

Genetic association of acidic mammalian chitinase with atopic asthma and serum total IgE levels

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Background: In view of the hygiene hypothesis and the involvement of acidic mammalian chitinase (CHIA) in the effector responses of IL-13 with asthma, *CHIA* (GeneID-27159) is a potential asthma candidate gene.

Objective: To investigate the association of *CHIA* polymorphisms with atopic asthma and serum total IgE levels.

Methods: Twenty-one single nucleotide polymorphisms were identified by sequencing DNA of 60 individuals. On the basis of linkage disequilibrium, 6 polymorphisms were selected and genotyped in unrelated atopic patients with asthma (N = 270) and controls (N = 292) and an independent pediatric cohort (patients, 150; controls, 101). Electrophoretic mobility shift assay and reporter gene assays were also performed.

Results: The rs3806448G/A promoter polymorphism showed significant association with atopic asthma ($P_{\text{adult}} = .00001$ and $P_{\text{pediatric}} = .0002$) and serum total IgE ($P < .05$). Also rs2282290G/A was associated with atopic asthma ($P_{\text{adult}} = .00009$ and $P_{\text{pediatric}} = .00003$), whereas the rs10494132C/T polymorphism was associated with serum total IgE in the patients ($P < .05$). We also showed that the promoter single nucleotide polymorphisms altered the transcriptional activity of *CHIA* promoter and the C to T substitution at rs10494132 abrogated the Octamer transcription factor-1 (*Oct-1*) binding site.

Conclusion: Our results establish a significant association of *CHIA* with atopic asthma and serum total IgE levels in the Indian population. (J Allergy Clin Immunol 2008;122:202-8.)

Key words: *CHIA*, SNPs, promoter, IgE, atopic asthma

Chitin, a major structural component of the outer coatings of many environmental organisms such as fungi,¹ parasitic nematodes,^{2,3} arthropods, and so forth,^{4,5} has been shown to redirect the immune system towards T_H1 polarization by suppressing T_H2-mediated IgE production and lung eosinophilia in allergic mice.⁶ On the other hand, chitin degrading enzyme, acidic mammalian chitinase (CHIA), has been speculated to shift the tissue

Abbreviations used

CHIA:	Acidic mammalian chitinase
EMSA:	Electrophoretic mobility shift assay
LD:	Linkage disequilibrium
OR:	Odds ratio
SNP:	Single nucleotide polymorphism
SPT:	Skin prick test
UTR:	Untranslated region

inflammation toward the T_H2 direction by eradicating chitin-containing pathogens.⁷ Alternatively, chitinase proteins may function directly as chemotactic agents or indirectly by inducing other chemokines that attract eosinophils and T cells to the sites of parasitic infection.⁸ The expression of CHIA is highly regulated in the lungs of mice. By using proteomics approach, a 5.7-fold change in the expression of CHIA was observed in the bronchoalveolar lavage fluid of mice with allergic airway inflammation.⁹ Recent microarray expression studies have also showed a substantial increase in the expression of *CHIA* gene in the allergic asthma model of mice.¹⁰ Also, its expression and activity are massively increased via a T_H2-specific, IL-13-mediated pathway in the bronchoalveolar lavage fluid and epithelial cells in the lungs of antigen-sensitized/challenged mice.⁷ Further, in-depth expression studies showed that it is predominantly expressed by nonmucus-producing Clara cell secretory protein-expressing cells of the distal airways and alveolar macrophages.^{11,12} Moreover, in human studies, *CHIA* mRNA was not detectable in the lungs of healthy controls, whereas its expression was highly induced in the epithelial and subepithelial cells of the lung tissues from the patients with asthma.⁷ CHIA neutralization ameliorated T_H2 inflammation, tissue eosinophilia, and airway hyperresponsiveness in mice, in part by inhibiting the IL-13 pathway and chemokine induction, reinforcing its role in effector cell chemotaxis and thus in asthma pathogenesis. Although all of the previous studies have suggested CHIA to be a positive mediator of allergic and asthmatic responses, recently Reese et al¹³ have reported that CHIA can prevent chitin-induced allergic innate immune responses in mice by inhibiting eosinophil and basophil recruitment to the lungs of chitin-challenged mice.

The gene for CHIA (GeneID-27159) is present on 1q13.1-21.3. It is secreted as a 50-kd product and is acid-stable.¹⁴ Recently Bierbaum et al¹⁵ have described the genetic association of exonic *CHIA* polymorphisms with bronchial asthma in a German pediatric population. Although the precise roles of the exonic single nucleotide polymorphisms (SNPs) remain to be elucidated, the biochemical and molecular studies mentioned indicate that its expression may be under the control of regulatory regions. Therefore, studying its genetic association in an ethnically diverse

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TABLE I. Demographic profile of the atopic patients with asthma and the control groups

	Adult cohort		Pediatric cohort	
	Cases	Control	Cases	Control
Native place*	North India	North India	North India	North India
Subjects recruited	270	292	150	101
Mean age (y)	31.08 ± (16.27)	25.41 ± (9.06)	10.15 ± (3.6)	10.87 ± (3.2)
Sex ratio (male vs female)	0.53:0.47	0.56:0.44	0.59:0.41	0.53:0.47
Familial history of asthma/atopy	All	None	All	None
Percent reversibility from baseline FEV ₁ (after 360-720 µg albuterol usage)	>15%	ND	>15%	ND
Smoking history†	None	None	None	None
SPT (positivity)	All	None	ND‡	None
Log ₁₀ mean total serum IgE (IU/mL)	2.93 ± (0.55)	2.17 ± (0.64)	2.88 ± (0.6)	2.48 ± (0.47)
Self-reported history of allergies	All	None	All	None

ND, Not done.

Parentheses contain the values for SD.

*Patients and controls were recruited from Delhi, Haryana, Punjab, and Gujarat.

†Patients and controls with a history of smoking or parasitic infections for the past 2 years were excluded from the study.

‡Atopic status was determined by allergen-specific serum IgE levels.

population would be very important to confirm its role in asthma pathogenesis.

Here we have performed case-control studies in 2 cohorts to establish its association with atopic asthma. For the first time, we report *CHIA* genetic variants primarily in the regulatory regions to be associated with atopic asthma and serum total IgE levels in the Indian population. The functional role of rs10494132C/T promoter polymorphism in differential binding of transcription factor *Oct-1* has also been established.

METHODS

Subjects and selection criteria

In a multicenter-based asthma genetic study program, unrelated adult patients (N = 270) and pediatric patients (N = 150) were recruited from various collaborating hospitals in North India (Indo-Aryan origin). Approval of the ethics committees of the participating centers was obtained. Written consents were obtained from all the participants. Asthma was defined by clinical history of asthma on the basis of National Asthma Education and Prevention Program (Expert Panel Report 2)¹⁶ and validated later by interviewed questions.¹⁷ All the probands fulfilled the following criteria: a positive skin prick test (SPT) result/high specific IgE¹⁸⁻²⁰; a positive response to 1 of 2 questions (Have you ever had attacks of breathlessness at rest with wheezing? Have you ever had an asthma attack?); associated with positive values for at least 2 out of the following parameters: pulmonary function test (using Morgan MS 10; Morgan Instrumentation Inc, Manchester, United Kingdom, portable spirometer following the American Thoracic Society guidelines for spirometer quality control), as measured under clinical supervision (FEV₁/forced vital capacity below 75% at the time of attack, and >15% improvement in FEV₁ by bronchodilator), hospitalization for asthma, or asthma therapy (Table I).

Healthy volunteers (N_{adult} = 292 and N_{pediatric} = 101) were recruited from the same geographical area and screened negative for the SPT and on the basis of the criteria of having no symptoms or history of allergic diseases. Individuals having a history of smoking or parasitic/helminthic infestations were excluded from the study (Table I). The genetic homogeneity between the patients and controls from both groups were confirmed by genotyping various loci (see this article's Online Repository at www.jacionline.org), as yet unlinked to asthma or related atopic disorders ($P \geq .05$, data not shown).

SPT and serum total and specific IgE estimation

Sixteen common environmental allergens with both negative and positive controls were used for the SPT (as described in the Online Repository).

Specific serum IgE was estimated for 6 common allergens by using the method described by Voller et al¹⁸ with slight modifications, and the OD values for different serum samples were analyzed according to the criteria laid down by Calenoff et al²¹ (as described in the Online Repository). Total serum IgE levels were estimated for all individuals by using ELISA as described,^{19,20} except few individuals (<10%) for whom sera were not available. Atopy was defined as a dichotomous variable, having a wheal reaction at least 3 mm greater than the negative control in cases in which SPT was performed, or high specific IgE²⁰ to at least 1 allergen in cases in which SPT was not performed.

Molecular methods

DNA was isolated from blood by using the modified salting out method.²² On the basis of functional relevance (in promoter, untranslated region [UTR], nonsynonymous or downstream of the gene) and existing linkage disequilibrium (LD) pattern, 6 selected SNPs were sequenced and genotyped by using primers shown in this article's Table E1 in the Online Repository (available at www.jacionline.org) by standard techniques.²³

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSAs) for *CHIA* rs10494132C and *CHIA* rs10494132T were performed as described in the Online Repository.

Reporter gene assay

Luciferase reporter gene constructs containing the *CHIA* promoter SNPs rs3806448 and rs10494132 were generated and the levels of expression of the luciferase were determined as described in the Online Repository.

Statistical analysis

Linkage disequilibria were evaluated by using Haploview (<http://www.broad.mit.edu/mpg/haploview/>).²⁴ Hardy-Weinberg equilibrium was assessed using FINETTI (<http://ihg.gsf.de/linkage/download/finetti.zip>). The Armitage trend test was performed, following the guidelines by Sasieni.²⁵ Multiple logistic regression analysis was performed to observe the effect of age and sex, if any. Haplotypes of each individual were inferred by using the algorithm developed by Stephens et al²⁶ (PHASE version 2.1). Differences in haplotype frequencies in cases and controls were compared by using a Monte Carlo approach as implemented in CLUMP 2.3 (<http://www.smd.qmul.ac.uk/statgen/dcurtis/software.html>).²⁷ Odds ratios (ORs) were calculated for haplotypes whose distribution was significantly different in the 2 groups studied. Bonferroni corrections were applied to correct for multiple

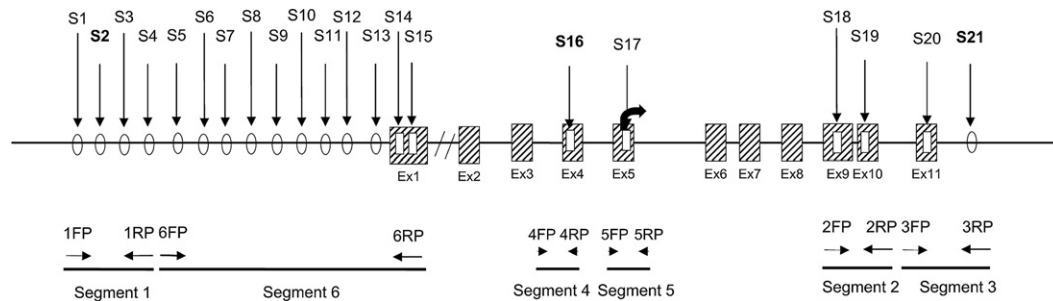


FIG 1. Gene map of *CHIA* along with SNPs in *CHIA* gene present on chromosome 1p13.1-21.3. Lower panel indicates the DNA segments amplified by using different PCR primer sets. *Ex*, Exon; *FP*, forward primer; *RP*, reverse primer.

testing (for n tests, α [α_{adj}] is adjusted to α/n). For serum total IgE levels, an ANOVA model was used for log-transformed values.

RESULTS

Polymorphism of *CHIA* and estimation of LD

For the genetic studies of the *CHIA* gene, we have sequenced the genomic DNA of 60 arbitrarily chosen individuals from both the study cohorts (as described in the [Online Repository](#)) by using the primer sets as detailed in [Table E1](#). We identified 16 SNPs in the promoter and the 5' UTR of the gene: 13 promoter variants (rs3806448 [S1], rs10494132 [S2], rs3806447 [S3], rs3806446 [S4], -907 A/G [S5], rs12023321 [S6], -762 C/A [S7], rs12033184 [S8], rs35042265 [S9], rs4546919 [S10], rs4554721 [S11], rs4442363 [S12], rs11102235 [S13], including 2 novel SNPs [S5 and S7]), and three 5' UTR polymorphisms (rs34698010 [S14], rs12026825 [S15], rs3818822 [S16]). In addition, 4 nonsynonymous SNPs (K17R [S17], rs2275253 [S18], rs2275254 [S19], rs2256721 [S20]) spanning exons 5 to 11 and a polymorphism (rs2282290 [S21]) 266 bp downstream of the gene were selected from the National Center for Biotechnology Information (NCBI) database on the basis of their reported allelic frequencies and the position in the gene and validated in our sequencing cohort ([Fig 1](#)). LD calculation using Haploview showed complete LD ($r^2 > 0.9$) among the 10 promoter polymorphisms and two 5' UTR polymorphisms. Complete LD also existed among 3 nonsynonymous SNPs, rs2275253, rs2275254 and rs2256721, generating 2 LD blocks (see this article's [Fig E1, A and B](#), in the [Online Repository](#) at [www.jacionline.org](#)). Thus, we decided to genotype the 6 distinct SNPs, rs3806448, rs10494132, rs3818822, K17R, rs2275253, and rs2282290, in our study population.

Association of *CHIA* polymorphisms with atopic asthma and serum total IgE

Genotype frequencies of the polymorphisms in cases and controls for the 2 cohorts are given in [Table II](#). All the polymorphisms in the control population were found to be in Hardy-Weinberg equilibrium in both cohorts ($P > .05$). The earlier reported SNPs, rs3818822 and K17R, were not found to be associated with atopic asthma in our study groups ([Table II](#)). However, the rs3806448 promoter polymorphism and the downstream SNP, rs2282290, were found to be significantly associated with atopic asthma ([Table II](#)). Allele A at rs3806448 was highly frequent in atopic patients with asthma (adult cohort, OR, 1.72, 95% CI, 1.35-2.19, $\chi^2 = 19.63$, $P = .00000$; pediatric cohort, OR, 1.95,

95% CI, 1.35-2.8, $\chi^2 = 12.86$, $P = .0002$) compared with the controls. Similarly, allele G of SNP rs2282290 was found to be the risk allele (adult cohort, OR, 1.6, 95% CI, 1.26-2.03, $\chi^2 = 15.19$, $P = .0001$; and pediatric cohort, OR, 2.05, 95% CI, 1.42-2.94, $\chi^2 = 15.58$, $P = .00003$). The association was found to be statistically significant even after applying the correction for multiple testing ($\alpha_{adj} = 0.008$). By using multiple logistic regression analyses, we confirmed the association of the rs3806448 and the rs2282290 polymorphisms with asthma, and it was found not to be influenced by sex and age (see this article's [Table E2](#) in the [Online Repository](#) at [www.jacionline.org](#)).

Serum total IgE levels were found to follow a log-normal distribution. Because increased serum total IgE level is one of the major characteristics of atopic asthma, the genetic effects of these polymorphisms were tested on serum total IgE levels. [Table III](#) shows the distribution of $\log_{10}$ IgE with respect to genotypes for all 6 polymorphisms. A significant effect was observed at the genotypic level for the rs3806448 polymorphism in both the patient cohorts (adult patients, $P = .003$; pediatric patients, $P = .008$) as well as in controls of both cohorts (adult controls, $P = .03$; pediatric controls, $P = .03$), whereas for the rs10494132 polymorphism, a significant effect was observed at the genotypic level in the patients of both the cohorts (adult patients, $P = .008$; pediatric patients, $P = .01$; [Table III](#)). This association was found to be highly significant when calculated in the total population without considering the disease status ($P < .00001$, data not shown). No significant difference with respect to $\log_{10}$ serum total IgE was observed for the rs2282290 polymorphism at the level of alleles or genotypes.

Altered transcriptional activity of *CHIA* gene by promoter SNPs

Generating 2 locus haplotypes by using the promoter SNPs rs3806448 and rs10494132, we observed that *GT* was the most frequent protective haplotype, whereas *AT* was found to be the risk haplotype ([Fig E2](#)). To correlate this functionally with its transcript level, we constructed reporter vectors containing these SNPs and measured their transcriptional activities by transient transfection assays in the human lung epithelial carcinoma cell line A549 (as described in the [Online Repository](#)). We observed that the construct containing the risk promoter haplotype *AT* resulted in a 3-fold increase in transcription ($P < .0013$) over that of the protective promoter haplotype *GT* ([Fig 2](#)), suggesting that these promoter SNPs are functionally associated with *CHIA* gene expression.

TABLE II. Genotype distribution of *CHIA* polymorphisms in adult atopic patients with asthma, pediatric patients with asthma, normal controls, and pediatric controls in an Indian population

Locus	Group	Genotype	P value*	P value†
rs3806448		AA		
	A _{adult}	60 (22.30%)		
	NC	34 (11.68%)		
	A _{pediatric}	38 (25.50%)		
rs10494132		AG		
	PC	13 (13.00%)		
	A _{adult}	150 (56.60%)		
	NC	166 (57.84%)		
rs3818822		GG		
	A _{adult}	6 (2.25%)		
	NC	12 (4.17%)		
	A _{pediatric}	1 (0.70%)		
K17R		AA		
	PC	1 (1.01%)		
	A _{adult}	216 (81.20%)		
	NC	223 (77.97%)		
rs2275254		AG		
	A _{adult}	137 (51.70%)		
	NC	137 (47.08%)		
	A _{pediatric}	72 (48.32%)		
rs2282290		GG		
	PC	46 (46.00%)		
	A _{adult}	49 (18.15%)		
	NC	94 (33.33%)		
CHIA-ht1[GTGA]		AA		
	A _{adult}	7 (2.68%)		
	NC	15 (5.36%)		
	A _{pediatric}	0 (0.00%)		
CHIA-ht2[ATGG]		AG		
	PC	8 (8.25%)		
	A _{adult}	17 (6.51%)		
	NC	8 (2.86%)		
		GG		
	A _{adult}	146 (54.07%)		
	NC	133 (47.16%)		
	A _{pediatric}	92 (61.33%)		
		AG		
	PC	48 (47.52%)		
	A _{adult}	72 (27.59%)		
	NC	108 (38.57%)		
		GG		
	A _{adult}	182 (69.73%)		
	NC	157 (56.07%)		
	A _{pediatric}	41 (29.29%)		
		AG		
	PC	34 (35.05%)		
	A _{adult}	34 (35.05%)		
	NC	34 (35.05%)		

A_{adult}, Adult atopic patients with asthma; NC, normal controls; A_{pediatric}, pediatric patients with asthma; PC, pediatric controls; CHIA, acidic mammalian chitinase. Values in boldface indicate a statistically significant association.

*Armitage trend test *P* values comparing normal controls vs adult atopic patients with asthma.

†Armitage trend test *P* values comparing pediatric controls vs pediatric atopic patients with asthma.

‡The association was found to be statistically significant even after applying the correction for multiple testing ($\alpha_{adj} = 0.01$ for the SNPs and $\alpha_{adj} = 0.007$ for the haplotypes).

Allele-specific binding of *Oct-1* to the *CHIA* rs10494132C/T polymorphism

To check the functional significance of the rs10494132 SNP, nuclear extracts were prepared from A549 cell line, and EMSA was performed by using oligonucleotides containing C or T variant, as detailed in [Methods](#) and the Online Repository. Distinct differences were observed in the binding of oligonucleotides containing either the rs10494132C or the rs10494132T variant of the *CHIA* promoter. Three distinct DNA-protein complexes (complex A, complex B, and complex C) were detected for the rs10494132C probe ([Fig 3, lane 2](#)), whereas for the rs10494132T, only 1 complex (complex B) was observed ([Fig 3, lane 9](#)). The specificity of these complexes was tested by using 100-fold excess of the unlabeled C or T probe ([Fig 3, lanes 3, 4, 10, and 11](#)) and an irrelevant nonspecific oligonucleotide

([Fig 3, lanes 6 and 12](#)). The unlabeled C probe inhibited the formation of these complexes, whereas the nonspecific oligo was unable to inhibit the formation of complex A only. Therefore, these data suggest that complex A formation is specific for the rs10494132C variant of the *CHIA* promoter. Further, our super-shift assay results (as described in the [Online Repository](#)) provide evidence that the transcription factor *Oct-1* physically interacts with the consensus *Oct-1* binding sequence upstream of the *CHIA* gene containing rs10494132 SNP.

DISCUSSION

To elucidate the genetic role of *CHIA* in asthma pathogenesis, we have undertaken an extensive genetic study focusing mainly on promoter polymorphisms and nonsynonymous variants.

TABLE III. Log₁₀ serum total IgE levels ($\pm$ SEs) in the context of genotypes in adult atopic patients with asthma, pediatric patients with asthma, normal controls, and pediatric controls

Locus	Group	Genotype			P value
rs3806448		AA	AG	GG	
	A _{adult}	3.18 $\pm$ 0.53 (37)	2.93 $\pm$ 0.50 (90)	2.81 $\pm$ 0.51 (48)	.003
	NC	2.41 $\pm$ 0.68 (22)	2.20 $\pm$ 0.60 (104)	2.05 $\pm$ 0.68 (116)	.03
	A _{pediatric}	3.03 $\pm$ 0.33 (26)	2.84 $\pm$ 0.45 (68)	2.62 $\pm$ 0.49 (22)	.008
rs10494132		TT	TC	CC	
	A _{adult}	2.97 $\pm$ 0.51 (113)	2.77 $\pm$ 0.57 (68)	2.62 $\pm$ 0.40 (15)	.008
	NC	2.19 $\pm$ 0.63 (133)	2.15 $\pm$ 0.68 (93)	2.34 $\pm$ 0.41 (18)	.5
	A _{pediatric}	2.92 $\pm$ 0.44 (72)	2.72 $\pm$ 0.48 (33)	2.55 $\pm$ 0.42 (11)	.01
rs3818822		AA	AG	GG	
	A _{adult}	2.65 $\pm$ 0.32 (3)	2.86 $\pm$ 0.08 (47)	2.96 $\pm$ 0.04 (113)	.39
	NC	1.95 $\pm$ 0.19 (11)	2.16 $\pm$ 0.07 (73)	2.18 $\pm$ 0.05 (161)	.51
	A _{pediatric}	2.05 $\pm$ 0.46 (1)	2.86 $\pm$ 0.09 (23)	2.82 $\pm$ 0.04 (88)	.24
K17R		AA	AG	GG	
	A _{adult}	2.94 $\pm$ 0.04 (154)	2.96 $\pm$ 0.1 (27)	3.32 $\pm$ 0.57 (1)	.77
	NC	2.17 $\pm$ 0.04 (194)	2.19 $\pm$ 0.09 (49)	2.35 $\pm$ 0.38 (3)	.89
	A _{pediatric}	2.84 $\pm$ 0.04 (95)	2.73 $\pm$ 0.10 (18)	2.46 $\pm$ 0.46 (1)	.48
rs2275254		AA	AG	GG	
	A _{adult}	2.98 $\pm$ 0.49 (20)	2.93 $\pm$ 0.55 (68)	2.91 $\pm$ 0.55 (92)	.88
	NC	2.16 $\pm$ 0.45 (15)	2.21 $\pm$ 0.65 (115)	2.13 $\pm$ 0.62 (115)	.65
	A _{pediatric}	2.84 $\pm$ 0.25 (8)	2.92 $\pm$ 0.40 (54)	2.76 $\pm$ 0.52 (54)	.16
rs2282290		AA	AG	GG	
	A _{adult}	3.06 $\pm$ 0.52 (26)	2.95 $\pm$ 0.54 (107)	2.82 $\pm$ 0.58 (50)	.187
	NC	2.21 $\pm$ 0.65 (82)	2.11 $\pm$ 0.68 (116)	2.31 $\pm$ 0.52 (43)	.19
	A _{pediatric}	2.83 $\pm$ 0.39 (13)	2.83 $\pm$ 0.51 (76)	2.84 $\pm$ 0.34 (28)	.99
	PC	2.41 $\pm$ 0.09 (19)	2.56 $\pm$ 0.07 (27)	2.44 $\pm$ 0.14 (8)	.39

A_{adult}, Adult atopic patients with asthma; NC, normal controls; A_{pediatric}, pediatric patients with asthma; PC, pediatric controls.

Values in parentheses denote number of individuals in each group. Values in boldface indicate a statistically significant association.

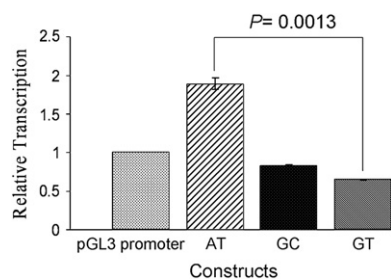


FIG 2. Transient transfection assays were performed in the human lung carcinoma cell line A549. Transcription of the *CHIA* promoter templates were separately normalized relative to the activity of the pGL3 promoter construct. Data points represent the average of 3 independent transfection experiments with error bars representing the SD.

Because of the existing strong LD among the polymorphisms in our sequencing cohort (60 individuals), we selected 6 SNPs for high throughput genotyping in 2 independent cohorts composed of 813 individuals. None of the nonsynonymous polymorphisms was found to be associated with asthma in both the cohorts. However, the promoter SNP, rs3806448, and the rs2282290 SNP were significantly associated with atopic asthma. Also, the rs3806448 and the rs10494132 SNPs were found to be significantly associated with the serum total IgE levels. Very recently, Bierbaum et al¹⁵ reported a significant association of the *CHIA* gene with bronchial asthma in the German population, but with results

varying from ours. They found significant association with a non-synonymous polymorphism, K17R variant (S17 in our case), and a noncoding SNP, rs3818822 (S16), whereas in our study, the most significant association was observed with the promoter polymorphisms and a downstream polymorphism (not included in the study by Bierbaum et al¹⁵). Various reasons including different genetic make-up could account for the varying results of the 2 studies. For example, the end phenotype chosen by them is bronchial asthma, which consists of both atopic and nonatopic asthma, in comparison with only atopic asthma in our case. Further, 1 of the 2 cohorts used by Bierbaum et al¹⁵ is not age-matched (normal controls and pediatric patients with asthma), and no multivariate analysis has been performed. In the current study, we have used 2 distinct age and ethnicity-matched study cohorts and also confirmed the associations by using multivariate analysis. We have also checked the genetic homogeneity between the patient and control groups by genotyping 39 microsatellite markers yet unlinked to asthma or related atopic disorders. Because the function of a gene can be modulated by SNPs present in the regulatory regions and/or SNPs present in the nonsynonymous coding regions, the study by Bierbaum et al¹⁵ and ours could be complementary.

Recent studies clearly showed that expression of *CHIA* is critically regulated in the lungs of individuals with asthma.⁷ Our study is important in this respect because it clearly demonstrates the association of the promoter SNPs with asthma and IgE levels,

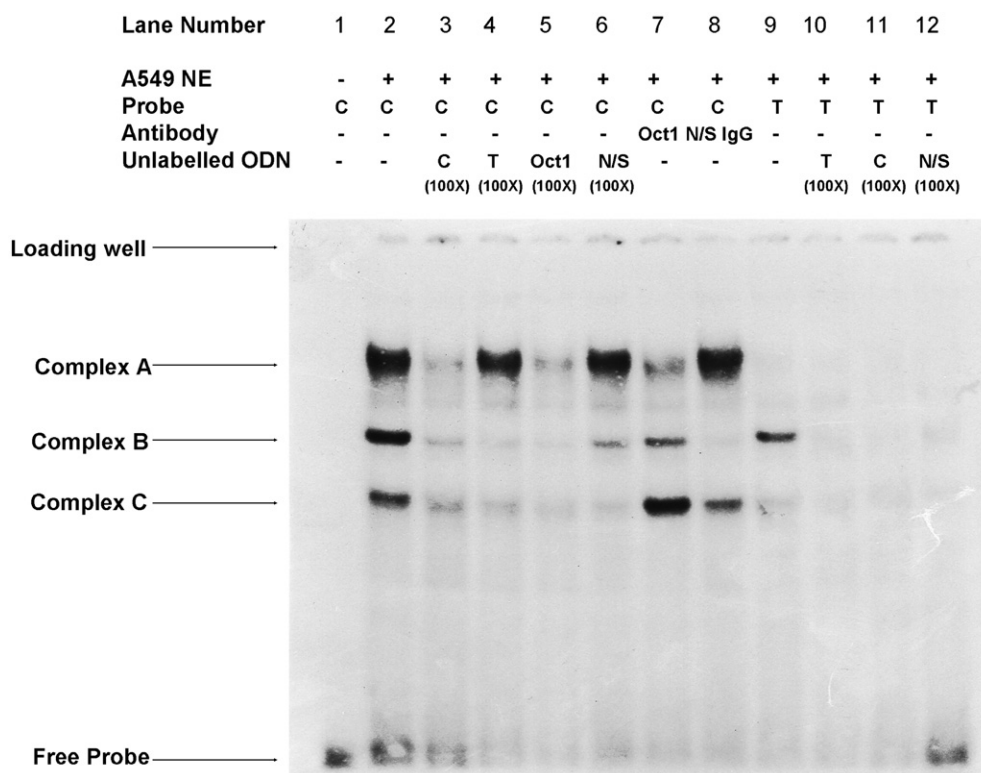


FIG 3. Analysis of differential binding of nuclear proteins to the C and T allele of *CHIA* rs10494132C/T polymorphism was performed by EMSA, as described in the Methods section. NE, Nuclear extract; ODN, oligonucleotide; N/S, nonspecific oligo.

which could probably contribute in the regulation of *CHIA* transcription and thus its gene expression. To verify the functional effect of the associated polymorphisms on its transcriptional levels, we performed real-time PCRs by using cDNAs prepared from leukocytes of 50 individuals but observed no detectable levels of *CHIA* transcript (data not shown). This result is not surprising because *CHIA* expression was primarily detected in the lungs of patients with asthma.⁷

Interestingly, our 2-locus haplotype association study containing the rs3806448 and rs10494132 promoter polymorphisms indicated that AT is the risk haplotype, whereas GT is the protective haplotype (see this article's Fig E2 in the Online Repository at www.jacionline.org). This observation was functionally confirmed by using reporter gene constructs containing the risk and the protective haplotypes (Fig 3). Also, the rs10494132 SNP, which was found to be significantly associated with the serum total IgE levels in both the asthmatic patient groups, showed a substantial change in transcription factor binding site in the presence of the C allele in our EMSA and supershift experiments. The C to T change at the rs10494132 SNP leads to the abolition of binding site of transcription factor, *Oct-1*. It is therefore likely that the differential binding of *Oct-1* to the promoter variant C may lead to a change in the *CHIA* expression. The possibility that other SNPs, present in LD with rs10494132 or the rs3806448 polymorphism, may influence the expression of *CHIA* cannot be excluded. Interestingly, by using the statistical software TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>), we observed that a few other promoter SNPs in complete LD with the rs3806448 SNP also showed potential transcription factor binding sites and hence

may be functionally significant in atopic asthma. However, functional analysis with allele-specific oligo is required to confirm our results.

Also, a downstream SNP, rs2282290, showed a statistically significant association with atopic asthma but not with serum total IgE levels in our study. We speculate that this SNP could be an important part of the enhancer/silencer binding site, regulating gene expression or present in LD with some other functional polymorphisms in the *CHIA* gene, modulating its expression/function. It is also possible that this SNP is in LD with other SNPs present in a nearby gene, which could be involved in asthma pathogenesis. For further understanding of the contribution of these genetic variants toward asthma, we also constructed 4-locus haplotypes (Table II; Online Repository). The haplotypic analyses were consistent with the single SNP association results (Table II), which further emphasized the functional role of rs10494132 SNP in asthma pathogenesis.

In summary, our results establish for the first time a significant association of *CHIA* with atopic asthma and serum total IgE levels. Moreover, we demonstrate that the rs10494132 polymorphism alters the binding of transcription factor *Oct-1*, and promoter polymorphisms modulate the regulation of *CHIA* gene expression. Thus, our results may initiate further research in elucidating the exact role of *CHIA* in asthma pathogenesis.

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Clinical implications: *CHIA* gene variants associated with atopic asthma and serum total IgE could be useful in identifying individuals at risk for developing asthma.

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METHODS

SPT and serum total and specific IgE estimation

Sixteen common environmental allergens with both negative and positive controls were used for the SPT (house dust mite, *Amaranthus spinosus*, *Brassica campestris*, *Cynodon dactylon*, *Parthenium hysterophorus*, *Propolis juliflora*, *Ricinus communis*, *Alternaria tenuis*, *Aspergillus fumigatus*, cockroach male, cockroach female, mosquito, moth, grain dust rice, hay dust, house dust). Specific serum IgE was estimated for 6 common allergens (house dust mite, 0.27; cockroach male, 0.144; mosquito, 0.23; moth, 0.03; grain dust rice, 0.05; hay dust, 0.04) by using the method described by Voller et al^{E1} with slight modifications. OD values for different serum samples were analyzed according to the criteria by Calenoff et al.^{E2} The upper limit of mean + 2SD was taken as the cutoff as an indication of raised specific IgE against respective antigen extracts. The OD values within parentheses above show the specific serum IgE cutoff values for each allergen, respectively.

Sequencing and genotyping of the polymorphisms

In an effort to identify functional SNPs in *CHIA*, we extracted information from the existing NCBI database single nucleotide polymorphism and identified 3 nonsynonymous SNPs in the exonic regions of the gene and 2 SNPs 1.4 kb upstream of the gene (putative promoter-enhancer region). Because no 3' UTR SNPs were identified, we also genotyped an SNP 266 bp downstream of the gene. We also included a novel nonsynonymous SNP and a 5' UTR, which had been reported by Bierbaum et al^{E3} to be associated with asthma. To verify the polymorphic status of these SNPs in the Indian population, we sequenced the genomic DNA of 60 individuals (15 adult patients with asthma, 15 normal controls, 15 pediatric patients with asthma, and 15 pediatric controls) by using the primer sets as detailed in Table E1, by the dideoxy chain termination method using the Big Dye Terminator cycle sequencing kit on an ABI 3100 sequencer (Applied Biosystems, Foster City, Calif). In our sequencing efforts, we further identified 13 promoter SNPs and 2 SNPs in the 5' UTR of the gene. On the basis of gene frequency and linkage disequilibrium, 6 variants were further genotyped in the total population.

The 6 polymorphisms were studied by using the SNaPshot ddNTP Primer Extension Kit (Applied Biosystems) as per the manufacturer's instructions. These samples were subsequently electrophoresed by using the ABI Prism 3100 Genetic Analyzer. The results were analyzed by using the ABI Prism GeneMapper v3.7 (Applied Biosystems).

Genetic homogeneity between the patients and controls. The genetic homogeneity between the patients and controls from both groups was confirmed by genotyping various loci, as yet unlinked to asthma or related atopic disorders ($P \geq .05$, data not shown; the panel of unlinked markers was D20S117, D6S1574, D20S196, D6S470, D12S368, D16S404, D6S446, D16S3136, D6S441, D8S264, D8S258, D8S1771, D8S285, D8S260, D8S270, D8S1784, D8S514, D8S284, D8S272, D5S406, D5S416, D5S419, D5S426, D5S418, D5S407, D5S647, D5S424, D5S641, D5S428, D5S2027, D5S471, D5S2115, D5S436, D5S422, D5S408, D6S281, D6S308, D6S264, and D6S287).

Cells and cell culture. The epithelial human lung carcinoma line A549 was procured from National Center for Cell Science, Pune, India. The A549 cell lines were grown and maintained in Dulbecco modified Eagle medium supplemented with 44 mmol/L NaHCO₃, 10% heat-inactivated FCS, and 1X Antibiotic-Antimycotic solution (Sigma, St Louis, Mo). For subculturing, the cells were dislodged by using 0.125% trypsin/0.01 mol/L EDTA solution in PBS (pH, 7.4). The viability of cells and cell count were determined by Trypan blue staining.

Preparation of nuclear extracts. Nuclear extracts were prepared by using a modification of previously published methods.^{E4} Briefly, A549 cells (2×10^6 cells/mL) were washed with PBS, dislodged using a cell scraper, and pelleted by centrifugation at 300g. The cells were resuspended in cell lysis buffer (10 mmol/L HEPES, pH 7.9; 1.5 mmol/L MgCl₂; 10 mmol/L KCl; 1 mmol/L phenylmethylsulfonyl fluoride; 1 mmol/L dithiothreitol; 0.5% Nonidet P40 [Shell International Petroleum Company Limited, London, UK]; 0.1 mmol/L ethyleneglycol-bis-[β -aminoethylether]-N,N,N',N'-tetraacetic acid

[EGTA]; and 0.1 mmol/L EDTA) and allowed to swell on ice for 10 minutes. This was followed by centrifugation at 3300g for 15 minutes. The supernatant was stored as cytoplasmic extract and the nuclear pellet resuspended in nuclear extraction buffer (20 mmol/L HEPES, 25% glycerol, 1.5 mmol/L MgCl₂, 420 mmol/L NaCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L dithiothreitol) and incubated for 30 minutes at 4°C. The extracted nuclei were pelleted at 25,000g for 15 minutes at 4°C, and the supernatant was collected as nuclear extract. The protein concentration was estimated by using the bicinchoninic acid method. The nuclear and cytoplasmic extracts were stored at -70°C.

Preparation of reporter constructs. Reporter constructs were generated by cloning 575 base pair promoter segments, 1.002 kb to 1.577 kb upstream from the putative transcription initiation site of the human *CHIA* gene into the luciferase reporter plasmid pGL3-Promoter (Promega, Madison, Wis). Promoter DNA was PCR amplified from genomic DNA of individuals having any of the 3 promoter haplotypes—AT (protective), GT (risk), or GC (nonassociated)—by using appropriate primer pairs (upstream primer for the promoter construct, 5'-CGGGGTACCTCTGCCATCGCTTCTGTCTCTGA-3'; downstream primer, 5'-CTAGCTAGCTTCCTTCCCTTTCACCCATTATTA-3'). PCR fragments were purified by gel extraction, digested with *KpnI* and *NheI* restriction enzymes for 16 hours at 37°C, and ligated into the pGL3-Promoter vector previously digested with the same enzymes.

Transient transfection assays. The activity of the *CHIA* promoter was determined by measuring the levels of expression of the luciferase reporter gene. Briefly, constructs (3 μ g *CHIA* promoter-containing vector or 3 μ g pGL3-Promoter plasmid, and 350 ng β -galactosidase expression plasmid) were cotransfected by using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif) into the human A549 cells. DNA was mixed with 6 μ L Lipofectamine 2000 (concentration, 1 mg/mL) in 244 μ L OptiMeM (Invitrogen) to a final volume of 500 μ L. The DNA complex was added to 1×10^6 cells and incubated for 5 hours at 37°C. After incubation, the media was discarded and 2 mL of fresh OptiMeM media was added. The cells were then plated and incubated for an additional 48 hours at 37°C. After incubation, cells were pelleted by centrifugation at 2000 rpm for 10 minutes and lysed in reporter lysis buffer (Promega, Madison, Wis). Cellular extracts were assayed for luciferase activity by using a luminometer (Orion Microplate Luminometer, Berthold Detection Systems, Pforzheim, Germany). Data were normalized to β -galactosidase expression (Promega), and relative transcription index was calculated by comparison with the activity of the pGL3-Promoter construct. The experiments were repeated 3 times.

EMSA

CHIA rs10494132C (5-CATTCCATGACTGTTATTATT-3 and 5-AATAA TAACAGTCATGGAATG-3) and *CHIA* rs10494132T (5-CATTCCAT GATTGTTATTATT-3 and 5-AATAATAACAATCATGGAATG-3) were synthesized, labeled with ³²P-ATP, and used for binding assays. *In vitro* binding reactions between ³²P-end-labeled double-stranded oligonucleotides and nuclear extracts were performed in a total volume of 20 μ L, containing 2 μ L 10X binding buffer (12 mmol/L HEPES, 50 mmol/L NaCl, 10 mmol/L TrisCl [pH 7.5], 10% glycerol, 1 mmol/L EDTA, and 1 mmol/L dithiothreitol), 1 μ L 1.0 μ g/ μ L poly dI-dC (Sigma), and 20 μ g nuclear protein. This reaction was allowed to proceed at 25°C to 28°C for 30 minutes before the addition of 2 μ L nondenaturing loading buffer (0.2% bromophenol blue, 20% glycerol). For supershift assay, 7.5 μ g rabbit anti-Oct-1 antibody or 7.5 μ g rabbit antihuman IgG (nonspecific antibody control) was added to the reactions. The samples were electrophoresed on 1.5-mm-thick 5% polyacrylamide gel by using TRIS-glycine buffer (pH 8.5) and visualized by autoradiography.

RESULTS

Haplotypic association of *CHIA* with atopic asthma and serum total IgE

Using the SNPs rs3806448, rs10494132, rs3818822 and rs2282290, which showed significant association with asthma or

IgE, a total of 14 four-locus haplotypes were generated. The distribution of the haplotypes with frequency >5% in the 4 groups is shown in Fig E3. When we analyzed the haplotype frequency difference in the 2 groups (adult patients vs normal controls) by using the Monte Carlo test with 1 million simulations, a highly significant association was observed (normal χ^2 [T1] 51.2, $df = 13$, $P = .000093$; and χ^2 from table after collapsing columns with small expected values together [T2] was 39.87, $df = 9$, $P = .000008$). The statistical significance was sustained when haplotypes of the pediatric patients and pediatric controls were compared (normal χ^2 [T1] 28.8, $df = 12$, $P = .0042$; and χ^2 [T2] 22.73, $df = 5$, $P = .00039$). The global test for the significance calculated by the expectation-maximization algorithm also revealed a significant association of the haplotypes with atopic asthma (for adult cohort, $P = .000085$; and for pediatric cohort, $P = .005$, using FAMHAP with 10,000,000 simulations). Haplotype *GTGA* was found to be the most frequent protective haplotype (adult patients vs normal controls, common OR = 0.60; pediatric patients vs pediatric controls, common OR = 0.52), whereas *ATGG* was found to be the risk haplotype (adult patients vs controls, common OR = 1.76; pediatric patients vs pediatric controls, common OR = 2.04; Tables II and E3). The association was found to be statistically significant even after applying the correction for multiple testing ($\alpha_{adj} = 0.007$).

When different haplotypes with frequency <5% were clubbed together and analyzed with respect to the log₁₀ serum total IgE levels, a highly significant difference was obtained (for adult patients, F ratio = 3.89, $df = 7$, $P = .0004$; for pediatric patients, F ratio = 2.94, $df = 7$, $P = .005$; for normal controls, F ratio = 2.51, $df = 7$, $P = .015$; for pediatric controls, F ratio = 2.39, $df = 7$, $P = .024$; and without considering the asthma status, F ratio = 3.8, $df = 7$, $P = .0004$ for adult cohort; and F ratio = 2.77, $df = 7$, $P = .007$ for pediatric cohort).

Next, we generated 2-locus haplotypes by using the promoter SNPs rs3806448 and rs10494132. A total of 4 two-locus haplotypes, *AT*, *GT*, *AC* and *GC*, were generated, of which *AC* was a minor haplotype (frequency <5% in both study cohorts) and hence was not considered for further analysis. When we analyzed the haplotype frequency difference in the 2 study cohorts, highly significant association was observed (adult cohort, $\chi^2 = 33.08$, $df = 3$, $P = .0001$; pediatric cohort, $\chi^2 = 15.63$, $df = 3$, $P = .001$). Haplotype *GT* was found to be the most frequent protective

haplotype (adult patients vs normal controls, common OR = 0.60; pediatric patients vs pediatric controls, common OR = 0.52), whereas *AT* was found to be the risk haplotype (adult patients vs controls, common OR = 1.54; pediatric patients vs pediatric controls, common OR = 2.04).^{E4} Haplotype *GC* was not found to be associated with asthma.

Allele-specific binding of *Oct-1* to the *CHIA* rs10494132C/T polymorphism

Software TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>) predicted that the T to C substitution would lead to the abrogation of various transcription factor binding sites. We tested this hypothesis by performing competition experiments with a 50-fold excess of various unlabeled oligonucleotides specific for *Oct-1*, CCAAT/enhancer binding protein, *GATA*, Yin-Yang-1, pre-B-cell leukemia transcription factor, and V-ets erythroblastosis virus E26 oncogene homolog transcription factor binding sites (data not shown). Interestingly, we observed that 50-fold or 100-fold excess of unlabeled *Oct-1* oligo was able to inhibit the binding of nuclear proteins with the C variant (Fig 3, lane 7), indicating that this region contains an overlapping *Oct-1* binding and the binding is diminished because of the C to T substitution at rs10494132. Moreover, the Oct-1/DNA complex was found to be diminished in the presence of Oct-1 antibody (Fig 3, lane 7), but not in the presence of a nonspecific control anti-IgG antibody (Fig 3, lane 8). Thus, our EMSA and supershift assay results provide evidence that the transcription factor *Oct-1* physically interacts with the consensus *Oct-1* binding sequence upstream of the *CHIA* gene.

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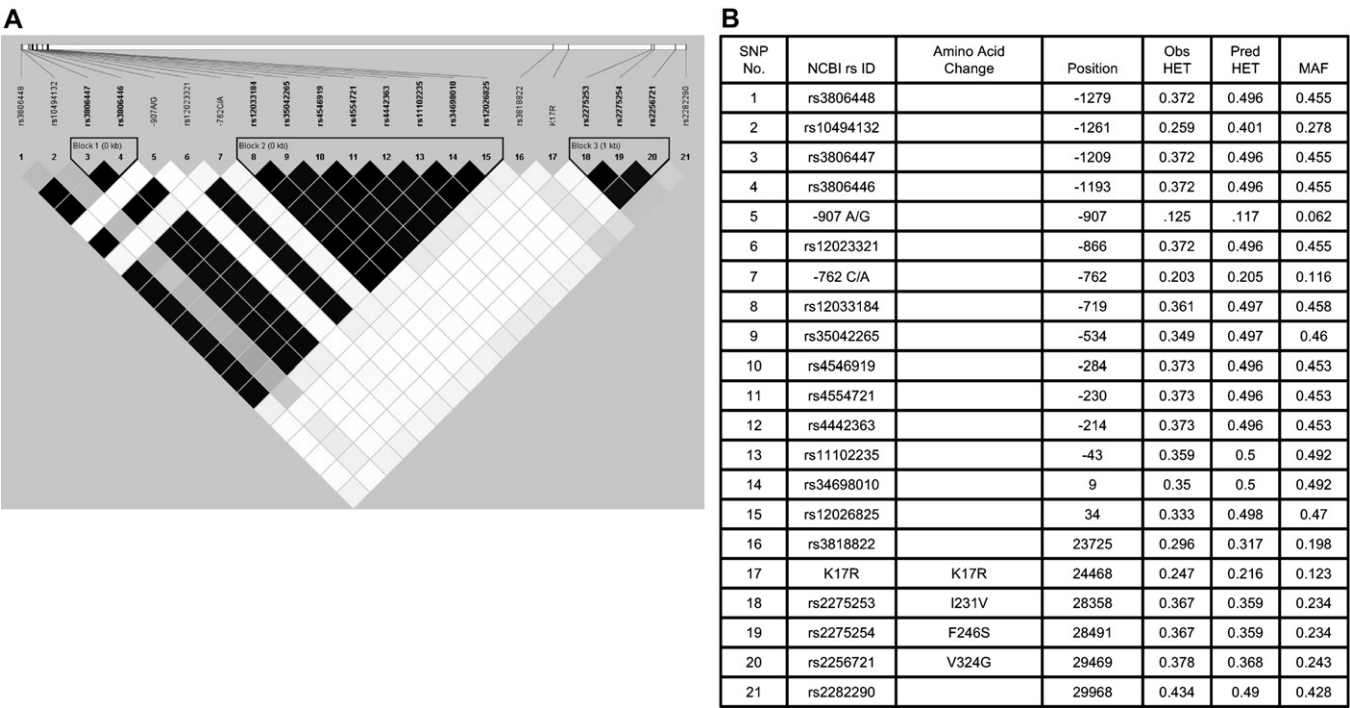


FIG E1. LD plot and SNP information in *CHIA*. **A**, LD map of *CHIA*. LD was calculated using Haploview. *Black boxes* indicated strong evidence of LD between the polymorphisms ($r^2 = 1$), whereas *white boxes* showed no or little LD between pairs ($r^2 = 0$). $0 < r^2 < 1$ Represented by *gray boxes*. **B**, General information about the SNPs from the sequencing cohort. *MAF*, Minor allele frequency. *Obs*, Observed; *Pred*, predicted; *HET*, heterozygosity.

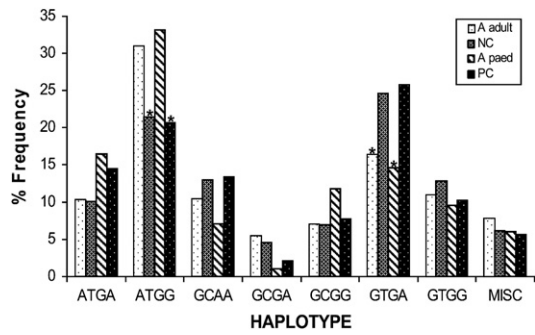


FIG E2. Haplotypes with frequency >5% constructed by PHASE v2.1 using 4 polymorphisms—rs3806448, rs10494132, rs3818822, and rs2282290—in the cohorts of adult atopic patients with asthma (*A adult*), normal controls (*NC*), pediatric patients with asthma (*A paed*), and pediatric controls (*PC*). *Haplotypes with significant frequency difference compared with controls.

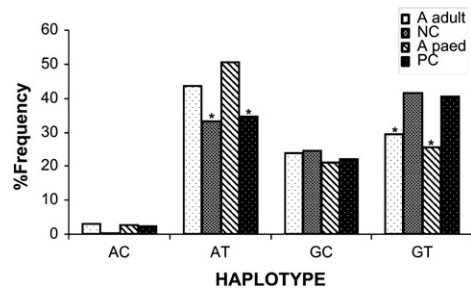


FIG E3. Haplotypes with frequency >5% constructed by PHASE v2.1 using the 2 promoter polymorphisms rs3806448 and rs10494132 in the cohorts of adult atopic patients with asthma (*A adult*), normal controls (*NC*), pediatric patients with asthma (*A paed*), and pediatric controls (*PC*). *Haplotypes with significant frequency difference compared with controls.

TABLE E1. *CHIA* polymorphisms, localization, and genotyping primers, and PCR cycling conditions

SNP	Location	Direction	PCR primer	Cycling conditions	Extension primer
rs3806448	Promoter	Fwd	5'-TCTGCCATCGCTTCTGTCTCTGA-3'	60°C; 40 cycles	5'-AAAAGTGATACTGCGTAGTTAA GATCATC-3'
		Rev	5'-TTCCTTCCCTTTGCACCCATTTA-3'		
rs10494132	Promoter	Fwd	5'-TCTGCCATCGCTTCTGTCTCTGA-3'	60°C; 40 cycles	5'-ATTCTTCCAAGGCCAATAATAACA-3'
		Rev	5'-TTCCTTCCCTTTGCACCCATTTA-3'		
rs3818822	5'UTR	Fwd	5'-GAAGTTTTTCAGTAGGGGAGG-3'	55°C; 35 cycles	5'-CCATTGGAGGCTGGAACTTC-3'
		Rev	5'-GATCACATGGGGCTAACTTG-3'		
K17R	Exon	Fwd	5'-GTCTCACCCCTGCCTTCTTTG-3'	55°C; 35 cycles	5'-TTTCATCACCTCAGTCATCA-3'
		Rev	5'-ACCCAATTCTCCTCGGAAAG-3'		
rs2275253	Exon	Fwd	5'-ACCCCAACTGGAGACATGAAAGAAGAA-3'	60°C; 40 cycles	5'-CCTTAATATCGAAGCTCTTGA-3'
		Rev	5'-GCAAGGGGGAGGTGGGAAGAC-3'		
rs2282290	Downstream	Fwd	5'-CGGGAACGGGAGCGGGAGTA-3'	64°C; 40 cycles	5'-GCAATTTTCCTCAGGAAGGAGCTGA-3'
		Rev	5'-CGAGCTGCAAATCTGAGGTGTGATAA-3'		

TABLE E2. Multiple logistic regression analysis of the *CHIA* polymorphisms for risk of asthma

Variable	Adult cohort			Pediatric cohort		
	LR χ^2	df	P value	LR χ^2	df	P value
Model 1						
rs3806448	16.72	2	.0003	8.96	2	.01
rs10494132	4.11	2	.13	0.51	2	.77
rs3818822	1.94	2	.37	0.12	2	.93
K17R	5.34	2	.06	1.71	2	.42
rs2275253	6.27	2	.04	3.83	2	.14
rs2282290	10.77	2	.01	10.93	2	.004
Age	17.7	1	.0001	1.18	1	.27
Sex	5.05	1	.28	0.96	1	.32
Model 2						
rs3806448	21.19	2	.0001	6.7	2	.03
rs10494132	7.65	2	.02	1.12	2	.57
rs3818822	1.08	2	.58	0.66	2	.717
K17R	8.38	2	.01	1.77	2	.41
rs2275253	7.85	2	.02	5.2	2	.07
rs2282290	15.05	2	.0005	14.86	2	.0006

Model 1: There was controlling for age and sex; model 2: There was no controlling for age and sex. All 6 SNPs were taken together in a single model for calculating logistic regression.

LR, Likelihood ratio.