the 16S rDNA clone libraries are indicated by arrowheads with their names listed in Table 1, except K* which indicates unidentified *Klebsiella* spp. Open and black arrowheads represent Gram-negative and Gram-positive bacteria respectively. Open, black, and outlined letters represent aerobic, facultatively anaerobic, and strictly anaerobic bacteria respectively. Bold lines under the sampling days indicate the period of antibiotic treatment. Electrophoresis was done in 8% polyacylamide gel with a denaturing gradient of 30–65%, where 100% corresponds to 7 M urea and 40% formamide. The gel was run at 100 V for 6.5 h at 60 °C and then stained in 1 × SYBR Gold (Molecular Probes, Eugene, OR).

anaerobic microbial ecosystem was observed in the succession of bacterial composition in the seven subjects (nos. 2, 5, 6, 10, 20, 24, and 25) without successive antibiotic treatment in the first four days. In the beginning, bands corresponding to aerobic Gram-negative bacteria such as *Pseudomonas* appeared and they were then replaced by facultatively anaerobic bacteria such as *Streptococcus*, *Enterococcus*, or *Staphylococcus epidermidis* and *Enterobacteriaceae*. Particularly, strong bands closely related to *Streptococcus parasanguis*, *Streptococcus cremoris* and *Streptococcus thermophilus* appeared on day 3 in many subjects. It is interesting to note that a large majority of the bacteria types such as *Streptococcus parasanguis*, *Streptococcus mitis*, *Streptococcus salivarius*, *Bifidobacterium dentium*, and *Veillonella parvula* detected in this period are regarded as oral-origin bacteria rather than intestinal species. This suggests that these oral-origin bacteria may transiently colonize the intestine during this period.

Bifidobacteria detected in the bottom part of the DGGE gel appeared within two months in most subjects. In infant no. 2, *Bifidobacterium pseudocatanulatum* colonized predominantly from day 3 and continued until 2 months of age. This subject was only the case which agreed with the finding of previous studies,[6,10,11] showing that bifidobacteria usually appear and become dominant within a week after birth. The appearance of bifidobacterial bands in infant no. 2 was concomitant with a decrease in *Enterobacteriaceae* bands, which appeared as dominant on day 3. A concomitant decrease in *Enterobacteriaceae* with an increase of bifidobacteria in breast-milk fed infants has been reported.[12] Bands related to *Clostridium butyricum* were also detected in infants nos. 6, 10, 20, and 24, in which they appeared earlier than bifidobacteria. The other strict anaerobes, *Veillonella parvula*-like bacteria, and *Ruminococcus* sp. were found only in infant no. 25. *Bacteroides uniformis* was detected only at day 5 in infant no. 5 (antibiotic treatment on day 0) who was the only subject brought up only on formula milk.

Infants nos. 1 and 33 treated with antibiotics in the first 3 days showed relatively simple microbiota, and the developmental patterns deviated remarkably from the trends observed in the other subjects without antibiotic treatment. In infant no. 1, a dominant band corresponding to *Micrococcus mucilaginosus*, which is not a common inhabitant of the intestine, appeared suddenly on day 5 and completely disappeared during month 1. In infant no. 33, a dominant band corresponding to *Enterococcus faecium* appeared on day 3 and disappeared during month 1. No bands corresponding to bifidobacteria and other strict anaerobes were found in the testing period in either baby and only bands corresponding to *Enterobacteriaceae* were found during month 1 and month 2, suggesting domination by *Enterobacteriaceae*. This was also indicated by the data of random sequencing of 16S rDNA clone libraries, which showed that all 30 clones sequenced from the month-1 and month-2 libraries of these two subjects belonged to *Enterobacteriaceae*. These data showed that antibiotic treatment at the beginning of life has strong

influence on the establishment of a normal microbial ecosystem in the intestine.

In conclusion, this molecular study indicates the stepwise development from aerobic to anaerobic microbial ecosystem with a variety of bacterial groups, although the process differed among individuals at the species level. The step of the bacterial colonization in the gastrointestinal tract is most likely a key to the developmental process. Strong antibiotic treatment interrupted the development of normal microbiota, including bifidobacteria. Further studies with modern molecular methods are needed for understanding of the environmental and host factors affecting the developmental process of neonatal microbiota.

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Functional promoter polymorphism in the *TBX21* gene associated with aspirin-induced asthma

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Abstract Asthma is a phenotypically heterogeneous disorder with many etiologic factors and clinical characteristics. T-bet, a Th1-specific transcription factor of T-box family, has been found to control interferon-γ (IFN-γ) expression in T cells. Mice lacking the T-bet gene (*tbx21*) demonstrate multiple physiological and inflammatory features reminiscent of human asthma. In order to examine whether polymorphisms in the candidate gene, *TBX21*, located on chromosome 17q21.32, are related to the risk of human asthma phenotypes, we have searched for genetic variations in the human *TBX21* gene and identified 24 single nucleotide polymorphisms (SNPs), including five novel SNPs, by direct sequencing in Japanese subjects. Among asthma phenotypes, a promoter −1993T → C SNP, which is in linkage disequilibrium with a synonymous coding 390A → G SNP in exon 1, is significantly associated with a risk of aspirin-induced asthma (AIA; $P=0.004$, $P_c=0.016$). This association has also been confirmed in additional independent samples of asthma with nasal polyposis ($P=0.008$), regardless of aspirin hypersensitivity. Furthermore, our data indicate that the −1993T → C substitution increases the affinity of a particular nuclear protein to the binding site of *TBX21* covering the −1993 position, resulting in increased transcriptional activity of the *TBX21* gene. Thus, in addition to the antigen-driven excess Th2 response, increased T-bet (and subsequent IFN-γ)

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production in human airways of individuals with the −1993T → C polymorphism could contribute to the development of certain asthma-related phenotypes, such as AIA.

Introduction

Asthma is defined as a chronic inflammatory lung disease that is characterized by airway hyperreactivity, eosinophil inflammation, and mucus hypersecretion resulting in intermittent airway obstruction (Busse and Lemanske 2001). A considerable increase has been noted in the incidence of allergic diseases including asthma in industrialized societies over the past three decades (Bach 2002; Woolcock and Peat 1997). The etiology of asthma is complex and multifactorial; development of the disease is controlled by both host genetic factors and a variety of environmental exposures. Although environmental influences, particularly a decrease in infections because of improved hygiene, might have increased allergic diseases, at least a dozen polymorphic genes have been calculated to regulate asthma, by controlling the inflammatory response, immunoglobulin E (IgE), cytokine, and chemokine production, and airway remodeling (Cookson 1999; Fahy et al. 2000; Umetsu et al. 2002).

Asthma is thought to arise from an imbalance in T helper type 1 (Th1)-Th2 immune regulation, resulting in the driving of the development of Th2-biased immune responses and the overproduction of cytokines such as interleukin 4 (IL-4), IL-5, IL-9, and IL-13, which mediate allergic inflammation (Renauld 2001; Umetsu et al. 2002). In contrast, Th1-type cytokine interferon-γ (IFN-γ) is essential for macrophage activation in cellular defense mechanisms, and IFN-γ-producing Th1 cells have been suggested to protect against allergic responses by dampening the activity of Th2 effector cells (Renauld 2001). However, the evidence from other in vivo studies of asthma conflicts with this hypothesis, suggesting a contribution of IFN-γ to asthmatic airway inflammation (Busse and Lemanske 2001; Salvi et al. 2001).

T-bet is a member of the T-box family of transcription factors that has been found to be expressed in IFN-γ-producing Th1, but not in Th2, cells. T-bet is a transcriptional regulator essential for the lineage commitment of Th1 cells by directly activating Th1-associated genetic programs and repressing Th2 cytokine production (Szabo et al. 2000). Recently, evidence has shown decreased numbers of $CD4^+$ T cells expressing T-bet in the airways of patients with allergic asthma, relative to control subjects (Finotto et al. 2002). Furthermore, deletion of the T-bet gene, *tbx21*, in mice results in airway eosinophilia, Th2 cytokine production, airway hyperresponsiveness (AHR), and changes of airway remodeling without allergen sensitization and challenge. Thus, T-bet-deficient mice demonstrate multiple physiological and inflammatory features reminiscent of human asthma (Finotto et al. 2002).

The human T-bet gene (*TBX21*) is located on chromosome 17q21.32, a region near to that linked with asthma in a genome screen for asthma and skin tests (Dizier et al. 2000; Zhang et al. 1999). Moreover, the region on mouse chromosome 11 that is syntenic to human chromosome 17q12-q22 has been linked to AHR (Zhang et al. 1999). So far, to our knowledge, there have been no reports showing disease-related polymorphism(s) in the *TBX21* gene. Based on these observations, we propose that genetic polymorphism contributes to susceptibility to human asthma and/or related phenotypes. To test this hypothesis, we have searched for polymorphisms in *TBX21* and then conducted a genetic association study in the Japanese population. Finally, we have investigated the functional consequences of disease-related polymorphisms.

Materials and methods

Subjects

We recruited 361 patients with childhood asthma (mean age 9.7 years, range 4–15 years, mean total serum IgE level, 1021 U/ml; 92% of whom were atopic), 313 adult patients with atopic asthma (mean age 49 years, range 20–81 years; mean total IgE, 775.7 U/ml), and 88 adult patients with non-atopic asthma (mean age 59 years, range 42–75 years; mean total IgE, 174.8 U/ml) from the Osaka Prefectural Habikino Hospital and the Miyatake Asthma Clinic. Patients with aspirin-induced asthma (AIA; mean age 53 years, range 24–73 years; 54% of whom were atopic; ≥50% had nasal polyposis) were recruited from the National Sagamihara Hospital. All patients with asthma were diagnosed according to the criteria of the National Institutes of Health, with minor modifications (National Heart, Lung, and Blood Institute, National Institutes of Health, 1997, http://www.nhlbi.nih.gov/guidelines/asthma/asthgdln.htm). The diagnosis of atopic asthma was based on the positive immunoassay test to common allergens (at least one of the following: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, and *Aspergillus fumigatus*) or a higher total serum IgE of ≥400 kU/l, as used in our previous study (Mao et al. 1996). The criteria for a diagnosis of non-atopic asthma was a total serum IgE of <400 kU/l and the absence of allergen-specific IgE (≤ 0.35 kU/l). All patients with AIA were documented to have histories of asthmatic attacks, such as severe bronchoconstriction and nasal symptoms, following the ingestion of more than two different kinds of non-steroidal anti-inflammatory drugs (NSAIDs) or to have had a positive reaction to aspirin systemic challenge. Of 72 AIA patients (58%), 42 were diagnosed on the basis of the aspirin challenge test, as previously described (Kawagishi et al. 2002; Mita et al. 2001). We also

independently recruited 42 asthmatic patients with nasal polyposis (AS/NP; mean age 54 years, range 23–75 years; 73% of whom were atopic). The NP was diagnosed on the basis of history, including nasal symptoms, clinical examination, nasal endoscopy, and sinus computerized tomography scanning. All adult asthmatics, except AIA patients, had no past history of aspirin hypersensitivity. Controls were 640 randomly selected healthy individuals with an age range 18–83 years from the same geographic areas who had neither respiratory symptoms nor history of asthma-related diseases and aspirin hypersensitivity. All subjects in this study were ethnically Japanese and gave written informed consent to participate in the study (or, for individuals less than 16 years old, their parents gave consent), according to the process approved by the Ethical Committee at the SNP Research Center, The Institute of Physical and Chemical Research (RIKEN), Yokohama.

Screening for polymorphisms and genotyping

To identify single-nucleotide polymorphisms (SNPs) in the human *TBX21* gene, we sequenced all six exons, including a minimum of 200 bases of flanking intronic sequence, 2.2 kb of the 5′ flanking region, and 2.5 kb continuous to the 3′ flanking region of the sixth exon from 24 asthmatic subjects (12 unrelated children and 12 adults). Eighteen primer sets were designed on the basis of the *TBX21* genomic sequence from the GenBank database (accession number AC003665; Table 1). For each polymerase chain reaction (PCR), 5 ng genomic DNA was amplified in a total reaction volume of 10 µl containing 12.5 pmol each primer, 3.9 mM $MgCl_2$, 1.25 mM each dNTP, 0.5 U *Taq* polymerase. Cycling conditions were an initial 95°C for 2 min, followed by 37 cycles at 94°C for 30 s, 58°C or 60°C for 30 s, and 72°C for 3 min, with a final extension of 7 min at 72°C. Each fragment amplified by PCR was sequenced by using the BigDye Terminator (Applied Biosystems, Foster City, Calif., USA) on an ABI Prism 3700 Genetic Analyzer (Applied Biosystems). The sequences were analyzed, and polymorphisms were identified by using the SEQUENCHER program (Gene Codes Corporation, Ann Arbor, Mich., USA). Four selected SNPs, viz., −1993T → C, 99C → G, 1298T → C, and 7725T → C, were genotyped by three methods: PCR-RFLP (PCR-restriction fragment lenght polymorphism; for −1993T → C and 1298T → C), Invader assay (for 99C → G), and direct sequencing (for 7725T → C). For PCR-RFLP analysis, we used mismatched primers for the −1993T → C SNP (5′-GGTCTTACTGAAAGCTCTCA-3′ and 5′- TCTCCTCCCCAACACCTTACGC-3′) and for the 1298T → C SNP (5′-GGCTAGTGCAGTAAAGCTTG-3′ and 5′-GGTTTTCACTGGACCAGCCGC-3′) where the changed nucleotides are underlined. The amplified products were digested with *Hha*I (−1993T → C) or *BstU*I (1298T → C) restriction enzymes (New England Biolabs, Bevery, Mass., USA) according to the manufacturer's instructions and were separated by electrophoresis on 4% agarose gels. Based on information available from the public JSNP database (http://snp.ims.u-tokyo.ac.jp), we generated the 99C → G SNP (IMS-JST000934) genotypes by using the Invader assay as previously described (Ohnishi et al. 2001). For the 7725T → C SNP, we performed direct sequencing with primers 5′-TTATCCAGGGTCATAGGGTAG-3′ and 5′-CCTCAGCCTTTAGAGAAGTTG-3′.

Luciferase assay

We generated luciferase reporter constructs, pGL3/−1993T and pGL3/−1993C, by cloning three concatenated copies of a 20-bp fragment of the *TBX21* gene into pGL3-Basic vector (Promega, Madison, Wis., USA) in the *Nhe*I site. The 20-bp primer sets carrying −1993T or −1993C alleles were 5′- CTAGCGGAGAAATGGTGGGTAAGGT T-3′ (forward) and 3′- GCCTCTTTACCACCCATTCCA AGATC-5′ (reverse) or 5′- CTAGC

Table 1 Primer sequences used in screening for SNPs of the human *TBX21* gene

F1	5′-TTTCCAGTAATAGCCGCTCCT-3′	R1	5′-CACAGCCTAGACACTGGTTC-3′
F2	5′-TTGCATAGTTACCATCCACCG-3′	R2	5′-GACCTTGGGATCCTTCACTAC-3′
F3	5′-AAGACTCCATTTGATCTTCAAC-3′	R3	5′-TTCACTCCACAAGGTGTCATG-3′
F4	5′-GTCAGGCTGGGACAGAAATG-3′	R4	5′-TGAGTTGGCTGCATCTTGTAG-3′
F5	5′-CTGGCTGCTGCTGATGCAG-3′	R5	5′-TGTCACTAGAGTCGCAGCGC-3′
F6	5′-AGTACTCGCCAAGAGCGTAG-3′	R6	5′-AAAAACAGACGAGACGTTCTTG-3′
F7	5′-TCGCGCTCAACAACCACCTG-3′	R7	5′-CTCAAAGTAAGACCGGAAAGG-3′
F8	5′-GGCTAGTGCAGTAAAGCTTG-3′	R8	5′-GACCAGAAGCTTGGGCTGTG-3′
F9	5′-CTCTGTTGTGTGGTCAGGAG −3′	R9	5′-TGAGAAGGTATGGAGGTAACC-3′
F10	5′-TTGAAGGAGGCAGTGGCTC-3′	R10	5′-AACACAGCTACCCAAAGTTATC-3′
F11	5′-TTATCCAGGGTCATAGGGTAG-3′	R11	5′-CCTCAGCCTTTAGAGAAGTTG-3′
F12	5′-TAACTTCCTCTACTTTTCCTGG-3′	R12	5′-AAACATCCTGTAGTGGCTGG-3′
F13	5′-TGCCTGGGCACTGTTGCAG-3′	R13	5′-GAAAAACGAACCTTCCTTCCTG-3′
F14	5′-CAACAATGTGACCCAGGTAG-3′	R14	5′-CAAGCTTTCCAACTCCAGTG-3′
F15	5′-GCCCTGTTTGTGCTGATACC-3′	R15	5′-CACAAGCAGAACCAGTCACC-3′
F16	5′-TGGGTTCAACTCAGCTTTGGT −3′	R16	5′-CTTTCATCATGTCATCTGCTC-3′
F17	5′-GCGAAGGAGACTCTAAGAGG-3′	R17	5′-TCTTGCTTCTTGAGATGTGGG-3′
F18	5′-CACGTATGTTATAACCATCAGC-3′	R18	5′-AGAGATAAAGGTGGAGGGCTG-3′

GGAGAAATGGCGGGTAAGGT T-3′ (forward) and 3′- GCCTCTTTACCGCCCATTCCA AGATC-5′ (reverse), respectively, where the added nucleotides for the *Nhe*I site are underlined. HEK293 or HeLa cells were cultured in growth medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2. Subconfluent cells cultured in 12-well plates were transiently co-transfected with 0.5 μg pGL3-Basic vector DNA or each reporter construct (pGL3/−1993T or pGL3/−1993C) and 10 ng pRL-TK vector DNA (Promega) as an internal control for transfection efficiency, by using 1.5 μl FuGENE six transfection reagent (Roche Diagnostics, Basel, Switzerland). After 24 h, we then lysed the cells and measured firefly and *Renilla* luciferase activities in a luminometer by using the Dual-Luciferase Reporter Assay System (Promega).

Electrophoretic mobility shift assay

Nuclear extracts were prepared from HEK293 and HeLa cells as described previously (Dignam et al. 1983). Double-stranded oligonucleotides −1993T and −1993C were obtained by annealing three concatenated copies of 5′-GAAATGGTGGGTAAG-3′ and 5′-GAAATGGCGGGTAAG-3′ with their respective complementary oligonucleotides. Electrophoretic mobility shift assay (EMSA) analysis was performed by using DIG gel shift kit (Roche). We prepared digoxigenin (DIG)-labeled double-stranded oligonucleotides corresponding to the sequence at position −2000 to −1986 of the *TBX21* promoter containing the −1993 polymorphism. For each binding reaction, we incubated DIG-labeled probes with nuclear extract (2–5 μg) in 1× binding buffer (20 mM HEPES, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM dithiothreitole, 30 mM KCl), 1 μg poly (dI-dC), and 0.1 μg poly L-lysine for 30 min on ice. For competition studies, we incubated unlabeled double-stranded oligonucleotide (100-fold molar excess) during preincubation. Reaction products were separated on 6% non-denaturing polyacrylamide gels in 0.3× TBE buffer (1× TBE buffer = 0.09 M TRIS-borate, 0.002 M EDTA, pH 8.3) and visualized by chemiluminescent detection. We scanned results into an LAS-3000 CCD camera system (Fuji Photo Film, Tokyo, Japan) and quantified each band intensity by using image analysis software Image Gauge Version 2.0 (Fuji Photo Film).

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 control subjects by using a 2×2 contingency χ^2 test with one degree of freedom or Fisher exact test and calculated odds ratios (ORs) with 95% confident intervals (95% CI). For multiple comparisons, *P*-values were corrected by the Bonferroni method. The linkage disequilibrium (LD) statistic *D′* was calculated by using the SNP Alyze statistical package (Dynacom, Chiba, Japan) as described elsewhere (Nakajima et al. 2002). Comparisons in reporter assays and EMSA experiments were performed with the Student's *t* test. A *P*-value of less than 0.05 was considered statistically significant.

Results

Screening for common polymorphisms in TBX21

Direct DNA sequencing of the indicated regions in 12 asthmatic and 12 healthy subjects (total 24 subjects) identified 24 biallelic SNPs in *TBX21*: three in the 5′ flanking region, three in the coding region (one non-synonymous and two synonymous), three in the 3′ untranslated region, and 15 in the intron (Table 2, Fig. 1). Five of these 24 SNPs (532G → C, 729G → T, 2839G → A, 9408C → A, and 10143C → A) are novel, and another 14 have been reported recently in Korean (Chung et al. 2003) and Finnish (Ylikoski et al. 2004) populations. Nucleotide position one (+1) is the first adenine of the initiation codon (ATG), and the positions for other SNPs are relative to the ATG on genome contig AC003665. A graphical overview of 24 SNPs identified in relation to the exon/intron structure of the human *TBX21* gene is given in Fig. 1. Since most of the SNPs were of relatively low frequency and in view of their location and LD with other sites, further genotyping and association studies in our asthma population focused on four SNPs: −1993T → C, 99C → G, 1298T → C, and 7725T → C. The distributions of all four SNPs were in Hardy–Weinberg equilibrium in the control group ($P > 0.05$). We calculated both *D′* and r^2 as statistical values for LD pair-wise between each SNP (Fig. 2). One of the three promoter SNPs (−1993T → C) and one synonymous coding SNP (390A → G, G130G) in exon one, were shown to be in strong LD.

TBX21 genotyping and association studies in asthma and related phenotypes

Initially, the association study was carried out on four clinical groups: child patients with asthma ($n = 361$), adult patients with atopic asthma ($n = 313$), adult patients with non-atopic asthma ($n = 88$), and adult patients with AIA ($n = 72$). Adult asthmatics, except AIA patients, had a negative reaction to the aspirin challenge or no past history of aspirin hypersensitivity. Allele frequencies of each selected SNP were compared between the patients and the normal controls by using a χ^2 test with 1 d.f. (Table 3). After correction for the number of SNPs investigated (Bonferroni correction), we found a significant association between the promoter SNP at −1993 and AIA in our Japanese cohort ($P = 0.004$;

Table 2 Locations and allele frequencies of *TBX21* SNPs in Japanese (*UTR* untranslated region)

Number	SNP	Location	Amino acid substitution	Minor allele frequency (%)	Primers
1	−1993T → C[a]	Promoter	–	8.3	F3R3
2	−1514T → C	Promoter	–	7.1	F4R4
3	− 1499G → A	Promoter	–	2.4	F4R4
4	99C → G[a]	Exon 1	H33Q	6.3	F6R6
5	390A → G	Exon 1	GI30G	8.3	F6R6
6	532G → C	Intron 1	–	2.2	F7R7
7	729G → T	Intron 1	–	4.2	F7R7
8	1298T → C[a]	Intron 1	–	16.7	F8R8
9	1662C → A	Intron 1	–	2.1	F8R8
10	2608G → A	Intron 1	–	2.1	F9R9
11	2839G → A	Intron 1	–	4.2	F9R9
12	7725T → C[a]	Intron 1	–	18.8	F11R11
13	8381A → T	Intron 1	–	16.7	F12R12
14	8762G → C	Intron 1	–	4.2	F12R12
15	9408C → A	Intron 2	–	2.3	F13R13
16	9882C → T	Intron 3	–	4.2	F14R14
17	9898C → T	Intron 3	–	4.2	F14R14
18	10143C → A	Intron 3	–	2.1	F14R14
19	10150T → C	Intron 3	–	4.2	F14R14
20	11268C → T	Intron 5	–	4.2	F16R16
21	11755G → A	Exon 6	P485P	2.1	F16R16
22	12077T → C	3′UTR	–	4.2	F17R17
23	12211G → A	3′UTR	–	2.1	F17R17
24	12403A → C	3′UTR	–	4.2	F17R17

[a]These SNPs were genotyped in a larger population

corrected P, $P_c = 0.016$). There was an increased risk for AIA associated with a C allele (OR = 1.93; 95% CI 1.22–3.06). Association analysis also demonstrated that a significant difference in allele frequency of −1993 SNP between AIA and other adult asthmatics who had no past history of aspirin hypersensitivity ($P = 0.001$). No other statistically significant association between disease status and genotype or any specific allele was detected from any of the other three case-control disease-association comparisons. We further analyzed the genotype and allele frequencies of the SNP at position 390, which was found to be in LD with SNP at −1993, in the AIA and control groups and then calculated the LD coefficient D' and r^2 between the −1993T → C and 390A → G SNPs ($D' = 0.92$; $r^2 = 0.85$). ORs of developing AIA at −1993T → C and 390A → G were 2.15 (95% CI 1.26–3.64) and 2.19 (95% CI 1.27–3.77), respectively, when the TC and TT (AG and GG) genotypes were compared with the wild-type TT (AA) genotype (Table 4). To determine whether these two SNPs in *TBX21* could also be associated with another AIA-related phenotype, we analyzed −1993T → C and 390A → G SNPs in 42 samples from independent adult AS/NP who either had a negative reaction to the aspirin challenge or no past history of aspirin hypersensitivity. Interestingly, comparison of the genotype and allele frequencies also revealed significant differences between the AS/NP group and the normal control group ($P = 0.008$ and 0.012, respectively). Furthermore, in the AIA case group, the C-allele frequency in AIA patients with NP tended to be much higher than that in AIA patients without NP (data not shown). Thus, although the sample size was small, we confirmed the *TBX21* SNP effect by using independent samples of AS/NP, regardless of aspirin hypersensitivity. We further analyzed two-loci haplotype distributions constituting the −1993T → C and 390A → G SNPs in the control, AIA, and AS/NP samples. Haplotype −1993T-390A was the

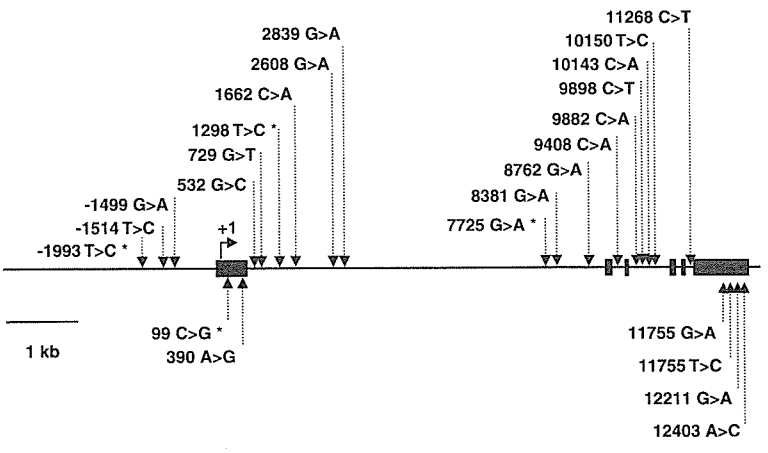

Fig. 1 Graphical overview of 24 SNPs identified in relation to the exon/intron structure of the human *TBX21* gene (*black boxes* five coding exons, *asterisks* SNPs genotyped in a larger population). Positions for SNPs are relative to the translation start site (+1)

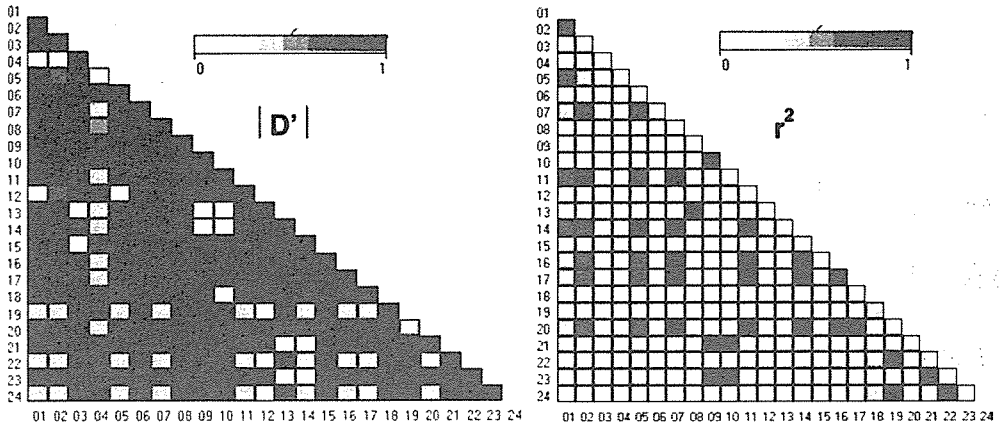

Fig. 2 Pair-wise linkage disequilibrium (*LD*) was measured by $|D'|$ and r^2 among the all of the SNPs identified in 24 sequenced samples. The blocks are *shaded* corresponding to the values obtained from the LD analysis program, SNP Alyze

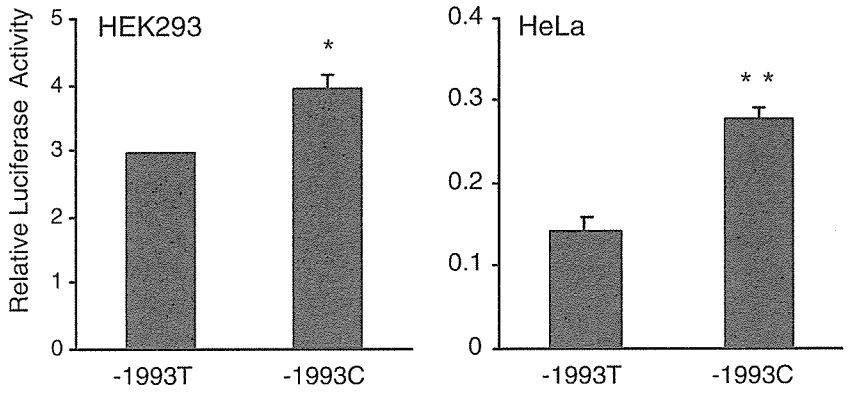

Fig. 3 Effect of the −1993T → C SNP on the transcription activity of the human *TBX21* promoter. HEK293 cells or HeLa cells were transiently cotransfected with pGL/−1993T or pGL/−1993C and pRL-TK vector. The relative luciferase activity of the *TBX21* reporter constructs is represented as the ratio of the firefly luciferase activity to that of *Renilla*. Each experiment was conducted in triplicate for each sample, and the results are expressed as mean ± SD for three independent experiments. $*P<0.001$; $**P<0.005$, as determined by the Student's *t* test

most common, followed by −1993C-390G and −1993C-390A. The two major haplotypes −1993T-390A and −1993C-390G were named haplotypes 1 and 2, respectively. The overall distribution of two loci haplotypes was not different between cases and controls (3 d.f.; $P=0.10$ for AIA, and $P=0.09$ for AS/NP), although the frequencies of two major haplotypes, haplotypes 1 and 2, were significantly different in a χ^2 test (1 d.f.; $P=0.014$ for AIA, and $P=0.016$ for AS/NP) (Table 4).

Transcriptional effect of TBX21 −1993T → C polymorphism

In functional assays, since the 390A → G polymorphism is a synonymous substitution, which is less likely to be directly associated with disease in general, we focused on the promoter SNP at position −1993, viz., −1993T → C. To understand the role of the T/C polymorphism at −1993 in the transcriptional regulation of the human *TBX21* gene, we performed transient expression of the −1993T and −1993C luciferase reporter constructs, pGL3/−1993T and pGL3/−1993C, in HEK293 and HeLa cells. Luciferase activity in cell extracts was analyzed after 24 h of transfection and was standardized against the internal control (*Renilla* activity). The results of this experiment showed that the −1993C construct had significantly higher luciferase reporter activity compared with the wild-type −1993T construct (33%–98% increase; $P<0.005$). These results suggest that the −1993C allele may be associated with the increased transcriptional activity of the *TBX21* gene in human lungs.

EMSA analysis

To examine whether −1993T → C affected interaction of a nuclear factor(s) with the *TBX21* sequence around −1993, we then performed EMSAs. We prepared 2000/−1986 double-stranded oligonucleotide probes containing either the T or the C allele at −1993 bp. HEK293 nuclear extract contained nuclear proteins binding specifically to this region of the *TBX21* promoter, resulting in the formation of one major and one minor complex. Competition with 100-fold to 200-fold excess of unlabeled −1993T of −1993C probes resulted in complete inhibition of complex formation. The single band corresponding to the −1993C allele was significantly more intense than that corresponding to the −1993T allele (21% increase; $P=0.02$ by Student's *t* test), suggesting the two different alleles had different affinities for a

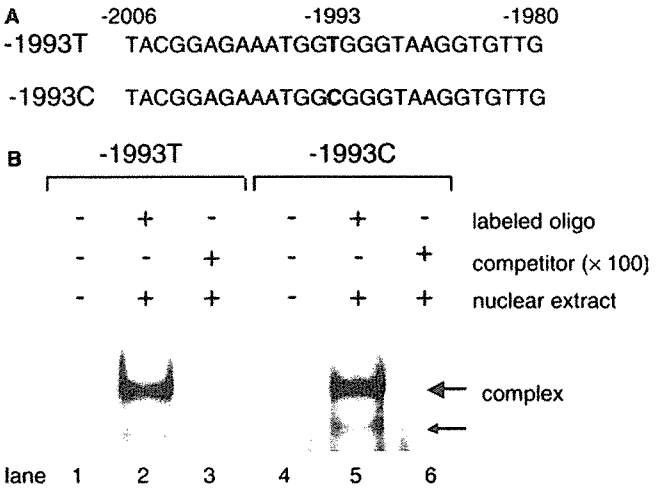

Fig. 4 EMSAs with nuclear extracts prepared from HEK293 cells. Extracts were incubated with DIG-labeled 27-bp double-stranded oligonucleotides corresponding to the −1993T or −1993C alleles of *TBX21*. Competition studies were performed by preincubating with a 100-fold excess of the unlabeled −1993T or −1993C double-stranded competitor oligonucleotides. **a** Oligonucleotide sequences containing T or C at −1993 bp (*bold*) and that were used as a probe or a competitor are shown. **b** Unknown nuclear protein of HEK293 nuclear extracts formed a much stronger complex with the −1993C oligonucleotide compared with the −1993T oligonucleotide (compare *lane 2* vs. *lane 5*; $P = 0.02$ by Student's *t* test). Binding complex was specifically competed by excess of unlabeled −1993T or C oligonucleotide (*lanes 3, 6*). Band intensity was quantified by using the LAS-3000 camera system and image analysis software Multi Gauge Version 2.0 (Fuji Photo Film). A representative result of three independent experiments is shown

particular nuclear factor. The same trend was also observed in HeLa cells (data not shown). Computer analysis of sequences covering −1993 bp, by using NSITE, available at http://www.softberry.com/berry.phtml?topic=nsite&group=programs&subgroup=promoter, indicated that the −1993T → C SNP is situated on a putative binding site for the E2F-1 transcription factor. To identify whether these putative consensus sites were involved in the transcriptional regulation of the *TBX21* gene, we performed a gel shift assay in the presence of specific anti-E2F-1 antibody (C-20; Santa Cruz Biotechnology, Calif., USA). However, preincubation with anti-E2F-1 antibody did not result in a supershift of the DNA-protein complexes (data not shown), suggesting that this protein (family) was not present in the complex binding to this region under these conditions. Together, these data indicate that the −1993T → C SNP in the human *TBX21* gene increases the affinity of an unknown nuclear protein to the binding site around −1993, leading to increased transcriptional activity and a higher expression of the T-bet protein.

Discussion

In the adaptive immune system, $CD4^+$ Th cells differentiate into at least two classes of effector cells, Th1 and Th2, in response to different pathogen-derived antigens. Th1 cells mediate cellular immunity and provide protection against intracellular pathogens and viruses, whereas Th2 cells produce IL-4, IL-5, and IL-13 and eradicate helminthes and other extracellular parasites (Mosmann and Coffman 1989). T-bet, a T box expressed in T cells, has recently been described as a master transcriptional regulator specific to IFN-γ-expressing lineages and is sufficient to induce IFN-γ and IL-12 receptor β2 expression, even under Th2-polarizing conditions (Afkarian et al. 2002; Szabo et al. 2000). Recent experiments have found that, without any allergic sensitization or challenge, the bronchi in mice lacking the T-bet gene, *tbx21*, are infiltrated with eosinophils and lymphocytes and exhibit signs of the airway remodeling and AHR to methacholine that are typical of allergic asthma (Finotto et al. 2002).

In order to examine whether polymorphisms in the candidate gene *TBX21* are related to the risk of human asthma phenotypes, we have characterized sites of

Table 3 Allele frequencies of *TBX21* SNPs in Japanese patients from different asthma groups and controls. Values are the number (%) of successfully genotyped chromosomes

Allele	Healthy controls ($n=640$)	Child patients with asthma ($n=361$)	P^a	Adult patients with					
				Atopic asthma ($n=313$)	P^a	Non-atopic asthma ($n=88$)	P^a	AIA ($n=72$)	P^a
−1993T → C									
T	1149 (89.8)	624 (89.1)		565 (90.3)		161 (93.6)		118 (81.9)	
C	131 (10.2)	76 (10.9)	0.67	61 (9.7)	0.74	11 (6.4)	0.11	26 (18.1)	0.004[b]
99C → G									
C	1127 (88.5)	617 (88.6)		509 (85.1)		143 (88.3)		116 (85.3)	
G	147 (11.5)	79 (11.4)	0.9	89 (14.9)	0.04	19 (11.7)	0.94	20. (14.7)	0.28
1298T → C									
T	1073 (83.8)	603 (83.5)		541 (87.0)		149 (84.7)		125 (86.8)	
C	207 (16.2)	119 (16.5)	0.86	81 (13.0)	0.07	27 (15.3)	0.78	19 (13.2)	0.35
7725G → A									
G	1062 (83.4)	577 (79.9)		502 (82.6)		139 (81.8)		122 (84.7)	
A	212 (16.6)	145 (20.1)	0.05	106 (17.4)	0.67	31 (18.2)	0.6	22 (15.3)	0.68

[a] *P*-value for the comparison with controls
[b] *P*-value statistically significant after Bonferroni correction (corrected $P=0.016$)

Table 4 Genotype, allele, and haplotype frequencies in Japanese AIA, AS/NP cases, and controls for the *TBX21* SNPs at—1993 and 390

Locus	Haplotype number		Controls ($n=640$)	AIA ($n=72$)	Uncorrected *P*	Odds ratio (95% CI)	AS/NP ($n=42$)	Uncorrected *P*	Odds ratio (95%)
−1993T → C		Genotype TT	519 (81.1)	48 (66.7)		1.0	27 (64.3)		1.0
		Genotype TC+CC	121 (18.9)	24 (33.3)	0.004	2.15 (1.26–3.64)	15 (35.7)	0.008	2.38(1.23–4.62)
		Allele T	1149 (89.8)	118 (81.9)		1.0	68 (81.0)		1.0
		Allele C	131 (10.2)	26 (18.1)	0.004	1.93 (1.22–3.06)	16 (19.0)	0.012	2.06(1.16–3.66)
390A → G		Genotpye AA	533 (83.3)	50 (69.4)		1.0	29 (69.0)		1.0
		Genotype AG+GG	107 (16.7)	22 (30.6)	0.004	2.19 (1.27–3.77)	13 (31.0)	0.019	2.23(1.12–4.44)
		Allele A	1165 (91.0)	120 (83.3)		1.0	70 (83.3)		1.0
		Allele G	116 (9.0)	24 (16.7)	0.004	2.01 (1.25–3.24)	14 (16.7)	0.021	2.01 (1.10–3.68)
[−1993]-[390]	1	T-A	1148 (89.7)	120 (83.3)			68 (80.9)		
	2	C-G	114 (8.9)	22 (15.3)	0.014[a]		14 (16.7)	0.016[a]	
	3	C-A	17 (1.3)	2 (1.4)			2 (2.4)		
	4	T-G	1 (0.1)	0 (0.0)	0.10[b]		0 (0.0)	0.09[b]	

[a] *P*-value for the comparison of the frequencies of haplotype 1 and 2
[b] *P*-value for the overall distribution of two loci haplotypes

genetic variation in selected genomic regions of *TBX21*. Among 24 SNPs identified (five are novel), four polymorphic sites were selected for further analysis. All SNPs fulfilled Hardy–Weinberg expectations in both asthmatic and non-asthmatic subjects, and our study showed a significant association between AIA and a SNP in the regulatory region −1993T → C of the human *TBX21* gene ($P_c=0.016$); this was found to be in strong LD with a synonymous coding SNP, 390A → G, located in exon 1 ($D'=0.92$; $r^2=0.85$). Consistent with recent data (Chung et al. 2003; Ylikoski et al. 2004), these four *TBX21* SNPs lack association with any other asthma phenotype in Japanese subjects.

The percent of the −1993C or 390G allele was much higher in AIA patients than normal controls. In an attempt to extend and support these findings, we further genotyped the −1993T → C and 390A → G SNPs in independent adult AS/NP patients and also found a significant association between these SNPs and AS/NP for the allele and genotype frequencies ($P=0.008$). Furthermore, our data indicated that the single base substitution corresponding to the −1993 *TBX21* polymorphic site produced differences in the transcriptional activity of the *TBX21* gene. Unexpectedly, the *TBX21*/−1993C reporter construct was transcriptionally more active than the wild-type −1993T construct in HEK293 and HeLa cells. In addition, EMSA analysis demonstrated that the −1993T → C substitution increased the affinity of a particular nuclear protein to the binding site of *TBX21* covering the −1993 position.

AIA refers to the development of bronchoconstriction following the ingestion of aspirin and other NSAIDs. This clinically distinct syndrome is characterized by aspirin hypersensitivity, bronchial asthma, and chronic rhinosinusitis with nasal polyposis, commonly called the "aspirin triad". AIA affects 5%–20% (about 10%) of adult asthmatics with a higher prevalence in women and is infrequently found in asthmatic children (Babu and Salvi 2000; Szczeklik and Stevenson 1999). Chronic persistent inflammation is the hallmark of patients with AIA. Recently, the importance of arachidonic acid metabolites in the pathogenesis of AIA has become apparent. The cyclo-oxygenase (COX) theory is widely accepted: AIA attacks are triggered by the specific inhibition of COX in the respiratory tract, which is followed by a reduction of prostaglandin E2 (a brake on leukotriene synthesis) and an overproduction of cysteinyl leukotrienes. Thus, cysteinyl leukotrienes have been recognized as the key mediators of AIA, but the precise molecular mechanism involved in AIA remains unclear.

Surprisingly, our results have shown a significant increase in the −1993C allele, the putative higher expression of T-bet, among patients with AIA or AS/NP, compared with controls in our Japanese cohort. An inappropriate or excess Th2-biased immune response to environmental antigens has generally been considered to play a crucial role in the development of asthma. Whereas Th2 cells promote asthmatic inflammation, Th1 cells, which secret IFN-γ, have been proposed to protect against asthma by dampening the Th2 response. However, the evidence from many studies of asthma in human and animal models conflicts with this interpretation (Busse and Lemanske 2001; Salvi et al. 2001). For example, IFN-γ production is elevated in the serum of patients with asthma (Corrigan and Kay 1990), in supernatants of bronchoalveolar lavage (BAL) cells (Cembrzynska-Nowak et al. 1993), in T cells themselves in BAL (Krug et al. 1996), and in whole blood culture (Magnan et al. 2000). By using an adaptive transfer system in mice, previous reports have shown that antigen-specific Th1 cells cause considerable airway inflammation instead of attenuating Th2-mediated lung disease (Hansen et al. 1999; Li et al. 1998; Randolph et al. 1999). IFN-γ has been demonstrated to activate eosinophils in vitro, not only with an increased expression of Fcγ receptors, CD69, HLA-DR, and intercellular adhesion

molecule-1, but also with increased viability (Busse and Lemanske 2001; Krug et al. 1996). Furthermore, therapy with IL-12, a Th1-inducing cytokine, fails to reduce AHR or the late asthmatic reaction (Bryan et al. 2000). These and other recent data (Ford et al. 2001; Sugimoto et al. 2004) suggest that IFN-γ contributes to the augmentation of allergic lung inflammation partly through the activation of eosinophils, highlighting the importance of both Th1 and Th2 cytokines in the development of asthma. Thus, the classification of allergic inflammation in asthma as a Th2-mediated disease is too simplistic (Busse and Lemanske 2001), and, as pointed out by recent work (Sugimoto et al. 2004), we propose that asthma may be classified roughly into at least two subgroups, Th2-type asthma and Th1/Th2 mixed-type asthma, including AIA.

Previous reports have suggested that Th1 cells can actually cooperate with Th2 cells in vivo and enhance Th2, eosinophil, and neutrophil recruitment by increasing the expression of TNF-α, chemokines, and adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) in the lungs (Randolph et al. 1999; Takaoka et al. 2001). Actually, aspirin sensitivity and AS/NP often coexist with severe asthma, and the airways of AIA patients with NP show signs of persistent inflammation with marked eosinophilia and enhanced VCAM-1 expression (Hamilos et al. 1996). The continuous airway inflammation in AIA could result from a non-IgE-mediated reaction to specific endogenous or exogenous antigens such as a virus (Szczeklik 1988; Szczeklik and Stevenson 1999). A latent or chronic viral infection has been shown to alter the expression of many cellular genes, including several constituents of the arachidonic acid pathway (Zhu et al. 1998), and virally infected cells are more prone to drug and drug-metabolite-related toxicity (Levy 1997; Nakagawa et al. 2001). Moreover, an antiviral drug, acyclovir, is reported to inhibit analgesic-induced bronchoconstriction and decrease the urinary levels of leukotrieneE4 in patients with AIA (Yoshida et al. 1998). Based upon these observations of AIA, virus-specific Th1 cells responding to a respiratory tract infection could alter the local lung environment sufficiently to increase Th2 and eosinophil recruitment, leading to strong Th2 responses to inhaled antigens induced by IL-4, 5, 13, and other mediators. We postulate that the $-1993T \rightarrow C$ SNP in the *TBX21* promoter causes a functional difference in T-bet expression, resulting in increased T-bet production and (viral-induced) an excessive Th1 inflammatory reaction in the lungs.

Asthma is a phenotypically heterogeneous disorder with many etiologic factors and clinical characteristics. Although we find no associations of *TBX21* SNPs with other asthma groups except for AIA, our data also indicate that the presence of the $-1993C$ allele increases the risk of AS/NP, regardless of aspirin sensitivity. Thus, in asthma phenotypes, the *TBX21* SNPs are probably not strictly associated with aspirin sensitivity itself. NP is a chronic inflammatory disease of the paranasal sinus mucosa, leading to the protrusion of edematous polyps into the nasal cavities (Mygind 1990). NP is commonly found in association with non-atopic asthma and aspirin sensitivity, and this association of NP with asthma might reflect the shared pathophysiology of these disorders of the upper and lower airways, respectively. Furthermore, previous studies have shown that NP-infiltrating T cells expressed a mixed Th1/Th2 pattern of cytokines (Hamilos et al. 1995; Sanchez-Segura et al. 1998). Together, our present data suggested that, in a variety of asthma-related conditions, the amplification of either side of the Th1/Th2 pathway, or both, could be adverse to the host. Churg-Strauss syndrome (CSS), also known as allergic granulomatosis and angiitis, is another asthma-related disorder characterized by systemic small vessel vasculitis. Indeed, analysis of the cytokine profile of T cell lines from patients with CSS has shown both type-1 cytokine and type-2 cytokine responses (Kiene et al. 2001). Of note, clinical signs of autoimmunity such as vasculitis have been observed in some patients with AIA (Szczeklik et al. 1995, 1997).

The human *TBX21* gene is located on chromosome 17q21.32, which has previously been linked with asthma and skin tests (Dizier et al. 2000). Moreover, the region on mouse chromosome 11, a region that has been linked to AHR, is syntenically homologous to human chromosome 17q12-q22 (Zhang et al. 1999). *TBX21* is likely to be a novel candidate gene in this region, in addition to other candidate genes such as eotaxin (*CCL11*). However, our data cannot exclude the possibility that $-1993T \rightarrow C$ is in LD with another polymorphism in *TBX21* or a neighboring gene. Further studies in larger or other populations will be required to confirm the effect of the *TBX21* polymorphism. To date, several candidate genes of the enzymes in the arachidonic pathway, such as *LTC4S* and *ALOX5*, have been proposed to increase the susceptibility to AIA (Choi et al. 2004; Kawagishi et al. 2002; Sanak et al. 1997); indeed many other genes, in addition to *TBX21*, probably contribute to the pathogenesis of AIA. Genetic epidemiology on larger numbers of AIA patients is an important future requirement in order to clarify the importance of our findings.

In conclusion, we have identified 24 SNPs (five novel) in the *TBX21* gene, and our studies demonstrate that the $-1993T \rightarrow C$ SNP in the *TBX21* promoter is likely to be associated with an increased risk for AIA in Japanese. This is the first report demonstrating a relationship between the *TBX21* SNPs and clinical features of human asthma. Furthermore, we have shown that the $-1993T \rightarrow C$ polymorphism affects the transcriptional activity of the gene and may contribute to an increase in T-bet expression. In certain asthma subgroups, such as AIA and AS/NP, this promoter SNP may cause inappropriate Th1 responses in the airway, leading to severe airway inflammation, in combination with antigen-specific Th2 responses. Our present data shed light on an important area of further study regarding the precise phenotype classification of asthma by using genotypes and also focus on the Th1 response in the pathogenesis of AIA.

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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

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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

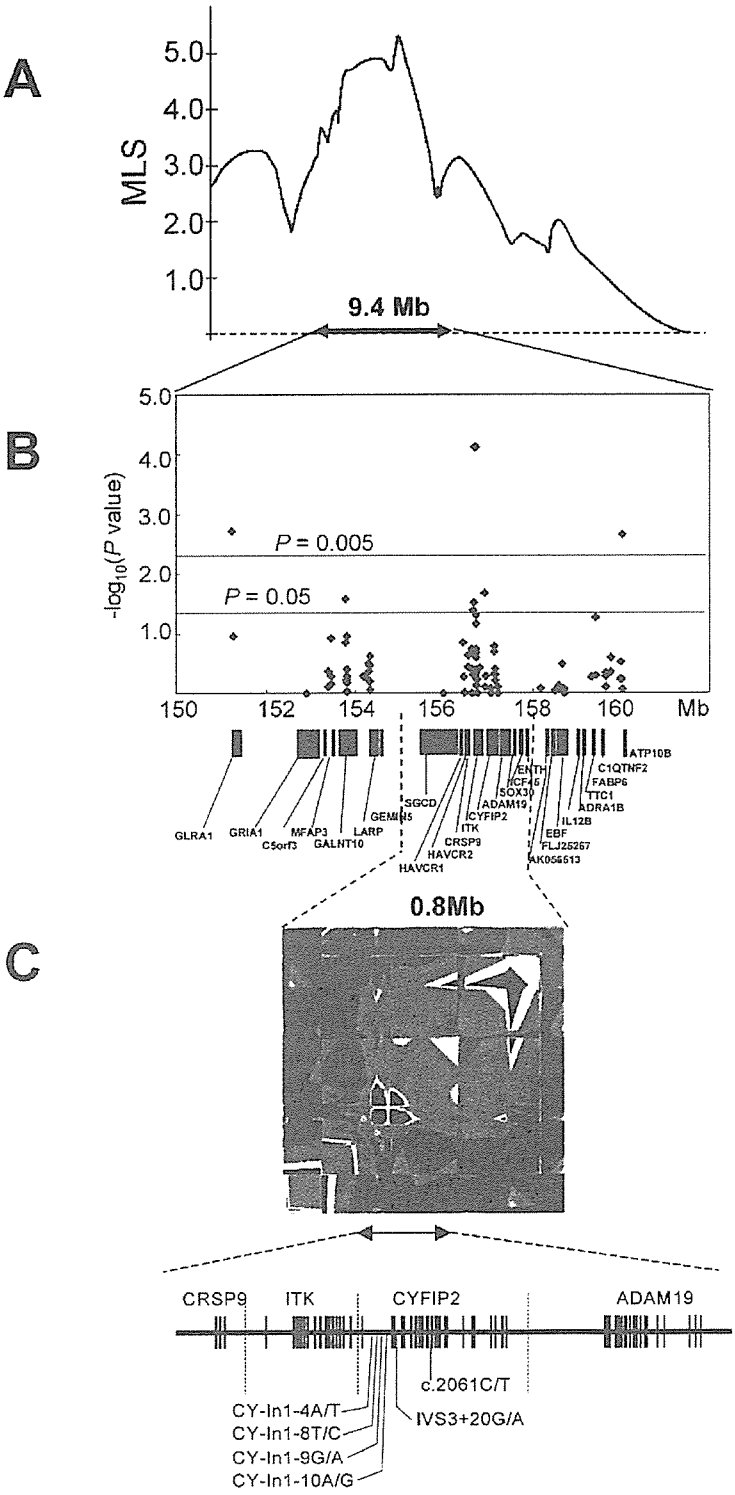

Figure 1. (*A*) Maximum logarithm of odds score (MLS) plot for asthma on human chromosome 5q33 in 47 families with asthma identified through children with mite-sensitive asthma. (*B*) Results of transmission disequilibrium test in the 95% confidence interval for the location of the asthma susceptibility 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 *FLJ25207*). 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