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Interleukin-4 and interleukin-13 pathway genetics affect disease susceptibility, serum immunoglobulin E levels, and gene expression in asthma

Jia Li, MM^{*}; Li-hui Lin, MM^{*}; Juan Wang, MM^{*}; Xia Peng, MM^{*}; Hui-rong Dai, BSM^{*}; Hui Xiao, MM^{*}; Fei Li, BSM^{*}; Yu-ping Wang, BSM^{*}; Zhi-jun Yang, PhD[†]; and Li Li, MD^{*}

^{*} Department of Laboratory Medicine, Shanghai First People's Hospital, Shanghai Jiao Tong University, Shanghai, China

[†] Shanghai Fosun Med-Tech Development Co, Ltd, Shanghai, China

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ABSTRACT

Background: Asthma is a common immune disorder characterized by increased IgE levels. The interleukin (IL)-4 and IL-13 pathway is central for IgE regulation, and previous studies have reported many genetic variants of IL-4/IL-13 signaling in relation to asthma, but few have focused on the gene-to-gene interactions that are likely to contribute to disease complexity.

Objective: To assess the combined effects of 7 functional single-nucleotide polymorphisms (SNPs) on asthma susceptibility, total serum IgE levels, and gene expression in children.

Methods: Seven SNPs (rs2243250, rs1800925, rs1805010, rs324011, rs2251746, rs2494262, and rs2427837) were genotyped children with asthma (n = 500) and a control group (n = 523), and total serum IgE levels and gene expressions were measured in children with asthma.

Results: Children with asthma had a likelier possibility of carrying more risk genotypes. Mean IgE levels increased from the minimum of 71.07 KU/L in children with no tested polymorphisms to a maximum of 901.7 KU/L in children carrying 7 risk genotypes. Gene expression analysis showed that patients with 4 SNPs (rs2243250, rs1800925, rs1805010, and rs3224011) had higher expression levels of IL-4, IL-13, and STAT6. Moreover, serum IgE level generally correlated well with IL-4 ($r = 0.236$, $P = .011$) and IL-13 ($r = 0.211$, $P = .021$) expressions; IL-4 expression correlated positively with IL-13 ($r = 0.962$, $P = .000$) and STAT6 ($r = 0.190$, $P = .022$) expressions, and STAT6 expression correlated with IL-4RA expression ($r = 0.904$, $P = .000$).

Conclusion: These data suggest that combinations of multiple SNPs might magnify the impact on disease risk. Only a combined analysis of the variants in the IL-4/IL-13 pathway could show the functional interplay of multiple genes in asthma.

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Introduction

Asthma is a typical atopic disease that is often a result of IgE-dependent immediate hypersensitivity reactions. The interleukin (IL)-4 and IL-13 pathway has been described as critical for isotype class switching from IgM to IgE in activated B lymphocytes and

essential for T-helper cell type 2 differentiation.¹ IL-4 and IL-13 operate through the IL-4 receptor (IL-4R) and IL-13R, respectively. These receptors share the IL-4R α chain and promote STAT6 homodimerization; then, the homodimers translocate to the nucleus and bind to the promoter regions, activating the transcription of target genes involved in IgE class switching.^{2,3} The first exposure to the antigen results in the production of specific IgE, which binds to its receptor (Fc ϵ R1 α) on the surface of mast cells. Further exposure results in the reaction of the antigen to the IgE bound to mast cells, leading to tissue damage caused by mast cell degranulation.⁴

Considering the important role of the IL-4/IL-13 pathway in the pathogenesis of asthma, genomewide association studies have identified many susceptibility loci within the pathway genes. However, it is difficult to replicate associations between genotypes and phenotypes across populations, and, even when successfully replicated, they typically have mild phenotypic effects.⁵

Reprints: Li Li, MD, Department of Laboratory Medicine, Shanghai First People's Hospital, Shanghai Jiao Tong University, 100 Haining Road, Shanghai 200080, China; E-mail: annylish@hotmail.com.

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Table 1
Characteristics of childhood asthmatics and controls

Variable	n	Age (y), mean $\pm$ SD	Girls/boys	IgE (log KU/L), mean $\pm$ SD
Asthma group	500	3–12 (8.4 $\pm$ 2.7)	259/241	2.45 $\pm$ 0.64
Control group	523	3–12 (7.9 $\pm$ 3.2)	271/252	1.54 $\pm$ 0.52
P value	—	.70	.75	<.001

Interestingly, when multiple variants synergize, the impact on disease risk can be magnified through gene-to-gene interactions.^{5,6}

For this study, 7 functional single-nucleotide polymorphisms (SNPs) in 5 major IL-4/IL-13 pathway genes (*IL-4*, *IL-13*, *IL-4RA*, *STAT6*, and *FCERIA*) were selected to assess the combined effect of SNPs on asthma susceptibility and total serum IgE levels. Because many previous studies have confirmed that these SNPs can alter gene expression,^{7–9} the authors previously proposed that these variants are expression SNPs, which are associated with transcription levels.¹⁰ Thus, they also determined mRNA expression levels of the 5 major IL-4/IL-13 pathway genes. The authors hypothesized that if these genetic variants regulate gene expression and are associated with asthma susceptibility and/or serum IgE levels, then the following causative link can be implied: DNA variant $\rightarrow$ gene expression change $\rightarrow$ asthma susceptibility $\rightarrow$ elevated serum IgE. Such evidence is much stronger than mere association between DNA variants and disease susceptibility and can lead to mechanistic insights.

Methods

Subjects

Five hundred children with asthma defined according to the revised criteria of the Global Initiative for Asthma¹¹ were recruited from hospital-based clinics associated with Shanghai First People's Hospital and Xinhua Hospital (Shanghai, China); 523 children with no history of allergies were included as controls. No significant differences were found with respect to population characteristics between the 2 groups; however, IgE levels were higher in children with asthma than in controls (Table 1). Informed written consent was obtained from all parents, and this study was approved by the ethics committee of Shanghai First People's Hospital (permit number 2013KY005).

SNP Selection, DNA Extraction, and Genotyping

The SNPs were chosen for their most replicated association with asthma phenotypes and previously reported functions.^{5–9}

Blood samples were obtained from all subjects, and genomic DNA was isolated using a genomic DNA kit (Axygen, Hangzhou, China). Genotyping of 7 polymorphisms in the respective genes was performed using polymerase chain reaction (PCR) and ligase

detection reaction (LDR). PCR primers and LDR probes were synthesized by Invitrogen (Shanghai, China) and the sequences are available online (eTables 1 and 2). For PCR, 1 μ L of each DNA sample was added to 19 μ L of PCR mixture containing 1 $\times$ PCR buffer, 1 $\times$ Q-Solution (Qiagen, Hilden, Germany), 3 mmol/L of MgCl₂, 0.2 mmol/L of each dNTP, 50 pmol/ μ L of primer, and 1 U of HotStar Taq polymerase (Qiagen). PCR cycling conditions were 15 minutes of denaturation at 95°C, 35 cycles for 30 seconds at 94°C, 30 seconds at 59°C, and 1 minute at 72°C, and a final 10-minute extension step at 72°C. PCR products were observed by agarose gel electrophoresis (3.0%), visualized with ImageMaster VDS (Pharmacia Biotech, Piscataway, New Jersey), and then used as templates in LDR.

The LDR reactions were carried out in 10- μ L reaction mixtures consisting of 1 $\times$ buffer, 12.5 pmol/ μ L of Probe mix, 2 U NEB Taq DNA ligase, and 100 ng/ μ L of PCR product. The reaction program was performed as follows: an initial heating at 95°C for 2 minutes, followed by 30 cycles for 15 seconds at 94°C and 25 seconds at 50°C. The products were submitted for sequencing on an ABI377 (ABI) sequencer, and the results were analyzed with GeneMapper 3.3 software (Applied Biosystems Incorporation, Foster City, California).

RNA Extraction and Gene Expression Profiling

The RNA samples were available only for the 500 children with asthma and not for the control group. RNA isolating details have been described previously,¹² and gene expression profiling was conducted by Multiplex Competitive RT-PCR Using Fluorescent Universal Primers, which has been described previously.¹²

Detection of Total Serum IgE

Total serum IgE levels were measured using a Siemens IgE kit (N Latex IgE mono, Siemens Healthcare Diagnostics Products, Marburg, Germany) on an automatic protein analyzer (Siemens BNII, Siemens Healthcare Diagnostics Products).

Statistical Analyses

Statistical analyses were performed with SPSS 17.0 software (SPSS, Inc, Chicago, Illinois). For Hardy-Weinberg equilibrium and the distribution of genotype and allele analyses, differences in groups were evaluated by the χ^2 test. The *FCERIA* haplotype was identified using Haploview 4.2 software (Broad Institute, Cambridge, Massachusetts). The comparison of risk genotypes between the 2 groups was conducted using the Wilcoxon rank-sum test. The association of SNPs and IgE level and the relation between SNPs and IL-4/IL-13 pathway gene expression were examined by 1-way analysis of variance. Correlations between IgE level and IL-4/IL-13 pathway gene expression were assessed by a Pearson correlation coefficient test. Stepwise logistic regression analysis with a forward approach was used to verify whether IL-4/IL-13 pathway SNPs

Table 2
Associations of IL-4/IL-13 pathway SNPs with asthma

Gene	SNP	Allele	Asthma ^a				Control ^a				Genotype χ^2 value	Genotype P value	Allele χ^2 value	Allele P value	Allele OR	95% CI
			0	1	2	MAF	0	1	2	MAF						
<i>IL-4</i>	rs2243250	T/C	324	150	17	0.19	338	144	21	0.18	0.02	.88826	0.02	.88685	1.017	0.811–1.274
<i>IL-13</i>	rs1800925	C/T	400	85	6	0.10	353	143	9	0.16	16.48	.00005 ^b	16.23	.00006 ^b	0.578	0.442–0.756
<i>IL-4RA</i>	rs1805010	A/G	125	243	126	0.50	115	259	123	0.51	0.10	.75250	0.1	.75408	0.972	0.815–1.159
<i>STAT6</i>	rs324011	C/T	285	167	36	0.24	290	183	29	0.24	0.06	.80528	0.06	.80173	1.027	0.836–1.261
<i>FCERIA</i>	rs2251746	T/C	443	50	0	0.05	476	42	0	0.04	1.26	.26104	1.2	.27272	1.264	0.831–1.924
	rs2494262	A/C	251	200	40	0.29	238	221	47	0.31	1.64	.20038	1.63	.20230	0.883	0.728–1.069
	rs2427837	G/A	445	49	0	0.05	468	48	0	0.05	0.11	.73950	0.1	.74593	1.07	0.711–1.609

Abbreviations: CI, confidence interval; IL, interleukin; MAF, minor allele frequency; OR, odds ratio; SNP, single-nucleotide polymorphism.

^aThe numbers 0, 1, and 2 represent the number of major, heterozygote, and minor genotypes, respectively.

^bP < .01.

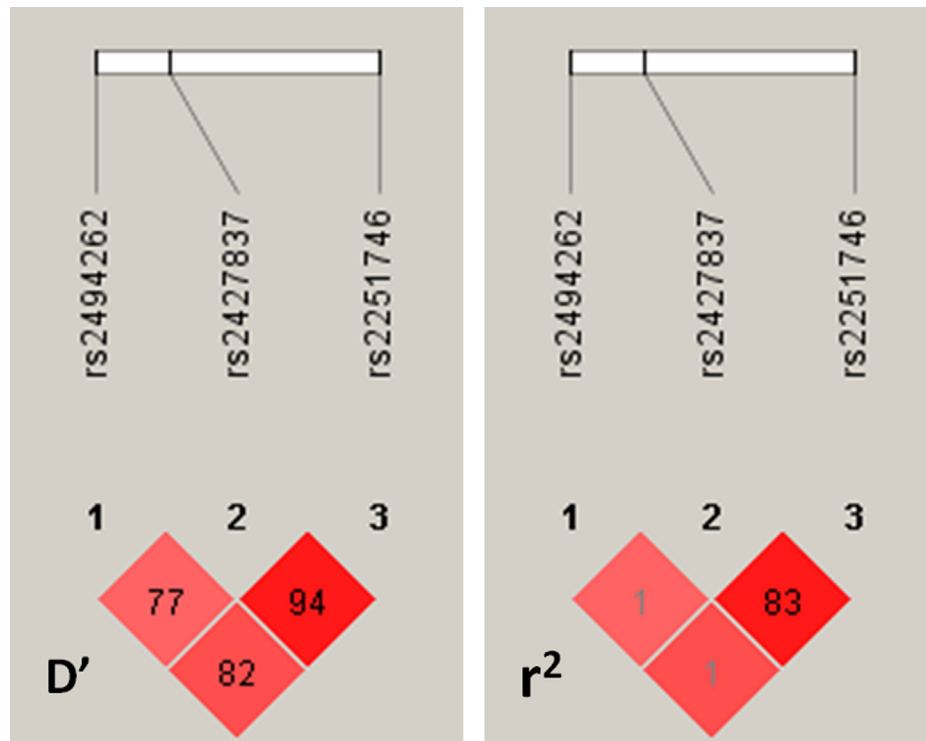


Figure 1. Linkage disequilibrium among the 3 FcεRIα polymorphisms.

could be considered predictors of high serum IgE levels. A *P* value less than .05 was considered statistically significant for all analyses.

Results

IL-4/IL-13 Pathway SNPs and Asthma Associations

Seven mutations in IL-4/IL-13 pathway genes (rs2243250, rs1800925, rs1805010, rs324011, rs2251746, rs2494262, and rs2427837) were assessed in this study. All analyzed SNPs were in Hardy-Weinberg equilibrium in the asthma and control groups (*P* > .05, data not shown).

As presented in Table 2, IL-13 rs1800925 was significantly associated with asthma, with the minor allele conferring decreased risk (odds ratio [OR] 0.578, 95% confidence interval [CI] 0.442–0.756). There was no other significant disease risk association. Haplotype analyses of SNPs rs2251746, rs2494262, and rs2427837 in the *FCER1A* gene are shown in Figure 1. The SNPs rs2427837 and rs2251746 were in high linkage disequilibrium (*D'* = 0.94, logarithm of odds 105.15, *r*² = 0.83).

In addition to the isolated effects of single alleles on the development of asthma, the combined effects of SNPs in IL-4/IL-13 pathway genes were investigated. The number of risk genotypes carried by each participant was summed; then, the percentages of children carrying the corresponding number of risk genotypes were calculated in each group; percentages were compared between the asthma and control groups. As shown in Figure 2, the frequency of participants carrying more than 3 risk genotypes was higher in the asthma group than in the control group (*Z* = 2.15, *P* = .03).

Effect of IL-4/IL-13 Pathway SNPs on Serum IgE Levels

Because RNA samples were available only for children with asthma, the following studies were carried out mainly in the asthma group. The effect of individual IL-4/IL-13 pathway SNPs on IgE levels was examined. The associations between the 7 SNPs and

serum IgE levels are presented in Figure 3. All 7 SNPs, except for IL-13 rs1800925, were associated with elevated serum IgE levels. The most significant SNPs in this association were rs1805010 in the *IL-4RA* gene (*P* = .0013) and rs2243250 in the *IL-4* gene (*P* = .0012); in these cases, the minor allele was associated with increased serum IgE concentrations.

Potential interaction effects of IL-4/IL-13 pathway SNPs on serum IgE concentrations were examined. As shown in Figure 3, participants carrying more risk genotypes of these 7 SNPs had significantly higher serum IgE levels (*P* < .0001). The mean serum IgE levels increased from the minimum of 71.07 KU/L (participants with no tested polymorphisms) to a maximum of 901.7 KU/L (participants carrying 7 risk genotypes).

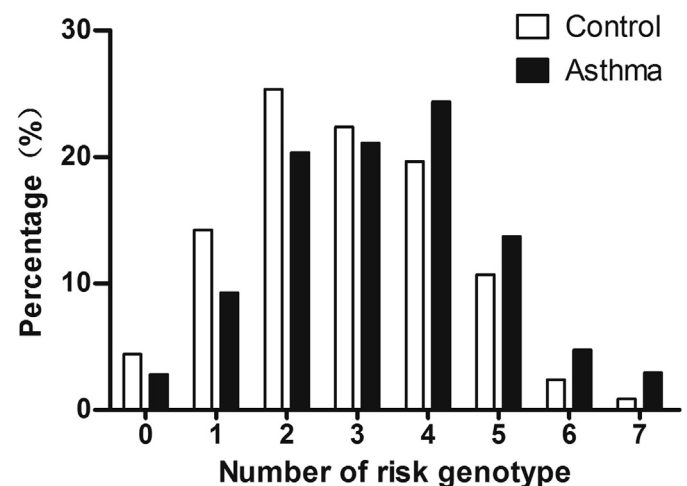


Figure 2. Distribution of risk genotypes in the asthma and control groups. The y axis represents the percentage of children carrying the corresponding number of risk genotypes.

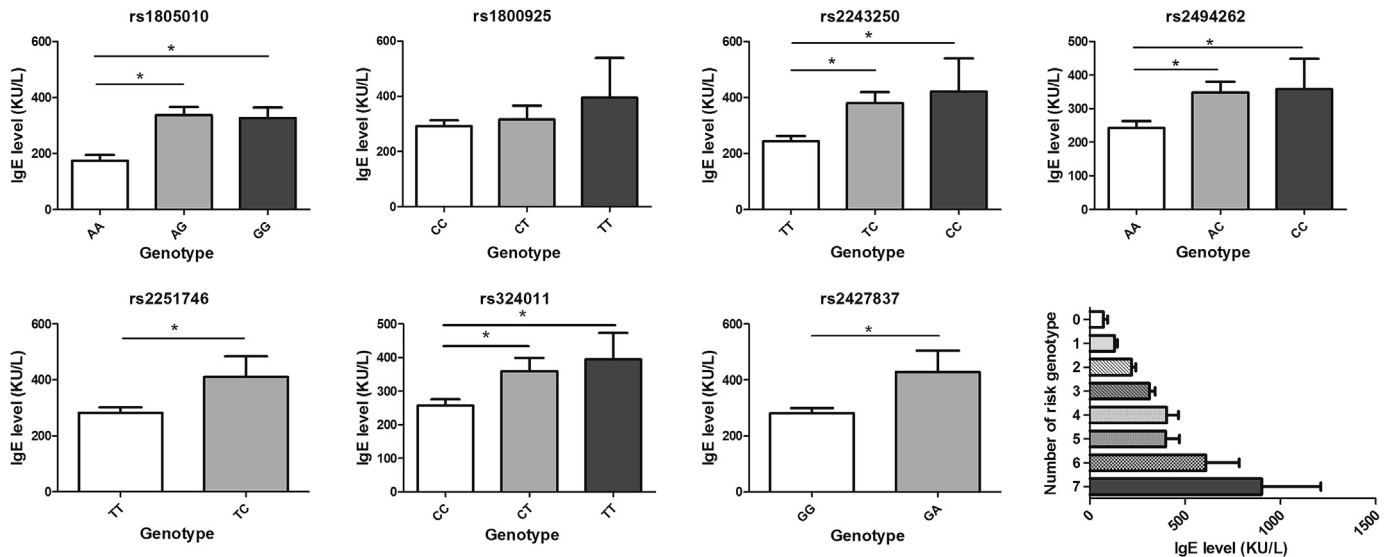


Figure 3. Effect of interleukin-4 and interleukin-13 pathway single-nucleotide polymorphisms on serum IgE levels. * $P < .05$.

IL-4/IL-13 Pathway SNPs Affecting Gene Expression

The isolated effects of single SNPs on the RNA expression of the respective genes are shown in Figure 4 and could be divided into 2 groups: *IL-4* rs2243250, *IL-13* rs1800925, *IL-4RA* rs1805010, and *STAT6* rs324011 were associated with elevated RNA expression ($P = .0068$, .041, .021, and .017, respectively); in contrast, the 3 variants in the *FCERIA* gene (rs2251746, rs2494262, and rs2427837) were associated with decreased RNA expression ($P = .029$, .000, and .0006, respectively).

These latter 3 loci were not included in the subsequent multiple SNP analysis. However, patients carrying more risk genotypes of the other 4 SNPs (*IL-4* rs2243250, *IL-13* rs1800925, *IL-4RA* rs1805010, and *STAT6* rs324011) appeared to have higher IL-4, IL-13, and STAT6 expression levels ($P = .0058$, .000, and .0022, respectively; Fig 5), but not higher IL-4RA expression ($P = .968$).

Associations between IL-4/IL-13 Pathway Gene Expression and Serum IgE Levels

As presented in Table 3, serum IgE levels (KU/L) showed a positive correlation with IL-4 and IL-13 expression levels ($r = 0.236$, $P = .011$, and $r = 0.211$, $P = .021$, respectively). A positive correlation also was observed between the IL-4 expression level and IL-13 and STAT6 expression levels ($r = 0.962$, $P = .000$, and $r = 0.190$, $P = .022$, respectively) and between IL-4RA and STAT6 expression levels ($r = 0.904$, $P = .000$).

Multiple Logistic Regression Analysis

To investigate which variant accounted for the high level of serum IgE, stepwise logistic regression analyses were performed that included the following covariates: age, sex, *IL-4* rs2243250, *IL-4RA* rs1805010, *STAT6* rs324011, *FCERIA* rs2494262, and rs2427837 (*FCERIA* rs2251746 and *IL-13* rs1800925 were not included because rs2251746 is in high linkage disequilibrium with rs2427837 and rs1800925 is not associated with elevated serum IgE levels). *IL-4* rs2243250 ($P = .030$, OR 1.58, 95% CI 1.05–2.40), *IL-4RA* rs1805010 ($P = .002$, OR 2.23, 95% CI 1.33–3.73), and *STAT6* rs324011 ($P = .010$, OR 1.79, 95% CI 1.13–2.83) were found to be independently associated with a serum IgE level of at least 100 KU/L.

Discussion

Immunoglobulin E is commonly regarded as a key player in allergic diseases because of its ability to activate mast cells through the high-affinity IgE receptor, FcεRI, thereby triggering immediate hypersensitivity reactions.^{13–15} It is characterized by its ε heavy chain and is produced by the immunoglobulin class switch recombination in activated B cells.¹⁶ The IL-4/IL-13 pathway has been shown to be critical for this isotype class switching to IgE. IL-4 and IL-13 operate through IL-4R and IL-13R, respectively. These receptors share the IL-4Rα chain and promote STAT6 activation and the induction of “germline” transcription at the Cε locus.^{17,18}

>Previous studies have identified many genetic variants of IL-4/IL-13 signaling associated with atopic disorders; however, few analyses have focused on the gene-to-gene interactions that are likely to contribute to the complexity of genetic diseases. A German population-based study of children demonstrated that when polymorphisms in *IL-4*, *IL-13*, *IL-4RA*, and *STAT6* were combined in a stepwise procedure, the risk for high serum IgE levels increased by 10.8-fold and the risk for the development of asthma increased by 16.8-fold compared with the maximum effect of any individual SNP⁶; however, thus far, these results have not been replicated in other ethnic populations.

In the present study, 7 functional SNPs in 5 major IL-4/IL-13 pathway genes were selected to observe the single and combined effects of SNPs on asthma susceptibility and total serum IgE levels. In addition, the mRNA expression levels of the 5 major IL-4/IL-13 pathway genes were detected to determine the associations between SNPs and gene expression. For single SNP analysis, the present findings did not exactly match those of previous association studies. *IL-13* rs1800925 was the only locus found to be associated with asthma, but was not associated with elevated serum IgE levels. This may be caused by the multiple roles of *IL-13* in the pathogenesis of asthma. *IL-13* is a T-helper cell type 2 cytokine implicated in (1) the recruitment of inflammatory cells from the blood to the lung, (2) IgE production by B cells, (3) the regulation of matrix metalloproteinases, and (4) the induction of mucin production and secretion. Based on these roles, *IL-13* may be associated with asthma by numerous ways, not only by regulating IgE production. Conversely, the inconsistency between the present results and those of previous studies also shows the instability of individual SNP analysis; it can be affected by gene-to-gene interactions and the relatively small number of subjects analyzed in studies.

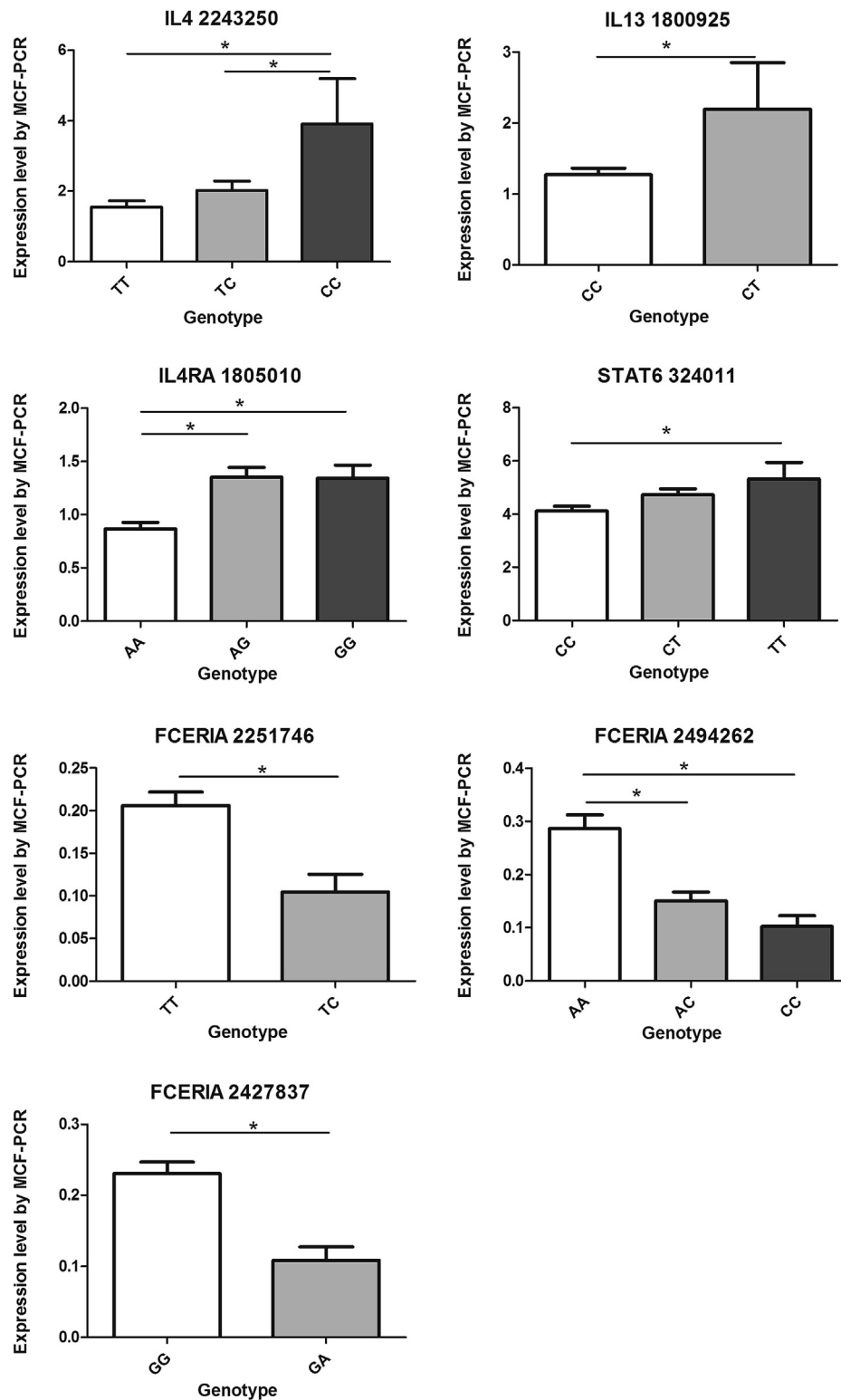


Figure 4. Isolated effect of interleukin-4 and interleukin-13 pathway single-nucleotide polymorphisms on respective gene expressions. * $P < .05$.

Therefore, the combined effect of single SNPs in the IL-4/IL-13 pathway was analyzed further and showed that children with asthma were more likely to carry a larger number of risk genotypes compared with the control group ($P = .03$). As indicated in the result (Figure 2), the difference is somewhat weak. The informative proportion of the study population decreased with the stepwise addition of SNPs (2.94% and 1.88% carriers of the 7 SNPs risk allele

combination in the asthma and control groups, respectively), and the analysis of 8 and 9 SNP interactions could not be performed confidently because of the small numbers. However, in this analysis the number of SNPs in each participant was summed and the potentially differential contributions of each SNP were not counted. In a future large-scale study, the authors will focus on the detailed contribution of SNPs.

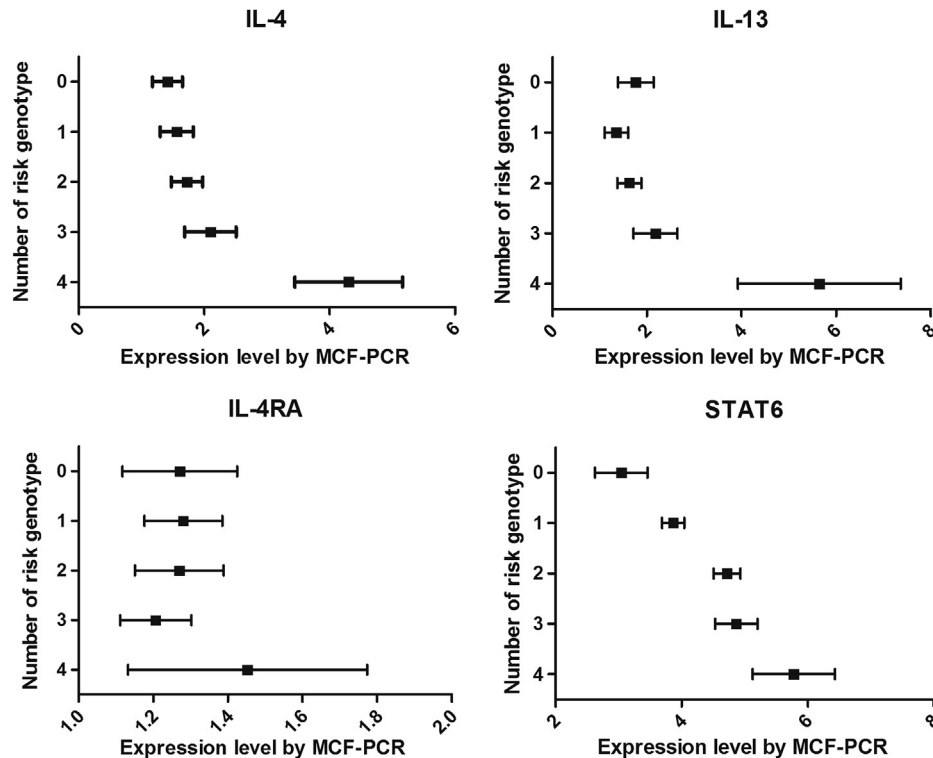


Figure 5. Combined effect of single single-nucleotide polymorphisms (*IL-4* rs2243250, *IL-13* rs1800925, *IL-4RA* rs1805010, and *STAT6* rs3224011) on respective gene expressions. MCF-PCR, Multiplex Competitive RT-PCR Using Fluorescent Universal Primers.

Each of the 7 SNPs included in this study has been reported to have a functional role in the respective gene. For *IL-4* rs2243250, the T allele was found to increase the binding of a transcription factor,¹⁹ the T allele of *IL-13* rs1800925 was found to alter gene expression,²⁰ *IL-4RA* rs1805010 was associated with increased *IL-4*–mediated downstream *STAT6* activation,^{6,21} the polymorphic

T allele at *STAT6* rs324011 was found to increase *STAT6* promoter activity,⁷ the preferential binding of the YY1 transcription factor to the *FCERIA* rs2494262 C allele resulted in lower transcriptional activity,⁸ the activity of the *FCERIA* promoter with a rs2251746 T allele was found to be stronger than with the C allele,⁹ and the G allele of *FCERIA* rs2427837 was significantly associated with increased *FCERIA* expression.²² In the present study, the effects of the SNPs on gene expression were shown using clinical samples. Combined analysis showed that patients carrying more risk genotypes of 4 SNPs (*IL-4* rs2243250, *IL-13* rs1800925, *IL-4RA* rs1805010, and *STAT6* rs3224011) had higher *IL-4*, *IL-13*, and *STAT6* expression levels, which may be a result of gene-to-gene interactions. However, because this combined effect was shown using clinical samples, further studies are needed to determine the biological mechanism in vitro and in vivo.

The authors also detected associations between the expression of *IL-4/IL-13* pathway genes and serum IgE levels. Indeed, the serum IgE level (KU/L) generally correlated well with *IL-4* and *IL-13* expression levels; *IL-4* expression correlated positively with *IL-13* and *STAT6*, whereas *STAT6* expression correlated with *IL-4RA* expression. These results are consistent with a role for the *IL-4/IL-13* pathway in IgE class switch recombination. *IL-4* and *IL-13* share many biological activities, which is in part a result of their shared use of the signal receptor, *IL-4Rα*.³ Ligation of *IL-4Rα* activates *STAT6*, whereas *IL-4Rα* signaling in B cells contributes to IgE class switch recombination and allergic inflammation.²³

This analysis elucidated the single and combined effects of *IL-4/IL-13* pathway SNPs on gene expression and serum IgE. This may help explain the complex diseases, such as asthma, that are affected by a combination of polygene heredity and the environment. The functional interplay between genetic variants within a pathway is likely to contribute to the complexity of genetic diseases. Each variant typically has a modest effect in isolation but synergizes effectively with other variants to magnify the impact on disease

Table 3
IL-4/IL-13 pathway gene expression and serum IgE level associations

Variable	IgE (KU/L)	IL-4	IL-13	IL-4RA	STAT6	FCERIA
IgE (KU/L)						
Pearson correlation coefficient	1					
(r)						
P value	—					
IL-4						
Pearson correlation coefficient	0.236 ^a	1				
(r)						
P value	.011 ^a	—				
IL-13						
Pearson correlation coefficient	0.211 ^a	0.962 ^b	1			
(r)						
P value	.021 ^a	.000 ^b	—			
IL-4RA						
Pearson correlation coefficient	−0.080	0.054	−0.043	1		
(r)						
P value	.389	.524	.610	—		
STAT6						
Pearson correlation coefficient	−0.014	0.190 ^a	0.136	0.904 ^b	1	
(r)						
P value	.881	.022 ^a	.099	.000 ^b	—	
FCERIA						
Pearson correlation coefficient	−0.033	0.077	0.026	0.143	0.115	1
(r)						
P value	.731	.379	.767	.097	.184	—

Abbreviation: IL, interleukin.

^a01 < P < .05.

^bP < .01.

risk. However, further large-scale replication studies are required before multiple SNPs in the IL-4/IL-13 pathway can be used as a predictor of the development of asthma.

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Supplementary Data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.anai.2014.05.004>.

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eTable 1

Sequence of primers used in polymerase chain reaction for genotyping

SNP	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon size (bp)
rs2243250	CTTGCCAAGGGCTTCCTTAT	GGTGGCATCTTGAAACTGT	220
rs1800925	AGGAAGTGGGTAGGGGAGAA	CACCACTCTCTGCAGGATCA	227
rs1805010	CCCCAGATCTGCTCACAT	AGCCACAGGTCCAGTGTAT	245
rs324011	GAGACCAGACCCACAGAGA	TCAGGCATTGACTCAGGATG	208
rs2251746	GTGGAGGTGGGATAGGGAGT	TCCTGGTGCTTACTGTGCTG	210
rs2494262	CCCCAAGGAGAAGAGAATCC	ACTCCAACAATCCCAACAG	189
rs2427837	GCTCTACTGCATCTTATCATTTCA	TGTTGTTTCTGCTGCTGCTT	247

Abbreviation: SNP, single-nucleotide polymorphism.

eTable 2

Sequence of probes used in LDR for genotyping

Probe name	Sequence (5'→3')	LDR length (bp)
rs2243250_modify	P-ACAAATGTTCTCCCAAGTTTATTTTTTTTTTTTTTTTTTTT-FAM	
rs2243250_C	TTTTTTTTTTTTTTTTTTCTCTCCTACCCAGCACTGGGGG	87
rs2243250_T	TTTTTTTTTTTTTTTTTTCTCTCCTACCCAGCACTGGGGA	89
rs1800925_modify	P-TTTCCTAGAAGTCTCCAGTTTTTTTTTTTTTTTTTTT-FAM	
rs1800925_C	TTTTTTTTTTTTTTTTTTTCGCTTTTCCTGCTCTCCCTCG	82
rs1800925_T	TTTTTTTTTTTTTTTTTTTCGCTTTTCCTGCTCTCCCTCA	84
rs1805010_modify	P-ACACGTGTGGGCTCTGCAGATTTTTTTTTTTTTTTTTT-FAM	
rs1805010_A	TTTTTTTTTTTTTTTTTTGGCTCCTGTCTCAGGGAT	77
rs1805010_G	TTTTTTTTTTTTTTTTTTGGCTCCTGTCTCAGGGAC	79
rs324011_modify	P-GTCCCTAGGAGGGCTATCTATTTTTTTTTTTTTTTTTTT-FAM	
rs324011_C	TTTTTTTTTTTTTTTTTTTTTTGGCCATGAGTGTTGGTGGGACG	102
rs324011_T	TTTTTTTTTTTTTTTTTTTTTTGGCCATGAGTGTTGGTGGGACA	104
rs2251746_modify	P-TCATATCTGGTTAACCCATCTTTTTTTTTTTTTTTTTTTT-FAM	
rs2251746_C	TTTTTTTTTTTTTTTTTTTTAGCAGAAGGAAATGTTTCTGTG	97
rs2251746_T	TTTTTTTTTTTTTTTTTTTTTTAGCAGAAGGAAATGTTTCTGTA	99
rs2494262_modify	P-AGGAATCTCAAAAGGATGATTTTTTTTTTTTTTTTTTTT-FAM	
rs2494262_A	TTTTTTTTTTTTTTTTTTTTTCTTACCTCTAGTAGCATGT	92
rs2494262_C	TTTTTTTTTTTTTTTTTTTTTCTTACCTCTAGTAGCATGG	94
rs2427837_modify	P-CATCATGGGGAGAAAGTCAATTTTTTTTTTTTTTTTTTTT-FAM	
rs2427837_A	TTTTTTTTTTTTTTTTTTTTTTTGCCTCAAGGATTATATAGTT	107
rs2427837_G	TTTTTTTTTTTTTTTTTTTTTTTGCCTCAAGGATTATATAGTC	109

Abbreviation: LDR, ligase detection reaction.