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**Functional variants in the thromboxane A2 receptor gene are
associated with lung function in childhood-onset asthma**

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Condensed title

TBXA2R genetic variants and lung function in asthmatics

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Abstract

Background: The thromboxane A2 receptor (*TBXA2R*) gene is associated with asthma,
but no functional genetic variations are known to associate with the disease or its related
phenotypes.

Objective: To investigate the association of *TBXA2R* polymorphisms with asthma

susceptibility and related phenotypes and to identify functionally relevant polymorphisms.

Methods: We performed comprehensive sequencing of the *TBXA2R* gene in 48 Japanese control subjects and found a set of variants (SNP1 G>T rs2238634, SNP2 T>G rs2238633, SNP3 C>T rs2238632, and SNP4 G>A rs2238631) in intron 1 in linkage disequilibrium with c.795 T>C rs1131882, which was previously reported to be associated with asthma and related phenotypes. To investigate the effect of four common haplotypes (H1, H2, H3, and H4) on transcriptional activity, we performed a luciferase assay in primary bronchial smooth muscle cells (BSMCs) and human airway epithelial cells (BEAS-2B). We also studied the haplotype association with lung function, *TBXA2R* mRNA levels, and eosinophil fraction/count in peripheral blood in childhood-onset asthma patients and/or controls.

Results: H2 and H4, containing minor alleles of SNP2 and SNP3, had significantly higher transcriptional activities than H1 consisting of major alleles ($P<0.001$ in BSMC and BEAS-2B). Homozygotes for redefined haplotype h2 corresponding to minor alleles of SNP2 and SNP3 were associated with lower lung function in childhood-onset asthma patients compared to other zygotes (baseline FEV1/FVC and %FEF_{25-75%}: $P=0.00201$ and 0.0128 , respectively, and post-bronchodilator FEV1/FVC

and %FEF_{25-75%}: $P=0.00224$ and 0.0393 , respectively). Haplotype h2 was also associated with higher mRNA levels in control peripheral blood cells and higher blood eosinophil fractions and counts in female controls.

Conclusions and Clinical Relevance: Genetic variants were identified in the *TBXA2R* gene that influenced transcriptional activity and were associated with asthma related phenotypes. Thromboxane pathways may therefore play important roles in airway inflammation and remodeling in asthma patients.

Keywords: childhood-onset asthma, eosinophil, haplotype, lung function, single nucleotide polymorphism, thromboxane A2 receptor gene

Abbreviations:

FEV1: Forced expiratory volume in one second

FVC: Forced vital capacity

FEF_{25-75%}: Forced expiratory flow between 25% and 75% of the FVC

Introduction

Asthma is a chronic airway inflammatory disease characterized by respiratory symptoms, variable airway obstruction, and airway hyperresponsiveness (AHR) [1]. It

has strong genetic and environmental components that interact and develop the disease phenotypes [2], and a large-scale genome-wide association study recently revealed the presence of several genetic loci that influence asthma susceptibility [3].

The thromboxane A2 receptor (TBXA2R) gene, located at 19p13.3, spans over 15 kb and contains three exons (Exons 1 to 3) and one alternative exon (Exon 1b) [4, 5].

TBXA2R is abundantly expressed at the mRNA and protein levels in tissues targeted by the TBXA2R ligand thromboxane A2 (TXA2), including erythroleukaemia cells, vascular and bronchial smooth muscle, uterus and placental tissue, endothelium, epithelium, trophoblasts, thymus, liver, and small intestine [6]. TBXA2R is a member of the seven-transmembrane G-protein-coupled receptor super family, which interacts with intracellular G proteins, regulates different downstream signaling cascades, and induces many cellular responses including the intracellular calcium influx, cell migration and proliferation, and apoptosis [7]. Activation of TBXA2R in bronchial smooth muscle cells by TXA2 leads to intracellular calcium mobilization with subsequent bronchoconstriction, contributing to bronchial smooth muscle hyperplasia and airway remodeling which occur in response to chronic airway inflammation in asthma [8].

Several studies have reported the association of single nucleotide polymorphisms

(SNPs) in the *TBXA2R* gene, c.795T>C (rs1131882) and c.924T>C (rs4523), with asthma and its related phenotypes in Asian populations. c.924T>C was found to be associated with adult asthma in a Japanese population [9] and childhood atopic asthma in a Chinese population [10], while c.795T>C was shown to be associated with atopic asthma in a Korean population [11]. Furthermore, these polymorphisms or their combination with other asthma-related gene polymorphisms such as *IL5*-746T>C, *HLA DPBI**0301, and *ALOX5*+21C>T were reported to associate with asthma subphenotypes, such as aspirin-intolerant asthma, and asthma-related quantitative phenotypes, such as lung function including %FEV1 and %FEF_{25-75%}, in patients with asthma [10, 12-14].

Although these findings suggest the association of these two polymorphisms with the pathogenesis of asthma and its related phenotypes, it is not known whether the SNPs themselves have a functional role *in vivo*. To our knowledge, no study has investigated the functional consequences of genetic variations associated with asthma or its related phenotypes. The SNPs c.795T>C and c.924T>C are synonymous and unlikely to influence the characteristics of the receptor protein. It is more likely that other genetic variations in linkage disequilibrium (LD) with these SNPs will affect the efficiency of transcription or translation [9, 10, 14].

The aim of this study was to seek functionally relevant polymorphisms of the *TBXA2R* gene and to investigate the association of these with asthma and asthma-related phenotypes in Japanese childhood-onset asthma patients. We performed comprehensive sequencing in the *TBXA2R* gene region to search for functional variants in LD with c.795T>C or c.924T>C. We assessed the effect of these variants on transcriptional activity and tested the association of putative functional variants with childhood-onset asthma, lung function, serum IgE levels, and blood eosinophil fractions in Japanese patients and controls.

Materials and methods

Subjects and phenotyping

We recruited 290 patients with childhood-onset asthma aged 6–25 years from five hospitals in the Kanto area, Japan: Chiba University Hospital, Chiba Children's Hospital, Shimoshizu National Hospital, Toho University Omori Medical Center, and Naruto General Hospital. All subjects enrolled in this study were Japanese. Of the 290 patients, 212 underwent a lung function test and 184 subsequently underwent a lung function test after inhaling a β_2 agonist. We measured lung function parameters (FEV₁, FVC, FEV₁/FVC ratio, and FEF_{25-75%}) with SUPER SPIRO D-21FXIITM (Chest, Tokyo,

Japan), CHESTAC-8800TM (Chest), or Autospiro AS-307/AS-507TM (MINATO, Osaka, Japan) and represented FEV₁, FVC, and FEF_{25-75%} values as a percentage of the predicted value. There was no significant difference in the mean or variance of measured values between these spirometers. Patient eosinophil fractions were those of the highest values known during the entry period.

Elementary school children aged 6–12 years were recruited as control subjects from Chiba City in Chiba Prefecture. Details of control subjects have been described previously [15]. Briefly, blood samples were taken from 411 children and genomic DNA was purified from all samples while serum IgE values were measured in 410 samples. A questionnaire was used to exclude subjects with asthma and congenital heart diseases, leaving 328 children eligible for the control group in this study. We were able to count peripheral blood cell types in 164 control subjects and total RNA was purified from total peripheral blood cells from 131 control subjects.

Asthmatic subjects were diagnosed by pediatricians who were experts on allergic diseases, based on a history of symptoms and reversible bronchoconstriction by inhalation of the β_2 -stimulant. Patients or their parents completed a daily diary of respiratory symptoms such as recurrent wheeze, dyspnea, exercise-induced symptoms, and symptoms causing waking at night, and these were checked by a doctor. None of

the patients showed aspirin intolerance. We defined subjects with childhood-onset asthma as those who presented with the disease at younger than 16 years of age.

Atopy was defined according to the presence of positive specific IgE (≥ 0.35 IU/ml) for at least one of the assessed allergens. Total and specific serum IgE levels were measured using the CAP-radio-allergosorbent test (Pharmacia Diagnostic, Uppsala, Sweden). Eight specific IgEs measured in this study were as follows: house dust mite (*Dermatophagoides pteronyssinus*), dog dander (*Canis familiaris*), cat dander (*Felis domesticus*), black mold (*Alternaria alternata*), cedar pollen (*Cryptomeria japonica*), orchard grass (*Dactylis glomerata*), egg white, and golden, black bellied, or djungarian hamsters (*Mesocricetus auratus*, *Cricetus cricetus*, *Phodopus sungorus*).

Written informed consent was obtained from every participant. This study was approved by the Ethics Committee of Chiba University Graduate School of Medicine (approval numbers: 150 (318), 209).

DNA preparation

Genomic DNA was extracted from the saliva of asthma patients using the Oragene[®] DNA collection kit (DNA Genotek Inc., Ottawa, Ontario, Canada) and from the peripheral blood of control subjects using the Gentra[®] Puregene[®] Blood Kit (Qiagen,

Valencia, CA). Whole-genome amplification was carried out using the Illustra™ GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's standard protocol.

Sequencing

We sequenced the *TBXA2R* gene region including 1.5 kb of the 5'-upstream region and 0.5 kb of the 3'-downstream region in 48 control subjects. Each sample was amplified by polymerase chain reaction (PCR) in a 10-μl aliquot of reaction mixture containing 5 ng of amplified template DNA, 0.5–1.5 U of TaKaRa ExTaq® HS (TaKaRa Bio Inc., Otsu, Japan), 1 μl of 10 × Vogelstein buffer, 1.5 mM of each dNTP, 4.7 mM of MgCl₂, and 0.5 μM of each of the PCR primers, using a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). The PCR thermocycle program was as follows: initial denaturation at 98 °C for 1 min followed by 40–45 amplification cycles at 98 °C for 10 s, 60 °C for 30 s and 72 °C for 1–2 min, with a final extension at 72 °C for 5 min. The touch-down PCR method was used for part of the intronic region to avoid amplification of nonspecific bands (initial denaturation at 98 °C for 1 min followed by 10 touchdown cycles at 98 °C for 10 s, 65 °C for 30 s with each cycle 0.5 °C lower than the preceding cycle, and 72 °C for 1 min, then 35 amplification cycles at

98 °C for 10 s, 60 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 5 min). Sequencing reactions were performed using the BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's protocol. Primer sets for PCR and sequencing are listed in Supporting Information Table S1.

Genotyping

Genotyping of SNPs in the *TBXA2R* gene was performed in 290 subjects with asthma and 328 controls, using the ABI PRISM[®] SNaPshot[®] Multiplex Kit (Applied Biosystems) or modified TaqMan[™] allele-specific amplification (TaqMan[™]-ASA) method [16] according to the manufacturer's protocol. The PCR template for the SNaPshot reaction was prepared using the same protocol as described in the "Sequencing" section. SNaPshot primers were designed to anneal adjacent to the SNP of interest and to contain an additional sequence with several (incomplete) repeats of the "acgt" sequence (indicated by lower case) at the 5'-end to obtain a convenient length to discriminate it from other SNaPshot products. Primer sequences for SNaPshot and TaqMan[™]-ASA are shown in Tables S2 and S3, respectively. The samples were analyzed with an ABI PRISM[®] 3100 Genetic Analyzer (Applied Biosystems) and the resulting data were analyzed with GeneMapper[®] v3.5 software (Applied Biosystems).

Reporter vector construction

To create test vector constructs, 605-bp DNA fragments including intron 1 of *TBXA2R* were PCR-amplified from genomic DNA of each subject with alternative haplotypes. Each PCR amplicon of four haplotypes was subcloned into the pGL4.23[luc2/minP] reporter vector, containing the firefly luciferase gene and a minimal promoter (Promega, Madison, WI), digested with *KpnI* using the In-FusionTM Advantage PCR Cloning Kit (TaKaRa Bio Inc., Otsu, Japan). The PCR primer set was as follows: forward,

5'-TGGCCTAACTGGCCGGTACCGGGCACCTTGTAGGGAGTTGAG-3'; reverse,

5'-GGCTAGCGAGCTCAGGTACCTTCTTTCTGGAAGGCGGACAGT-3'

(underlined sequences indicate the 20-bp homology extension for In-Fusion cloning into vector pGL4.23). DNA sequences of each construct were confirmed by sequencing.

Luciferase assay

Primary human bronchial smooth muscle cells (BSMCs; obtained from LONZA, Walkersville, MD) were cultured in Smooth Muscle Basal Medium (SmBM; LONZA) with human epidermal growth factor (hEGF), insulin, human fibroblast growth factor (hFGF)-B, fetal bovine serum (FBS), and gentamicin/amphotericin-B. Human bronchial

epithelial cells (BEAS-2B cells) were cultured in Bronchial Epithelial Basal Medium (BEBM; LONZA) with bovine pituitary extract, hydrocortisone, hEGF, epinephrine, insulin, triiodothyronine, transferrin, gentamicin/amphotericin-B, and retinoic acid. For the luciferase assay, 5×10^4 cells per well were seeded in 24-well dishes and cultured with 5% CO₂ at 37°C with no stimulation. After 24 h of incubation, either the test vector construct (500 ng) or pGL4.23 empty vector (500 ng) and 12.5 ng of pGL4.74[hRluc/TK] vector (Promega) encoding *Renilla* luciferase gene as an internal control for transfection efficiency were transiently cotransfected into cells using LipofectamineTM LTX and PLUSTM Reagent (Invitrogen, Carlsbad, CA). The ratios of LipofectamineTM LTX (μ l= μ g) to test and empty vector (μ g) were 6:1 in BSMCs and 2.5:1 in BEAS-2B cells.

Luciferase reporter assays were performed using the Dual-Luciferase[®] Reporter Assay System (Promega). Twenty-four hours after transfection, cells were washed with phosphate-buffered saline (PBS), and lysed with 100 μ l of Passive Lysis Buffer (Promega) per well. Each 20 μ l aliquot of lysate was assayed for luciferase activity and luminescence intensity was measured in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Transfection efficiency was normalized by calculating the ratio of firefly to *Renilla* luciferase activity. There was no significant difference in transfection

efficiencies between the two cell types or among cells transfected with each haplotype.

Relative luciferase activity was represented as the ratio of the normalized luciferase activity of each haplotype to that of pGL4.23 empty vector. Each independent experiment was performed four or five times in triplicate.

Quantitative real-time PCR

Total RNA was extracted from peripheral blood cells of control subjects with the RiboPureTM-Blood kit (Ambion, Austin, TX) according to the manufacturer's instructions. cDNA was synthesized from total RNA using the ReverTra Ace[®] qPCR RT Kit (TOYOBO, Osaka, Japan). Real-time PCR was performed using SYBR[®] Premix Ex TaqTM II (TaKaRa) with the Mx 3000P QPCR System (Stratagene Japan, Tokyo, Japan). Relative *TBXA2R* mRNA levels were calculated using the threshold cycle (Ct) method and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The PCR primer sets for *TBXA2R* and *GAPDH* were as follows: *TBXA2R*: forward, 5'-ACCCTGTGCCACGTCTACCAC-3'; reverse, 5'-GGTTTCGCAGCACTGTCTGG-3' and *GAPDH*: forward, 5'-GCATCCTGGGCTACACTGAG-3'; reverse, 5'-TGCTGTAGCCAAATTCGTTG-3'.

Statistical analysis

We calculated allele frequencies and tested their agreement with the Hardy-Weinberg equilibrium using a χ^2 goodness-of-fit test at each locus. All genotypes in case and control subjects were in Hardy-Weinberg equilibrium ($P>0.01$). Pair-wise LD was calculated as r^2 using genomic DNA from 48 control subjects. Haplotype blocks were defined by the solid spine of LD using the Haploview 4.1 program. Haplotype frequencies for multiple loci were estimated using SNPalyze[®] ver.4.1 Pro software (DYNACOM, Mobara, Japan). We compared differences in allele frequency and genotype distribution of each polymorphism using a contingency χ^2 test in co-dominant, dominant, recessive, and allele models. Differences in haplotype frequencies between cases and controls were evaluated both by the χ^2 test of haplotype against the others (haplotype-wise test) and by permutation test with 1000 iterations using SNPalyze[®]. Odds ratios (ORs) with 95% confidence intervals (CIs) for subjects with one or two minor alleles were calculated. A general linear model was used to investigate the association of genetic variants with lung function in an additive or recessive genetic model, including age and/or sex as covariates. We applied Bonferroni's corrections for multiple testing by multiplying each raw P value by the number of tests.

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Analysis of gene-sex interactions for asthma-related quantitative traits was performed using a general linear model. Quantitative traits used in this analysis were baseline and post-bronchodilator FEV1/FVC, total serum IgE levels, and blood eosinophil fractions/counts in peripheral blood cells derived from asthmatics and/or controls. The general linear model was applied to investigate the association of genetic variants with total serum IgE, blood eosinophil fractions/counts, or TBXA2R mRNA expression levels, including age, sex, and/or the presence of atopy as covariates. A recessive genetic model was applied to these analyses. Values of total IgE and eosinophil fractions/counts were log-transformed to obtain a normal distribution for statistical analysis. Comparisons in reporter assays were performed using the *t*-test.

A *P* value of less than 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 17.0 software (SPSS Japan Inc, Tokyo, Japan) unless otherwise stated.

Results

Subject characteristics

Subject characteristics are shown in Table 1. Subjects with asthma had significantly higher male to female ratios, atopy fractions, serum IgE levels, and blood eosinophil fractions than control subjects without asthma ($P < 0.0001$). The fraction of

asthma patients who had childhood-onset asthma before 4 years of age was 85.1%. The rate of atopy in control children was compatible to recent data on Japanese children [17].

Association of c.795T>C and c.924T>C with asthma and lung function

We first investigated c.795T>C and c.924T>C SNPs of *TBXA2R* in our childhood-onset asthma cases and controls because of their previous significant results. The findings of the association study of the SNPs with asthma are shown in Table S4. In the case of c.924T>C, subjects with the C allele (minor allele) showed a trend for a decreased risk of asthma in a co-dominant ($P=0.0497$), a dominant (OR 0.65, 95% CI=0.46-0.92, $P=0.0143$), and an allele (OR 0.69, 95% CI=0.51-0.94, $P=0.0195$) model. However, after correcting for multiple comparisons (Bonferroni's correction) by the number of SNPs tested multiplied by the number of genetic models, P values for all genetic models became insignificant (corrected P (P_{cor})=0.398, 0.114 and 0.156, respectively). c.795T>C showed no trend to associate with asthma in any genetic model.

We next tested for association between these two SNPs and lung function in patients with childhood-onset asthma. The results of this study are shown in Table S5. The C allele (minor allele) of c.795T>C showed a trend to associate with lower baseline

and post-bronchodilator FEV1/FVC ($P=0.00776$, 0.0363 , respectively). After correction for multiple testing, however, P values again became insignificant ($P_{cor}=0.124$, 0.581 , respectively). c.924T>C was not associated with any lung function.

SNPs and haplotype blocks in TBXA2R

To identify polymorphisms in LD with c.795T>C and/or c.924T>C, we performed comprehensive sequencing of *TBXA2R* including 1.5 kb of the 5' flanking region and 0.5 kb of the 3' flanking region in 48 Japanese control samples. We detected a total of 49 polymorphisms with minor allele frequencies of more than 0.01 (Table S6). To create an LD map, we selected 43 polymorphisms with minor allele frequencies of more than 0.05. The LD map of *TBXA2R* is shown in Figure 1. We found a haplotype block in intron 1 composed of four SNPs (SNP1G>T: rs2238634, SNP2T>G: rs2238633, SNP3C>T: rs2238632, and SNP4G>A: rs2238631) and in moderate LD with c.795T>C. The LD values between these four SNPs and c.795T>C are shown in Figure 1. We also identified an SNP (SNP5: rs5756) in the 3' UTR (Exon 3) in strong LD with c.924T>C. However, no putative microRNA (miRNA) interacting with the sequence containing this SNP could be identified using an online database (www.microrna.org) (data not shown). Thus, there is no information that strongly suggests that this and/or other SNP(s) in LD

with c.924T>C is functional.

Functional analysis of genetic variants associated with c.795T>C

TBXA2R is composed of three major exons, E1–E3, and an additional exon, E1b, located in intron 1 [4]. Three individual promoters, P1, P2, and P3, located 5' of E1, E1b, and E2, respectively, have been identified [18]. As the four SNPs we identified are located in intron 1, between the 3'-downstream of P1 and the 5'-upstream of P2, we hypothesized that they might affect the enhancer and/or promoter activity of the gene. We determined haplotype frequencies by genotyping the four SNPs in control subjects and identified four common haplotypes (H1, H2, H3, and H4) and their frequencies, as shown in Figure 2a.

Luciferase reporter vectors with a minimal promoter were constructed by cloning DNA fragments containing each haplotype (Figure 2b). We transfected each construct into BSMCs and BEAS-2B cells, and performed luciferase transcriptional assays. In BSMCs, haplotypes H2, H3, and H4 showed significantly higher transcriptional activity than haplotype H1 (H2, H3, and H4 vs. H1: $P=0.0013$, 0.036 , and 0.00016 , respectively) (Figure 2c). In BEAS-2B cells, haplotypes H2 and H4 showed significantly higher transcriptional activity than H1 (H2 and H4 vs. H1: $P=0.00087$ and

0.0024, respectively) (Figure 2c). Haplotypes H2 and H4, associated with increased luciferase activity in both cell types, commonly contain minor alleles of SNP2 and SNP3, suggesting that SNP2 and/or SNP3 are functional variations.

Transcription factor binding protein analysis

To identify a putative transcription factor binding protein which interacts with the sequence containing SNP2 or SNP3, we used the following internet tools: TFSEARCH (www.cbrc.jp/research/db/TFSEARCH.html) and TESS (www.cbil.upenn.edu/cgi-bin/tess/tess). Analysis by these programs revealed that SNP2 could locate in cis-elements recognized by a sterol regulatory element binding protein (SREBP) and CACCC binding protein and that SNP3 could locate in a cis-element recognized by cyclic AMP response element binding (CREB) protein. In both SNPs, conversion of the wild (major) to mutant (minor) allele resulted in a higher binding score, suggesting that these minor alleles may enhance the transcriptional activity level in *TBXA2R*.

Association of the haplotype in intron 1 with TBXA2R expression in peripheral blood cells

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Because SNP2 and SNP3 were suggested to affect expression of *TBXA2R*, we focused on the association of a redefined haplotype consisting of SNP2 and SNP3 for further analyses. Four types of haplotypes, h1 (SNP2:T; SNP3:C), h2 (SNP2:G; SNP3:T), h3 (SNP2:G; SNP3:C), and h4 (SNP2:T; SNP3:T), were identified in study subjects (see Table 2), where h2 corresponded to the high luciferase activity allele. To assess whether the h2 haplotype affects *TBXA2R* mRNA levels *in vivo*, we used quantitative real-time PCR to measure mRNA purified from peripheral blood cells of control subjects (n=131). As shown in Figure 3, there was a trend for increased expression levels of *TBXA2R* mRNA in subjects with the h2 haplotype (h2/h2 + h2/others vs. others/others: mean relative expression 0.824 vs. 0.680; $P=0.0498$).

Association of the haplotypes in intron 1 with asthma and lung function

We next investigated the association of the haplotype with clinical phenotypes in study subjects, but no significant association was found between the haplotype and asthma, suggesting that this haplotype has little impact on disease development (Table 2).

The results of the tests of association between the haplotype and lung function in asthma patients are shown in Table 3. Association tests were performed in a recessive

model (h2/h2 vs. h2/others + others/others) and there was a significant decline in both baseline and post-bronchodilator FEV1/FVC and %FEF_{25-75%} in h2/h2 homozygotes compared with other zygotes (baseline FEV1/FVC and %FEF_{25-75%} $P=0.00201$ and 0.0128 , respectively, and post-bronchodilator FEV1/FVC and %FEF_{25-75%} $P=0.00224$ and 0.0393 , respectively). After stringent correction for multiple testing (multiplied by 8, the number of lung function tests), the P values for baseline and post-bronchodilator FEV1/FVC remained significant ($P_{cor}=0.0161$ and 0.0179 , respectively). A graphical representation of the relationship between genetic variation and baseline FEV1/FVC is shown in Figure 4 and clearly reveals the recessive pattern in terms of the h2 haplotype.

Association of the h2 haplotype with eosinophil fraction/count and IgE level

We assessed whether the h2 haplotype associated with other asthma-related phenotypes including blood eosinophil fractions and total serum IgE levels in peripheral blood samples from asthma patients ($n=226$ and 247 , respectively) and control subjects ($n=164$ and 328 , respectively). There was no significant association with blood eosinophil fractions in either asthma patients (h2/h2 vs. h2/others + others/others: mean $\log_{10}$ [eosinophil fractions (%)] : 0.93 vs. 0.99 ; $P=0.174$) or control subjects (0.53 vs. 0.39 ; $P=0.267$); however, the interaction between the haplotype and sex was significant

for blood eosinophil fractions in control subjects ($P=0.0355$), and showed a trend in asthma patients ($P=0.102$).

We therefore repeated the analysis in sex subgroups (male and female). As shown in Figure 5a, the h2/h2 homozygotes showed significantly higher blood eosinophil fractions than the other zygotes in female control subjects ($n=82$; h2/h2 vs. h2/others + others/others: 0.73 vs. 0.39; $P=0.0146$), but not male control subjects ($n=82$; 0.26 vs. 0.39; $P=0.396$). As shown in Figure 5b, the h2/h2 homozygotes showed a trend for lower blood eosinophil fractions than the other zygotes in female asthma patients ($n=80$; 0.85 vs. 1.01; $P=0.0486$), but not male asthma patients ($n=146$; 0.97 vs. 0.98; $P=0.894$). In control subjects, a clearer trend for association was observed in blood eosinophil “counts” in the female subgroup ($n=82$; mean $\log_{10}$ [eosinophil counts (cells/ μ L)]: 2.64 vs. 2.25; $P=0.00956$).

No significant associations were observed between the haplotype and total serum IgE levels in either asthma patients (h2/h2 vs. h2/others + others/others: mean $\log_{10}$ [total IgE (IU/mL)]: 2.86 vs. 2.80; $P=0.636$) or control subjects (2.01 vs. 1.89; $P=0.930$), and no interactions between haplotype and sex were observed for total serum IgE levels in the two groups ($P=0.553$ and 0.915, respectively).

Discussion

In this study, the c.924T>C SNP of the *TBXA2R* gene showed a trend of association with asthma susceptibility in co-dominant, dominant, and allele tests. The *P* values for these results were significant if multiple testing was ignored, as seen in previous papers [9, 10] that claimed a significant association between c.924T>C and asthma. However, they failed to clear the level of significance after correction. The rate of the c.924T>C C allele in asthma patients was less than in controls, which agreed with the result of a previous association study in a Japanese adult population [9], but contradicted that of a Chinese child population study [10]. Further work is therefore necessary to conclude the importance of this association.

We also found a trend of association of c.795T>C with lung function in asthma patients. The C allele of c.795T>C was associated with lower FEV1/FVC, which was in the same direction of effect as observed previously [13, 14]. Some studies reported that while a single c.795T>C or c.924T>C had a weak or no association with asthma and its related phenotypes, a combination of c.795T>C or c.924T>C and other genetic variations [12, 13] and both SNPs [11, 14] showed significant association, suggesting that the association of c.795T>C or c.924T>C alone with the disease and its related phenotypes might not be strong enough to detect. Together, these data suggest that

c.795T>C and c.924T>C show weak but consistent association with asthma and its related phenotypes. Since c.795T>C and c.924T>C are located in the coding exon and cause no amino acid change, we sought functional variations that were in LD to these SNPs.

Haplotypes H2 and H4, consisting of minor alleles of SNP2 and SNP3, showed significantly higher transcriptional activities than H1, consisting of major alleles of the two SNPs in BSMCs and BEAS-2B. In BSMCs, the H3 haplotype showed a significantly higher transcriptional activity than H1. Although this difference did not reach statistical significance in BEAS-2B, H3 showed a trend of higher transcriptional activity than H1. It is therefore possible that SNP2 alone has a small effect on transcriptional activity in cells. Since H2 or H4 showed even higher activity than H3, it is likely that both SNP2 and SNP3 affect transcriptional activity. To our knowledge, these variations in intron 1 are the first to show experimental functional differences in *TBXA2R*. Transcription factors expressed in BSMCs have previously been reported as different from those in BEAS-2B in asthmatic subjects [19]. For example, while the CACCC-binding protein was expressed in both BSMCs and BEAS-2B in basal and asthmatic conditions, SREBP and CREB were expressed in BSMCs alone [19]. These findings may explain the difference of luciferase assay results between BSMCs and

BEAS-2B.

Although none of the intron 1 haplotypes showed a significant association with asthma *per se*, haplotype h2 showed a significant association with a lower FEV1/FVC in pre- and post-bronchodilator conditions in childhood-onset asthma patients. Haplotype h2 was also associated with an increased *TBXA2R* mRNA level, which together suggests that individuals with higher *TBXA2R* expression have more severe airway inflammation and/or remodeling. Progression of lung function decline and AHR has previously been associated with airway remodeling [20] and an increase in airway smooth muscle mass is one of the main ways of airway remodeling in asthma [21]. The mitogen-activated protein kinase (MAPK) signaling pathway is known to be associated with proliferation and migration in airway structural cells of asthma patients [22] and mitogenic signaling of the MAPK pathway is related to *TBXA2R* in vascular [23] and uterine [24] smooth muscle cells. Moreover, *TBXA2R* stimulation by its agonist was reported to enhance proliferation of human BSMCs via extracellular signal-regulated kinase activation [25-27]. These findings together suggest that *TBXA2R* is implicated in airway remodeling via the MAPK pathway. We speculate that the *TBXA2R* genetic variants in intron 1 affect the airway remodeling process in asthma patients.

It is known that *TBXA2R* agonists enhance contraction [25] and proliferation [26]

of airway smooth muscle cells in a dose-dependent fashion. A dexamethasone-induced decrease in TBXA2R was reported to reduce vascular responsiveness to a TBXA2R agonist [28, 29]. Moreover, TBXA2R densities in overloaded myocytes and atherosclerotic coronary arteries were increased compared to corresponding non-disease tissues [30], while TBXA2R-mediated contractions in vascular smooth muscle cells decreased in parallel with reduced TBXA2R mRNA and protein expression [31]. We propose that the TBXA2R expression level impacts on airway responsiveness and/or remodeling, which explains at least part of our genetic association results.

We also found that female control h2/h2 homozygotes had a significantly higher blood eosinophil fraction and count than subjects with other zygotes, while h2/h2 female asthma patients had a slightly lower fraction. Although it is too soon to draw any firm conclusions from these data, it is possible that the effect of the h2 haplotype is modified under higher eosinophil fraction conditions in asthma. Thus, the positive effect of the h2 haplotype on TBXA2R expression may be difficult to see under such conditions. Data for explaining the sex-specific association are currently lacking, but the available results suggest the involvement of thromboxane pathways in controlling eosinophil proliferation and/or mobilization.

Several lines of evidence support the association of TBXA2R with the physiology

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and pathophysiology of eosinophils. A murine asthma model previously showed that the TXA2 synthase inhibitor OKY-046 and the TBXA2R antagonist S-1452 inhibited cytokine production and subsequently reduced eosinophil infiltration into lung tissue [32]. Similarly, the TBXA2R antagonist AA-2414 inhibited cytokine expression and eosinophil infiltration in bronchial tissues of asthmatic patients [33]. Further, Suzuki et al. [34] recently showed that TBXA2R was related to eosinophilic inflammation by inducing CCL11 expression in BSMCs. Our observation provides a new line of evidence for the regulatory roles of TXA2/TBXA2R pathways on eosinophil proliferation/mobilization in humans.

Our association study has some limitations. The subject number was relatively small so the study should be replicated using a larger sample size. Since we only analyzed subjects with childhood-onset asthma for the association study of lung function, it is not known whether the association between genetic variants in *TBXA2R* and lung function would be specific for childhood-onset asthma. Association studies are therefore warranted in different groups including subjects without asthma, patients with alternative asthma subgroups such as aspirin-intolerant asthma, and patients with other respiratory diseases such as chronic obstructive pulmonary disease (COPD).

Additionally, we did not experimentally investigate the functional aspects of

SNP5 (rs5756) in the 3' UTR of *TBXA2R*, which leaves open the possibility that it is also functionally relevant. The 3' UTRs of eukaryotic mRNAs are often involved in posttranscriptional regulatory pathways and have been implicated in mRNA stability [35].

In summary, we identified functional genetic variations in *TBXA2R* that influence transcriptional activity and associate with lung function, eosinophil fraction, and *TBXA2R* mRNA levels in Japanese childhood-onset asthma patients and/or controls. Our findings offer a new line of evidence for the implication of thromboxane pathways in the pathophysiology of asthma and its related phenotypes.

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Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Fig. 1.

Structure and linkage disequilibrium (LD) map of the *TBXA2R* gene.

Exons are indicated by boxes (black and white boxes denote coding regions and untranslated regions, respectively). Seven single nucleotide polymorphisms (SNPs) investigated in this study (SNP1, SNP2, SNP3, and SNP4 in intron 1; c.795T>C, c.924T>C in exon 3; SNP5 in 3'-UTR) are represented above the gene map. The bold numbers on the gene map correspond to the number sign for each polymorphism in Table 6S. The numbers for seven SNPs are shown in black squares. The number in each cell represents the LD parameter r^2 ($\times 100$) calculated by the Haploview 4.1 program. Black and white cells denote $r^2 = 1$ and $r^2 = 0$, respectively, with a gradient color in

each cell according to the strength of LD. Four SNPs forming one haplotype block in intron 1 are in LD with c.795T>C, and SNP5 in 3'-UTR shows LD with c.924T>C.

Fig. 2.

Effect of haplotypes on transcriptional activity.

(a) Haplotype frequencies consisting of four SNPs using 328 control samples. (b) DNA fragments containing one of each haplotype were subcloned into reporter plasmids with a minimal promoter (minP) upstream of the luciferase gene. Numbers below the DNA fragment represent distance from the translation start site. (c) The results of the luciferase assay in BSMCs and BEAS-2B cells. Relative luciferase activities are represented as the ratio of the normalized luciferase activity of each haplotype to that of the empty control and are expressed as mean $\pm$ Standard error (SE) in four (BSMC) or five (BEAS-2B) independent experiments. Each experiment was performed in triplicate. Statistical analyses were performed to compare H1 with the other haplotypes using the *t*-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig.3

Effect of haplotypes on TBXA2R mRNA expression.

Total RNA was extracted from a total of 131 whole peripheral blood samples in control subjects, and was reverse-transcribed into cDNA. Real-time PCR was performed to

quantitate each mRNA level. Relative TBXA2R mRNA expression levels are represented as the ratio of expression levels of TBXA2R to that of GAPDH calculated by the Ct method. Each experiment was performed in duplicate. Statistical analyses were performed using the general linear model including age, sex and the presence of atopy as covariates. Data (error bar) represent mean ($\pm$ SE).

Fig. 4

Association between haplotype and baseline and post-bronchodilator FEV1/FVC.

We tested for association between diplotype (haplotype pairs) and lung function in asthma patients in a recessive model (h2/h2 vs. h2/others+others/others). Statistical analyses were performed using the general linear model including age and sex as covariates. Data (error bar) represent mean ($\pm$ SE).

Fig. 5.

Association between haplotype and eosinophil fractions.

We evaluated the interaction between haplotype and sex for blood eosinophil fractions in (a) control subjects (n=164) and (b) asthma patients (n=226). We next analyzed the association of the haplotype with blood eosinophil fractions separately in sex subgroups (male and female). Statistical analyses were performed using the general linear model including age, sex and/or the presence of atopy as covariates. Data (error bar) represent

mean ($\pm$ SE).

Table 1. Characteristics of study subjects

Characteristic	Asthma	Control
Number of subjects, n	290	328
Age, years (range)	12.6 $\pm$ 3.4 (6-25) [†]	9.2 $\pm$ 1.7 (6-12)
Male, n (%)	186 (64.1%)*	162 (49.4%)
Atopy, n (%)	290 (100.0%)*	235 (71.6%)
Total IgE, IU/ml (range)	718.0 (3-65000) [†]	227.5 (5-3670)
Eosinophil fraction, % (range)	9.8 (2.6-49.3) [†]	2.6 (0.2-15.4)
Onset before age 4 years, n (%)	182/214 (85.1%)	
<i>Baseline lung function</i>		
FEV1 (% predicted)	89.5 $\pm$ 14.5	N.D.
FVC (% predicted)	92.8 $\pm$ 13.7	N.D.
FEV1/FVC (%)	84.8 $\pm$ 8.3	N.D.
FEF _{25-75%} (% predicted)	83.7 $\pm$ 26.2	N.D.
<i>Post-bronchodilator lung function</i>		
FEV1 (% predicted)	94.0 $\pm$ 14.3	N.D.
FVC (% predicted)	93.8 $\pm$ 14.0	N.D.
FEV1/FVC (%)	88.2 $\pm$ 6.6	N.D.
FEF _{25-75%} (% predicted)	98.7 $\pm$ 26.9	N.D.

Total IgE was measured in 247 asthma patients and 328 control subjects. Eosinophil fractions were measured in 226 asthma patients and 164 control subjects. Age at asthma onset was known in 214 asthma patients. Baseline and post-bronchodilator lung function tests were performed in 212 and 184 of asthma patients, respectively. Values for total IgE and eosinophil fractions are medians; other values (before plus-minus) are means. Values after plus-minus represent standard deviation (SD). Total IgE and eosinophil counts were log-transformed to show normal distributions for statistical analysis.

N.D.: not determined

* $P < 0.0001$ compared with controls by Chi-square test

[†] $P < 0.0001$ compared with control by t-test

Table 2. Association between haplotypes in *TBXA2R* and asthma

Haplotype	SNP2-3	Haplotype frequency		Odds ratio (95% CI)	<i>P</i> value	Permutation
		Asthma	Control			<i>P</i> value
h1	T-C	318 (0.548)	381 (0.580)	1.14 (0.91-1.43)	0.250	0.251
h2	G-T	178 (0.306)	193 (0.294)	0.94 (0.74-1.20)	0.627	0.690
h3	G-C	82 (0.142)	79 (0.121)	0.83 (0.60-1.16)	0.275	0.331
h4	T-T	2 (0.004)	3 (0.005)	1.33 (0.22-7.97)	0.558	1.000

CI: confidence interval.

Table 3. Association between haplotypes in *TBXA2R* and lung function

Haplotype	Diplotype	N	%FEV1	%FVC	FEV1/FVC	%FEF _{25-75%}
<i>Baseline lung function</i>						
h2	h2/others+others/others	191	89.82 ± 14.63	92.54 ± 14.01	85.39 ± 8.33	85.17 ± 26.14
	h2/h2	21	86.85 ± 12.87	95.28 ± 9.96	79.84 ± 6.82	70.44 ± 23.11
	<i>P</i> value		0.345	0.373	0.00201	0.0128
	Corrected <i>P</i> value*		> 1	> 1	0.0161	0.103
<i>Post-bronchodilator lung function</i>						
h2	h2/others+others/others	164	94.08 ± 14.63	93.33 ± 14.42	88.62 ± 6.43	100.08 ± 26.05
	h2/h2	20	93.57 ± 11.09	97.58 ± 9.78	84.36 ± 7.33	87.36 ± 31.74
	<i>P</i> value		0.874	0.163	0.00224	0.0393
	Corrected <i>P</i> value*		> 1	> 1	0.0179	0.314

Lung function measures represent mean ±SD.

“Others/others” includes h1/h1, h1/h3, h1/h4 and h3/h3; “h2/others” includes h1/h2 and h2/h3.

N represents number of subjects.

General linear model was applied to association study in a recessive model, adjusted for age and sex.

*Corrected *P* values were calculated by multiplying raw *P* values by 8 (number of lung function tests)

Significant *P* values (<0.05) are in boldface.

Fig. 1.

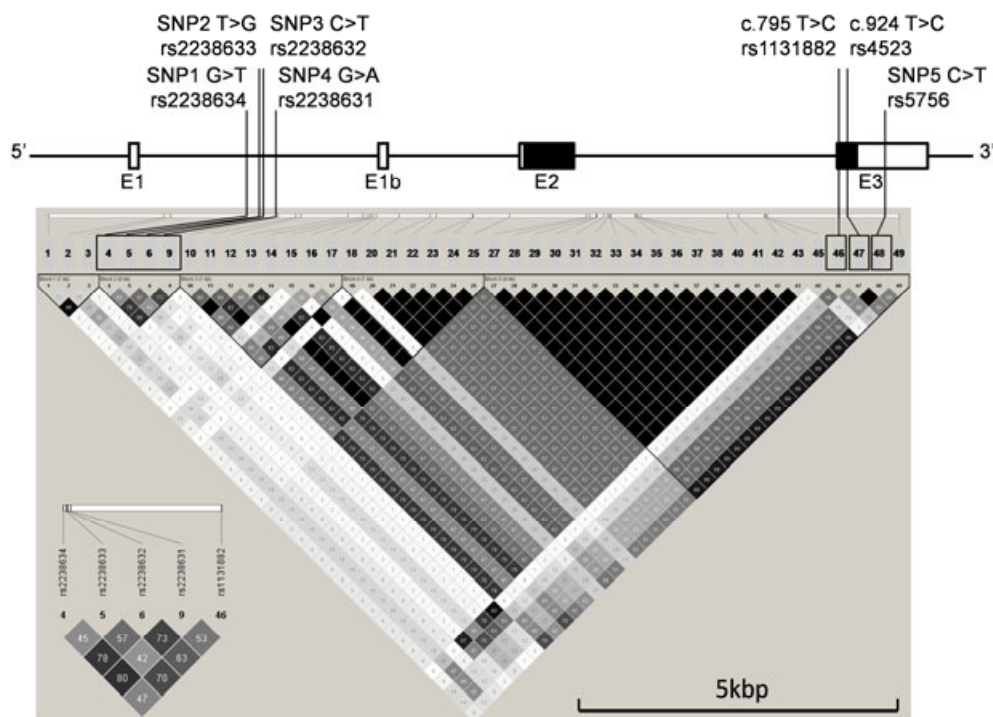
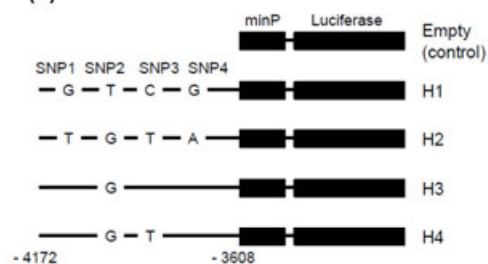


Fig. 2.

(a)

	SNP1 rs2238634	SNP2 rs2238633	SNP3 rs2238632	SNP4 rs2238631	Freq.
H1	G	T	C	G	0.558
H2	T	G	T	A	0.223
H3	G	G	C	G	0.119
H4	G	G	T	G	0.063
others					0.037

(b)



(c)

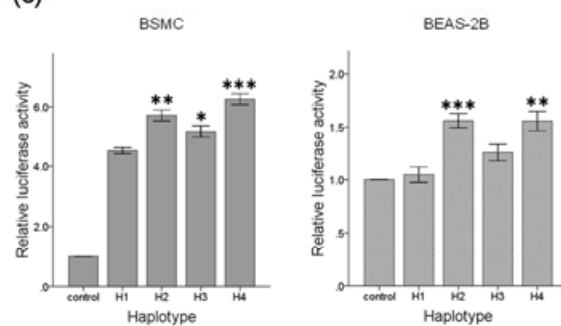


Fig. 3.

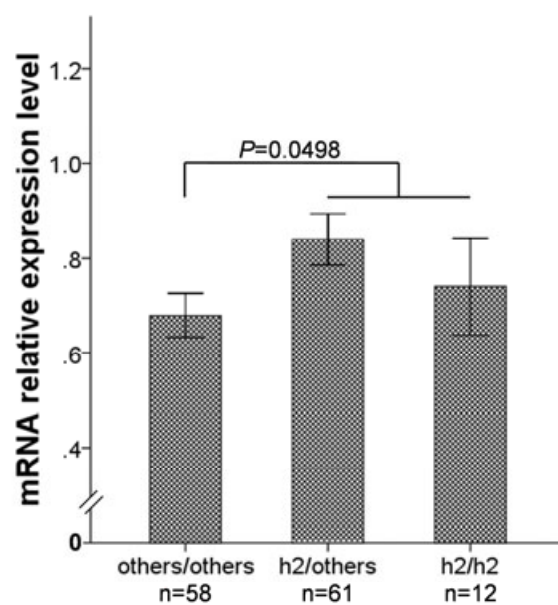


Fig. 4.

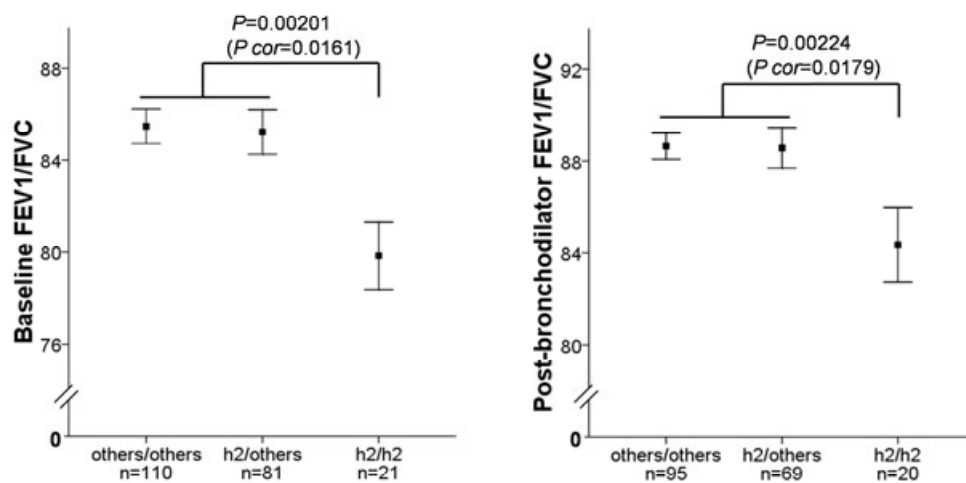
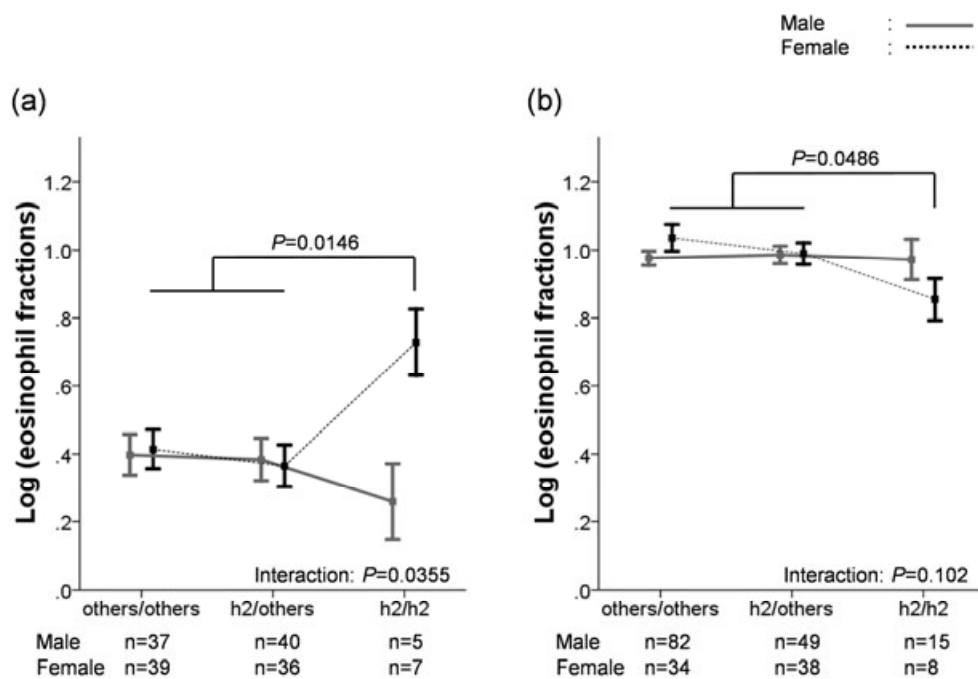


Fig. 5.



Supporting Information

Table S1. Primers for PCR and sequencing

Location	Method	Direction	Sequence
5'-upstream	PCR	Forward	5'-TCCTAGCTCACCTCTGGGCTTC
	PCR	Reverse	5'-GGCTTCCTTGGCCTTGTTTGTA
	Sequencing	Reverse	5'-GGCTTCCTTGGCCTTGTTTGTA*
5'-upstream	PCR	Forward	5'-GCTGGGAATACCCCTCACTCAC
	PCR	Reverse	5'-GGAACGCGGCACACAGTAGTAG
	Sequencing	Reverse	5'-GGAACGCGGCACACAGTAGTAG*
5'-upstream	PCR	Forward	5'-CTGCTCTGGGACCACCTTTCTC
	PCR	Reverse	5'-CTCGCTCTCTCCGTCCAGTCTC
	Sequencing	Reverse	5'-CTCGCTCTCTCCGTCCAGTCTC*
Exon1	PCR	Forward	5'-GCTGAGGCTCCCGAGGAA
	PCR	Reverse	5'-GCGACCTCACCTTCAGAGACCT
	Sequencing	Forward	5'-GCTGAGGCTCCCGAGGAA*
Exon1	PCR	Forward	5'-CTGATGAGAGACAGCGGGAGAC
	PCR	Reverse	5'-GAGTCTGGGACCCCCATTAC
	Sequencing	Reverse	5'-GAGTCTGGGACCCCCATTAC*
Intron1	PCR	Forward	5'-ATGCACTGCTCTGGTGTGAGC
	PCR	Reverse	5'-CAAAGCTCCATTTCCGAGAACA
	Sequencing	Forward	5'-ATGCACTGCTCTGGTGTGAGC*
	Sequencing	Forward	5'-TCTCTGGGAGTGAACCATGCAT
	Sequencing	Reverse	5'-ACTCGAGACACACAGACACAGCC
	Sequencing	Reverse	5'-CAAAGCTCCATTTCCGAGAACA*
Intron1	PCR	Forward	5'-TGCATGTTGGTGGGAATCACTGT
	PCR	Reverse	5'-CCTCCCTCGAGAGCTGGTATGT
	Sequencing	Forward	5'-TGCATGTTGGTGGGAATCACTGT*
	Sequencing	Forward	5'-GGGCACCTTGTAAGGAGTTGA
	Sequencing	Reverse	5'-TGCGGCTCTTGGGAAAATTACT
	Sequencing	Reverse	5'-CCTCCCTCGAGAGCTGGTATGT*

Intron1	PCR	Forward	5'-GTGGTCGAGGAGACCCTGAGAC
	PCR	Reverse	5'-AGTCACCCAGGAGGCTAGGATG
	Sequencing	Forward	5'-GTGGTCGAGGAGACCCTGAGAC*
	Sequencing	Forward	5'-GAGATGGAGTCTCGCTCTGTCTG
	Sequencing	Reverse	5'-TCGAGACCATCCTGGCTAACAC
	Sequencing	Reverse	5'-AGTCACCCAGGAGGCTAGGATG*
Intron1	PCR	Forward	5'-CATCTCGCACACAGTCCCATTTC
	PCR	Reverse	5'-TTCCTCCATTTTCATGGTGTCTG
	Sequencing	Forward	5'-CATCTCGCACACAGTCCCATTTC*
	Sequencing	Forward	5'-CACCTGGCCGTTTTGTTTTCTT
	Sequencing	Reverse	5'-TTCCTCCATTTTCATGGTGTCTG*
Exon2	PCR	Forward	5'-GAACCTGCCATTCCGAAGAAGT
	PCR	Reverse	5'-CCGTCTCTCCTCCAGGGTAATG
	Sequencing	Forward	5'-GAACCTGCCATTCCGAAGAAGT*
	Sequencing	Reverse	5'-CCGTCTCTCCTCCAGGGTAATG*
Exon2	PCR	Forward	5'-AATCTTCTCTCGCCTCCCAGGT
	PCR	Reverse	5'-TGATACCCAGGTAGCGCTCTGA
	Sequencing	Forward	5'-AATCTTCTCTCGCCTCCCAGGT*
Exon2	PCR	Forward	5'-CTGCCGTCTCTGTCTGCTTCAT
	PCR	Reverse	5'-GAGGTCCAGTCAGGAGGGTAGC
	Sequencing	Forward	5'-CTGCCGTCTCTGTCTGCTTCAT*
Intron2	PCR	Forward	5'-ACCCTGTGCCACGTCTACCAC
	PCR	Reverse	5'-AAGGTGCGTCTGCAGGAAGTG
	Sequencing	Forward	5'-ACCCTGTGCCACGTCTACCAC*
	Sequencing	Forward	5'-AGCCAGACATGGTGGCATGTG
	Sequencing	Reverse	5'-GTTTTATTGGCCACAGCCACG
	Sequencing	Forward	5'-TGCAGTGAGCCGAGATCACGT
Intron2	PCR	Forward	5'-CATGGGTCAACCCTTGCTCTAA
	PCR	Reverse	5'-GCCTGGCAGGAAATTTTTAAACTA
	Sequencing	Forward	5'-CATGGGTCAACCCTTGCTCTAA*
	Sequencing	Forward	5'-TTTCATCGTGTTAGCCAGGATG
	Sequencing	Reverse	5'-GCCTGGCAGGAAATTTTTAAACTA*

Intron2	PCR	Forward	5'-TAGTTTAAAAATTCCTGCCAGGC
	PCR	Reverse	5'-ATAGGTCAGGATTGATGGGAAA
	Sequencing	Forward	5'-TAGTTTAAAAATTCCTGCCAGGC*
Intron2	PCR	Forward	5'-TTTCCCATCAATCCTGACCTAT
	PCR	Reverse	5'-CGGCCAGTGTCCACTCTGTTAC
	Sequencing	Forward	5'-TTTCCCATCAATCCTGACCTAT*
	Sequencing	Reverse	5'-CGGCCAGTGTCCACTCTGTTAC*
Intron2	PCR	Forward	5'-GCCTGGCTAAGAGTGCCTAAT
	PCR	Reverse	5'-ATGAAGACCTGCAAAGGGGAGA
	Sequencing	Forward	5'-GCCTGGCTAAGAGTGCCTAAT*
Exon3	PCR	Forward	5'-AAAACGGAGTGGACCCTGGAT
	PCR	Reverse	5'-GGGAGCTCTGGATGGGAAAAA
	Sequencing	Reverse	5'-GGGAGCTCTGGATGGGAAAAA*
Exon3	PCR	Forward	5'-GGCTGCAGTAGGAAGTGGACAG
	PCR	Reverse	5'-TGACACACAGATTCAAAGGTGGTTT
	Sequencing	Forward	5'-GGCTGCAGTAGGAAGTGGACAG*
	Sequencing	Forward	5'-TAGCTGGGACTATAGGCGCGC
	Sequencing	Reverse	5'-ATGCCTGTGATCCCAGCACTTT
	Sequencing	Reverse	5'-TGACACACAGATTCAAAGGTGGTTT*
3'-downstream	PCR	Forward	5'-CTTGCTGGGGTACAGTTGCTTC
	PCR	Reverse	5'-CACTCCACGCTTTTGCATCTCT
	Sequencing	Forward	5'-CTTGCTGGGGTACAGTTGCTTC*
	Sequencing	Forward	5'-CCTGTAATGGGAGGGCTACAGG
	Sequencing	Reverse	5'-CACTCCACGCTTTTGCATCTCT*

* The sequencing primer was the same as the primer used for PCR.

Table S2. Primers for SNaPshot reaction

Location	Method	Direction	Sequence
Intron1	PCR	Forward	5'-TGCATGTTGGTGGGAATCACTGT
	PCR	Reverse	5'-CCTCCCTCGAGAGCTGGTATGT
	SNaPshot (SNP1)	Forward	5'-GAGCCAGGGAGGCCTGAGCT
	SNaPshot (SNP2)	Reverse	5'-gactGACTACAAGTCCCCTCTCCTCAGCAC
	SNaPshot (SNP3)	Reverse	5'-gactgactgactgCTCCCCCACTCCCTGGAGC
	SNaPshot (SNP4)	Reverse	5'-gactgactgactgacCGATGCCACCCCTGACAGGGTA
Exon3	PCR	Forward	5'-TCTCCCCTTTGCAGGTCTTCAT
	PCR	Reverse	5'-AGCAGCTCCTTCTCCGTGGT
	SNaPshot (795T>C)	Forward	5'-TCTCCCCTTTGCAGGTCTTCAT

Table S3. Primers and probe for TaqMan-ASA method

SNP	Primer/probe	Direction	Sequence
924T>C	T allele	Forward	5'-CAGATCCTGGACCCCTGGGTGTCT
	C allele	Forward	5'-CAGATCCTGGACCCCTGGGTGTTC
	Common	Reverse	5'-CGGGTGCTGAGGCGAGG
	TaqMan probe		5'-CGCCGTGCTCCGGCGTCTC

Table S4. Association between SNPs in *TBXA2R* and asthma

SNP	Genotype/allele		Frequency		Odds ratio (95% CI)	<i>P</i> value	Corrected
			Asthma	Control			<i>P</i> value*
c.795T>C	Genotype	T/T	116 (0.400)	139 (0.424)		0.780 ^a	>1
		T/C	139 (0.479)	148 (0.451)	1.10 (0.80-1.52) ^b	0.549 ^b	>1
		C/C	35 (0.121)	41 (0.125)	0.96 (0.59-1.56) ^c	0.871 ^c	>1
	Allele	T	371 (0.640)	426 (0.649)	0.96 (0.76-1.21) ^d	0.721 ^d	>1
		C	209 (0.360)	230 (0.351)			
c.924T>C	Genotype	T/T	218 (0.752)	217 (0.662)		0.0497 ^a	0.398
		T/C	67 (0.231)	103 (0.314)	0.65 (0.46-0.92) ^b	0.0143 ^b	0.114
		C/C	5 (0.017)	8 (0.024)	0.70 (0.23-2.17) ^c	0.537 ^c	>1
	Allele	T	503 (0.867)	537 (0.819)	0.69 (0.51-0.94) ^d	0.0195 ^d	0.156
		C	77 (0.133)	119 (0.181)			

a: Co-dominant model

b: Dominant model

c: Recessive model

d: Allele model

CI represents confidence interval.

* *P* values after Bonferroni's correction by multiplying raw *P* value by 8 (number of SNPs tested × number of genetic models)Significant *P* values (<0.05) are in boldface.

Table S5. Association between SNPs in *TBXA2R* and lung function

SNP	Genotype	N	Baseline				N	Post bronchodilator			
			%FEV1	%FVC	FEV1/ FVC	%FEF ₂₅ - 75%		%FEV1	%FVC	FEV1/ FVC	%FEF ₂₅ - 75%
c.795 T>C	T/T	9	90.52 ±	94.18 ±	84.23	84.08 ±	8	96.43 ±	95.71 ±	88.33	101.05 ±
		3	14.24	14.52	± 8.19	25.98	1	14.18	15.01	± 6.26	23.25
	T/C	9	89.20 ±	90.99 ±	86.49	85.97 ±	8	92.21 ±	91.63 ±	88.81	98.55 ±
		2	14.36	13.10	± 8.21	25.30	1	14.50	13.51	± 6.64	27.70
	C/C	2	87.18 ±	94.29 ±	81.28	74.74 ±	2	91.85 ±	94.71 ±	85.15	90.57 ±
		7	15.76	12.17	± 8.24	28.86	2	12.87	11.31	± 7.52	35.27
	<i>P</i> value		0.490	0.232	0.0077	0.157		0.145	0.183	0.0363	0.319
					6						
	Corrected										
	<i>P</i> value*		> 1	> 1	0.124	> 1		> 1	> 1	0.581	> 1
c.924 T>C	T/T	1					1				
		5	90.21 ±	93.79 ±	84.64	84.36 ±	3	95.61 ±	95.19 ±	88.34	100.50 ±
		7	14.47	13.61	± 8.53	26.42	5	14.60	14.13	± 6.65	26.81
	T/C	5	87.28 ±	89.33 ±	85.83	83.52 ±	4	89.73 ±	89.85 ±	87.89	95.01 ±
		0	14.22	13.45	± 7.90	25.83	6	12.75	13.49	± 6.62	26.91
	C/C	5	90.26 ±	96.98 ±	81.01	65.44 ±	3	88.15 ±	91.29 ±	84.45	73.95 ±
			17.70	14.16	± 6.29	18.10		5.04	6.84	± 8.34	21.40
	<i>P</i> value		0.454	0.117	0.250	0.273		0.0569	0.126	0.530	0.218
	Corrected										
	<i>P</i> value*		> 1	> 1	> 1	> 1		0.910	> 1	> 1	> 1

Lung function measures represent mean ±SD.

N represents number of subjects.

General linear model was applied to association study in an additive model, adjusted for age and sex.

* *P* values after Bonferroni's correction by multiplying raw *P* value by 16 (number of SNPs tested ×number of lung function tests)

Significant *P* values (<0.05) are in boldface.

Table S6. Location and allele frequencies of polymorphisms in *TBXA2R* gene

#	Position*	rs number	Polymorphism	Genomic location	Minor allele frequency
1	-7050	rs4807491	T/C	5'-upstream	0.417
2	-5329	rs12460249	T/C	intron1	0.198
3	-5232	rs114599143	T/G	intron1	0.406
4	-4097	rs2238634	G/T	intron1	0.198
5	-3917	rs2238633	T/G	intron1	0.354
6	-3866	rs2238632	C/T	intron1	0.240
7	-3796	rs35230357	- /A	intron1	0.031
8	-3707	-	G/A	intron1	0.010
9	-3667	rs2238631	G/A	intron1	0.188
10	-3633	rs2238630	A/G	intron1	0.083
11	-3372	rs3786989	C/G	intron1	0.052
12	-3296	rs3786990	G/C	intron1	0.083
13	-2595	rs34620121	G/A	intron1	0.063
14	-2367	rs113462322	G/T	intron1	0.052
15	-2294	rs35424656	A/G	intron1	0.063
16	-2259	rs35296805	T/C	intron1	0.156
17	-2230	rs113604036	C/T	intron1	0.063
18	-2173	rs28416788	A/G	intron1	0.063
19	-1965	-	C/T	intron1	0.031
20	-1833	rs56043676	A/G	intron1	0.063
21	-1370	rs61413740	C/T	intron1	0.063
22	-1279	rs57466064	G/A	intron1	0.063
23	-737	rs55878364	A/T	intron1	0.063
24	-735	rs55694642	A/ -	intron1	0.063
25	-192	rs36092560	C/A	intron1	0.063
26	435	rs5748	G/A	exon2	0.042
27	956	rs10409504	G/A	intron2	0.104
28	1010	rs10415853	A/G	intron2	0.104
29	1098	rs10407626	C/T	intron2	0.104
30	1104	rs10409244	G/A	intron2	0.104
31	1205	rs10415389	A/G	intron2	0.104
32	1266	rs10407192	C/T	intron2	0.104

33	1299	rs111330275	T/A	intron2	0.104
34	1306	rs12980948	T/A	intron2	0.104
35	1671	rs34376331	C/T	intron2	0.104
36	1693	rs35419703	A/T	intron2	0.104
37	1715	rs71339052	A/G	intron2	0.104
38	1754	rs34243734	G/C	intron2	0.104
39	2851	rs113119048	T/A	intron2	0.021
40	3048	rs8112115	A/G	intron2	0.104
41	3210	rs71166915	CAAA/ -	intron2	0.104
42	3602	rs35976116	C/G	intron2	0.089
43	3617	rs35283199	G/T	intron2	0.104
44	3625	rs10417834	C/G	intron2	0.042
45	3641	rs6510762	G/T	intron2	0.198
46	4710	rs1131882	T/C	exon3	0.302
47	4839	rs4523	T/C	exon3	0.167
48	5395	rs5756	C/T	exon3	0.167
49	5601	rs5758	G/A	exon3	0.115

* +1 corresponds to the translation start site.