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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 non-stratified 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	o	×	×	o	×	o	o	×
TNF	−1037C>T	×	o	o	o	×	o	×	×
IL4RA	Ile50Val	×	o	×	×	o	o	×	×
	Gln551Arg	×	×	×	×	×	×	×	o
ADAM33	Met764Thr	×	×	o	×	×	o	×	o
	13236C>T	×	o	×	×	o	×	×	o
Group 2									
GSTM1	Ins/del	×	o	×	×	×	o	×	o
IL10	571C>A	×	×	×	×	×	×	×	o
LTC4S	−444A>C	o	×	×	o	×	×	o	×
AAA1	522363G>C	×	o	×	×	×	×	×	o
NOS1	GT repeat (187 allele)	×	o	o	×	×	×	×	o
	GT repeat (183 allele)	×	×	o	×	×	×	×	×
CCL5	−28C>G	o	×	×	×	×	×	×	×
Group 3									
MMP9	2127G>T	×	o	×	×	×	o	o	×
	5546G>A	×	o	×	×	o	×	o	×
IL12B	−6415ins/del	o	o	×	o	o	×	×	o
	1146 C> A	o	o	×	o	o	×	×	o
C3	Block 2 (haplotype 6)[a]	o	×	×	o	o	×	×	×
	Block 4 (haplotype 1)[a]	×	o	o	×	o	×	×	×
SOCS1	−1478CA>del	×	×	o	×	o	×	×	×

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 Inoue *et al.*[11]
Polymorphisms with a *P*-value ⩾0.05 in all association tests of the four genetic models are not shown.
[o]*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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