ECM glycoprotein tenascin-C (TNC) has been accepted as a histopathological marker, beneath the asthmatic airway, for evaluating the severity and the therapeutic effects of drugs in bronchial asthma (7,8) because of its tightly controlled expression pattern. TNC expression is prominently increased around airway basement membranes of asthmatic patients (8), and two independent microarray experiments, including our own, identified TNC as one of the IL-4- or IL-13-induced genes in human bronchial epithelial cells (9,10). Recent studies showed that the fibronectin-III (Fn-III) domain of TNC has molecular elasticity (11) and mechanical strain can induce TNC expression (12), so we consider TNC to be more than just a marker for asthmatic pathology.

In the present study, we show the genetic association between an adult asthma and an SNP in exon 17 (44513A/T) causing amino acid substitution in the fibronectin type III-D (Fn-III-D) domain region of TNC gene (13). We carried out protein structure modeling of the Fn-III-D domain and found that the amino acid replacement Leu1677Ile could affect the structural stability of the Fn-III-D domain, which might affect the elasticity of the domain. In addition, TNC expression in lung fibroblasts was increased with IL-4 or IL-13 stimulation. The aim of our study was to test the association between the coding SNP in the TNC Fn-III-D domain and asthma and to determine how the SNP may affect the pathophysiology of asthma.

RESULTS

Identification of TNC genetic polymorphisms and selection of representative SNPs

We detected 62 genetic polymorphisms within the TNC region (Supplementary Material, Table S1) by resequencing samples from 24 Japanese individuals (12 asthmatics and 12 controls). Of these, we selected 23 SNPs whose minor allele frequency (MAF) was >20%. To check the intragenic linkage disequilibrium (LD) pattern in the TNC gene, pairwise LD was measured by r among the 23 SNPs (Fig. 1A). We selected 10 representative SNPs on the basis of location and LD with other sites; the positions of the 10 SNPs are shown in Figure 1B.

Case–control association study using asthmatic patients

We carried out a case–control association study using a Japanese asthmatic population. Clinical characteristics of the bronchial asthma patients are presented in Table 1. The severity of asthma before treatment was classified by the Global Initiative for Asthma Guideline (14). All 10 investigated SNPs were within the Hardy–Weinberg equilibrium. The overall success rate for genotyping was 99.1%. Of these 10, an SNP in exon 17 (44513A/T) had a significant association with adult bronchial asthma in our Japanese cohort under a recessive model [χ^2 test, 44513TT versus AT + AA, raw P-value 0.00019, Odds ratio (OR) 1.76, 95% confidence interval (95% CI) = 1.31–2.36] (Table 2). Stronger association was observed when we limited case subjects to non-smoking asthmatics (44513TT versus AT + AA, raw P-value 0.000025, OR 2.06, 95% CI = 1.45–2.87). There was no correlation between the severity of asthma and the TNC genetic association (data not shown).

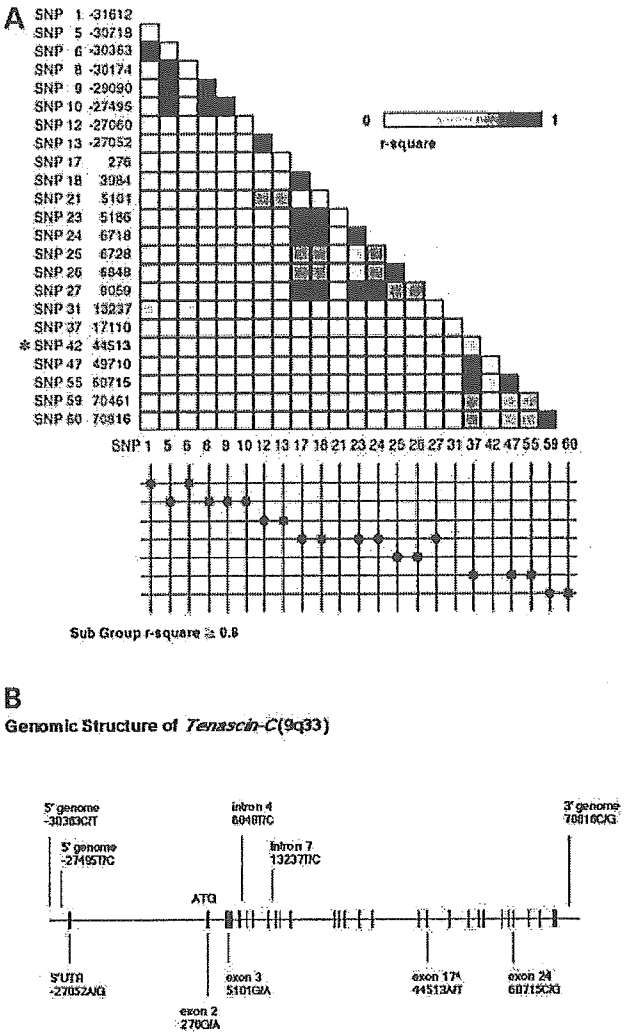

Figure 1. Pairwise LD map and SNP map in the TNC genomic region. (**A**) Pairwise LD in the TNC gene, as measured by the r^2 value between all pairs of SNPs examined. The position of 44513A/T is indicated with an asterisk and the remaining nine SNPs genotyped were indicated with red color. (**B**) The complete coding region of TNC, intron/exon boundaries, the intronic sequence, ~3 kb of 5′ genomic DNA and 1 kb of 3′ genomic DNA are shown. Twenty-seven exons are indicated by closed squares. Position 1 is the start codon of the TNC gene. An asterisk indicates the 44513A/T SNP.

LD mapping around the TNC gene

To exclude the possibility that our results reflected the association of other genes near the TNC locus with asthma, we constructed an LD map around the TNC gene locus using 48 SNPs (MAF > 10%). The results indicated that 44513A/T (indicated by an asterisk in Fig. 2) was located in the LD block extended from intron 8 of the TNC gene to the 3′ genome region of TNC (30 kb upstream and 20 kb downstream of this SNP) and that there were no other genes in this block (Fig. 2).

Table 1. Clinical characteristics of the bronchial asthma patients

Pretreatment: severity of disease[a]	Frequency of attack before treatment	Mean age	Mean age at onset	Mean duration of asthma (years)
Class 1: intermittent	Less than once a week	31.1	14.4	14.0
Class 2: mild persistent	More than once a week, less than once a day	45.9	30.6	16.0
Class 3: moderate persistent	Symptoms daily	47.1	36.5	19.8
Class 4: severe persistent	Symptoms daily, frequent nocturnal asthma	54.2	40.7	15.2

[a]Severity of disease was classified by Global Initiative for Asthma Guideline (14).

Table 2. Genotype frequencies for TNC SNPs and asthma susceptibility

SNP location	Control ($n = 658$) (%)			Bronchial asthma ($n = 446$) (%)			P-value[a]	P-value[b]	P-value[c]
	1	2	3	1	2	3			
−30363C/T	464 (71)	178 (27)	16 (2)	313 (71)	124 (29)	5 (0)	NS	NS	NS
−27495T/C	268 (41)	306 (47)	79 (12)	182 (41)	205 (47)	55 (12)	NS	NS	NS
−27052A/G	254 (39)	318 (49)	79 (12)	171 (39)	207 (47)	64 (14)	NS	NS	NS
276G/A	302 (46)	275 (42)	73 (11)	213 (48)	191 (43)	37 (9)	NS	NS	NS
5101G/A	301 (46)	299 (46)	56 (9)	197 (44)	200 (45)	49 (12)	NS	NS	NS
6848T/C	212 (32)	323 (49)	118 (18)	164 (37)	203 (46)	75 (17)	NS	NS	NS
13237T/C	340 (52)	269 (41)	46 (7)	229 (52)	180 (41)	29 (7)	NS	NS	NS
44513A/T	169 (26)	303 (46)	183 (28)	125 (29)	233 (53)	79 (18)	0.037	NS	0.0019
60715C/G	237 (36)	301 (46)	116 (18)	134 (30)	218 (49)	90 (20)	NS	NS	NS
70816C/G	279 (43)	284 (44)	87 (13)	178 (41)	193 (45)	61 (14)	NS	NS	NS

P-value adjusted with Bonferroni correction (raw P-values were multiplied by 10); NS, not significant.
[a]Allele1 versus allele 2.
[b]Genotype11 versus genotype 12 + 22.
[c]Genotype11 + 12 versus genotype 22.

Haplotype analysis

We carried out haplotype analysis of four representative SNPs in the LD block containing the 44513A/T SNP. Estimated frequencies of the four-locus haplotype were compared between cases and control subjects. The results of association studies for each haplotype showed a significant association between haplotype 1 and asthma (Table 3) (raw P-value = 0.004); however, the association was not stronger than that observed for the single locus (44513A/T).

Immunohistochemistry of TNC

Paraffin sections of asthmatic lungs were immunostained with a rat anti-TNC monoclonal antibody. Subepithelial deposition of TNC protein was observed beneath the bronchial epithelium in the asthmatic lung of a 65-year-old male (Fig. 3A). No apparent TNC staining was observed in the control lung of a 68-year-old male (Fig. 3B).

Computer modeling of the TNC Fn-III-D domain structure

We derived a protein structure model of the TNC Fn-III-D domain with MOE software (Fig. 4) to examine the possible effects of the substitution of the 1677th amino acid. The major allele in the normal population 44513-T encodes 1677Leu, whereas 44513-A, common in asthmatic patients, encodes 1677Ile. The 1677th amino acid is located at the beta-strand, which makes up the outermost side of the beta-sheet (Fig. 4A and B). The amino acid faces to the inside of the beta-sheet structure and there is a hydrophobic interaction between Phe1636, Leu1638, Leu1652, Ile1654 and Leu1680 (Fig. 4C, shaded region). The substitution of Leu1677Ile could result in steric hindrance with Phe1636 because of its side chain (Fig. 4D).

Identification of TNC variant expression in normal human lung fibroblasts by RT–PCR and western blotting

To confirm the expression of the TNC mRNA variant containing SNP 44513A/T in exon 17, RT–PCR (reverse transcription–polymerase chain reaction) was performed with a forward primer in exon 10 and a reverse primer in exon 19. The PCR results showed bands of 1969, 607 and 331 bp with normal human lung fibroblasts (NHLF) cDNA (Fig. 5A, left). The PCR products were subcloned and then sequenced. Larger bands (1969 and 607 bp) contained the Fn-III-D domain, including SNP 44513A/T. The cell lysate of NHLF was electrophoresed and immunoblotted with the rat anti-TNC antibody. A 250 kDa variant of TNC, corresponding to the largest mRNA, was dominantly expressed in NHLF, and both IL-4 and IL-13 could upregulate the 250 kDa TNC protein expression (Fig. 5B).

DISCUSSION

In the present study, asthmatic patients were recruited on the basis of the clinical asthma findings (14). We selected well-controlled cases after asthma treatments, (for class 2, 3 and

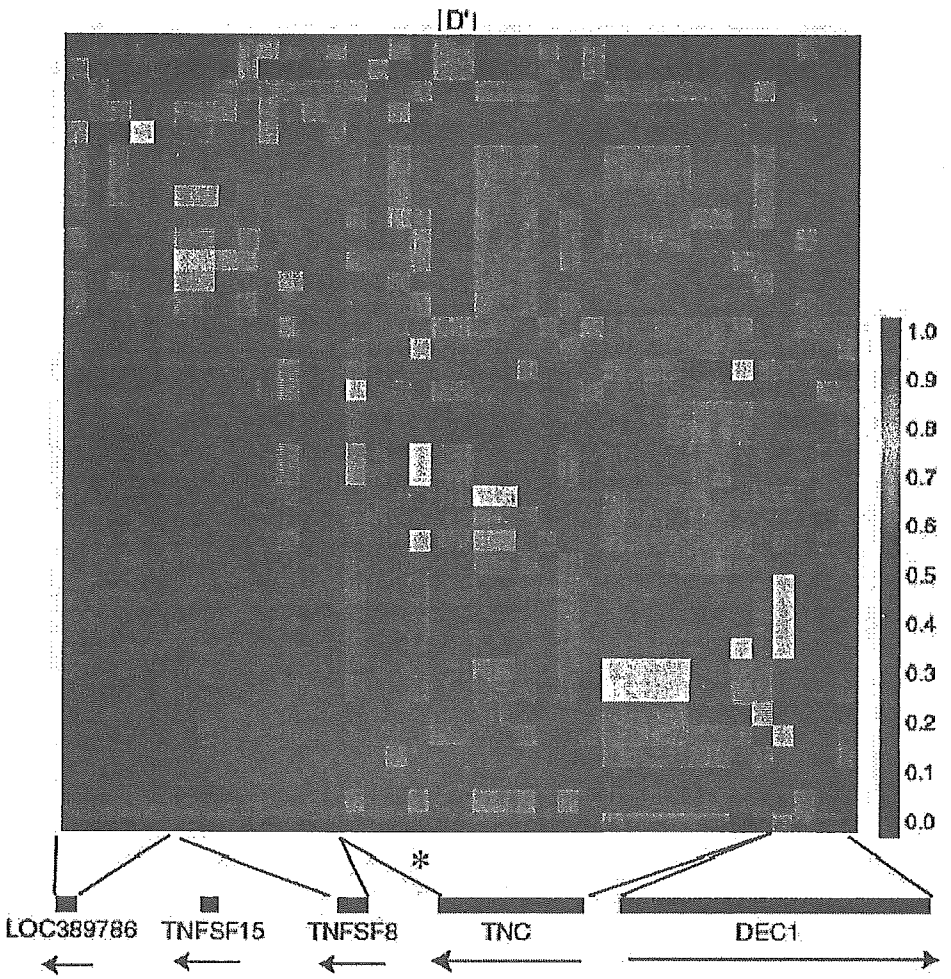

Figure 2. Pairwise LD map around TNC locus, as measured by D' value or r value. Pairwise LD map around the TNC locus, as measured by D' value or r value between all pairs of SNPs examined. The position of 44513A/T is indicated with an asterisk. Arrows indicate the direction of transcription of the genes.

4 cases with known amounts of an inhaled steroid: beclomethasone dipropionate; BDP), to ensure the reversibility of lung functions (Table 1). We took care to exclude possible COPD (chronic obstructive pulmonary disease) cases by spirometric analysis to check the reversibility of airflow obstruction and by X-ray/CT examinations. We found a genetic association between SNP 44513A/T in exon 17, coding the 1677th amino acid in the Fn-III-D domain, and adult bronchial asthma (Table 2). The genetic association between 44513A/T and asthma became stronger when we limited the analysis to the non-smoker asthmatic subpopulation. This result suggested that the association was not the consequence of secondary impairment of lung function due to smoking. The LD map around the TNC gene region showed that SNP 44513A/T was located in the LD block that extended from intron 8 to the 3′ genomic region of the TNC gene, and there were no other genes in the block (Fig. 2). Therefore, we concluded that the strong association observed with the SNP 44513A/T originated from the TNC gene itself. We intensively searched SNPs around the 44513A/T by resequencing and using public SNP databases, but we could not find any SNPs showing tight LD with 44513A/T. Weak LD was observed with 60715C/G (a coding SNP in exon 24) but there was no association between that SNP and bronchial asthma (Table 2), and the association between the four-SNP haplotypes including these two SNPs and asthma was not stronger than that of the 44513A/T single locus (Table. 3). Therefore, we selected SNP 44513A/T as the target for further analysis.

The TNC gene was chosen as a candidate gene for asthma on the basis of our previous GeneChip experiment (9). According to the results, TNC was one of the few genes constantly upregulated in bronchial epithelial cells in response to Th2 cytokines. We analyzed several candidate genes on the basis of the GeneChip results and found a significant

Table 3. Haplotype structure and frequency in TNC

Haplotype	SNP position				Haplotype frequency		*P*-value[a]	OR
	13237T/C	44513A/T	60715C/G	70816C/G	Controls	Cases		
Haplotype 1	T	T	C	C	0.33	0.27	0.004	1.33
Haplotype 2	T	A	G	G	0.26	0.27	0.725	1.04
Haplotype 3	C	T	C	C	0.17	0.17	0.816	1.03
Haplotype 4	C	A	G	G	0.08	0.08	0.933	1.01

OR, odds ratio.
[a]Analysis using a 2×2 table for each haplotype against all others combined in cases and controls.

association with the TNC gene. Furthermore, one previous genome-wide linkage study by Wjst *et al.* (15) showed that D9S1784 and D9S195 markers at chromosome 9q33 could be linked to asthma. TNC genes were located between these two markers (~9.7 Mb to D9S1784 and 5 Mb to D9S195). On the basis of these results, TNC seemed to be a good candidate gene for affecting susceptibility to asthma.

Our immunohistochemical staining of asthmatic airways showed TNC deposition around the basement membrane (Fig. 3A). Both bronchial epithelial cells and lung fibroblasts under the basement membrane may produce TNC. *In situ* hybridization experiments with the developing human lung (16) and respiratory distress syndrome (17) have shown that myofibroblasts under the epithelium express TNC mRNA. Therefore, we suppose that TNC in the asthmatic lung is predominantly produced by lung fibroblasts. It should be noted that the TNC Fn-III domain has both molecular elasticity (11) and essential roles for airway branching (18,19). We considered that TNC around the airway might have homeostatic roles for maintaining the integrity of airways in stressed conditions like bronchial asthma.

The structural model of the TNC Fn-III-D domain showed that the Ile1677 variant caused instability of the beta-sheet in the domain (Fig. 4D). Thus, Ile1677, a common variant among adult asthmatic patients, may alter the molecular elasticity of the TNC Fn-III domain. Airway resistance measurements of the asthmatic patients with or without allele 44513-A to investigate genotype–phenotype association are now ongoing.

It is known that a part of the TNC Fn-III domain, Fn-III-A1 through Fn-III-D, (Fig. 5A), is alternatively spliced (13). We checked the alternative splicing exon–intron junction for SNPs that might affect the splicing sites (20), but we could not find any SNPs that showed a significant association with asthma. Previous reports showed that the large form of TNC, including the alternative splicing region, was the predominant form in developing rat lung (19). Thus, it is likely that the large form of TNC is the main variant in the lung. Our monoclonal antibody could not distinguish between the large and small forms of TNC in immunohistochemistry, so we further analyzed the TNC variants by RT–PCR and by western blotting using NHLF. We showed that 250 and 190 kDa TNC variants contained the alternatively spliced Fn-III-D domain in NHLF (Fig. 5A) and either IL-4 or IL-13 treatment could preferentially induce the 250 kDa variant (Fig. 5B). We also found that the induction of TNC mRNA by IL-4 and IL-13 was not the consequence of non-specific inflammation because STAT6 activation could upregulate TNC mRNA expression (Supplementary Material, Fig. S3). From these findings, we conclude that it is highly likely that SNP 44513A/T in the TNC Fn-III-D domain is functional, especially under the influence of Th2 cytokines.

There are a few studies analyzing the role of TNC in pathologic conditions, some of which showed homeostatic roles of TNC protein (21,22). Habu snake-venom toxin induces glomerulonephritis phenotype in TNC knockout mice with more severe disease than that in congenic control mice (23). We suppose that TNC is a molecule with homeostatic functions emergent under stressful conditions. The TNC molecule may also have homeostatic roles in asthmatic conditions and the instability of the Fn-III-D structure caused by this SNP may hence affect the pathophysiology of asthma.

In conclusion, we found a genetic association between the SNP encoding the Fn-III-D domain of the TNC molecule and the adult bronchial asthma. The coding SNP causes instability of the Fn-III-D domain structure. Under the influences of Th2 cytokines, the expression and functional impact of the TNC molecule increase. The coding SNP might be a useful marker for evaluating the risk for adult asthma and provides insights into the precise functional roles of TNC in the pathogenesis of asthma. Further study is needed.

MATERIALS AND METHODS

Materials

The rat anti-human TNC monoclonal antibody (clone 3–6) was described previously (24). A horseradish peroxidase (HRP)-conjugated goat anti-rat IgG antibody and precast Tris–glycine polyacrylamide gels were purchased from Invitrogen (Carlsbad, CA, USA). Recombinant human IL-4 and IL-13 were purchased from Peprotec (London, UK).

Cell culture

NHLF were purchased from BioWhittaker (Walkersville, MD, USA) and cultured with the fibroblast basal medium from the same company according to the manufacturer's protocol.

Subjects

The adult asthmatic patients were recruited from approximately 4000 outpatients who were diagnosed as having bronchial asthma at the Miyatake Asthma Clinic or at the Osaka Prefectural Habikino Hospital by asthma specialists using

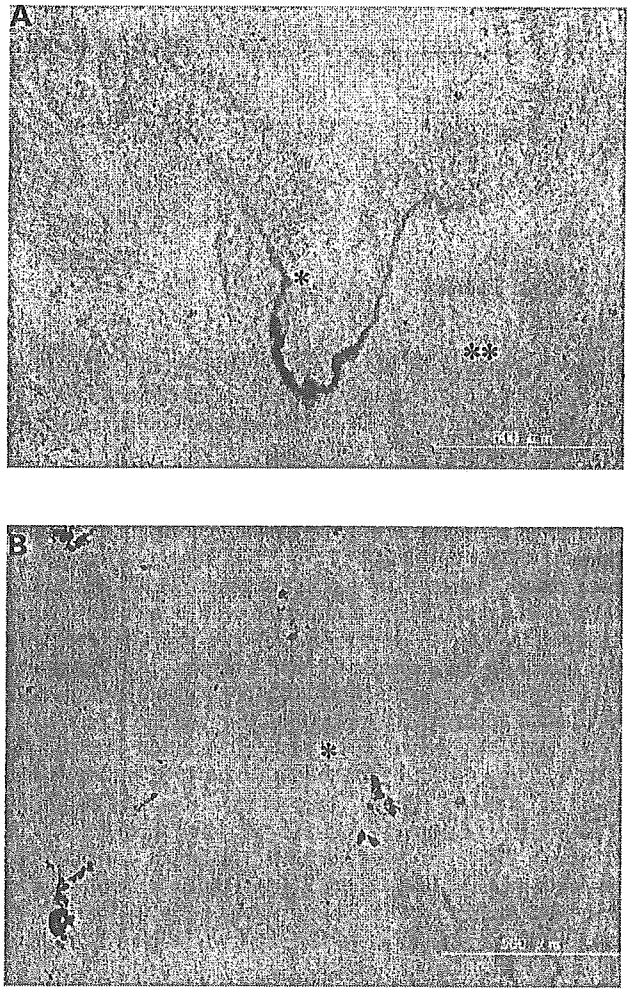

Figure 3. Immunohistochemical analysis of asthmatic lung with TNC antibody. Paraffin sections of asthmatic and control lungs were immunostained with the anti-TNC monoclonal antibody and visualized with indirect immunoperoxidase staining. Intense subepithelial staining is observed in the asthmatic lung (**A**) but not in the control lung (**B**) * indicates bronchial epithelium and ** indicates airway smooth muscle. Dark black spots in the control lung are foreign particles in the lung.

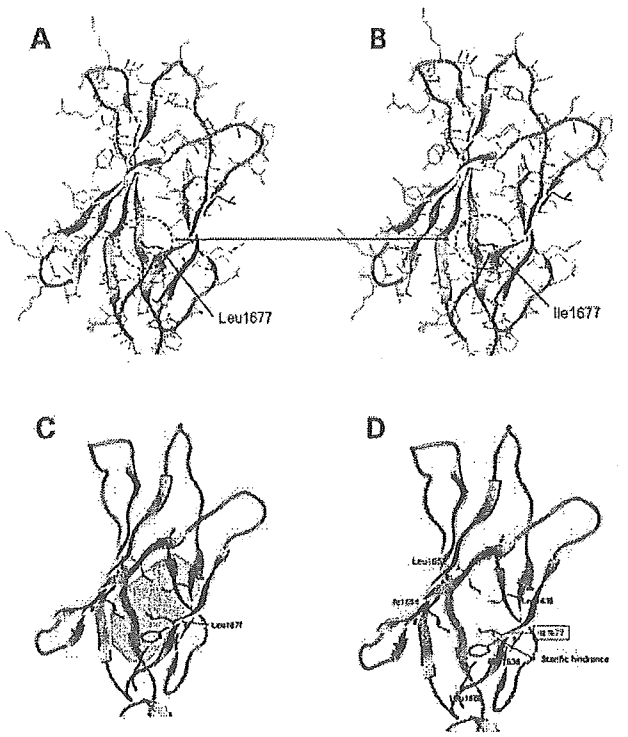

Figure 4. Computer modeling of TNC Fn-III-D domain and effect of 1677 Leu-Ile substitution. The 2.25 Åcrystal structure of chicken TNC (PDB accession no. P24821) was used as a template for homology modeling of the human TNC Fn-III-D domain. (**A**) The amino acid Leu1667 located in the fifth beta-sheet (yellow arrow, downward, indicated by black arrow) of the TNC Fn-III-D domain is shown with a green bar. (**B**) Amino acid Ile1677 located in the fifth beta-sheet of the TNC Fn-III-D domain is shown with a red bar. (**C**) Leu1677 makes a hydrophobic interaction plane (shaded region) among the hydrophobic amino acids. (**D**) The change of the amino acid from Leu to Ile caused steric hindrance with Phe1636 inside the beta-sheet.

the American Thoracic Society criteria as previously described (25,26). We selected 446 adult bronchial asthma patients (mean age 46.9, 16–70 years; male:female ratio, 1.0:1.2; mean serum IgE level, 741.3 U/ml; mite RAST positive 64.9%) satisfying the following symptoms and physical examination criteria: (i) those who showed episodic breathlessness, wheezing and chest tightness before treatment, (ii) the asthmatic symptoms were well controlled with known amounts of inhaled steroids. Among them, 105 patients were smokers or ex-smokers but not heavy smokers judged by the Fagerstrom Tolerance Questionnaire (26). Detailed information about the patients, including the severity of asthma (14) is summarized in Table 1. Peak expiratory flow analysis, spirometry, chest X-ray and CT scan were performed for the patients in need of differential diagnosis for COPD. Bronchial hyper-responsiveness was not tested. Peripheral blood was obtained from these 505 adult bronchial asthma patients. As a healthy control group, we analyzed 625 randomly selected population-based individuals (mean age 42.0, 18–69 years; male:female ratio, 2.5:1.0). We excluded the presence of asthma, atopic dermatitis and nasal allergies in the control population through careful interviews by physicians. All individuals were of Japanese origin and gave written informed consent to participate in the study, according to the process committee at SNP Research Center, RIKEN.

SNP discovery and genotyping in TNC gene

The TNC region targeted for SNP discovery included a 5 kb continuous region 5′ to the gene and 28 exons, each with a minimum 200 bp of a flanking intronic sequence. Forty primer sets were designed on the basis of TNC genomic sequences (Supplementary Material, Table S2). Each PCR was performed with 5 ng of genomic DNA from 24 individuals (12 asthmatic patients and 12 controls). The PCR product was reacted with BigDye Terminator v3.1 (Applied

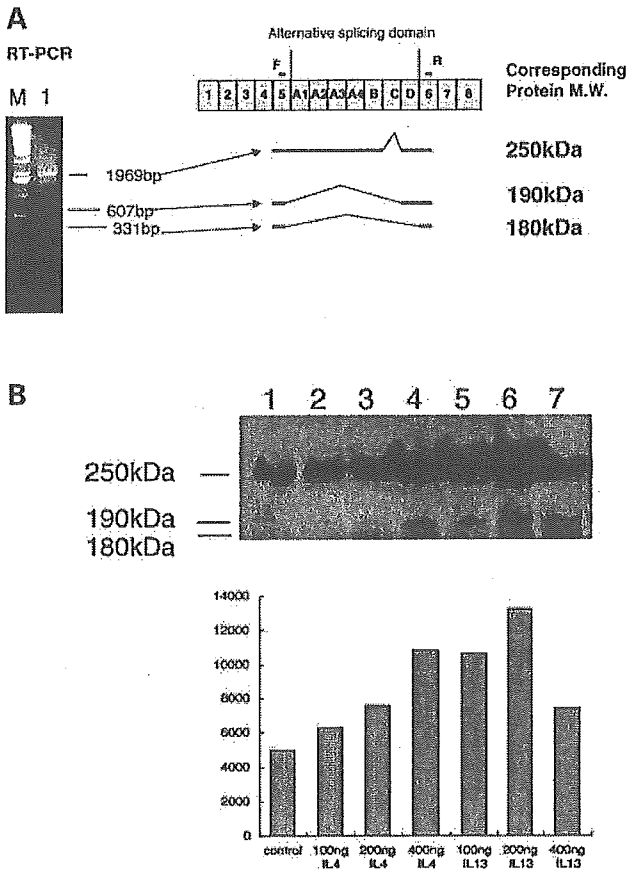

Figure 5. RT–PCR and western blot analysis for TNC variants in NHLF with Th2 cytokine stimulation. (**A**) TNC mRNA expression in NHLF was examined with RT–PCR. Subconfluent NHLF were stimulated with 100 ng/ml IL-13 for 72 h. After mRNA extraction and cDNA synthesis, PCR was performed with a pair of primers designed to differentiate alternatively spliced mRNA of TNC. The PCR products were electrophoresed on 1% agarose gel. M: 1 kb DNA marker and Lane 1: NHLF (left side). The structure of the FN-III domain in the human TNC gene is shown (center). F: forward primer and R: reverse primer. Corresponding protein molecular weights (M.W.) are indicated to the right. (**B**) Western blot analysis of NHLF culture samples stimulated with IL-4 or with IL-13 for 72 h. The samples were electrophoresed in 4–20% Tris–glycine gels and electrotransferred in PVDF membrane. Immunoblotting was performed with the rat anti-TNC monoclonal antibody. The relative intensity of TNC protein was quantified with NIH Image and is shown at the bottom. Lane 1 represents NHLF without stimulation. Lanes 2–4 represent NHLF stimulated with IL-4 at the concentrations of 100, 200 and 400 ng/ml, respectively. Lanes 5–7 represent NHLF stimulated with IL-13 at the concentrations of 100, 200 and 400 ng/ml, respectively.

Biosystems). We also utilized the SNP information from the database of SNPs by Japanese Science and Technology Agency database (JSNP). Intragenic pairwise LD in the TNC locus was examined by measuring r^2 among 22 SNPs. The pairwise LD and haplotype were evaluated using the SNPAlyze 3.1 software (Dynacom Co. Ltd, Chiba, Japan). Position SNPs were numbered according to their position relative to the published genomic sequence containing the TNC region (GenBank accession no. AL162425), and position 1 is the adenine of the first methionine of TNC. The panel of 10 SNPs was genotyped with the multiplex PCR-Invader assay or Taqman genotyping system as described previously (27). To investigate the pattern of LD in and around the TNC locus, pairwise LD coefficients were calculated and expressed as |D′| or r. We evaluated the LD extension of the TNC genomic region with 48 SNPs registered in JSNP by genotyping 1041 general Japanese subjects.

Statistical analysis

Allele frequencies in bronchial asthma and controls were compared by the contingency χ^2 test. A P-value of less than 0.01, after Bonferroni adjustment in case of multiple comparisons, was considered to be statistically significant. The OR and 95% CI were also calculated. Haplotype frequencies were estimated by the expectation-maximization algorithm.

TNC immunohistochemistry

TNC immunohistochemistry was performed essentially as previously described (24). Fresh human lung tissues were obtained and embedded in paraffin from patients undergoing surgery; informed consent was obtained. Asthmatic lung specimen was obtained from autopsied lung. The sections were deparaffined and endogenous peroxidase activity was quenched with 0.3% H_2O_2 in methanol for 20 min. Non-specific staining was blocked with blocking buffer [10% normal goat serum and 1% bovine serum albumin in phosphate-buffered saline (PBS)] for 30 min. The rat anti-human TNC antibody (10 μg/ml) was applied and reacted overnight at 4°C. After PBS washing, slides were incubated with HRP-conjugated anti-rat IgG antibody for 30 min. The slides were developed with DAB (Dojindo, Kumamoto, Japan) in Tris-buffered saline with 0.05% H_2O_2.

Computer modeling of TNC Fn-III-D protein structure

To examine the effect of amino acid substitution at position 1677 in the Fn-III-D domain, protein structural modeling was performed using MOE software (Chemical Computing Group Inc., Montreal, Canada). The coordinates of the 2.25 Å crystal structure of the chicken TNC Fn-III domain (PDB accession no. P24821) were used as a template for homology modeling of the human TNC Fn-III-D domain. The two structures were further minimized with AMBER 94 using MOE software. Both Leu1677 Ile variants of the TNC Fn-III-D domain were built up using the same program.

RT–PCR and western blotting analysis for TNC variants detection

Subconfluent NHLF were stimulated with 100 ng/ml IL-13 for 72 h and mRNA was isolated using a QuickPrep micro mRNA purification kit (Amersham, Uppsala, Sweden). cDNA was made with the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) using oligo$(dT)_{20}$ primer. RT–PCR was carried out for 5 min at 95°C for initial denaturing, followed by 35 cycles of 95°C for 60 s, 52°C for 60 s, and 72°C for 120 s, in the GeneAmp PCR System 9700 (Applied Biosystems). The primer TNC-3089: ACCGCTACCGCCT

CAATTACA and TNC-5331: GGTTCCGTCCACAGT TACCA were set to distinguish mRNA variants due to alternative splicing (13). The PCR products were electrophoresed in 1% agarose gel and distinct bands were excised. DNA was extracted from the excised bands with a DNA Gel Extraction Kit (Millipore, Tokyo, Japan) and subcloned into pCR II-TOPO cloning vector (Invitrogen). The subcloned inserts were read by sequencing. For western blotting, subconfluent NHLF were stimulated either with IL-4 or with IL-13 for 72 h at the concentration indicated in Fig. 5. The NHLF were solublized with SDS sample buffer (50 mM Tris–HCl pH6.8, 2% SDS, 20% glycerol, 0.4% bromophenol blue, 50 mM DTT). SDS–PAGE and subsequent immunoblotting were performed as previously described (21).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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Association of Transforming Growth Factor-β1 Single Nucleotide Polymorphism C-509T with Allergy and Immunological Activities

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Key Words
Immunological activities · Single nucleotide polymorphisms · T-cell proliferation · TGFβ1

Abstract
Background: A single nucleotide polymorphism (SNP) C-509T within the tumor growth factor β1 (TGFβ1) gene has been associated with atopic asthma and asthma severity. To further understand the mechanisms involved, the association of C-509T with allergy, T-lymphocyte proliferation and plasma TGFβ1 concentration has been explored in a case-control study with allergic and nonallergic subjects. ***Methods:*** The recruited subjects including allergic (n = 38) and nonallergic (n = 25) participants have been genotyped for C-509T using allele discrimination assay. Association of C-509T with allergy status was examined using logistic regression analysis in both dominant and recessive models. Association of C-509T with T-cell proliferation in control and antigen-stimulated peripheral blood mononuclear cells (PBMCs), plasma TGFβ1 and total IgE level were tested by multiple regression analysis. ***Results:*** Individuals with homozygous mutant TT genotype showed a higher risk of allergy (TT: odds ratio = 5.099, 95% confidence limit: 1.355–19.190, p = 0.016) after covariates were adjusted. A trend to increased plasma TGFβ1 in subjects with T allele has been discovered. In the meantime, the T allele is associated with lower T cell proliferation in controls and maximum response to above antigens. A low T-cell proliferation is correlated with higher plasma TGFβ1 concentration ($p < 0.01$). The in vitro studies confirmed the suppressing effect of TGFβ1 on T-cell proliferation at physiological range. A significant inhibitory effect on IL-4 production was also observed. ***Conclusions:*** A C to T base change in TGFβ1 SNP C-509T has been associated with a higher risk of allergy. The mechanisms are not clear. Elevated TGFβ1 levels associated with the C-509T polymorphism might suppress immune activation as well as Th2 cytokine production.

Introduction

Allergic diseases such as asthma, allergic rhinitis and atopic dermatitis have been considered as complex polygenic disorders resulting from a combination of genetic and environmental factors. In past years, a lot of efforts have been pursued to look for possible genetic components contributing to allergies and asthma [1–4]. Studies from our group and others have indicated that tumor

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growth factor β1 (TGFβ1) is a promising candidate gene whose genetic polymorphisms may play a key role in allergic diseases [5–10]. Among reported genetic polymorphisms in the TGFβ gene, a C to T base exchange at –509 position in the promoter region was associated with elevated levels of total IgE in allergic asthma patients in our previous study [8]. Two subsequent independent case-control studies in relatively large populations reported the association of variant T allele with asthma, and even with asthma severity [5, 6]. The exact role and possible mechanisms involved are still not clear.

TGFβ1 is a 25-kDa disulfide-linked homodimeric multifunctional cytokine. With respect to its diverse activities in normal cell growth and development, TGFβ1 might be the most important growth factor in immunomodulatory effects because knockout mice died of massive inflammatory lesions [7]. Both anti-inflammatory and inflammatory properties have been reported [8]. In the present case-control study including allergic and non-allergic subjects, we have been able to further study the significances of the TGFβ1 C-509T genetic polymorphism in allergic diseases, its association with TGFβ1 production, immune modulation activity reflected from antigen-stimulated T-cell proliferation in peripheral blood mononuclear cells (PBMCs), and even regulatory effects on cytokine production.

Patients and Methods

Subjects

Adult volunteers either with a history of atopy or allergic diseases, e.g. allergic rhinitis, atopic dermatitis, and asthma, or no allergy (healthy individuals) have been recruited. Atopy or allergy status was determined based on identified allergen through positive skin test or allergen-specific IgE test, and allergic symptoms. To avoid possible confounding effects for T-cell proliferation to antigen, patients with other diseases especially an immune deficiency, allergic patients with a history of immunotherapy, and patients who have taken steroids or other immunosuppressive agents at least 3 days before blood drawing met the exclusion criteria. All subjects signed informed consent with National Jewish Institutional Review Board approval before being enrolled and donating blood.

Genotyping

Peripheral venous blood was obtained from each subject. Genomic DNA was extracted from whole blood using the QIAamp blood kit (QIAGEN, Santa Clarita, Calif., USA). Briefly, blood cells were first lysed, and supernatant containing genomic DNA was mixed with ethanol and applied to QIAamp spin column. After washing several times, purified genomic DNA attached in column was eluted finally in TE buffer.

The allelic discrimination assay using the ABI PRISM 7700 Sequence Detection System (AB Applied Biosystems, Foster City, Calif., USA) has been developed for genotyping of TGFβ1 C-509T (HUgn0007040). Primers used for amplification of DNA fragments are: forward primer at position –572~–551 (-CATGGGAGGT-GCTCAGTAAAGG-), reverse primer at position –437~–454 (-GGTAGGAGAAGAGGGTCTGTCAAC-). Probe 1 containing allele C at position –519~–494 was linked with reporter dye FAM (6-carboxyfluorescein) at the 5′ end and black hole quencher (Biosearch Technologies) at the 3′ end: -CCCTTCCATCCCT-CAGGTGTCCTGTT-. Probe 2 containing allele T instead of C was labeled with reporter dye 6-JOE (6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein). Each reaction contains 25 μl of Taqman Universal Master Mix (AB Applied Biosystems), 900 n*M* of forward and reverse primers in concentration, 100 n*M* of probe 1 and probe 2 in concentration, and 200 ng of genomic DNA in 50 μl total volume. Thermal cycling conditions for allelic discrimination assay are: 2 min at 50°C, 10 min at 95°C for AmpliTaq Gold Activation, and 40 cycles of 15 s at 95°C for denaturing and 1 min at 60°C for annealing/extension. Rn for a given reaction tube, defined as the ratio of the emission intensity of the reporter dye over the emission intensity of the passive reference, was obtained. The relative Rn value of FAR to JOE reporter dye for each reaction was utilized to separate CC (1.1–1.43), CT (0.74–1.1) and TT (0.30–0.74) after adjustment with non-template control based on the 95% confidence limits in each group. Duplicate experiments have been performed confirming reproducibility. Three distinct groups for CC, CT and TT have been recognized based on our setup thresholds.

Cell Culture and Cell Proliferation

PBMCs were isolated from human heparinized venous blood by density gradient centrifugation on Ficoll-Paque (Amersham, Uppsala, Sweden), and washed three times in HBSS (Gibco, Grand Island, N.Y., USA). Purified PBMCs were suspended in complete RPMI 1640 medium (Gibco), which was supplemented with 5% heat-inactivated fetal calf serum (Gemini Bio-Products, Calabasas, Calif., USA), 200 U/ml penicillin, 200 μg/ml streptomycin, 50 μg/ml gentamicin, 4 m*M* *L*-glutamine (Gibco), 10 m*M* HEPES (Sigma), 1 m*M* sodium pyruvate (Gibco), 0.1 m*M* nonessential amino acid (Gibco), and 50 μ*M* 2-mercaptoethanol.

To assess lymphocyte proliferation response, PBMCs were cultured at a concentration of 10^5 cells/well in 96-well flat-bottom plates in a volume of 200 μl. PBMCs were incubated with medium control; tetanus toxoid (Aventis Pasteur, Swiftwater, Pa., USA) at concentrations of 0.02, 0.07, and 0.2 lac-forming units (LFU)/ml; timothy (pollen) *Phleum pratense* allergic extract (Bayer, Elkhart, Ind., USA) at concentrations of 4, 40, and 400 PNU/ml, and standardized cat hair allergic extract (Fel d1; ALK, Horsholm, Denmark) of 1, 10, and 100 BAU/ml. On day 6, 1.0 μCi/well [^{3}H] thymidine (Amersham, Arlington Heights, Ill., USA) was added to 100 μl of cell culture and incubated for 6 additional hours. Cells were finally harvested onto filter paper disks via a multiwell harvester and radioactivity (counts/min) counted with a 1450 Microbeta Plus Liquid Scintillation Counter (Wallace, Orange, Calif., USA). All samples were run in triplicate.

To assess the effect of TGFβ1 on cell proliferation, PBMCs were also cultured at a concentration of 10^5 cells/well in 96-well flat-bottom plates in a volume of 200 μl for 6 days. Human recombinant TGFβ1 (R&D Systems, Minneapolis, Minn., USA) was added as indicated in the Results. Radioactivity was counted as described above.

To assess the effect of TGFβ1 on cytokine production, PBMC were cultured at a concentration of 10^6 cells/well and treated as described in the Results. Supernatant was collected at 48 h, and IL-2, IFNγ, IL-4 were measured using ELISA assays.

Plasma TGFβ1 Measurement

Plasma was first isolated from heparinized venous blood by density gradient centrifugation on Ficoll-Paque and further centrifuged at 3,000 *g* for 10 min to eliminate platelets, and then frozen at −70°C. Plasma TGFβ1 was measured using the TGFβ1 ELISA Kit (Biosource, Nivelles, Belgium). Based on the manufacturer's instruction, latent TGFβ1 was first extracted from platelet-depleted plasma, and incubated with biotinylated anti-TGFβ1 in TGFβ1-antibody-coated wells for 3 h as well as TGFβ1 standard and diluent control. Streptavidin-HRP working solution was added for 30 min after thorough washing. Stabilized chromogen solution was finally added to each well for 30 min and terminated by stopping solution before absorbance (optical density, OD) was detected at a wavelength of 450 nm. The concentration of plasma TGFβ1 was calculated after deriving from a standard curve. The minimum detectable dose of TGFβ1 is 15.6 pg/ml. Duplicate experiments have been performed.

Plasma Total IgE Measurement

Plasma total IgE was measured using the ELISA kit from ALerCHEK (Portland, Maine, USA). According to the manufacturer's instructions, 20 μl of standard IgE and plasma sample were diluted with 80 μl of specimen diluent and incubated in anti-human IgE-coated microwells for 60 min at room temperature. After thorough washing with washing buffer, 100 μl of HRP-conjugated goat anti-human IgE was added for another 60 min. Finally, 100 μl of TMB/peroxide substrate were incubated for 30 min after another thorough washing and terminated by 0.5 *N* sulfuric acid before OD at 450 nm was determined. Total IgE concentration (IU/ml) in plasma was derived from the constructed standard curve. Duplicate experiments have been performed.

Cytokine Assays

IL-2, IFNγ and IL-4 production in cell culture supernatants was measured using ELISA kits (Biosource) with a sensitivity of <5 pg/ml, 0.03 IU/ml, and 2 pg/ml, respectively. According to the instructions, standard, control and samples from supernatant were incubated in specific-antibody-coated microwells together with antibody conjugate for 2 h at room temperature on a horizontal shaker set. After thorough washing, 200 μl of freshly prepared chromogenic solution were pipetted into each well and incubated for 15 min at room temperature on a horizontal shaker set before 50 μl of stop solution were added. The OD value at 450 nm was read, and plasma concentrations of cytokines were calculated based on standard curves finally.

Statistical Methods

Statistical analyses were performed using SAS software version 9.1.3. Balance of categorical variables regarding gender, race, ethnicity, genotype and allele frequency between allergy and non-allergy groups was tested using the χ^2 test or Fisher's exact test in PROC FREQ. The Hardy-Weinberg equilibrium for TGFβ1 C-509T allele frequency was calculated according to Thompson et al. [9]. Logistic regression was utilized to find the association between the TGFβ1 C-509T genotype and allergy status, while gender (male or female), race (White, Asian or Black) and TGFβ1 C-509T genotype (CC and CT, or TT only) has been considered in model building. Backward selection with slentry = 0.2, slstay = 0.2 options was used to remove low-risk factors. To adjust for possible confounding influences in the association of the C-509T genotype with numerical variables such as T-cell proliferation, stepwise-wise selection with slentry = 0.2 and slstay = 0.2 has been run with race, gender, allergy status, and TGFβ1 C-509T genotype entered in multiple regression models. Regression diagnostics have been performed to make sure that no violation of assumption occurs. Pearson's correlation coefficient has been used to estimate correlations between two numerical variables. Nonparametric approaches (the Kruskal-Wallis test and Wilcoxon's two-sample test) have been applied for multiple comparison among groups. p values less than 0.05 are considered as statistically significant.

Results

We recruited 63 volunteers including 38 with a history of allergic or atopic diseases and 25 normal subjects. All of them are nonsmokers. The properties of the recruited population are summarized in table 1. There is no significant difference between allergy and non-allergy groups regarding the composition of gender, race, ethnicity (χ^2 test or Fisher's exact test, $p > 0.05$) and age (Wilcoxon's rank sum test, $p > 0.05$). The allele frequency of TGFβ1 C-509T is consistent with the Hardy-Weinberg equilibrium ($\chi^2 = 3.93$, $p > 0.1$). Except for medium and phytohemagglutinin (PHA) control, T-cell proliferation stimulated by tetanus toxoid and timothy allergy is significantly higher in the allergy group than in the non-allergy group. There is a trend towards an increase in T-cell proliferation for cat hair allergen in the allergy group although it is not statistically significant ($p = 0.0710$).

Association of the TGFβ1 C-509T Genotype with Allergy

The association of the TGFβ1 C-509T genotype with allergy was examined using logistic regression. The possible covariates such as gender (male or female) and race (White, Asian or Black) have been considered and were removed according to the selection criterion (see Statistical Analysis). In the recessive model, the allergy status was associated with the TT genotype (odds ratio = 5.099, 95% confidence limits 1.355–19.190, $p = 0.016$). However, there is no significant association between allergy status and CC in the dominant model (odds ratio = 0.823, 95% confidence limits 0.26–2.383, $p = 0.7199$). This suggests that the TT variant of the TGFβ1 gene at promoter −509 is associated with increased risk of allergy in humans.

Table 1. Subject characteristics (mean and SD)

	Allergy	No allergy	Total	p value
Subjects	38	25	63	
Age, years	36 (10)	38 (12)	36 (11)	0.5737
Sex, %				
Female	58	52	56	0.6450
Race, %				
White	79	68	75	
Asian	16	32	22	
Black	5	0	3	0.2725
Ethnicity. %				
Non-hispanic	89	100	94	
Hispanic	11	0	6	0.0937
TGFβ1 C-509T genotype, %				
CC	37	44	40	
CT	53	20	40	
TT	11	36	20	0.0158
TGFβ1 C-509T allele frequency, %				
C	63	54	60	
T	37	46	40	0.9114
Timothy allergy, %				
Yes	84	0	51	<0.0001
Cat allergy, %				
Yes	58	0	35	<0.0001
CPM for control	8.49 (1.64)	8.12 (1.34)	8.34 (1.52)	0.2243
CPM for tetanus toxoid	9.70 (1.13)	9.04 (1.12)	9.44 (1.16)	0.0216
CPM for cat hair allergen	9.19 (1.36)	8.59 (1.13)	8.95 (1.30)	0.0710
CPM for timothy allergen	9.36 (1.15)	8.79 (1.08)	9.13 (1.15)	0.0409
CPM for PHA	11.45 (0.66)	11.45 (0.51)	11.45 (0.60)	0.8088
Total IgE	1.58 (0.75)	0.84 (0.72)	1.32 (0.82)	0.0009
Plasma TGFβ1, ng/ml	3.10 (0.47)	3.16 (0.41)	3.12 (0.45)	0.6014

Racial and ethnic classification is according to the standard used in Census 2000. For age and other numerical data, means (SD) are shown. Values for CPM and total plasma IgE are natural logarithmic transformed. Wilcoxon's rank sum score test was used for comparisons between allergy and non-allergy groups.

Association of TGFβ1 C-509T with Plasma TGFβ1 Level

To understand the possible mechanisms associated with the above finding, the association of C-509T with plasma TGFβ1 levels has also been estimated using multiple regression. The results indicated that there is a trend to higher plasma TGFβ1 concentration in the presence of T at the C-509T allele (CC: 3,008.34 ± 369.96, CT: 3,143.76 ± 529.17, and TT: 3,312.66 ± 386.55 pg/ml). However, statistically significant differences could not be shown in either the dominant model (CC only: p = 0.1198) or the recessive model (TT only: p = 0.1346) while possible covariates such as allergy, gender, age and race have been adjusted for. The hypothesis that a higher concentration of plasma TGFβ1 in the TT genotype accounts for the association of C-509T with allergy may need to be verified in larger studies in the future.

Association of the TGFβ1 C-509T Genotype with T-Cell Proliferation in Human PBMCs

With respect to the importance of TGFβ1 in the immunomodulation of allergen-induced cell proliferation, the association of the TGFβ1 C-509T genotype with antigen-stimulated cell proliferation in human PBMCs has also been examined using multiple regression analysis. Based on selection rules, the adjusted variables are listed in table 2 as indicated. Outcomes including unstimulated cell proliferation (medium control) and cell proliferation induced by maximum tetanus toxin, cat hair allergen and timothy allergen extracts showed a negative relationship

Table 2. Association of the TGFβ1 C-509T genotype with T-cell proliferation and IgE (n = 63)

Outcomes (ln value)	Adjusted variables	Regression coefficients for TT or CC (mean ± SE)	t value	p value
TT (recessive)				
CPM for medium control	White	−1.26±0.48	−2.64	0.0127
CPM for tetanus toxoid	Black	−1.04±0.34	−3.08	0.0031
CPM for cat hair allergen	cat allergy, White	−1.02±0.41	−2.50	0.0152
CPM for timothy allergen	grass allergy, White	−0.93±0.36	−2.55	0.0134
CPM for PHA	age, White	−0.07±0.192	−0.37	0.7132
Plasma IgE	allergy	0.003±0.26	0.01	0.9902
CC (dominant)				
CPM for medium control	age, White, Asian	1.20±0.37	3.25	0.0019
CPM for tetanus toxoid	age, allergy	0.70±0.28	2.48	0.0159
CPM for cat hair allergen	White, cat allergy	0.67±0.32	2.07	0.0429
CPM for timothy allergen	White, grass allergy	0.84±0.29	2.95	0.0047
CPM for PHA	age, Asian	0.03±0.16	0.19	0.8464
Plasma IgE	allergy	0.07±0.20	0.34	0.7376

with TT ($p < 0.05$) in the recessive model except for PHA. Conversely, the T-cell proliferation had a positive relationship with CC in the dominant model ($p < 0.05$) except for PHA. These results indicate that the C-T mutation is associated with reduced T-cell proliferation in general. We have used the stimulation index (ratio of antigen-stimulated cell proliferation over control) as response variable for tetanus toxin, cat allergen and timothy allergen. Neither the TT nor the CC genotype was correlated with antigen-specific cell proliferation (data not shown), suggesting that suppression might not be antigen specific.

The analysis for the association of the TGFβ1 C-509T genotype with plasma total IgE indicates that IgE is apparently not associated with the TGF C-509T genotype ($p = 0.9902$ for TT and $p = 0.7376$ for CC). The C-509T genotype does not exhibit an allergy-status-dependent difference ($p = 0.5676 > 0.05$ for TT and $p = 0.6718 > 0.05$ for CC) in IgE level.

Effect of TGFβ1 on T-Cell Proliferation

We also tested whether higher concentration of plasma TGFβ1 is correlated with lower T-cell proliferation capacity (fig. 1). From the graphs, negative associations of the plasma TGFβ1 concentration with natural log-transformed value of control, tetanus toxoid, cat allergen, timothy allergen, and even PHA-stimulated cell proliferation were found. Total IgE seems not to be associated with plasma TGFβ1 amounts. After adjusting for possible covariates such as gender, race and age in multiple regression models, the linear correlation still exists (table 3). These results support our hypothesis that human subjects with the T allele in the TGFβ1 C-509T genetic polymorphism suppressed immunological activity resulting probably from higher TGFβ1 production.

Table 3. Regression analysis of T-cell proliferation and IgE with plasma TGFβ1 concentration adjusted for gender, age, and race (n = 63)

Outcomes (ln value)	Regression coefficients for TGFβ1 (mean ± SE), $\times 10^{-3}$	t value	p value
CPM for medium control	−1.573±0.4251	−3.70	0.0005
CPM for tetanus toxoid	−0.808±0.3354	−2.41	0.0196
CPM for cat hair allergen	−1.239±0.3711	−3.34	0.0016
CPM for timothy allergen	−0.970±0.3317	−2.92	0.0051
CPM for PHA	−0.356±0.1924	−1.85	0.0705
Plasma IgE	−0.138±0.2291	−0.6	0.5485

Furthermore, recombinant human TGFβ1 was added at three different concentrations (32 and 3.2 ng/ml and 32 pg/ml) simultaneously in in vitro studies while PBMCs were cultured in the presence of tetanus toxoid (0.02 LFU/ml) as a stimulator. TGFβ1 significantly inhibited cell proliferation at 32 ng/ml ($p = 0.012$) with a dose-response trend (fig. 2) consistent with a suppressing effect of TGFβ1.

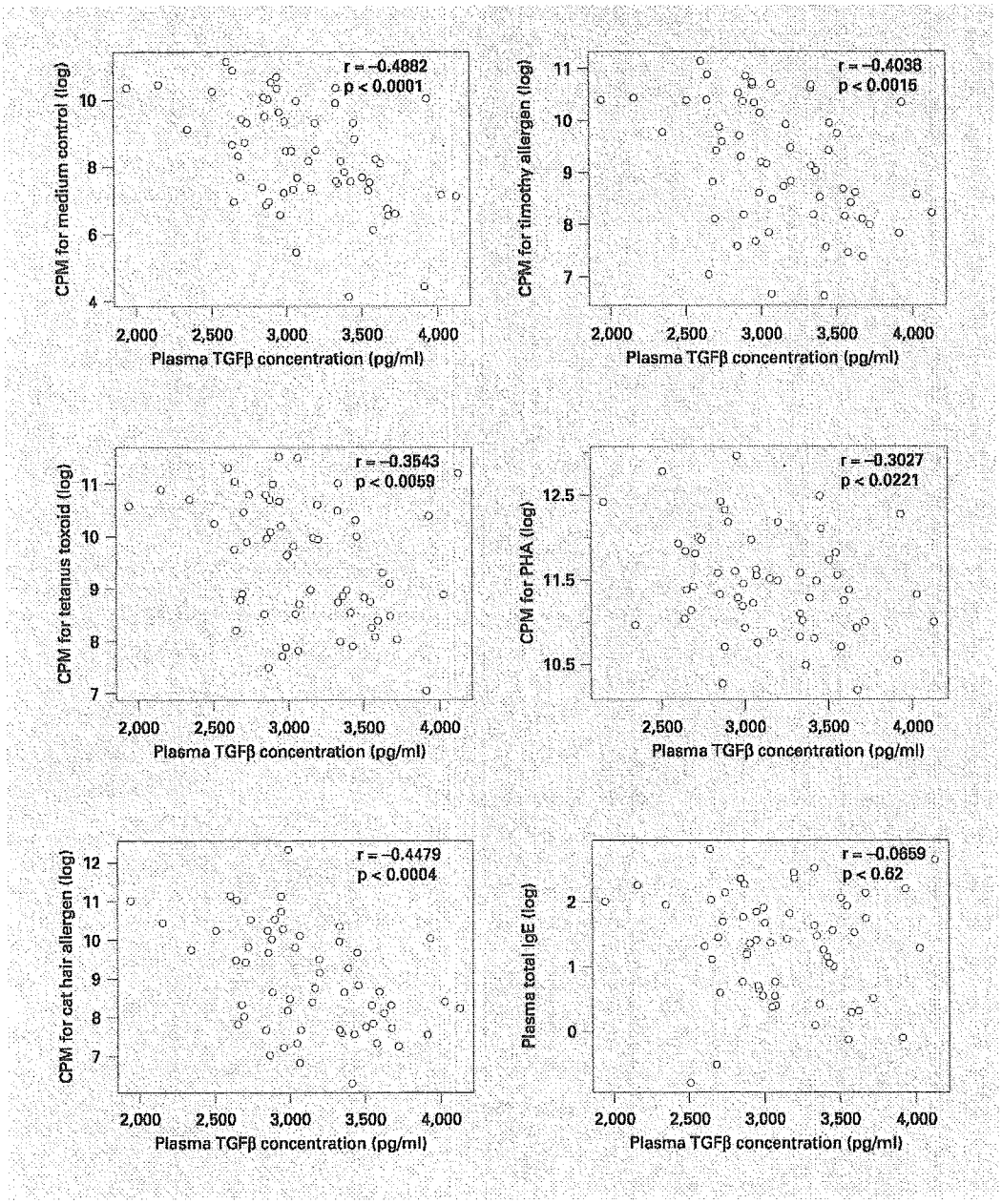

Fig. 1. Scatter plot of plasma TGFβ1 with plasma total IgE and T-cell proliferation in response to antigens in 63 subjects. Counts per minute (CPM) and total IgE are the natural log-transformed value of maximum T-cell proliferation in medium control, tetanus toxoid, cat allergen, timothy allergens, PHA-stimulated PBMCs and plasma IgE level. r = Correlation coefficient.

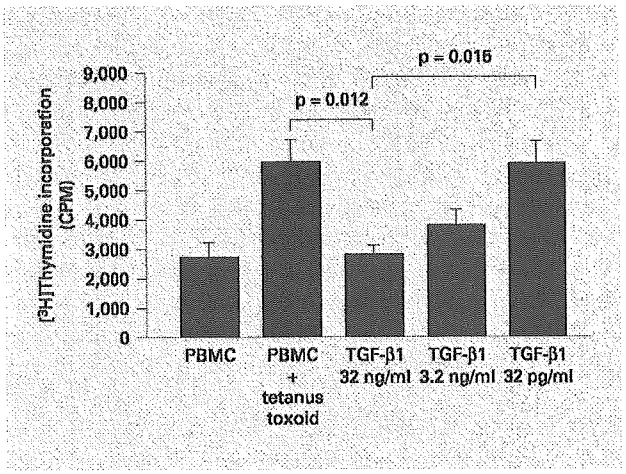

Fig. 2. Inhibition of T-cell proliferation by TGFβ1. [^{3}H]thymidine uptake (counts/min. CPM) of T-cells stimulated with 0.02 LFU/ml tetanus toxoid in the presence of TGFβ1 for 6 days (n = 8).

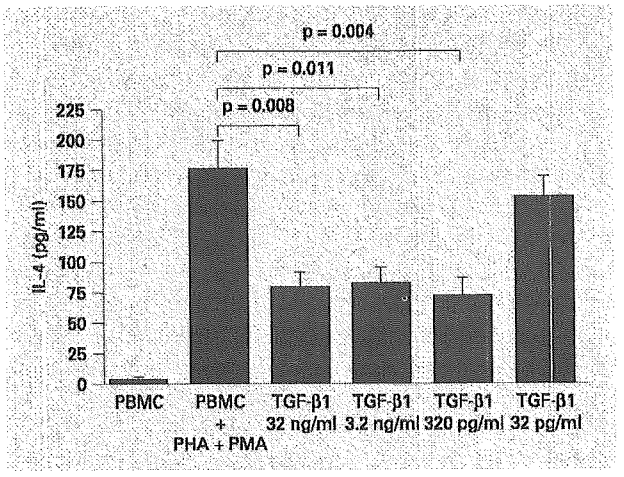

Fig. 3. Inhibition of IL-4 synthesis by TGFβ1. PBMCs were stimulated with PHA 10 μg/ml and 10 ng/ml phorbol 12-myristate 13-acetate (PMA) in the presence of TGFβ1 (n = 5).

Inhibiting Effect of TGFβ1 on Cytokine Production

Cytokines have been considered to be very important in regulating T-cell proliferation. The inhibitory effect of TGFβ1 on cytokine production was further studied in vitro. PBMCs were incubated in the presence of 10 μg/ml PHA and 10 ng/ml phorbol 12-myristate 13-acetate with TGFβ1 at four different concentrations (32 and 3.2 ng/ml, and 320 and 32 pg/ml) for 48 h. Supernatant was collected and measured for IL-4, IL-2 and IFNγ using ELISA. Figure 3 showed that TGFβ1 significantly inhibited IL-4 synthesis even at 320 pg/ml ($p = 0.004$). However, the effects of TGFβ1 on IL-2 and IFNγ production were not statistically significant (data not shown). The suppressing effect of TGFβ1 on cytokine production, especially of the Th2 type, may be involved in the association of the TGFβ1 genotype with reduced immunological activities.

Discussion

TGFβ belong to a super family of about 40 structurally related polypeptide growth factors [10]. Perturbation of the TGFβ superfamily has been implicated in various human diseases, e.g. cancer, angiogenesis, fibrotic diseases, and atherosclerosis, due to their mediation of many key events in normal growth and development including cellular differentiation, proliferation, motility, organization and death [11]. TGFβ1 is one of the best-studied factors in mammalian tissues. The role of TGFβ1 in asthma and allergic diseases has also been well documented in the literature [12]. Increased TGFβ1 expression in asthma has been repeatedly reported. Overexpression of TGFβ1 mRNA and proteins in bronchial biopsies from subjects with severe and moderate asthma has been observed compared with controls without asthma [13–15]. In asthmatic airways, TGFβ1 mRNA expression mainly produced from eosinophils was related to the degree of disease severity, subepithelial fibrosis and airway remodeling [13, 14, 16].

The genetics of atopy and asthma has been a very interesting research area in past years. Deciphering secrets in genetic variations related to allergy and asthma development may greatly benefit our efforts in the prevention and treatment of asthma and allergic diseases. The candidate gene approach, in which genes of relevance to the physiological changes of the disease process are tested for direct involvement with the disease, has been successfully applied to link genetic variation in the promoter region of a number of cytokine genes to asthma and allergic diseases. The promoter region of the TGFβ1 gene has been characterized showing regulatory impacts on transcriptional processes [17]. Our previous work demonstrated that a TGFβ1 promoter polymorphism at C-509T with the TT homozygote genotype is linked to allergic and asthmatic patients with elevated serum IgE [18]. Two case-control independent studies in relatively large populations of white individuals also found the association of

variant T allele with asthma, even with severity of asthma [5, 6, 19]. In our present study, the T variant of the genetic polymorphism TGFβ1 C-509T is significantly associated with allergy status. These results consistently support our hypothesis that the TGFβ1 T allele genotype is an important risk factor in allergy development.

C to T base change is located in the region of the promoter associated with Yin Yang-1 activator consensus sequence. It has been reported to increase promoter function using luciferase constructs [20]. The T allele has been demonstrated to enhance the YY1 binding site on the TGFβ1 promoter region and may account for the increased transcription of TGFβ [6]. The C-509T polymorphism was significantly associated with increased concentration of latent TGFβ1 in serum in a human study [21]. Our present results also showed an increased trend of plasma TGFβ1 to the T allele. Limited sample sizes and differences in population properties may explain the lack of statistical significance in this study. It is reasonable to deduce that subjects with the T allele may display a higher level of TGFβ1 in other tissues such as respiratory airways or skin. The observed overexpression of TGFβ1, e.g. in bronchial biopsies of asthmatic patients as discussed before, may at least partly result from their enhanced capacity to produce TGFβ1 determined by their genetic components such as the T allele mutation in C-509T, although a feedback mechanism from the inflammatory reaction in asthmatic patients cannot be excluded.

It has been well known that immunological activity plays a crucial role in initiating and developing allergic reactions in response to allergens. In mice, knockout studies with TGFβ1 indicated that TGFβ1 might be one of the most important growth and regulatory factors in the immune system because the knockout mice died of massive inflammatory lesions [22]. However, the evidences about its effects in the immune system seem confusing and contradictory. Its anti-inflammatory effect has been demonstrated by inhibiting the proliferation of T and B lymphocytes [23, 24]. On the other hand, TGFβ1 has been found to be a growth factor by inducing proliferation of macrophages, dendritic cells, and even T cells [25, 26]. Our present study indicates that a C to T mutation at the TGFβ1 genetic polymorphism C-509T is correlated with suppressed T-cell proliferation in vivo. This suggests that subjects with the T allele in the absence or presence of allergy demonstrated reduced lymphocyte response after exposure to allergen, even at the basic level. Increased production of TGFβ1 seems to be one of the reasons, since the plasma TGFβ1 concentration is negatively correlated with the degree of T-cell proliferation, and TGFβ1 suppresses T-cell proliferation in vitro at physiological concentrations. Human subjects with the T allele may own a relatively lower immune activity due to more TGFβ1 inside their body.

Allergic diseases are aberrant immune responses to antigen mainly mediated by IgE. Our previous study indicated that the C to T base exchange is associated with elevated IgE in atopic asthmatic patients. However, other studies did not confirm our findings [6]. Our present study failed to show a significant association with the total IgE level while other possible covariates have been considered. Lack of adjustment of covariates, e.g. the allergy status, in our previous report might be one reason for the elevated IgE in atopic asthma patients. These results implied a complicated relationship between TGFβ1 and total IgE. As we know, TGFβ1 is believed to inhibit IgE predominately in immunity. It might be related to its downregulatory effect on various cell surface molecules including CD23 and MHC class II antigen in human cells, which may be very important in promoting IgE synthesis [27, 28]. TGFβ1 could also inhibit e-germline transcription [29] directly through transcription factors or through inhibiting Th2 cytokines such as IL-4. Elevated TGFβ1 in TT allele subjects is expected to reduce the IgE level. However, as IgE synthesis is a very complicated process involving lots of cytokines (e.g. IL-13 and IL-10), even suppression of IgE is environment directed. It has been reported that at a later stage of differentiation, IgE B cells are refractory to the inhibitory effect of TGFβ, as shown by the slight but significant increase in the spontaneous secretion of IgE by PBMC of atopic patients [30]. Finally, TGFβ1 downregulation of IgE may be a mechanism where Th2 inflammation may still be prominent, with no increase in IgE, a situation that might be seen in intrinsic asthma for example. Therefore, we may not be surprised to see the lack of association of this genetic polymorphism with the IgE level in plasma in our present study.

Both T-cell proliferation and production of IgE and cytokines, including Th2 and Th1, are believed to play an important role in allergic inflammation. In our study, the Th2 cytokine IL-4 was depressed rather than IL-2 and IFNγ, implying that Th2 cytokines are crucial in explaining the observed suppressing effect of TGFβ1 on T-cell proliferation. However, the suppressing effect of TGFβ1 on the Th1-type reaction or initiation of T-cell reactions may still exist. This is because the TGFβ1-induced downregulation of the IL-2 receptor at both protein and mRNA level has been reported [29]. TGFβ1 has been shown to inhibit IL-2-induced tyrosine phosphorylation, activation of Jak-1 and Stat 5, and IL-2-induced expression of

α and β chains of IL-2R [30]. Interaction of IL-4 and IFNγ has been reported. Reduction of IL-4 by TGFβ1 may reduce the forces to inhibit IFNγ production. These may be some reasons that we failed to observe the significant effect on IFNγ. With respect to the cytokine IL-4, T-cell proliferation in the allergic reaction, an increased TGFβ1 concentration in T-allele subjects may play a role in the suppression of the allergic reaction or inflammatory effect rather than an increase in the risk of allergic status. The mechanisms involved are still not clear. More studies in larger patient cohorts and on the mechanisms regarding its role in allergic development may be worth of much more attention in the future. Other roles than cell proliferation for TGFβ1 may be hidden behind the association of TGFβ1 C-509T with allergy. As an example, TGFβ1 is an important stimulant of fibrosis, inducing formation of the extracellular matrix, and promoting wound healing and scar formation. Therefore, the profibrotic effect of TGFβ1 may play a more important role in asthma development. Another important function for TGFβ1 is their regulatory activity in cell differentiation. IL-3 and M-CSF in cooperation with TGFβ induces granulocyte macrophage colony-stimulating factor-independent differentiation of human CD34+ hematopoietic progenitor cells into dendritic cells with features of Langerhans' cells. TGFβ1 may play an important role in the differentiation and maturation of dendritic cells and monocyte differentiation. TGFβ1 may play an important role in regulating some cell cycle arrest rather than others, as indicated in CD34+ CD38-c-kit(high) vs. c-kit(low/–) cells. Due to the complexity in the development of allergic diseases, the T allele may be combined with other genetic variations, which produce a different environment leading to the allergic status.

In summary, our study demonstrated the modulatory effects of TGFβ1 and its polymorphism on cell proliferation and cytokine production. Further investigations with larger sample sizes could further solidify our findings. Clarification of the association of the TGFβ1 C-509T polymorphism with allergic diseases may raise new and better methods for diagnosis, and more importantly, the potential for new pharmacological interventions related to the products of the candidate genes.

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