es to earlier relevant studies, and the recent AR prevalence in these studies ranged from 23 to 28% [14]. The International Study of Asthma and Allergies in Childhood in 1997 reported that the prevalence of rhinoconjunctivitis varied across centers from 0.8 to 14.9% in 6- to 7-year-olds and from 1.4 to 39.7% in 13- to 14-year-olds [15]. In an Aberdeen population study on 3,537 subjects, the prevalence of hay fever increased significantly from 1994 (13%) to 1999 (15%) [16]. In Japan, Sakurai et al. [9] showed that the prevalence rates of AR, seasonal rhinitis and JCP were 36, 29, and 11%, respectively, and age was a negative risk factor for all allergic conditions. The subjects of the study consisted of 2,307 male railway employees who underwent a health examination from February to May 1995 (mean age, 41.4 years; range, 19–65 years). In the study, AR was determined from self-reported AR or from the seasonal nasal symptoms, and JCP was defined as the presence of cedar-specific IgE positivity among subjects with seasonal rhinitis. The prevalence of AR in this study was 44.2% (681 of the 1,540 subjects), which is higher than in previous reports. However, there was no difference of prevalence between 20 and 49-year-olds. Interestingly, the prevalence of AR in subjects aged 30–39 years was 42.7% in a study conducted in 1995 [9]. These subjects aged 30–39 years in 1995 were 40–49 years old in 2005. The prevalence of AR in this study for subjects from 40 to 49 years of age was 43.9%, and there was no difference in the prevalence between the studies. The prevalence among this age group did not markedly increase during the last 10 years. Further etiological studies in independent populations or those aged less than 20 years and elderly populations are needed to determine the effects of age on the susceptibility to AR.

In the present study, a total of 859 subjects (859/1,540, 55.8%) had no symptoms of AR; however, among them, 392 subjects (392/859, 45.6%) were already sensitized to one or more of the 7 test aeroallergens. It is generally recognized that sensitization to any allergen is an important risk factor for developing allergic diseases; however, those sensitized subjects had no symptoms of AR.

The present study has shown that a total of 167 of 681 subjects with AR (24.5%) were sensitized to JC pollen and not to the other 6 test aeroallergens. Allergen-specific immunotherapy is established as an effective treatment for patients with IgE-mediated reactions, and it has been widely used as a desensitizing therapy for AR [17, 18]. Specific immunotherapy retrospectively reduces new sensitization in monosensitized subjects suffering from AR [19]. Subjects with monoallergen sensitization appear to be good candidates for immunotherapy.

Among the 681 subjects with AR, 451 (66.2%) were sensitized to multiple (two or more) aeroallergens, and 385 (56.5%) were sensitized to dust mites. Although our data strongly indicated an important role of JCP in AR, a significantly higher prevalence of sensitization to dust mites was observed in younger subjects. Dust mites, an indoor allergen, have a predominant impact on asthma, and a recent population-based study has shown that dust mite sensitization is a significant risk factor for developing the disease [20]. Another recent study, a long-term (23-year) follow-up study of university students, has shown that sensitization to pollen leads to an increased risk of developing asthma [21]. A limitation of our study was the lack of longitudinal data. To clarify factors that increased the risk of developing new AR or bronchial asthma, further cohort analyses should be conducted regarding the involvement of the sensitized allergens in airway allergic inflammation.

A recent etiological study in an unselected rural Chinese population tested sensitization to 14 allergens, including 5 aeroallergens (dust mite, cockroach, *Alternaria tenuis*, dog epithelia, and cat hair) by skin prick tests. 2,118 subjects whose ages ranged from 11 to 71 years were tested (43.3% were children between 11 and 17 years old) [22]. The study showed that 41.1% of the children were sensitized to 1 or more aeroallergens, and 36.5% of the adult subjects aged ≥18 years were sensitized [22]. The most common sensitizing aeroallergen in the Chinese study was dust mites (30.6%) [22]. In meta-analyses using data from 12,687 subjects aged 20–44 years in the European Community Respiratory Health Survey conducted in 2002, the highest prevalence of sensitization was found for the house dust mite (20.2%) [23]. In the present study, of the 1,540 subjects, 1,073 (69.7%) were sensitized to at least 1 of the 7 aeroallergens, and 855 (55.5%) and 649 (42.1%) were sensitized to Japanese cedar pollen and dust mites, respectively.

Several limitations of this survey should be mentioned. The survey is likely to be fraught with a certain recruitment bias. In general, individuals affected by a specific disease are more willing and interested in a study. However, only 13 subjects (0.84%) did not agree to participate in this survey whereas 1,540 subjects agreed to assays of serum total IgE and specific IgE for the 7 aeroallergens and to answer the questionnaire in the present study. Hospital workers, nursing and medical students might not be representative of the general population and there might have been a population selection bias with regard to socioeconomic status and higher education. Previous studies in various countries have reported an increased

occurrence of asthma among specific groups of healthcare workers [24–26]. Thus, selection bias might have had an influence on the higher prevalence of sensitization to 1 or more aeroallergens (69.7%) and of AR (44.2%) in our study.

Although a population selection bias might reduce the generalizability of the study, we showed here that the prevalence of AR has increased and that Japanese cedar pollen and dust mites were the predominant allergen sources among the 7 tested allergen sources in the Japanese population. However, further study is needed using larger, more representative samples.

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ORIGINAL ARTICLE

A functional polymorphism (−603A→G) in the tissue factor gene promoter is associated with adult-onset asthma

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Tissue factor (TF) is important for initiation of coagulation and for the increased thrombin activity observed at sites of inflammation. Thrombin activity is induced by allergen challenge in asthmatic airways and is involved in the pathogenesis of asthma. A −603A→G polymorphism (rs1361600) in the promoter region of the *TF* gene has been associated with serum TF levels and with the development of cardiovascular diseases. The aim of this study was to determine whether the functional −603A→G polymorphism has genetic influences on the development of asthma. Case–control analysis was performed of the association between six common single-nucleotide polymorphisms (SNPs), including the −603A→G polymorphism, at the *TF* gene, and the development of asthma, using two unrelated Japanese populations. In the primary population (n=826), the GG genotype at the −603A→G polymorphism was associated with adult-onset asthma (onset at ⩾21 years of age) (odds ratio (OR) 2.886, P=0.0231). A second population showed a similar tendency (n=1654, OR 1.602, P=0.064). Transcriptional activity of promoters with −603A→G genotypes were examined using luciferase promoter assays. The −603G allele was associated with higher promoter activity (P<0.05). The association between the functional polymorphism (−603A→G) in the *TF* gene promoter and adult-onset asthma indicates that *TF* is a candidate gene contributing to asthma susceptibility.
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Keywords: asthma; polymorphism; tissue factor

INTRODUCTION

Several previous reports suggest that activation of coagulation is involved in the pathogenesis of inflammatory pulmonary diseases,[1–4] although the precise role of coagulation abnormalities in pulmonary pathology is still unclear. It has been established that the coagulation system is activated in human asthma and that the levels of thrombin, the enzyme generated by the activation of coagulation, are consequently increased in the airways.[5,6] Thrombin has been shown to induce a variety of cell responses involved in the pathogenesis of asthma, such as mast cell degranulation[7] and airway hyperreactivity[8] and remodeling.[9] Genetic polymorphisms of key coagulation factor genes are associated with an increased risk of adverse outcomes in illnesses such as deep venous thrombosis and stroke, and, in some cases, of acute lung injury and pulmonary fibrosis.[10–12] Recently, we showed that polymorphisms in the plasminogen-activator inhibitor-1 gene, which has shown a significant association with cardiovascular diseases in several studies, are associated with the development of asthma.[13] These results strongly suggest that activation of the coagulation system in the airway has a significant role in the pathogenesis of asthma.

Tissue factor (TF) is a 43-kDa transmembrane cell surface glycoprotein, which, after forming a complex with factor VIIa, activates the extrinsic pathway of the coagulation system. In response to tissue injury and stress, TF has an important role in the initiation of coagulation and in the increase in thrombin activity at the site of inflammation. In a murine model, when coagulation is blocked at the TF-activated factor VII level during experimental sepsis or acute respiratory distress syndrome, inflammatory responses in the lung are attenuated,[14–16] indicating that activation of coagulation by TF is involved in the pathogenesis of inflammatory lung diseases, including asthma. The human *TF* gene is located on chromosome 1, spans 12.4 kb, and is organized into six exons separated by five introns. Polymorphisms with potential functional relevance have been identified in the *TF* promoter.[10] Of these polymorphisms, the −603G allele has been analyzed most extensively and has been associated with increased *TF* gene mRNA expression in human monocytes[17] and with higher serum levels of TF.[10,11] Further evidence of the functional importance of these variants lies in the significantly increased risk of myocardial infarction[11] and venous thrombosis[10] in the carriers of risk alleles.

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Given the potential association of an activated coagulation system with airway inflammation, particularly the role of TF as an important initiator of the coagulation system, the *TF* gene is an excellent candidate for asthma susceptibility. In this study, using a case–control analysis, we evaluated the association of *TF* polymorphisms in correlation with the development of asthma. In particular, the −603A→G polymorphism, which has been associated with the development of cardiovascular diseases, was correlated to the development of asthma. To gain further insight into the possible molecular basis of this disease association, we also examined the functional consequences of this polymorphism at the regulatory region of the *TF* gene *in vitro*.

MATERIALS AND METHODS

Study populations

Two independent populations of Japanese asthmatic patients and control subjects were recruited. The demographic characteristics of the primary population (primary study population) and the replicate population (second study population) are presented in Table 1.

Primary study population

A total of 826 unrelated Japanese subjects, including 437 patients with asthma and 389 healthy volunteers, were recruited as the primary study population (Table 1). All patients with asthma were recruited from the pulmonary clinic of the First Department of Medicine, Hokkaido University Hospital. Asthmatic patients were defined as patients with recurrent episodes of at least two of three asthma symptoms (coughing, wheezing and dyspnea) and with a demonstrable reversible airflow limitation (15% variability in forced expiratory volume in 1 s, or in peak expiratory flow rate, either spontaneously or with an inhaled short-acting β2-agonist) and/or with increased airway responsiveness to methacholine as previously described.[18,19] Individuals who visited our clinic for annual routine physical examinations, as well as healthy students at the Hokkaido University School of Medicine, were recruited as non-asthmatic controls if they had no history of asthma or any other chronic pulmonary diseases. Total serum immunoglobulin-E (IgE) levels (IU ml^{-1}) were measured in all subjects, and specific IgE responses to 10 common inhaled allergens, including *Der f*, grass pollens, animal danders and molds, were assessed. Atopic status was defined as a positive response to at least 1 of the 10 common inhaled allergens, as previously described.[18,19] An increase in specific IgE antibody levels (IgE CAP RAST >0.35 UA ml^{-1}, or MAST >1.0 lumicount) was considered to be a positive response. All subjects were unrelated and were Japanese. Written informed consent was obtained from all the subjects for enrollment in the study. The Ethics Committee of the School of Medicine, Hokkaido University, approved the study.

Replicate study population

A total of 1654 unrelated Japanese subjects, including 932 patients with asthma and 722 healthy volunteers, were used as the replicate study population (Table 1). All subjects with asthma were diagnosed according to the criteria of the National Institute of Health as previously described.[20,21] The diagnosis of atopic asthma was based on one or more positive skin scratch test responses to seven common aeroallergens in the presence of a positive histamine control and a negative vehicle control. The seven aeroallergens were house dust, *Felis domesticus* dander (Feld), *Canis familiaris* dander, *Dactylis glomerata*, *Ambrosia*, *Cryptomeria japonica* and *Alternaria alternate*. A total of 722 healthy individuals, without any respiratory symptoms or history of asthma-related diseases, were recruited on the basis of a physician's interview, in which they were asked whether they had been diagnosed as having asthma and/or atopy. All asthmatic patients were atopic and no information was obtained regarding the atopic status of healthy controls. No information regarding the smoking status or the total serum IgE level was obtained for this population. All subjects were unrelated Japanese who provided their written informed consent for enrollment in the study. The Ethics Committee of the SNP Research Center, The Institute of Physical and Chemical Research (RIKEN), approved the study.

Genotyping

We initially genotyped the −603A→G polymorphism in the primary population because of reports linking this polymorphism as functionally relevant to several cardiovascular diseases.[10,11,17] We selected an additional five single-nucleotide polymorphisms (SNPs) for genotyping on the basis of the frequency and location of SNPs and the linkage disequilibrium structure in and around the *TF* gene. We initially obtained genotyping data for 17 HapMap SNPs (spanning 15 kb around the gene) from the International HapMap Project

Table 1 Characteristics of the subjects

	Primary population			
		Patients with asthma		
	Control (N=389)	*Case (N=437)*	*Child-onset (N=135)*	*Adult-onset (N=302)*
Age in years[a]	31.0 (18–69)	48.0 (16–81)	26.0 (16–73)	54.0 (21–81)
Sex male, *N* (%)	253 (65)	194 (43.4)	78 (57.7)	116 (38.4)
Age of onset, years[a]		34.0 (1–77)	5.0 (1–20)	46.0 (21–77)
Smokers (%)				
Never/ex/current	68.6/4.4/27.0	55.2/21.7/23.1	59.3/15.5/25.2	53.3/24.5/22.1
Atopy, *N* (%)	221 (56.8)	316 (72.3)	124 (91.9)	192 (63.6)
	Replicate population			
		Patients with asthma		
	Control (N=722)	*Case (N=932)*	*Child-onset (N=196)*	*Adult-onset (N=736)*
Age in years[a]	52.0 (19–87)	53.0 (20–75)	32.5 (20–75)	56.0 (26–75)
Sex male, *N* (%)	525 (72.7)	145 (42.7)	101 (51.5)	297 (40.4)
Age of onset, years[a]		40 (0–75)	6.0 (0–20)	46 (21–75)
Atopy, *N* (%)		804 (86.3)	181 (92.3)	623 (84.6)

[a]Median (range).

(available online at http://www.hapmap.org/). To select tagSNPs in this region, we used the multimarker predictor method implemented in the Tagger program.[22] The Tag set was generated (using a threshold r^2 of 0.8) using five common SNPs with a minor allele frequency of more than 0.1 in the Japanese population.

Genotypes of subjects were identified by an assay that combined kinetic real-time quantitative PCR with allele-specific amplification as previously described.[18,19] Real-time PCR was performed using the SYBR Green I Master Mix (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM TM 7700 Sequence Detection System (Applied Biosystems). In addition, 25 samples were directly sequenced to confirm the accuracy of genotyping using allele-specific PCR. The sequences of the primers used for allele-specific PCR and direct sequencing are shown in Supplementary Tables S1 and S2. The −603A→G polymorphisms of the replicate population were genotyped using the TaqMan system (Applied Biosystems).

Cell culture

BEAS-2B cells were cultured in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in 5% CO_2-containing humidified air. Human umbilical vein endothelial cells were obtained from Cambrex (Walkersville, MD, USA) and were cultured in endothelial cell basal medium (EBM-2, Cambrex) maintained at 37 °C in 5% CO_2 and supplemented with 5% fetal bovine serum, penicillin/streptomycin and endothelial cell growth supplement (SingleQuots, Cambrex).

Construction of *TF* reporter vectors

Reporter plasmids containing the *TF* −603A or −603G alleles were constructed as follows. A PCR product of 864 bp was generated from the −603A or −603G alleles using genomic DNA templates from individuals homozygous for either A or G at position −603. The PCR products, including the −603A→G polymorphism, were digested with *Kpn*I/*Bgl*II, and then directionally cloned into the *Kpn*I and *Bgl*II sites of the promoterless, enhancerless luciferase (luc) reporter plasmid, pGL4.10 (Promega, Madison, WI, USA), generating −603A-luc and −603G-luc, respectively. The pGL4.10 plasmid was denoted by pGL4.10basic. The constructs were verified by direct sequence analysis and prepared for transfection using the EndoFree Plasmid Maxi kit (Qiagen, Valencia, CA, USA).

Transfection and dual-luciferase assay

The cell lines BEAS-2B or human umbilical vein endothelial cells were individually plated in the wells of six-well plates. The plasmids −603A-luc, −603G-luc or pGL4.10 (1.0 μg) were transiently co-transfected into the same cell as the control Renilla luciferase reporter plasmid (pGL4.74, 1.0 μg; Promega) using Superfect (Qiagen). Cells were then incubated at 37 °C in 5% CO_2. Following 24 h incubation in growth medium, luciferase activity was measured using the Dual-Luciferase Reporter Assay system (Promega) and a TD 20/20 luminometer (Promega). The results were normalized for Renilla activity and expressed as relative luciferase activity.

Electrophoretic mobility shift assay

Nuclear extracts were prepared from BEAS-2B cells using the Nuclear Extraction Kit (Chemicon, Temecula, CA, USA) and were frozen at −80 °C. The protein concentration of the extract was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Nuclear extract (10 μg protein) was mixed for 30 min at room temperature with biotin-labeled, double-stranded oligonucleotides using an EMSA kit (Panomics, Redwood, CA, USA) according to the manufacturer's instructions. The oligonucleotides used corresponded to the promoter region of *TF* spanning −603A (5′-TCAAGAATACTTGG CCTGCC-3′ and 5′-GGCAGGCCAAGTATTCTTGA-3′) and −603G (5′-TCA AGAATACCTGGCCTGCC-3′ and 5′-GGCAGGCCAGGTATTCTTGA-3′), and were synthesized by Hokkaido System Science (Sapporo, Japan). Nuclear extract (10 μl) was subjected to 6% polyacrylamide gel electrophoresis at 120 V for 55 min in Tris-borate EDTA buffer (Bio-Rad), followed by transfer to a nylon transfer membrane (Pall biodyne B nylon membrane, Pall, East Hills, NY, USA) using the Trans-Blot SD semi-dry transfer cell system (Bio-Rad). The membranes were then ultraviolet cross-linked (CL-1000; Funakoshi, Tokyo, Japan) for 3 min. Detection was performed according to the manufacturer's instructions after the membranes were exposed on Hyperfilm ECL (Amersham Biosciences, Chandler, AZ, USA). For the super-shift and competition assays, 2 μl of the antibodies E2F-1 or USF-1, E47 or NF-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or 2 μg of the respective non-labeled synthesized oligonucleotides were added to 10 ng of the biotin-labeled oligonucleotide (Hokkaido System Science).

Transcription factor binding site identification

Transcription factor binding sites were predicted using the TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) and TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess) databases.

Statistical analyses

The χ^2-test was used to compare qualitative risk factors (sex, smoking status and atopic status) among the cases and controls. One-way analysis of variance was used to compare the age of the subjects. The Haploview program[23] was used to compare the observed number of genotypes with the number of genotypes expected from the Hardy–Weinberg equilibrium. Statistical analyses were based on the calculation of odds ratio (OR) to provide estimates of the relative risk of asthma. Logistic regression analysis was used to estimate OR adjusted for sex, age and smoking status (current, ex or never). The linkage disequilibrium structure was examined using the Haploview software (http://www.broad.mit.edu/mpg/haploview).[23] For haplotype analysis, the Haplo.score program was used, which adjusts for covariates and calculates *P*-values for each haplotype.[24] A study-wide analysis combining two case–control populations was performed using the Cochran–Mantel–Haenszel method. To further examine the relationship between age at the onset of asthma and the genetic effects of the −603A→G allele, we performed survival analyses with age at the onset of asthma as the primary outcome using all subjects. The subgroups stratified according to the *TF* −603A→G genotypes were analyzed for the time taken for development of asthma using the standard Kaplan–Meier method, which plots the proportion of the population that is asthma-free based on the subjects' age at the time of evaluation. Relative promoter activity in the luciferase reporter assay was compared between the −603A→G genotypes using an unpaired *t*-test. These statistical analyses were performed on a personal computer with the statistical packages SYSTAT for Windows (version 10.2, Systat, Chicago, IL, USA) and SPSS for Windows (SPSS Japan, Tokyo, Japan).

RESULTS

Analysis of the primary population

The demographic characteristics of the 389 healthy controls and 437 patients with asthma from the primary population are listed in Table 1. The median age of subjects with asthma was significantly higher compared with the healthy control subjects ($P<0.0001$). There were significantly more females in the asthma group than in the control group ($P<0.0001$). Over one half of the subjects with asthma were never smokers, which was a lower number than that for controls (55 vs 68%; χ^2-test, $P<0.01$). More than 50% of the control subjects had atopic status, a result consistent with recent findings that the prevalence of atopy (as indicated by specific IgE against common inhaled allergens) among the Japanese population is increasing.[25,26]

Supplementary Table S3 shows the genotype frequencies of six *TF* gene polymorphisms for all 826 subjects in the primary population. No significant deviation from Hardy–Weinberg equilibrium was observed in healthy control subjects ($P>0.05$). Of the SNPs examined, there was a significant association only of the −603A→G polymorphism with asthma (χ^2-test, $P<0.05$, Supplementary Table S3). As these results were in accordance with the results of the initial screen of the association analysis and with several previous studies that showed an association of this polymorphism with cardiovascular diseases,[10,11] we therefore further analyzed the association of this polymorphism with asthma.

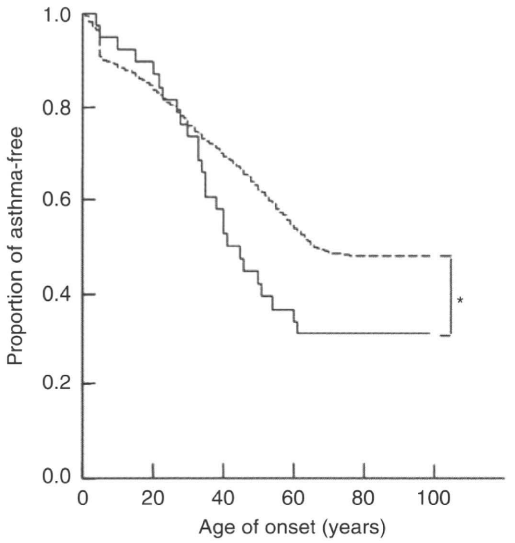

Figure 1 Kaplan–Meier survival analysis by *TF* −603 genotype (primary population). The plots show the effect of the *TF* −603 genotype on age of asthma onset. (solid line: GG, dotted line: AA or AG). *P=0.033.

The linkage disequilibrium structure was examined using the Haploview software.[23] Analysis of the data from the six SNPs using this program identified a haplotype block (Supplementary Figure S1) in the primary case–control population. Haplotype block I comprised three SNPs in the promoter and coding region (−1953AG [rs958587], −603A→G [rs1361600] and 1973AG [rs696619]). The frequency of the *TF* haplotype is shown in Figure 1. Haplotype analysis was performed in block I that contained the −603A→G polymorphism. However, none of the haplotypes in block I was associated with asthma (Supplementary Table S4).

The normal aging process alters the blood coagulation system in humans.[27] It is interesting that epidemiologic studies show that adult-onset asthma, age of onset ⩾21 years in particular, is associated with cardiovascular diseases,[28] carotid atherosclerosis,[29] coronary heart disease and stroke,[28] in which TF has a significant role during disease development. Therefore, we analyzed the possible effects of the −603A→G genotypes on the age of asthma onset using the Kaplan–Meier method of estimation. Figure 1 shows a plot of the proportions of subjects that were asthma-free versus their age of asthma onset for control (*TF* −603GG) and −603 AG/AA genotypes. In the plot for the proportion of asthma-free subjects, the slope for the −603GG carriers becomes steeper around 20 years of age. Statistical analyses indicated a significant difference between the two estimated survival curves for the −603A→G genotypes (P<0.05). In light of the results of the Kaplan–Meier curve and the interest in the associations between cardiovascular diseases and adult-onset asthma (age of onset ⩾21 years), in this study, we divided the patients with asthma into two groups: child-onset asthma (age of onset <21 years, n=135) and adult-onset asthma (age of onset ⩾21 years, n=302). Patients with child-onset asthma were highly atopic as a population compared with healthy controls (Table 1). Compared with child-onset asthma, patients with adult-onset asthma had a lower level of mean total serum IgE and a lower frequency of atopy. The GG genotype was significantly associated with adult-onset asthma (OR 2.886; 95% confidence interval 1.156–7.202; P=0.023). In contrast, there were no significant differences in genotype distribution of the −603A→G polymorphism between healthy controls and patients with child-onset asthma (OR 1.72; P=0.377) (Table 2 and Supplementary Table S5).

Table 2 Association of the genotype of the *TF* −603A→G polymorphism with the development of asthma

	Primary population			*Replicate population*		
Genotype	*OR*[a]	*95% CI*[a]	*P-value*[a]	*OR*[b]	*95% CI*[b]	*P-value*[b]
Child-onset BA						
AA or AG	1.00			1.00		
GG	1.72	0.516–5.736	0.377	1.31	0.591–2.913	0.505
Adult-onset BA						
AA or AG	1.00			1.00		
GG	2.89	1.156–7.202	0.0231	1.60	0.973–2.639	0.064

Abbreviations: BA, bronchial asthma; CI, confidence interval, OR, odds ratio.
[a]Multivariate logistic regression: adjusted for sex, age, smoking status and atopic status.
[b]Multivariate logistic regression: adjusted for sex and age.

Analysis of the replicate population

To confirm this association between the −603A→G polymorphism and adult-onset asthma, we determined whether we could replicate the association in an independent population. The demographic characteristics of the 722 healthy controls and 932 patients with asthma from the replicate population are shown in Table 1. The median age of subjects with asthma was significantly higher compared with healthy controls (P<0.0001). There were significantly more females in the asthma group than in the control group (P<0.0001). Genotype frequencies of the −603A→G polymorphism are presented in Supplementary Table S5. No significant deviation from the Hardy–Weinberg equilibrium was observed in healthy controls (P>0.05).

Patients with asthma were divided into two groups consisting of 196 child-onset and 736 adult-onset asthma as defined in the primary population (Table 1). Although it was not statistically significant, a similar tendency of association was observed in this second population, in which the frequency of individuals carrying the −603 GG genotype was higher in the adult-onset asthmatic group (logistic regression analysis, P=0.064) (Table 2 and Supplementary Table S5).

Analysis of the combined populations

Analysis of the combined populations by the Cochran–Mantel–Haenszel test showed that the OR for the GG genotype (AA or AG vs GG) was 1.595 (95% confidence interval 1.082–2.351; P=0.018) (Table 3). This genotype was consistently associated with adult-onset asthma in a pooled sample when the analysis was adjusted for age and sex using the logistic regression model. The OR for the GG homozygotes of the −603A→G polymorphism was 1.736 compared with the A allele (95% confidence interval 1.130–2.667; P=0.0117) (Table 3). In contrast, there were no significant differences in genotype distribution of the −603A→G polymorphism between healthy controls and patients with child-onset asthma (OR 1.207, P=0.559) (Table 3).

The −603A→G polymorphism modulates the transcription of the *TF* gene

To directly confirm the allele-specific effects of the *TF* −603A→G polymorphism on native promoter activity, two luciferase reporter gene constructs were generated, spanning 864 bp of the *TF* promoter region (736 bp of the *TF* 5′-flank and 128 bp of the 5UTR), with an

Table 3 Association of the genotype of the *TF* −603A→G polymorphism with the development of asthma (pooled population)

	Cochran–Mantel–Haenzsel			*Multivariate logistic regression*		
Genotype	*OR*	*95% CI*	*P-value*	*OR*[a]	*95% CI*[a]	*P-value*[a]
Child-onset BA						
AA or AG	1.00			1.00		
GG	1.149	0.631–2.091	0.650	1.207	0.643–2.266	0.559
Adult-onset BA						
AA or AG	1.00			1.00		
GG	1.595	1.082–2.351	0.018	1.736	1.130–2.667	0.0117

Abbreviations: BA, bronchial asthma; CI, confidence interval, OR, odds ratio.
[a]Adjusted for sex, age and population.

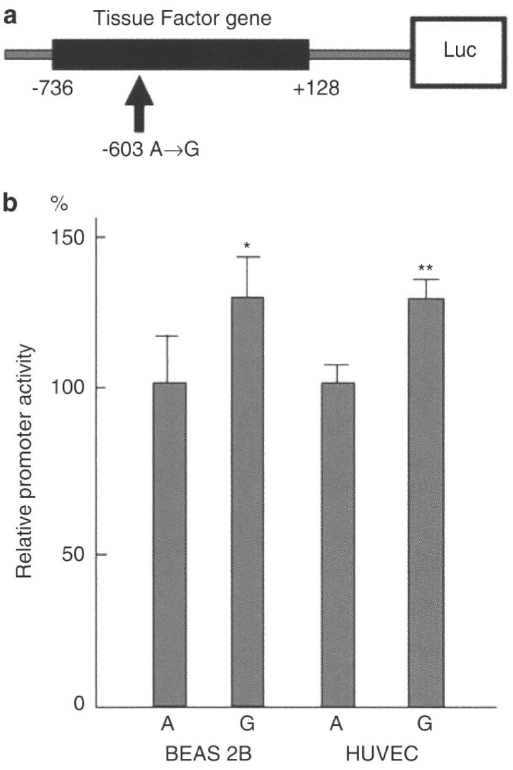

Figure 2 Comparison of the luciferase activity of the A-allele and G-allele promoter-reporter constructs after transient transfection. (**a**) Schematic outline of the reporter constructs in the pGL4 vector (Luc, luciferase). (**b**) BEAS 2B (n=10) and human umbilical vein endothelial cells (n=8) were transfected with 1.0 μg of pGL4.74 and with an A-allele (A) or G-allele (G) promoter-reporter plasmid and were harvested 24 h later. The data are presented as the means fold increase ± s.d. relative to the luciferase activity of a Renilla luciferase reporter plasmid (pGL4.74). *P=0.032, **P=0.0015.

A or G at the −603 polymorphic site (Figure 2a). The promoter region contained within these constructs has three known sequence variants other than −603A→G. Variances of all other known SNPs in the promoter region of *TF* were excluded from these constructs so that the data would reflect the functional impact of the −603A→G polymorphism. These constructs were transiently transfected into BEAS-2B and human umbilical vein endothelial cells. As shown in Figure 2b, the relative luciferase activities driven by the mutant G-allelic *TF* promoter were 30% of those driven by the A-allelic *TF* promoter in both of the cell lines examined (P<0.05). These results clearly indicated that the G-allelic *TF* promoter is associated with increased transcriptional activity.

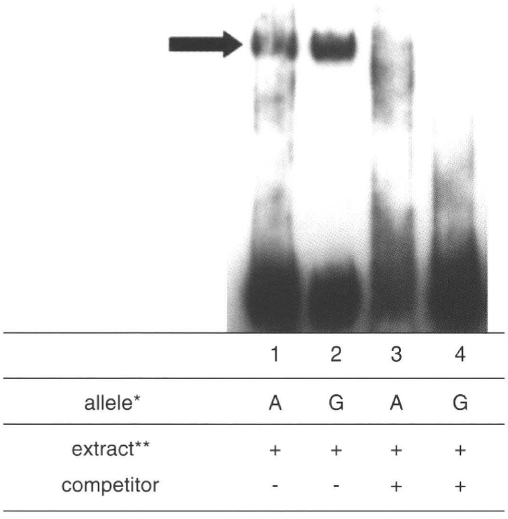

Figure 3 Electrophoretic mobility shift assay of fragments of the −603A→G region of the *TF* gene with nuclear extracts from BEAS 2B. Genomic fragments containing −603A or −603G were synthesized and used as DNA probes. A 200-fold molar excess of cold oligonucleotides was added in lanes 3 and 4. Increased formation of a specific DNA–protein complex was observed with the G-allele when compared with the A-allele (black arrow). *A:A allele, G:G allele, **BEAS-2B cells.

Allele-specific binding of nuclear proteins to the *TF* promoter

Electrophoretic mobility shift assay, using probes corresponding to the 20-bp sequence surrounding the −603A→G site, confirmed the binding of several transcription factors, and the varying affinity of A and G alleles for these factors, at this polymorphic site. Electrophoretic mobility shift assay was performed using nuclear extracts from BEAS-2B cells that were incubated with biotin-labeled oligonucleotide probes corresponding to the −603A or −603G allele. A specific DNA/nuclear extract complex was generated by both the A-allele and G-allele probes. Higher levels of this complex were observed with the −603G allele probe than with the −603A allele probe (Figure 3), suggesting that the −603G allele had a higher affinity for this binding factor and that the −603A→G transition might inhibit or eliminate this binding site. This DNA–protein complex was completely eliminated by 200-fold unlabeled −603A-allele or G-allele probes, indicating that this DNA–protein binding is likely specific for the −603A allele. As the factor does bind, although more weakly, to the −603A allele, this further suggests that the −603A→G transition might inhibit or eliminate this binding site (Figure 3). Two website databases (TESS and TFSEARCH) predicted that sequences surrounding the −603A→G polymorphic site in the promoter region of the *TF* gene could potentially bind the transcription factors E2F-1, E47, NF-1 or USF-1. However, none of the antibodies to these transcription factors revealed a supershifted complex in the presence of the −603A or −603G allele (data not shown).

DISCUSSION

In this study, we showed that the −603A→G polymorphism at the promoter region of the *TF* gene is associated with the development of

adult-onset asthma in a Japanese population. Further evidence of the importance of the −603G allele in the pathogenesis of asthma was provided by the observed significant association of this allele with increased transcriptional activity and strong binding of transcription factor(s) *in vitro*. Among several molecules involved in the coagulation system, TF has an important role in the initiation of coagulation and in the increase in thrombin activity at the site of inflammation.[30,31] TF binds activated factor VII, resulting in activation of factor IX and factor X and ultimately leading to thrombin and fibrin formation. In addition, TF has a role in tissue fibrosis through G-protein-coupled protease-activated receptors.[32] *In vivo* activation of protease-activated receptors leads to proinflammatory responses,[33,34] bronchoconstriction[35] and airway remodeling.[36,37] The level of TF in the sputum is higher in asthma patients than in healthy controls.[5] The combined data suggest that TF might be involved in the pathogenesis of asthma through its participation in a number of different pathways. Our findings in this genetic study are in line with this contention.

Asthma is a phenotypically heterogeneous disorder that results from complex interactions between environmental and genetic factors.[38] A precise definition of the asthma phenotype is now becoming more relevant not only to increase our understanding of pathophysiologic mechanisms but also to ascertain the specific genes associated with these phenotypes. The Kaplan–Meier analysis identified an age of 20 years at onset as the cutoff point for assigning subjects to a child-onset group and an adult-onset group. This approach made it possible to avoid prespecification of an arbitrary threshold and may be suitable for identification of homogeneous subsets for association studies. A cutoff age of 20 years is consistent with the observation that child-onset asthma and adult-onset asthma have distinct pathobiologic mechanisms. Patients with child-onset asthma were shown to have a significantly greater likelihood of allergic sensitization and clinical responses to triggers than patients with adult-onset asthma. Genetic factors related to atopy, including genes on chromosomes 5q31 and 11q13, are involved in child-onset asthma.[39–42] In contrast, asthma that develops during adulthood is more heterogeneous and a family history of asthma is often nonexistent. Although the role of genetic predisposition in adult-onset asthma is less clear than in atopic childhood-onset asthma, a specific host genetic factor might be important in the development of adult-onset asthma. It is noted that we previously reported a functional polymorphism in the *CCL5/RANTES* gene promoter that was associated with late-onset asthma, that is, starting after 40 years of age, in a Japanese population.[43] This study may have identified a new genetic factor involved in the development of adult-onset asthma.

Several epidemiologic studies showed that adult-onset asthma (age of onset $\geqslant$21 years) in particular is associated with cardiovascular diseases,[28] carotid atherosclerosis,[29] coronary heart disease or stroke.[28] Asthma may predispose an individual to atherosclerosis through specific pathobiologic pathways, perhaps linked to the chronic inflammatory responses of this disorder. Alternatively, the association between asthma and atherosclerosis may be caused by an inherent joint susceptibility to both diseases through shared inflammatory pathways and genetic factors. It is noted that the −603G gain-of-function allele in the *TF* gene has been associated with an increased risk of myocardial infarction[11] and venous thrombosis.[10] These data, together with the data presented in this study, suggest that *TF* might be a common genetic factor for the development of both asthma and several cardiovascular diseases. Further studies that incorporate precise data regarding the presence of cardiovascular diseases will help to clarify this speculation.

Several environmental factors have been identified, such as strong inducers of *TF* gene expression, including viruses,[44] bacteria,[45–47] other microbial pathogens such as cytomegalovirus, *Chlamydia pneumoniae*, *Streptococcus sanguis* and *Plasmodium falciparum*,[48–50] endotoxin[51] and oxidative air pollutant particulate matter.[52] In this study, we showed that the G allele in the −603 position is associated with a higher promoter activity than the A allele *in vitro*. Therefore, in individuals possessing the potentially functional −603GG genotype, *TF* may be expressed to a greater extent in response to an exogenous stimulus, ultimately leading to an exaggerated inflammation of the airways. Prolonged and repeated exposure to exogenous stimuli may be required for the −603GG genotype to manifest its genetic effects in airways, which may in part explain why the association of this allele was found only with adult-onset asthma but not with child-onset asthma. It has also been noted that the normal aging process alters the blood coagulation system in humans.[27] The plasma concentration of several coagulation factors increases in healthy humans in parallel with the physiological aging process.[53] In mice studies, stress-induced expression of TF in several tissues was substantially higher in aged mice than in young mice.[54] Accordingly, *TF* expression in subjects with the −603GG genotype may be enhanced to a greater extent by aging when compared with the carriers of other genotypes, and adult-onset asthma subsequently develops in these individuals, which is an alternative explanation for the heterogeneity of the genetic effects of the *TF* gene according to age at onset of the disease.

Sequence analysis indicated that, at the site of the −603A→G polymorphism, the A allele creates a potential E2F-1 or NF-1 binding site that might function in suppression of transcription according to the TESS database, and that the G allele creates a potential E47 or USF-1 binding site that might function in enhancement of transcription according to the TFSEARCH database. A specific DNA/nuclear protein complex was formed by the A-allele and G-allele probes. Furthermore, the G-allele showed enhanced formation of this specific DNA–protein complex compared with the A-allele. However, no difference in specific binding of these nuclear factors to the −603A→G polymorphism could be observed. Further studies are required to identify the specific transcription factor(s) that bind to this region, which may enable a demonstration of different binding affinities and thereby explain the varying transcriptional activity induced by this SNP.

A similar trend for association of the −603A→G polymorphism in the *TF* gene and adult-onset asthma was observed in the second population, although it was not statistically significant (P=0.064). Differences in several confounding factors between the two populations, which were recruited from geographically different areas of Japan, might explain this observation, as asthma is a complex disease associated with gene–gene and gene–environmental factor interactions. The age and sex distribution, as well as the frequency of atopic subjects, differed between the two populations. Information concerning smoking status was also lacking in the second population. Therefore, given the genetic heterogeneity underlying the pathogenesis of asthma, especially for patients who develop the disease in their adulthood, the existence of such similar trends in both populations supports the result obtained in the primary population.

Case–control studies based on questionnaires are susceptible to recall bias. Definitions of physician-diagnosed asthma have been shown to be specific but somewhat lacking in sensitivity and probably exclude milder cases of asthma.[55] In this study, to judge age at onset of asthma as accurately as possible, patients were asked about episodes of dyspnea, wheezing or coughing during childhood and puberty. In cases of uncertainty, the time of earliest respiratory symptoms was

designated as age at onset of asthma symptoms, which was used for the calculation of the duration of asthma. A recent study of age at onset of asthma in 4335 Japanese adult patients with asthma found that 26.3% developed the disease before the age of 20 years, and 73.8% developed the disease at 20 years or older.[56] This variation in age at onset of adult asthma is similar to that found in this study and is evidence that the self-reported data on age at onset of asthma obtained in this study are reliable.

In conclusion, the results of this case–control study and *in vitro* functional analysis, combined with the known important roles of the coagulation system in inflammatory lung diseases, suggest that TF is involved in the pathogenesis of asthma. Further study to determine the mechanism by which risk for adult-onset asthma is mediated by specific genetic variations in *TF* should improve our understanding of the molecular basis of asthma.

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ORIGINAL ARTICLE

Replication of genetic association studies in asthma and related phenotypes

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In asthma genetics, the association of highly replicated susceptibility genes lacks consistency across populations. To identify genuine associations, we investigated the reproducibility of the 23 most promising asthma and asthma-related candidate genes in a moderately sized sample from the Japanese population. We compared the frequency of 33 polymorphisms in unrelated cases and controls and tested for their association with asthma, atopy and serum total IgE levels using allele frequency, codominant, dominant and recessive genotype models. On the basis of the consistency of our findings with previous meta-analyses and large replication studies, *IL13*, *TNF*, *ADAM33*, *IL4RA* and *TBXA2R* might represent common major asthma and asthma-related trait genes. Individual gene assessment was extended to the interactions between two polymorphisms using our original method. Interactions between *TBXA2R* and *ADAM33*, and *IL4RA* and *C3* were suggested to increase the risk for childhood and all asthma (adult and childhood asthma combined). The confirmation of previously reported associations between gene polymorphisms and phenotypes was problematic when as few as several hundred samples per group were used. Stratification of the subjects by environmental factors or other confounding factors may be necessary to improve the sensitivity and reliability of association results.
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INTRODUCTION

Asthma is a heritable trait[1] and investigations to determine the genetic components underlying asthma using linkage mapping and the candidate gene approach have been carried out. By 2006, more than 100 genes were associated with asthma and asthma-related phenotypes;[2] 25 of these genes have been replicated in more than six independent association studies. In 2008, this list was complemented with an additional three genes, *FLG*, *NAT2* and *CCL15*.[3] However, no single polymorphic marker or gene locus has been unanimously labeled as a strong and independent genetic determinant of asthma, and the results for the highly replicated genes have been inconsistent across the tested populations.[3]

To identify true associations, it is of critical importance to comprehensively replicate the initial finding.[4] To this aim, we investigated whether the 23 most replicated genes for asthma and asthma-related phenotypes were positively associated with extrinsic childhood and adult asthma, atopy and total serum IgE levels in a moderately sized sample drawn from the Japanese population.

We also tested eight genes that were significantly associated with asthma in our subjects: *IL13*,[5] *TBXA2R*,[6] *GSTP1*,[7] *ADAM33*,[8] *MMP9*,[9] *IL12B*,[10] *C3*[11] and *SOCS1*.[12] The re-evaluation of these associations is conditioned by the limitations of the original reports in which childhood asthma included subjects with nonatopic asthma and those who were <4 years of age. The adult asthma cases also included nonatopic asthma in some of these reports. Moreover, the comparison of the childhood asthma group was with an adults-only control group. In this study, we redefined the atopic asthma patients, introduced age-matched child controls and re-evaluated the association of these genes with the asthma phenotypes, atopy and total serum IgE levels.

Further, we extended the assessment of individual genes to identify potential interactions between the genes, as increasing knowledge about biological pathways and gene networks implies that gene–gene interactions are important and must be taken into account when estimating the genetic risk of a disease.[13]

MATERIALS AND METHODS

Study population

The asthma population was restricted to extrinsic asthma patients with subsequent distinction between childhood and adult asthma by cutoff age of below or above 16 years old regardless of the age of the disease onset. We recruited 325 subjects with childhood atopic asthma, 367 adults with atopic

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asthma and 646 adult controls from Osaka City, Japan. The details of these subjects have been described elsewhere.[10,12,14] For childhood controls and the investigation of total and specific IgE levels, we recruited children attending an elementary school in Chiba City, Japan. The clinical characteristics of this population as well as inclusion and exclusion criteria have been described previously.[15] In brief, after the exclusion of questionnaire-assessed asthma and/or atopic dermatitis subjects and those with congenital heart diseases and lung diseases caused by premature birth, 336 children having a complete set of information on the total and eight specific IgE levels, genotypes and environmental factors were assigned to the child control group. The mean ages (range) of the four groups were as follows: childhood asthma, 9.9 (4–15); adult asthma, 45 (16–83); child controls, 9.3 (6–12); and adult control, 43.7 (20–75) years. Written informed consent was received from all participants and the study was approved by the ethics committees of Chiba University Graduate School of Medicine and RIKEN.

Gene and polymorphism selection

The list of candidate gene polymorphisms included in this study, their location within the gene and corresponding rs numbers are given in Supplementary Table S1. In this table, we also included the allele frequency in child and adult control populations.

The most replicated genes were selected based on the list created by Ober and Hoffjan.[2] From the 25 cited genes, we intentionally excluded *HLA-DRB1* and *HLA-DBP1* from our analysis due to the high number of variants linked to asthma and asthma-related phenotypes, the genotyping of which would surpass our capacity. The remaining 23 genes were tested for association based on the most positively reported polymorphisms and are represented by Group 1 (>10) and Group 2 (6–10), depending on the number of their replications (Supplementary Table S1). We included four genes (*MMP9*, *IL12B*, *C3* and *SOCS1*), which were not in the original 23 genes, but were found to be associated with asthma in our previous studies (Supplementary Table S1, Group 3). These were tested for association with the same positive polymorphisms as in the initial reports.

DNA extraction and genotyping

Genomic DNA was prepared from peripheral blood samples using the standard protocols. Whole genome amplification was carried out using the Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. Genotyping was carried out by means of allele-specific amplification, single-nucleotide primer extension reaction or fragment analysis of the PCR product. The genotyping methods used for each polymorphism are given in Supplementary Table S1 and the primer sequences are shown in Supplementary Tables S2, S3a, S3b and S4. The results obtained by these molecular assays were analyzed on ABI PRISM 3100 Genetic Analyzer and ABI PRISM 7000 Sequence Detector Systems (Applied Biosystems, Foster City, CA, USA), or by using Chromo4 Real-Times System (Bio-Rad Laboratories, Hercules, CA, USA). The detailed genotypic protocols are described in Supplementary Methods.

Statistical analysis

A case–control study design was used to analyze the associations between gene variants and the dichotomous phenotypes. We calculated allele frequencies and tested their agreement with Hardy–Weinberg equilibrium (HWE) using a χ^2 goodness-of-fit test. We compared differences in the allele frequencies and genotype distribution of each polymorphism between the case and control subjects by using a 2×2 (allele) or 2×3 contingency χ^2-test (dominant, codominant and recessive genotype models) with 1 or 2 degree of freedom. Total serum IgE values were logarithm transformed to approximate a normal distribution and analyzed as a quantitative trait using two different tests: linear regression analysis with age and sex as covariates; and analysis of variance (ANOVA). All statistical analyses were carried out with SPSS Statistics 17.0 (SPSS Japan Corporation, Tokyo, Japan). The statistical power of this study was calculated using SamplePower 2.0 (SPSS Corporation). *P*-values <0.05 were judged to be significant; as there was an *a priori* hypothesis with all tested polymorphisms, we did not adjust the significance levels for multiple testing.

Interactions between two polymorphisms were screened using a program written in Excel VBA (Microsoft Japan, Tokyo, Japan). This program identified every possible pairing of polymorphisms, calculated the χ^2 and *P*-values of contingency tables between the genotype of one polymorphism and the disease after stratification by the genotype of the other polymorphism. The program then calculated the χ^2 and *P*-values for the goodness-of-fit statistics of the distribution pattern of genotype vs disease table with stratification against no stratification. Single-nucleotide polymorphisms (SNPs) in the same gene were not tested for this interaction because most of them showed linkage disequilibrium.

RESULTS

Statistical power of this study

We estimated the frequency difference between the case and control groups, and odds ratio (OR) for our samples with different genotype/allele frequencies according to sample size. We set our detection power at 80% with an α level of 0.05.

Supplementary Table S5a shows the results of this calculation for the asthma cases, when estimation was carried out for given genotype/allele frequencies among their respective control peers. Adult asthma group had slightly better power than the childhood asthma group because of almost twice bigger number of adult controls compared with the child controls. In the case of adult asthma, enough (>80%) power could be expected for polymorphisms with an OR of 1.4 when the minor genotype frequency was from 30–60%. In the allele frequency comparison, there was slightly more detection power than for the genotype comparison, as the number of alleles was twice that of the genotype. When we estimated the genotype/allele frequencies in child asthmatics for given adult control genotype/allele frequencies (Supplementary Table S5b), as expected, some increase in power could be observed with ORs getting closer to those of childhood asthma vs child control estimation. In case of calculation based on the comparison between combined asthma subjects and combined controls (Supplementary Table S5b), ORs of 1.3 and 1.2 could be detected for minor genotype and allele frequencies of 30–60%, respectively.

The estimated power to detect an association between atopy and genetic polymorphisms (Supplementary Table S6) was comparatively inferior to that of asthma with an OR of 1.9.

Association of the 23 genes with childhood and adult atopic asthma

The genotype frequencies of all gene variants, except for *GSTM1* ins/del, were in Hardy–Weinberg equilibrium (goodness-of-fit χ^2-test, $P>0.01$) in adult and child controls. We could not test the *GSTM1* ins/del variant for Hardy–Weinberg equilibrium because the genotyping method did not distinguish between ins/del heterozygotes and homozygotes.

The summary of results for basic comparison between cases and controls is presented in Table 1, in which only polymorphisms with *P*-value <0.05 in at least one genetic model test are shown. When the frequency of polymorphisms in childhood asthmatics were compared with those of asthma free children, the most strong candidate variant was *IL13* −1112C>T, which was significantly associated in all but dominant genotype model; *LTC4S* was positively associated in the allele frequency and dominant model tests and *CCL5* −28C>G resulted in significant difference only in the dominant genotype model. In the adult samples, there were significant differences noted between the controls and the asthma patients for *TNF*, *ADAM33* and *NOS1*. *TNF* showed positive results in the allele frequency, codominant and dominant models. Association of *ADAM33* Met764Thr variant with adult asthma was significant for allele frequency and dominant genotype models. There was strong association of both *NOS1* variants with the disease in all tests except for GT repeat intron

Table 1 Association of genotyped polymorphisms with the asthma phenotypes (basic comparisons)

		Case–control study P-*value*											
		Childhood asthma vs child control				*Adult asthma vs adult control*				*All asthma vs all controls*			
Gene	*Polymorphism*	*Allele*	*Codom.*	*Dom.*	*Rec.*	*Allele*	*Codom.*	*Dom.*	*Rec.*	*Allele*	*Codom.*	*Dom.*	*Rec.*
Group 1													
IL13	Arg110Gln	×	×	×	×	×	×	×	×	×	×	×	×
	−1112C>T	0.014	0.011	×	0.003	×	×	×	×	×	×	×	×
TNF	−1037C>T	×	×	×	×	0.001	0.003	0.001	×	0.003	0.014	0.005	×
ADAM33	Met764Thr	×	×	×	×	0.008	×	0.005	×	0.029	×	0.035	×
	13236C>T	×	×	×	×	×	×	×	×	0.038	×	×	×
Group 2													
LTC4S	−444A>C	0.023	×	0.026	×	×	×	×	×	×	×	×	×
NOS1	GT repeat intron 2 (187 allele)	×	×	×	×	0.0003	0.002	0.001	0.011	0.026	×	×	×
	GT repeat intron 2 (183 allele)	×	×	×	×	0.007	0.022	0.007	×	0.045	×	×	×
CCL5	−403A>G	×	×	×	×	×	×	×	×	×	×	×	×
	−28C>G	×	×	0.048	×	×	×	×	×	×	×	×	×
Group 3													×
MMP9	2127G>T	×	×	×	×	×	×	×	×	×	×	×	×
	5546G>A	×	×	×	×	×	×	×	×	×	×	×	×
IL12B	−6415CTCTAA>GC	0.010	0.006	×	0.001	×	×	×	×	0.012	0.009	×	0.002
	1146 C> A	0.019	0.010	×	0.003	×	×	×	×	0.003	0.003	×	0.001
C3	Block 2 (haplotype 6)[a]	0.017	×	×	×	×	×	×	×	0.015	0.022	0.010	×
	Block 4 (haplotype 1)[a]	×	×	×	×	0.001	0.002	0.024	0.002	0.001	0.005	0.003	0.019
SOCS1	−1478CA>del	×	×	×	×	0.0005	0.002	0.002	0.015	0.002	0.005	0.006	0.017

Abbreviations: Allele, χ^2-test of allele frequency; codom., 2×3 codominant model genotype χ^2-test; dom., 2×2 dominant model genotype χ^2-test; rec., 2×2 recessive model genotype χ^2-test.
[a]For haplotype description please refer Inoue *et al*.[11]
Polymorphisms with a *P*-value ⩾0.05 in all association tests of the four genetic models are not shown.
×$P \geqslant 0.05$.

(183 allele) in the recessive genotype model. In the association test between the polymorphisms and all asthma, that is child and adult asthma combined, significant associations were observed for the same genes as in adult asthma. *TNF* and *ADAM33* Met764Thr remained positively associated with asthma in the allele frequency, codominant and dominant models alike in the single adult asthma group comparison. The association of *NOS1* variants became weaker with significance only in allele frequency model. Additional significant association was observed only for *ADAM33* 13236C>T in the allele frequency model.

Conditioned by the inherent characteristics of our case and control samples, we extended our analysis further (Table 2). In our child control group, we recognize the potential presence of asthma susceptibility gene variants carriers who might become asthmatics later in life, and consequently, to become a source of bias. Addressing this issue, we tested childhood asthmatics also with adult controls, considering the later ones as more reliable asthma 'free' subjects. Paradoxically, the previous significant associations found in the comparison for child asthma vs child control disappeared; instead, *TNF*, *IL4RA*, *ADAM33*, *GSTM1*, *AAA1* and *NOS1* showed significant results. The substantial difference in results between childhood asthma vs child control and childhood asthma vs adult control tests might be due to some confounding effects of hidden genetic and environmental heterogeneities between child and adult control groups.

Furthermore, as the natural history of asthma in some adults starts during childhood, we characterized the association results after stratifying adult asthma cases by age at the onset (Table 2). Using the same cutoff age (<16 years) as for discerning childhood asthma from adult asthma, we obtained 118 adults (32% of adult asthma), who have reported physician diagnosed asthma during their childhood, and 249 adults with newly onset bronchial asthma. To elucidate the genes associated with differential onset of asthma in children, we merged the child-onset adult subgroup with childhood asthma group (all L16 adult atopic asthma (BA)) and tested with either child or adult controls by applying the same strategy as used in the nonstratified adult asthma. In the association test with child control, besides the previously detected gene variants in the comparison of child asthma vs child control, *TNF* showed at least one significant association in four association tests, whereas the significance of *CCL5* disappeared. When the combined child-onset asthma group was compared with adult controls, the observed associations in child asthma vs adult control comparison could be confirmed for only *IL4RA* Ile50Val and *ADAM33* 13236C>T polymorphisms. We next examined the new-onset adult asthma, in which *TNF* and *ADAM33* Met764Thr have retained their significant associations found with the onset nonstratified adult asthma. In addition, *IL13*, *IL4RA* and *GSTM1* have emerged as genes associated with asthma establishment during adulthood. Interestingly, the two *ADAM33* polymorphisms were associated with different asthma phenotypes when comparisons were carried out with adult control. *ADAM33* Met764Thr was significantly associated with adult asthma, whereas *ADAM33* 13236C>T variant was significantly more frequent in childhood asthma and all child-onset asthma groups.

We also compared the distribution of polymorphisms between the two control groups; it showed a relative homogeneity with significant

Table 2 Summary of the association results between polymorphisms and asthma when case and control groups were compared in various combinations

		Two group comparison							
Gene	*Polymorphism*	*Child BA vs child CO*	*Child BA vs adult CO*	*Adult BA vs adult CO*	*All L16 BA vs child CO*	*All L16 BA vs adult CO*	*M16 adult BA vs adult CO*	*Child CO vs adult CO*	*Child BA vs adult BA*
Group 1									
IL13	Arg110Gln	×	×	×	×	×	×	×	×
	−1112C>T	°	×	×	°	×	°	°	×
TNF	−1037C>T	×	°	°	°	×	°	×	×
IL4RA	Ile50Val	×	°	×	×	°	°	×	×
	Gln551Arg	×	×	×	×	×	×	×	°
ADAM33	Met764Thr	×	×	°	×	×	°	×	°
	13236C>T	×	°	×	×	°	×	×	°
Group 2									
GSTM1	Ins/del	×	°	×	×	×	°	×	°
IL10	571C>A	×	×	×	×	×	×	×	°
LTC4S	−444A>C	°	×	×	°	×	×	°	×
AAA1	522363G>C	×	°	×	×	×	×	×	°
NOS1	GT repeat (187 allele)	×	°	°	×	×	×	×	°
	GT repeat (183 allele)	×	×	°	×	×	×	×	×
CCL5	−28C>G	°	×	×	×	×	×	×	×
Group 3									
MMP9	2127G>T	×	°	×	×	×	°	°	×
	5546G>A	×	°	×	×	°	×	°	×
IL12B	−6415ins/del	°	°	×	°	°	×	×	°
	1146 C> A	°	°	×	°	°	×	×	°
C3	Block 2 (haplotype 6)[a]	°	×	×	°	°	×	×	×
	Block 4 (haplotype 1)[a]	×	°	°	×	°	×	×	×
SOCS1	−1478CA>del	×	×	°	×	°	×	×	×

Abbreviations: Child BA, childhood atopic asthma; child CO, child controls; adult BA, adult atopic asthma; adult CO, adult controls; all L16 adult BA, childhood atopic asthma and adult atopic asthma at onset age <16 years combined; M16 adult BA, adult atopic asthma at onset age ⩾16.
[a]For haplotype description please refer Incue *et al.*[11]
Polymorphisms with a *P*-value ⩾0.05 in all association tests of the four genetic models are not shown.
°*P*-value <0.05 in at least one association test of the four genetic models.
×None of the *P*-value in the four genetic models was <0.05.

difference only for *IL13* and *LTC4S*. It indicates that there would be little bias created using either sample as a control group for any of the case groups and justifies our combining of the two control groups to form a single one in the comparison for all asthma vs all control. In the comparison test for childhood asthma vs adult asthma, *IL4RA*, *ADAM33*, *GSTM1*, *IL10*, *AAA1* and *NOS1* genes were found significantly different, which is coherent with the case–control comparison results.

Association of the 23 genes with atopy

ADAM33 13236C>T, *NOS1* GT repeat 183 bp allele and *TBXA2R* 795T>C were significantly associated with atopy in the recessive or dominant models (Supplementary Table S7). *NOS1* and *TBXA2R* also showed significant association in the allele frequency test, whereas *ADAM33* did not.

Association of the 23 genes with total serum IgE

Supplementary Table S8 summarizes the relationship between the mean of $\log_{10}$-transformed total serum IgE level and gene variants. There was a significant association between *GSTP1* and total IgE level as determined by ANOVA that remained significant after adjusting for age and gender as covariates in linear regression analysis.

Re-evaluation of the associations previously discovered in our subjects

We confirmed the association of the *IL13*, *ADAM33*, *MMP9*, *IL12B*, *C3* and *SOCS1* polymorphisms with atopic asthma (Tables 1 and 2). The association of *TBXA2R* with asthma was not replicated; instead, we identified an association of *TBXA2R* with atopy (Supplementary Table S7). Comparatively to our previous studies, in this study we also investigated the polymorphisms for association with serum IgE level. Significant results were observed for *GSTP1* as described above and for *MMP9* 5546G>A (Supplementary Table S8).

Screening of gene–gene interactions

From the 40 genetic polymorphisms, polymorphism pairs with a *P*-value for association with the disease of <0.05 and a *P*-value for interaction <0.01 are listed in Table 3. A pair of *LTA* and *TNF* SNPs showed a very small *P*-value; however, these two genes are physically close and these SNPs are known to be in linkage disequilibrium. Thus, this combination was omitted from the table. Interactions between *TBXA2R* and *ADAM33* and between *IL4RA* and *C3* were suggested for childhood asthma and all asthma. These interactions were our most robust results.

Table 3 Screening of gene–gene interactions

			Effect of SNP 1 on association between SNP 2 and disease[a]			*Association between SNP 2 and disease after stratification by SNP 1*		
SNP 1	*SNP 1 genotype*	*SNP 2*	χ^2	*d.f.*	P-*value*	χ^2	*d.f.*	P-*value*
Childhood asthma								
LTA −735G>A	A/A	*IL12B* 1146C>A	7.12	1	0.0076	18.1	2	0.00012
IL4RA Ile50Val	Ile/Ile	*C3* block2 (haplotype 6)[b]	11.63	1	0.00064	13.42	1	0.00025
TBXA2R 924C>T	C/T	*ADAM33* 313236C>T	9.51	2	0.0086	14.02	2	0.0009
SPINK5 Lys420Glu	G/G	*ADAM33* 313236C>T	12.93	2	0.0016	12.24	2	0.0022
AAA1 522363G>C	C/C	*IL4* 33C>T	10.98	2	0.0041	8.88	2	0.012
AAA1 522363G>C	C/C	*IL4* −590T>C	9.31	2	0.0095	7.67	2	0.022
CCL5 −28C>G	G/G	*IL13* Arg110Gln	7.2	1	0.0073	4.69	1	0.03
CD14 −550C>T	T/T	*IL10* −571C>A	7.1	1	0.0077	4.11	1	0.043
Adult asthma								
SOCS1 −1478CA>del	CA/del	*CTLA4* −318C>T	7.38	1	0.0066	6.36	1	0.012
TNF −1037C>T	T/T	*CCL5* −403A>G	8.51	1	0.0035	5.25	1	0.022
LTA −753G>A	A/A	*SPINK5* Lys420Glu	8.65	1	0.0033	6.95	2	0.031
CD14 −550C>T	T/T	*IL4* −590T>C	11.01	2	0.0041	6.45	2	0.04
All asthma								
TBXA2R 924C>T	C/T	*ADAM33* 313236C>T	12.76	2	0.0017	11.83	2	0.0027
IL4RA Ile50Val	Ile/Ile	*C3* block2 (haplotype 6)[b]	7.29	1	0.0069	6.62	1	0.01
ADAM33 313236C>T	C/T	*TBXA2R* 924C>T	8.05	1	0.0046	8.99	2	0.011
TNF −1037C>T	T/T	*CD14* −159C>T	7.04	1	0.008	7.34	2	0.026
STAT6 GT repeat	172/172	*CC16* 38A>G	11.01	2	0.0041	6.9	2	0.036

Abbreviations: d.f., degree of freedom; SNP, single-nucleotide polymorphism.
[a]Polymorphism combination that demonstrates the interactions with $P<0.01$ and an association with $P<0.05$ are shown.
[b]For haplotype description please refer Inoue *et al.*[11]

DISCUSSION

We conducted our study in three phases. We first, under the assumption of the common disease–common variant hypothesis, investigated whether the 23 most promising asthma/atopy candidate genes retained their association in a Japanese population set. We considered the gene as the unit of our replication, and the gene was judged as positively replicated if demonstrated a statistically significant association with one or more phenotypes (atopic asthma, atopy and total serum IgE level) in at least one of four genetic models. In our samples, among the highly replicated genes (>10 positive associations), *IL13*, *TNF*, *IL4RA* and *ADAM33* maintained their reputation as robust asthma and asthma-related candidate genes. From the genes with a lower replication rate (6–10), we confirmed the associations of *GSTM1*, *GSTP1*, *LTC4S*, *AAA1*, *NOS1*, *CCL5* and *TBXA2R*. In the second phase of the study, we screened our initial significant asthma associations to *IL13*, *TBXA2R*, *GSTP1*, *ADAM33*, *MMP9*, *IL12B*, *C3* and *SOCS1*. These associations were replicated for all genes, except for *TBXA2R* and *GSTP1*, which were associated with atopy and total serum IgE level, respectively. In the final phase, we explored the potential multigenic effect of all 27 candidate genes (the three groups of genes combined) in the expression of asthma phenotypes based on a pairwise method.

If we summarize our findings from the replication study, interesting patterns of associations could be observed. Except for *IL13* and *IL4RA*, we found no association for the prominent genes implicated in innate immunity and immunoregulation with asthma or asthma-related phenotypes, that is, *CD14*, *IL10* and *TGFB1* as well as the Th2 cytokines and their receptors represented by *IL4*, *STAT6* and *MS4A2*. Instead, the genes secreted from airway epithelial cells (for example, *CCL5* and *AAA1*), and genes known to affect lung function, mediate inflammatory conditions and participate in airway remodeling (for example, *TNF*, *ADAM33*, *GSTM1*, *GSTP1*, *LTC4S*, *NOS1* and *TBXA2R*) demonstrated statistically significant associations. This observation may indicate the relatively higher predisposing effect that these two groups of genes exert on the development of asthma-related phenotypes in the Japanese population. Our postulation is supported by the hypothesis that not the dysregulated immune response, but the inherently abnormal respiratory epithelium of asthmatics and the reactivation of the epithelial–mesenchymal trophic unit leading to pathological airway wall remodeling has a major role in the disease.[16,17] Another interesting finding was the distinct partition of genes between adult and child asthma. The association of *LTC4S*, *AAA1* and *CCL5* specifically in the child samples might reflect the differing etiopathogenetic background of childhood asthma.[18,19] For example, *CCL5* is a key chemokine recruiting Th1 and Th2 proinflammatory cells, and its expression in epithelial cells is induced by the respiratory syncytial virus (RSV).[20,21] This is in line with the evidences that the epithelial barrier in young asthmatics is inherently abnormal[22] and that RSV bronchiolitis is a more important risk factor for the development of asthma and atopy up to the age of 7 years than heredity or environmental factors.[23] Moreover, the candidate genes showing significant association with both the phenotypes, when the case groups were compared with adult controls separately (childhood asthma vs adult control and adult asthma vs adult control), were the same as those observed in the combined analysis (all asthma vs all controls). Thus, *TNF*, *ADAM33* and *NOS1* might represent the common susceptibility gene for adult and childhood asthma. When we categorized asthma cases by the age at onset, the number of shared

significant genes increased (*IL13*, *TNF*, *IL4RA* and *ADAM33*), making in the overall no substantial differences in the genetic determinants between child-onset and adult-onset asthmas. The exception was for *LTC4S* and *GSTM1*, suggesting the former gene to be related with the development of asthma during childhood and the later one during adulthood. However, as *GSTM1* was significantly associated with childhood asthma in the comparison of childhood asthma vs adult control, it is difficult to conclude whether the polymorphism in this gene affected the susceptibility to new-onset adult asthma.

Our replication results were in agreement with several large-scale studies. A recent review of the literature revealed that five asthma candidate genes, *ADAM33*, *TNF*, *TBXA2R*, *CD14* and *LTC4S*, were the focus of several meta-analyses in which *ADAM33* and *TNF* had a modest association with asthma.[24] The first genome-wide replication study of 39 asthma candidate genes generated *IL4RA* results that were consistent with our observations.[25] In the most comprehensive replication study carried out to date, the reproducibility of 93 genes previously associated with asthma and/or asthma intermediate traits was tested.[26] *IL13* was associated with asthma, and *TBXA2R* was associated with atopy, as we also observed in this study. Our replication rate of 48% (11 genes out of 23; OR 1.15–1.62, if the outlier OR of 3.01 (95% confidence interval, 1.40–6.51) for *IL13* −1112C>T is excluded) was higher than that reported in previous large association studies (for OR see Table 4); the study of Daley *et al.*[26] (unrelated case–control sample of $N=5565$) and a genome-wide screen of 422 nuclear families using SNP arrays had low replication rates of 13% (12 out of 93 tested genes, OR <1.4) and 15.4% (6 out of 39 at SNP-level replication, OR 1.4–1.7), respectively. This better replication rate might be attributed to our sample size, as it is well documented that smaller studies have a tendency to have more favorable outcomes than larger ones.[27] Daley *et al.* concluded that many published associations for asthma and atopy may be false-positive results. Whereas Rogers *et al.*[25] suggested that the poor coverage of genome-wide association study genotyping platforms and lack of statistical power due to insufficient sample size were the main reasons for their low replication. We are more inclined to suspect the 'contextual' bias explaining our failure to replicate all candidate genes. By that we mean the confounding effect of the whole complex network of gene–gene and gene–environmental interactions. This can be seen from the controversy in the findings between this current study and our previous one. In this study, *CD14* −550C>T and *IL4RA* Ile50Val were not associated with total serum IgE level. Whereas, in our recent association study carried out on the same school children, these two gene variants had a modifying effect on the levels of total IgE later in life depending on the children's attendance of day care before 2 years of age.[15] This association could be detected because the day care attendance was taken in consideration as an environmental factor and the effect of a gene was investigated simultaneously with the effect of the other one.

In the gene–gene interaction analysis, we identified some statistical interactions that asserted the weak associations found in the individual gene assessment. Among them, significant interaction between *C3* and *IL4RA* and between *ADAM33* and *TBXA2R* were observed for both childhood and all asthma groups. Although straightforward functional evidences of such paired interactions are lacking, some plausibility can be inferred. C3 or complement component 3 is an important part of the innate immunity recognizing exogenous and endogenous molecular patterns. Some functions of its C3a subtype indicate a possible role for the complement system in asthma pathogenesis.[28] In allergen-sensitized mouse model of pulmonary allergy deficient in *C3* or in its receptor *C3aR*, Drouin *et al.*[29,30] have observed that in the mutant mice the characteristic manifestations of asthma were significantly attenuated compared with wild-type animals and that in the lung the number of interleukin 4 (IL4)-producing cells was decreased; whereas Kawamoto *et al.*[31] showed that the absence of *C3aR* in mice results in significantly increased level of Th2 cytokines (IL4, IL5 and IL10). In spite of the fact that the two groups' results are contradictory calling for further examination, the observed functional relationship clearly indicates a modulator role of C3 on IL4 cytokine expression. IL4 signal transduction is mediated through the α subunit of the IL4 receptor (IL4RA), which is IL4 specific. Thus, the *C3-IL4-IL4RA* axis might be one of the plausible models for the interaction between *C3* and *IL4RA*. With regard to *ADAM33* and *TBXA2R*, one common feature that could indicate their putative interaction is their involvement in angiogenesis, a process frequently underestimated in the pathophysiology of asthma.[32] Novel findings on *ADAM33* showed that its catalytic domain promoted endothelial cell (EC) proliferation *in vitro*, and formation of new vessels *ex vivo* and *in vivo*.[33] *TBXA2R* is also known to be implicated in neovascularization but in an opposed way: suppresses EC migration and angiogenesis by inhibiting the effector pathways of the vascular endothelial growth factor (VEGF), a key angiogenic and chemotactic regulator of EC.[34] Although the exact mechanism by which *ADAM33* exerts its proangiogenic effect is yet to be elucidated, the involvement of VEGF is likely to take place. In that case, the above findings will suggest interactive effect of *ADAM33* and *TBXAR* on VEGF regulation and consequently on angiogenesis and microvascular remodeling of conductive airways in asthma.

Nevertheless, the significant results of our replication study as well as of the gene–gene interactions investigation should be interpreted with caution for inflation of type 1 errors. We have presented our findings based on the nominal α threshold of <0.05 without taking into account multiple testing. Relative to the replication study, this study is not an exploratory study aimed to find a 'significant' gene from multiple candidates but rather to test for confirmation of previously well-established hypotheses. Indeed, the genes from Group 1 and Group 2 are the top asthma and allergy related genes, each replicated in at least six or more independent populations, meaning they all have a high previous probability to show true associations even in the case of a relaxed threshold value for significance. However, if we adjusted for multiple comparisons by the Bonferroni method, none of our significant findings would survive this stringent level of correction. It is obvious that the power is enough to detect genetic effect with OR of around 1.4 with the current sample size, but not if we consider multiple testing. The same is for the results obtained from the screening of the interactions between two polymorphisms. If we strictly applied Bonferroni correction, the significant *P*-values would need to be in the order of 9.46×10^{-5} (0.05/528) because we carried out $33C^2=528$ tests for each phenotype; no *P*-value reached this value. Thus, our findings for the potential gene–gene interactions must be evaluated physiologically or by analyses of other sets of samples to validate these observations.

There are other limitations to this study. We focused on the effect of genetic polymorphisms on dichotomous phenotypes and ignored clinical severity and environmental factors. There was also a delay between the recruitment of child asthma cases and child control samples, which could be a source of bias due to differences in DNA processing as well as in environmental exposure. Although population stratification was not controlled in this study, we consider the confounding effect of this factor to be of a lesser extent in comparison to studies conducted on North American[35,36] or Western European[37,38] populations. From the genetic point of view, this

Table 4 Odds ratio and 95% CI of significant polymorphisms found in the basic association studies

		OR (95%CI)		
Gene	*Polymorphism*	*Allele*	*Dom.*	*Rec.*
Childhood asthma vs child control				
Group 1				
IL13	Arg110Gln	×	×	×
	−1112C>T	1.40 (1.07–1.84)	×	3.01 (1.40–6.51)
ADAM33	Met764Thr	×	×	×
	3236C>T	×	×	×
Group 2				
LTC4S	−444A>C	1.40 (1.05–1.88)	1.47 (1.05–2.06)	×
CCL5	−403A>G	×	×	×
	−28C>G	×	1.43 (1.00–2.05)	×
Group 3				
IL12B	−6415CTCTAA>GC	1.33 (1.07–1.66)	×	1.78 (1.25–2.55)
	1146 C> A	1.30 (1.04–1.61)	×	1.73 (1.21–2.48)
C3	Block 2 (haplotype 6)	1.92 (1.12–3.31)	×	×
	Block 4 (haplotype 1)	×	×	×
Adult asthma vs adult control				
Group 1				
TNF	−1037C>T	1.53 (1.20–1.96)	1.62 (1.22–2.16)	×
ADAM33	Met764Thr	1.47 (1.10–1.96)	1.57 (1.14–2.15)	×
	13236C>T	×	×	×
Group 2				
NOS1	GT repeat intron 2 (187allele)	1.42 (1.20–1.71)	1.55 (1.19–2.03)	1.56 (1.10–2.19)
	GT repeat intron 2 (183allele)	1.29 (1.07–1.55)	1.48 (1.11–1.97)	×
Group 3				
C3	Block 2 (haplotype 6)	×	×	×
	Block 4 (haplotype 1)	1.34 (1.14–1.64)	1.38 (1.04–1.84)	1.60 (1.19–2.14)
SOCS1	−1478CA>del	1.73 (1.27–2.36)	1.69 (1.20–2.37)	3.93 (1.20–12.86)
All asthma vs all controls				
Group 1				
TNF	−1037C>T	1.32 (1.10–1.59)	1.36 (1.10–1.68)	×
ADAM33	Met764Thr	1.26 (1.02–1.56)	1.28 (1.02–1.61)	×
	13236C>T	1.19 (1.01–1.40)	×	×
Group 2				
NOS1	GT repeat intron 2 (187allele)	1.17 (1.02–1.35)	×	×
	GT repeat intron 2 (183allele)	1.15 (1.00–1.33)	×	×
Group 3				
IL12B	−6415CTCTAA>GC	1.20 (1.04–1.37)	×	1.44 (1.14–1.81)
	1146 C> A	1.24 (1.08–1.42)	×	1.49 (1.18–1.88)
C3	Block 2 (haplotype 6)	1.53 (1.08–2.15)	1.58 (1.11–2.25)	×
	Block 4 (haplotype 1)[a]	1.27 (1.10–1.45)	1.39 (1.21–1.73)	1.31 (1.05–1.63)
SOCS1	−1478CA>del	1.47 (1.16–1.96)	1.43 (1.11–1.86)	3.09 (1.17–8.16)

Abbreviations: Allele, χ^2-test of allele frequency; CI, confidence interval; dom., 2×2 dominant model genotype χ^2-test; OR, odds ratio; rec., 2×2 recessive model genotype χ^2-test.
[a]For haplotype description please refer Inoue *et al*.[11]
Polymorphisms with a *P*-value ⩾0.05 in all association tests of the four genetic models are not shown.
×$P \geqslant 0.05$.

assumption is based on the fact that our control subjects were residents of the mainland of Japan, the population of which belongs to the genetically homogeneous Hondo cluster,[39] and also on the results of genomic control analysis[40] that showed the populations from the Kinki and Kanto regions (where we recruited our samples and controls) do not differ in the allele frequency of the null marker. In terms of stratification determined by an individual's socioeconomic position, we would refer to the specific egalitarian characteristic of the Japanese society in support of our claim.[41]

In conclusion, our findings and previous studies suggest that *IL13*, *TNF*, *IL4RA*, *ADAM33* and *TBXA2R* might represent the major asthma and asthma-related traits genes common across populations. *GSTM1*, *GSTP1*, *LTC4S*, *AAA1*, *NOS1* and *CCL5* along with *MMP9*, *IL12B*, *C3* and *SOCS1* might be additional susceptibility genes, which have stronger effects in the Japanese population. Despite our failure to replicate the other genes, our results were not strong enough to eliminate them from the candidate gene list because we did not investigate all known variations in these genes and we did not consider

the effects of environmental factors. Replication studies of genotype–phenotype associations with sample sizes ranging from several hundred to several thousand are not exempt from inconsistencies in findings and have low replication rates. Given the present limited availability of biobanks, methodologically irreproachable studies that integrate more detailed clinical information and that explore the effects of genes in their entirety by dissecting the direct and interactive effects from environmental factors and other genes are required to improve the power and reproducibility of genetic association studies.

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S2554X mutation in the filaggrin gene is associated with allergen sensitization in the Japanese population

To the Editor:

Atopic diseases such as atopic dermatitis (AD) and allergic rhinitis are some of the most common diseases in developed societies, and the number of patients with these diseases is increasing. These diseases are caused by interactions between genetic and environmental factors; some patients develop various atopic diseases concurrently, whereas others show a gradual progression from one manifestation of allergy to the next (atopic march),[1] thereby indicating common genetic/environmental features among atopic diseases.

Skin barrier dysfunction may contribute to the allergen penetration responsible for AD and predate the development of asthma and allergic rhinitis.[2] Filaggrin (filament-aggregating protein; FLG) has been reported to play an important role in skin-barrier formation and hydration. FLG aggregates the keratin cytoskeleton to facilitate the collapse and flattening of keratinocytes in the outermost skin layer. Null mutations in *FLG* are associated with AD in various populations, and some mutations showed associations with rhinitis and allergen sensitization in white pediatric populations.[3] In a recent study, *Flg*-deficient mice showed a predisposition to sensitization after percutaneous exposure to an allergen and developed cutaneous inflammatory infiltration and allergen-specific immune responses after allergen sensitization.[4]

TABLE I. Characteristics of the study population (1499 subjects)

Characteristic	
Sex (male:female)	466:1033
Age (y), mean ± SD	32.3 ± 9.7
Asthma (current and past)	104/1093
Allergic rhinitis	575/1093
Sensitization	1014/1499
Total IgE (IU/mL), geometric mean	65.6 (range, <5-24,000)
Specific IgE (U_A/mL),	**Prevalence (range, U_A/mL)**
Mite	39% (<0.34-100)
Japanese cedar	62% (<0.34-100)
Dactylis glomerata	26% (<0.34-100)
Ambrosia artemisiifolia	12% (<0.34-32)
Candida albicans	6.5% (<0.34-35.8)
Aspergillus	3.3% (<0.34-9.1)

Total IgE values of <5 IU/mL have been considered as 0.1 IU/mL for the sake of calculations. The prevalence of specific IgE was calculated as the proportion of subjects with specific IgE titer of >0.70 U_A/mL.

We previously reported that the null allele of *FLG* showed statistically significant association with AD, and this association was stronger in the Japanese patients with the only-AD phenotype—that is, patients with AD without other atopic diseases.[5] However, the effects of *FLG* null mutations on other atopic conditions have not been investigated in Asian populations. In the current study, we genotyped 4 *FLG* null mutations in the Japanese general population and studied the association between these mutations and atopic phenotypes.

Between 2003 and 2007, 1575 hospital workers and university students were invited to participate in this study. All the participants were of Japanese origin and were residents of Fukui prefecture, Japan. The characteristics of the study population are shown in Table I. Asthma was diagnosed on the basis of whether patients answered that they had ever been diagnosed with asthma by a doctor. Allergic rhinitis was diagnosed on the basis of a positive history of rhinitis during the pollen season and/or all seasons, and high levels of allergen-specific IgE antibodies in the serum (RAST score ≥class 2). Total and specific IgE (produced in response to Japanese cedar, *Dermatophagoides*, *Dactylis glomerata*, *Ambrosia artemisiifolia*, *Candida albicans*, and *Aspergillus*) were measured by using the CAP-RAST method (Pharmacia Diagnostics AB, Uppsala, Sweden), and positive allergic sensitization was defined if the levels of 1 or more specific IgE molecules were greater than or equal to 0.70 IU/mL (class2). All the participants gave their written informed consent to participate in the study. The study was approved by the ethical committees of the University of Tsukuba and the University of Fukui, Japan.

Genomic DNA was extracted from whole-blood samples by using a DNA-isolation kit (QuickGene-810; Fuji, Tokyo, Japan). The 3321delA genotype was determined by sizing a fluorescently labeled PCR fragment on an Applied Biosystems 3100 DNA Sequencer (Applied Biosystems, Foster City, Calif) as described previously.[6] S2554X, S2889X, and S3296X were genotyped on TaqMan Assay-by-Design system for single nucleotide polymorphism genotyping (Applied Biosystems). The accuracy of genotyping was confirmed by using direct sequences/restriction fragment length polymorphism analysis[7] of samples obtained from all carriers and selected noncarriers of the null mutations.

TABLE II. Results of association study with atopic phenotypes in 98 subjects with filaggrin null mutations

Phenotype	AA	Aa	aa	*P* value	OR (95% CI)
Asthma/without asthma					
3321delA	104/976	0/13	0/0	.98	NA
S2554X	104/980	0/9	0/0	.98	NA
S2889X	99/947	5/42	0/0	.86	1.09 (0.42-2.85)
S3296X	104/980	0/9	0/0	.98	NA
Combined	99/917	5/71	0/1	.36	0.65 (0.25-1.65)
Allergic rhinitis/without allergic rhinitis					
3321delA	567/513	8/5	0/0	.53	1.43 (0.46-4.41)
S2554X	569/515	6/3	0/0	.37	1.89 (0.47-7.61)
S2889X	552/494	23/24	0/0	.60	0.85 (0.48-1.54)
S3296X	571/513	4/5	0/0	.61	0.70 (0.18-2.66)
Combined	535/481	39/37	1/0	.90	0.97 (0.61-1.55)
Sensitization/without sensitization					
3321delA	1000/483	14/2	0/0	.078	3.81 (0.86-16.89)
S2554X	1001/485	13/0	0/0	.013	NA
S2889X	977/467	37/18	0/0	.99	0.99 (0.56-1.77)
S3296X	1004/480	10/5	0/0	.94	0.96 (0.32-2.85)
Combined	941/460	72/25	1/0	.11	1.47 (0.91-2.35)

AA, Wild-type for the mutation; *Aa*, heterozygote for the mutation; *aa*, homozygote for the mutation or a compound heterozygote for mutations; *NA*, not applicable; *OR*, odds ratio. The number of subjects with a particular genotype is represented in the following form: number of subjects with the phenotype (eg, asthma)/number of subjects without the phenotype (eg, asthma).

The genetic effects of the association between the case-control status and each individual single nucleotide polymorphism were estimated by logistic regression analysis after adjusting for sex and age; the analysis was performed by using R version 2.7.0 (http://www.r-project.org/). The Fisher exact test was applied when the genotype counts were 0 and logistic regression could not be performed.

Among the 1575 participants, we obtained DNA samples and information on total and specific IgE levels for 1499 participants. Among these, 1093 subjects completed the asthma/rhinitis questionnaire, whereas 98 subjects (6.5%) had at least 1 *FLG* null mutation, and 1 subject was a combined heterozygote for null mutations. There was no deviation from the Hardy-Weinberg equilibrium ($P > .05$). The results of the association study are shown in Table II. *FLG* mutations were not associated with asthma and allergic rhinitis ($P > .05$). However, allergen sensitization was more common in the S2554X carriers ($P = .013$), and the trend toward association was observed in the 3321delA carriers ($P = .078$). However, the more common null mutation, S2889X, was not associated with allergen sensitization ($P > .05$), resulting in the disappearance of the association in the combined results ($P > .05$).

This is the first population-based study on the effects of *FLG* null mutations in the atopic phenotypes in Asian populations, and specific null mutations were found to be associated with allergen sensitization. In large population-based studies, a detailed clinical diagnosis of asthma and rhinitis cannot be easily performed, and careful examination of the subjects can yield stronger estimates of associations. Allergic rhinitis showed high prevalence in the subjects in this study; nearly half of the subjects were affected. This is a result of the high prevalence (31.4% to 39.1%) of Japanese cedar pollinosis among adults age 20 to 49 years.[8]

The reason for the association between specific null mutations and allergen sensitization remains to be determined, but our AD case-control samples also showed a similar trend; the odds ratios for the development of AD were 5.0 in the case of S2554X and 2.27 in the case of S2889X (see this article's Methods and Table E1 in the Online Repository at www.jacionline.org). Similar trends were also observed in white populations.[9] *FLG* consists of 10 to 13 *FLG* tandem homologous sequence units, and each null mutation is linked to a particular *FLG* homologous unit. Therefore, we speculated that the effects of *FLG* null mutations vary according to their locations and units.

The current study has a number of limitations. In a majority of the subjects with *FLG* null mutations, the mutations did not show an association with allergen sensitization. Furthermore, our study population contained only a small number of subjects with S2554X null mutations. In addition, the lack of independent general populations for use in replication analysis may weaken our observations. Future studies should aim to identify the precise mechanisms of *FLG* null mutations in the development of atopic phenotypes.

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