

Association of the –1072G/A Polymorphism in the *LTC4S* Gene with Asthma in an Indian Population

Amrendra Kumar Shilpy Sharma Anurag Agrawal Balaram Ghosh

Molecular Immunogenetics Laboratory and Center of Excellence for Translational Research in Asthma and Lung Disease, CSIR-Institute of Genomics and Integrative Biology, Delhi, India

Key Words

Asthma • Case-control study • Family-based analysis • Haplotypes • Leukotriene pathway • Single nucleotide polymorphisms

Abstract

Background: Atopic asthma, the most common chronic disease affecting children and young adults, is a complex disorder with variable phenotypes. Cysteine leukotrienes (Cys-LTs) are powerful bronchoconstrictors and play a critical role in airway inflammation and remodeling that are characteristic of asthma. **Objective:** To investigate the association of *ALOX5*, *LTC4S* and *CysLTR2* gene polymorphisms with atopic asthma in an Indian population. **Methods:** A total of 19 single nucleotide polymorphisms (SNPs) within these genes were genotyped in a family-based cohort (n = 239) and a case-control cohort (139 cases and 194 controls) followed by association analyses. **Results:** We found a significant association of the –1072G/A (rs3776944) SNP with atopic asthma in the family-based association analysis (p = 0.0004). These results were also replicated in the case-control cohort (p = 0.009). The allele A was negatively associated with atopic asthma. We also noted a significant association in the two-locus (rs3776944G/A and rs730012A/C) haplotypic analysis of this gene both in the family-based (p = 0.03) and the case-

control (p = 0.02) analyses. **Conclusion:** This study supports the role of the *LTC4S* gene polymorphism in genetic susceptibility to atopic asthma in an Indian population.

Copyright © 2012 S. Karger AG, Basel

Introduction

Asthma is a complex multifactorial disease where complex gene-gene and gene-environment interactions are proposed to bring about phenotypes characteristic of the disease [1]. Since different combinations of genes and environment factors may lead to the same phenotype, it is expected that genetic risk factors for asthma would vary across the globe due to genetic as well environmental differences. Due to significant contribution by the genetic factors in asthma, genetic studies have been conducted across the world unraveling several genetic modifiers that may influence disease susceptibility, progression and/or severity [1]. Cysteinyl leukotrienes (Cys-LTs) are the most potent bronchoconstrictors and also modulate inflammation; consequently, their role in asthma pathogenesis has been well documented [2, 3]. Higher levels of Cys-LTs have been found in urine, exhaled breath condensate and sputum samples of patients in comparison to normal controls [4]. Also, LT receptor antagonists have been shown to

Table 1. Characteristics of the patients and controls (case-control study) and the families (family-based study) of north/northwest India (native place)

	Patients (n = 147)	Controls (n = 199)	Probands (n = 247)	Parents
Age, years ¹	38.5 ± 15	37.0 ± 13.5	15.6 ± 11.2	43.7 ± 10.4
Sex ratio (male:female)	48:52	50:50	44:56	50:50
Family history of asthma/atopy ²	all	none	all	all
Smoking history ³	none	none	none	none
Reversibility from baseline FEV ₁ (after β ₂ -agonist usage), %	>15	not done	>15	>15 (all affected) ⁴
log ₁₀ serum total IgE, IU/ml ¹	2.86 ± 0.57	2.44 ± 0.6	2.84 ± 0.63	2.65 ± 0.57
Atopy/self-reported history of allergies and SPT ⁵ positivity	all	none	all	all (affected)

Patients, controls and families were recruited from Delhi, Lucknow (UP), Jaipur (Rajasthan), Shimla (HP), Chandigarh (Punjab) and Mumbai (Maharashtra). ¹ Means ± SD. ² Control individuals were subjected to a questionnaire to eliminate all individuals having atopic disorders or a family history of atopic disorders. ³ Patients and controls known to have experienced smoking in the past or suffering from parasitic infections were excluded from the study. ⁴ According to the design, at least one of the par-

ents had to be affected (atopic asthmatic). ⁵ Fifteen common environmental allergens (house dust mite, *Amaranthus spinosus*, *Brassica campestris*, *Cynodon dactylon*, *Parthenium hysterophorus*, *Propolis juliflora*, *Ricinus communis*, *Alternaria tenuis*, *Aspergillus fumigatus*, cockroach, mosquito, moth, grain dust rice, hay dust and house dust) were used for the skin prick test (SPT).

ameliorate asthma features in a subset of asthmatics [4]. However, studies of genetic variations in this pathway in Indians and their association with asthma are lacking.

Cys-LTs are generated by the 5-lipoxygenase (ALOX5) pathway, and several genes from this pathway have been shown to harbor polymorphisms that might alter the expression/regulation of the gene products, thus influencing genetic susceptibility to asthma [5]. For instance, a mutation in the *ALOX5* gene, which is the initial enzyme in the biosynthesis of LTs, in the upstream region (–176 to –146 bp) of the ATG (in a GC-rich region) codon with deletion of one, or two or addition of one zinc finger (Sp1/Egr-1) motif resulting in reduced Sp1/Egr-1 transcription factor binding and gene transcription has been identified. The mutations in the Sp1 repeat were found to be associated with airway hyperresponsiveness although no association with asthma was noted [6]. This polymorphic repeat has also been suggested to be a predictor of disease severity [7]. Recently, a polymorphic allele for the coding region polymorphism 760 G→A (E254K) in exon 6 was only noted in bronchial asthma patients but not in a cohort of non-allergic subjects, indicating that this polymorphism may be associated with bronchial asthma [8]. The LTC₄S enzyme conjugates reduced glutathione (GSH) with LTA₄ to form LTC₄ [4]. In the *LTC4S* gene, two promoter polymorphisms [–1072G/A (rs3776944) and –444T/C (rs730012)] have been studied in various populations [5, 9]. The rs730012T/C polymorphism was associated with asthma or asthma severity in different studies while no association with the rs3776944 SNP was reported in any study so far [9]. Another recently identi-

fied 10 G→A (Glu4Lys) polymorphism was associated with asthma and other allergic diseases [10]. Similarly, a number of studies have identified associations with various polymorphisms in the *CysLTR2* gene and asthma in different populations [9].

In this study, we have attempted to investigate the role of polymorphisms in *ALOX5*, *LTC4S* and *CysLTR2* genes with asthma in Indians. We report for the first time the association of the –1072G/A polymorphism in the *LTC4S* gene with asthma in our study population.

Patients and Methods

Subjects

The criteria for the inclusion of study subjects, asthmatics, normal controls and families, have been described previously [11] and detailed in the online supplementary material (www.karger.com/doi/10.1159/000336675). Briefly, asthmatic patients (n = 147, 38.5 ± 15 years), normal controls (n = 199, 37.0 ± 13.5 years) and families 247 (15.6 ± 11.2 years) ascertained through probands were recruited from our collaborating centers following clinician's diagnosed asthma (table 1) and a set of inclusion/exclusion criteria (online suppl. table 1).

Gene and SNP Selection

We selected two previously reported asthma-associated SNPs (rs3776944 and rs730012) and 3'UTR rs28365149 in the *LTC4S* gene. Since the *ALOX5* gene is bigger in size (82 kb) and not very well studied with atopic asthma, we sought to comprehensively genotype markers spanning the whole gene. Therefore, HapMap-validated SNPs were selected such that the gap between adjacent SNPs was 4–5 kb. We also included some previously studied SNPs in this gene even if they were not present in the HapMap data-

base. Subsequently, a total of 24 SNPs (rs2228064, rs2228065, rs10751382, rs10751383, rs10900213, rs10900215, rs10900216, rs11239505, rs11239523, rs12264801, rs12765320, rs1487562, rs1565096, rs2029253, rs2115819, rs3780894, rs3780908, rs3780914, rs3824612, rs4948671, rs4948672, rs7090328, rs7918542 and rs7099684) in the *ALOX5* gene were selected for genotyping. Similarly, in the *CysLTR2* gene, we selected two HapMap-validated SNPs (rs912278 and rs1323552) for our study. Note: we did not include *CysLTR1* in this study due to methodological issues since it is present on the X chromosome.

Genotyping and Data Cleaning

A detailed description of SNP selection in genes and OPA (Oligonucleotide Pool All) design has been provided in the online supplementary methods. SNPs in the *LTC4S* gene were genotyped using the SNaPShot ddNTP Primer Extension Kit (Applied Biosystems, Foster City, Calif., USA) while SNPs in *ALOX5* and *CysLTR2* were genotyped using the Illumina's Bead Array system/Golden Gate Assay (Illumina Inc., San Diego, Calif., USA) in accordance with the manufacturer's protocol [12]. The methodologies are provided in detail in the online supplementary methods. We retained markers for further analysis if the call rate was >90%, a maximum of one reproducibility error (5% replicates were used), a maximum of five mendelian errors and showed consistency with the Hardy-Weinberg equilibrium at the level of $p < 0.05$.

Data/Statistical Analyses

A detailed description of the statistical methods is provided in the online supplementary methods. Briefly, Hardy-Weinberg calculations were performed using PLINK (<http://pngu.mgh.harvard.edu/~purcell/plink/>) [13]. Pairwise linkage disequilibrium (LD) calculations and TagSNP selection was done using Haploview v 4.2 [14]. Single marker (Armitage trend test and genotypic association analysis) and haplotypic association studies were done using PLINK and FBAT (additive and genotypic model) [15, 16]. Odds ratios (OR) were also calculated using 2×2 contingency tables (<http://home.clara.net/sisa/>). Logistic regression analysis was performed to evaluate the effect of age and sex, if any, on the association of various genotypes on asthma or serum total IgE levels. A description of the combined p value approach using Fisher's method and multiple testing using Bonferroni's correction is to be found in the online supplementary methods. For the single gene analysis, a total of 19 SNPs were included in association analysis and therefore the SNPs showing a combined $p \leq 0.0026$ were considered to be significantly associated with asthma or total serum IgE.

Results

Genotyping and Data Cleaning

The rs28365149 SNP in the 3'UTR of *LTC4S* could not be genotyped due to technical problems of PCR amplifications (multiple primer sets and different strategies were used; data not shown). For the other two loci (rs3776944 and rs730012) in the *LTC4S* gene, 201 families were genotyped. In the *ALOX5* gene, rs7099684 had poor clustering

in Golden gate assay analysis and was, therefore, discarded. Since rs2228064 and rs2228065 had minor allele frequency <5% (in normal controls), they were also not included for further analysis. Of the 1,368 samples, 17 samples had a call rate <95% and were therefore removed. Furthermore, 8 triads were consistently showing transmission errors and were discarded. In total, therefore, 1,326 individuals [239 pedigrees with 993 individuals and case-control cohort (139 cases + 194 controls)] finally passed the quality control. After data cleaning, all the SNPs had a call rate >99%.

LD Plots and TagSNPs

The SNPs that passed the quality control criterion were used for genewise LD calculation and TagSNP selection. We used the LD values (r^2) to identify TagSNPs using the tagger as implemented in Haploview. Using tagger (gene wise), we selected 19 TagSNPs for our further association analyses (table 2).

Gene-Wise Association Analysis with Asthma

We noted a significant association of the rs3776944G/A (–1072G/A) polymorphism with asthma. Polymorphic allele A was found to confer a protective role in both family-based [OR 0.40 (0.23–0.67); $p = 0.0004$] and case-control analysis [OR 0.52 (0.31–0.85); $p = 0.009$]. Also, the combined p value obtained by Fisher's method was statistically significant ($p = 0.00005$; table 2). There was a significant difference in the transmission of the genotypes to the affected offspring in family-based association analysis (global $\chi^2 = 12.6$; $p = 0.002$). The wild-type homozygous genotype GG was significantly over-transmitted compared to affected offspring ($p = 0.0004$), while the heterozygote GA was significantly under-transmitted ($p = 0.0005$; table 3). In case-control analysis, the risk associated with the heterozygous GA genotype was significantly lower [OR 0.49 (0.28–0.83); $p = 0.008$] in comparison to the wild-type GG homozygote (table 3). None of the other SNPs showed a statistically significant association. The rs11239523 in the *ALOX5* gene showed a significant difference in the family-based study ($p = 0.014$) but not in the case-control cohort ($p > 0.5$). Also, the combined p value obtained by Fisher's test was not significant after Bonferroni correction ($p > 0.5$; table 3).

Gene-Wise Association Analysis with $\log_{10}$ Total Serum IgE

Total serum IgE levels follow a log normal distribution, and when cases and controls were compared with respect to log serum IgE levels, a statistically significant

Table 2. Results of family-based and case-control association analyses together with combined p values

Gene (Chr.)	SNP	Location	ALL (2>1)	Mn. ALL	Family-based analysis					Case-control analysis					p value	
					ALL FRQ	n	Z'	OR	p	F A	F U	χ^2	p	OR	com.	corr.
ALOX5 (10)	rs10751382	intron	A>G	G	0.411	198	-0.541	0.943	0.5887	0.38	0.37	0.086	0.7692	1.049	0.81157	1
	rs10900213	intron	C>A	A	0.413	190	-1.296	0.863	0.1950	0.42	0.46	1.020	0.3126	0.852	0.23150	1
	rs11239505	intron	A>G	G	0.261	174	-1.066	0.893	0.2866	0.32	0.27	2.673	0.1021	1.325	0.13260	1
	rs11239523	intron	A>G	G	0.085	78	-2.518	0.620	0.0118	0.12	0.11	0.148	0.7006	1.101	0.04795	0.9
	rs12264801	intron	G>A	A	0.467	170	-1.969	0.791	0.0490	0.50	0.49	0.056	0.8131	1.038	0.16824	1
	rs1487562	intron	G>A	A	0.183	136	-1.521	0.816	0.1284	0.21	0.18	0.882	0.3478	1.203	0.18345	1
	rs1565096	intron	A>G	G	0.378	197	-0.166	0.976	0.8682	0.34	0.35	0.038	0.8462	0.968	0.96120	1
	rs2029253	intron	A>G	G	0.328	176	0.456	1.054	0.6485	0.32	0.37	1.273	0.2592	0.829	0.46784	1
	rs2115819	intron	A>G	G	0.493	198	0.688	1.076	0.4914	0.46	0.47	0.024	0.8770	0.976	0.79374	1
	rs3780894	intron	A>G	G	0.19	142	-1.263	0.860	0.2066	0.23	0.19	2.056	0.1516	1.317	0.13981	1
	rs3780914	intron	G>A	A	0.408	193	1.095	1.135	0.2733	0.39	0.42	0.627	0.4286	0.881	0.36835	1
	rs3824612	intron	G>A	A	0.319	167	-0.774	0.898	0.4391	0.32	0.36	1.106	0.2930	0.839	0.39249	1
	rs4948671	intron	A>G	G	0.412	199	1.436	1.178	0.1510	0.37	0.39	0.424	0.5151	0.900	0.27645	1
	rs4948672	intron	G>C	C	0.454	209	0.317	1.023	0.7516	0.47	0.47	0.006	0.9390	0.988	0.95170	1
	rs7090328	intron	G>A	A	0.346	185	-0.705	0.924	0.4807	0.31	0.31	0.030	0.8634	1.030	0.78001	1
CysLTR2 (13)	rs1323552	FL 3'UTR	C>A	A	0.408	199	0.143	1.023	0.8862	0.46	0.42	0.889	0.3458	1.161	0.65096	1
	rs912278	3'UTR	G>A	G	0.495	205	-0.321	1.035	0.7484	0.49	0.51	0.099	0.7533	0.952	0.88688	1
LTC4S (5)	rs3776944	FL 5'UTR	G>A	A	0.079	48	-3.543	0.396	0.0004	0.06	0.11	6.71	0.0089	0.498	0.00005	0.001
	rs730012	FL 5'UTR	A>C	C	0.218	86	-0.471	0.932	0.6374	0.32	0.32	0.02	0.8923	1.020	0.88654	1

Chr = Chromosome; ALL (2>1) = alleles (2 is wild type and 1 is minor or polymorphic allele); Mn. ALL = minor allele; ALL FRQ = allele frequency (minor allele); n = number of informative families; Z' = Z' statistics; F A/F U = frequency of affected (cases)/unaffected (normal controls); FL = flanking; com./corr. = combined/corrected p value (see suppl. material for further information). Family-based analysis was done using FBAT and TDT (PLINK), while case-control analysis was done using PLINK. Cells with bold font across the rows represent SNPs which were found to be significantly associated with asthma in this study.

Table 3. Results of genotypic association analyses of rs3776944 (*LTC4S*) in family-based and case-control analysis

Family (FBAT)										Case-control				
geno-types	genotype freq.	n	S	E(S)	Var(S)	Z	p	global χ^2	global p	F A	F U	test	OR (95% CI)	p
G/G	0.844	48	47	32.8	16.4	3.51	0.0004			0.881	0.781	G/A		
G/A	0.154	48	19	33	16.5	-3.45	0.0006	12.558	0.002	0.114	0.208	vs.	0.4	0.008
A/A	0.002	1	NA	NA	NA	NA	NA			0.005	0.011	G/G	(0.28-0.83)	

freq. = Frequency; n = number of informative families; E(S)/S = expected/actual score of transmission; Var (S) = variance in the score of transmission; Z = Z score statistics; NA = test not performed due to inadequate number of informative families; F A/F U = frequency of affected (cases)/unaffected (controls). The

FBAT additive model is used. In the biallelic test, the p value has been calculated based on Z' score statistics (1 degree of freedom). In the multi-allele test, the p value has been calculated using χ^2 statistics (2 degrees of freedom). p values are nominal and not corrected for multiple testing.

difference was obtained ($p < 0.0001$; data not shown). In our association analyses, none of the markers showed a statistically significant association after the multiple testing corrections ($p > 0.05$).

Haplotype Association Analyses

In the haplotype-based association analyses, we found two marker haplotypes (rs3776944G/A and rs730012A/C) in the *LTC4S* gene to be associated with asthma both in family-based as well as case-control studies in an omni-

Table 4. Results of two-locus (rs3776944G/A and rs730012A/C) haplotypic association analyses of the *LTC4S* gene in family-based and case-control analyses

Family (FBAT)										Case-control					
Haplo.	Haplo. freq.	n	S	E(S)	Var (S)	Z	p ¹	global χ^2	global p	F A	F U	χ^2	p ¹	global χ^2	global p
G_A	0.724	99	205	196.3	42.1	1.34	0.18	9.1	0.03	0.64	0.63	0.09	0.98	9.639	0.02
G_C	0.196	80	78	76.6	34.9	0.23	0.82			0.3	0.26	1.73	0.41		
A_C	0.13	7	1	4.3	1.9	-2.43	0.02			0.03	0.06	5.8	0.03		
A_A	0.68	35	18	24.7	12.1	-1.92	0.05			0.03	0.05	1.32	0.52		

Haplo. = Haplotypes; freq. = frequency; n = number of informative families; E(S)/S = expected/actual score of transmission; Var (S) = variance in the score of transmission; Z = Z score statistics; F A/F U = frequency of affected (cases)/unaffected (controls).

¹ p values listed are permutation p values (refer to online suppl. material for methods).

bus, multiallelic test of association (table 4). Since the frequency of some of the haplotypes was very low, we used the Monte Carlo permutation approach as implemented in FBAT (hbat -p option) for the family analysis and Haploview for the case-control analysis to assess the association of individual haplotypes. The permutation procedure resulted in a statistically significant association both in families and case-control analyses (table 4). Haplotype A_A was negatively associated with asthma in families ($p = 0.05$) but not in the case-control ($p > 0.05$) cohort (table 4). On the other hand, the haplotype A_C showed a negative association in both families ($p = 0.02$) as well case-control ($p = 0.03$) analysis (table 4). There was no significant association in the haplotypic analysis of the *ALOX5* and *CysLTR2* genes (data not shown). Furthermore, no significant association was observed in haplotypic analyses with $\log_{10}$ total serum IgE in any of the genes (data not shown).

Discussion

Apart from being powerful bronchoconstrictors, LTs have been shown to play an important role in airway inflammation leading to asthma [4, 5]. Since heritable genetic changes in genes coding for the LT pathway genes could affect their levels and/or functions, identification of the genetic modifiers, through population genomics approaches, is one of the major areas of investigation. However, in comparison to aspirin-intolerant-asthma and responsiveness to LT-modifying drugs (pharmacogenetics) the studies investigating an association with allergic asthma are few and wanting [5, 9]. Due to their role in promoting airway inflammation and remodeling, it is

very relevant to conduct association studies with LT pathway genes and allergic/atopic asthma [17]. Our study focuses on three key candidate genes from this pathway (*ALOX5*, *LTC4S* and *CysLTR2*) where we have selected various SNPs across the genes in order to assess the role of these genes in genetic susceptibility to asthma using powerful family-based and case-control approaches.

The rs3776944G/A polymorphism showed statistically significant results with asthma in our population in single marker association analysis (tables 2, 3). The polymorphic allele A was shown to have a protective effect with respect to asthma susceptibility. Bizzintino et al. [18] showed that urinary LTE₄ (uLTE₄) was influenced in a genotype-specific way where GG individuals were shown to be associated with higher uLTE₄ levels in comparison to AG or AA genotypes. LTE₄ formed by enzymatic catalysis of LTC₄ is also a potent bronchoconstrictor [2]. In our study, individuals with AG genotypes had a lower risk for asthma than individuals with GG genotypes. Therefore, it may be possible that this polymorphism may be in LD with some other functional polymorphism or may play an independent role in *LTC4S* gene regulation thereby modulating the levels of Cys-LTs (LTC₄, LTD₄ or LTE₄). The other polymorphism rs730012A/C found to be associated in other studies [9] did not show any association signal in our study. In our two-locus haplotypic analysis of the *LTC4S* gene (rs3776944G/A and rs730012A/C), we did find an association signal in both case-control ($p = 0.02$) and family-based association studies ($p = 0.03$; table 4). Sayers et al. [19] examined the role of the haplotypes in influencing basal-level transcription in a reporter gene assay and detected no statistically significant difference in transcriptional activity between the four haplotypes identified. Subsequently, it was

reported that the haplotypes G_A, A_C and G_C induce 50% more transcription of the *LTC4S* gene upon dexamethasone induction, while no induction was observed with the constructs having A_A haplotype [20]. In our study, the A_C haplotype did show a negative association with asthma both in family-based and case-control analysis. The A_A haplotype also showed a protective role in family-based analysis while there was no association in the case-control analysis. The other two haplotypes G_A and G_C did not demonstrate any statistically significant association while it should be noted that G_C was found with higher frequency in asthmatics than normal controls in the case-control analysis. We were unable to measure serum LTC₄ levels in humans which could have further clarified the functional role of these haplotypes. Also, as per results of Sayers et al. [20], due to differential promoter activity observed upon corticosteroid treatment, these polymorphisms may have important pharmacogenetic implications.

The non-association of the rs730012A/C SNP in our study could be due to reasons such as phenotypic heterogeneity or ethnic differences for example. In this regard, it would also be valuable to note that our study may be underpowered to draw conclusive inferences for some of the genes which might confer only modest risk. Nevertheless, the 239 trios included in our study would have nearly 80% power to detect an OR ranging from 1.9 to 2.2, which are comparatively high for complex trait genetics. Furthermore, we did not find an association of any of the *ALOX5* and *CysLTR2* polymorphisms with asthma in our study. To the best of our knowledge, this is the first study investigating polymorphisms spanning the whole *ALOX5* gene and asthma. The previously associated rs2228065G/A (760G/A or Glu254Lys) [9] was non-polymorphic in our study population. Therefore, it may be reasonable to infer that this gene may play a relatively minor role, if at all, with respect to asthma susceptibility. In the *CysLTR2* gene, the two polymorphisms genotyped did not provide any evidence of an association.

The bias in our results due to population stratification is highly unlikely. The genetic homogeneity of our asthmatics and normal controls has been verified in our laboratory by genotyping loci (microsatellite markers) that are yet unlinked to asthma [11, 21]. Also, recently, subsets of individuals from the cases and controls have been genotyped on genome-wide association studies and no evidence of stratification (based on the analysis of 10,000 randomly selected markers) was observed [unpubl. data] as such family studies are inherently robust against population stratification [21]. Furthermore, a positive asso-

ciation signal in both family-based and case-control analyses is more likely to reject the null hypothesis. Our conclusion is further strengthened by the combined probability testing method as it provides a way to integrate information from more samples and a resultant increase in power.

We were also interested to know if there was any interaction effect among these genes with respect to asthma susceptibility using logistic regression analyses as implemented in PLINK [13]. However, statistically significant interactions were absent (data not shown). We also performed interaction analysis using a second model [22] implemented in R package where SNPs with significant main effects were selected. Two SNPs rs3776944G/A and rs11239523A/G showing a significant main effect (combined and uncorrected $p < 0.05$) were therefore selected for the analysis. However, no interaction effect was noted among these SNPs.

In summary, we report here for the first time the association studies of the three LT pathway genes *ALOX5*, *LTC4S* and *CysLTR2* with atopic asthma in an Indian population, and rs3776944G/A was associated with atopic asthma in family-based and case-control cohorts. We conclude that *LTC4S* may play an important role in genetic susceptibility to asthma in Indians.

Acknowledgments

The authors acknowledge the financial assistance of the Council of Scientific and Industrial Research, Government of India (NWP0033). A.K. and S.S. acknowledge the fellowship received from CSIR. We thank all participating clinicians; Drs. S.K. Sharma, V.K. Vijayan, P.V. Niphadkar, B. Lahker, A. Sinha and U. Mabalirajan, and volunteers for their help in performing this study. We also thank Sanobar Nahid and Deepti Maan for their technical assistance. Help provided by the genotyping facility and cluster computation facility, IGIB, CSIR, is duly acknowledged.

References

- 1 Guerra S, Martinez FD: Asthma genetics: from linear to multifactorial approaches. *Annu Rev Med* 2008;59:327–341.
- 2 Nicosia S, Capra V, Rovati GE: Leukotrienes as mediators of asthma. *Pulm Pharmacol Ther* 2001;14:3–19.
- 3 Werz O, Steinhilber D: Therapeutic options for 5-lipoxygenase inhibitors. *Pharmacol Ther* 2006;112:701–718.
- 4 Lam BK: Leukotriene C(4) synthase. *Prostaglandins Leukot Essent Fatty Acids* 2003;69:111–116.

- 5 Tantisira KG, Drazen JM: Genetics and pharmacogenetics of the leukotriene pathway. *J Allergy Clin Immunol* 2009;124:422–427.
- 6 Kim SH, Bae JS, Suh CH, Nahm DH, Holloway JW, Park HS: Polymorphism of tandem repeat in promoter of 5-lipoxygenase in ASA-intolerant asthma: a positive association with airway hyperresponsiveness. *Allergy* 2005;60:760–765.
- 7 Kalayci O, Birben E, Sackesen C, Keskin O, Tahan F, et al: ALOX5 promoter genotype, asthma severity and LTC production by eosinophils. *Allergy* 2006;61:97–103.
- 8 Bai C, Matsui E, Ohnishi H, Kimata K, Kasahara K, Kaneko H, Kato Z, Fukao T, Kondo N: A novel polymorphism, E254K, in the 5-lipoxygenase gene associated with bronchial asthma. *Int J Mol Med* 2008;21:139–144.
- 9 Duroudier NP, Tulah AS, Sayers I: Leukotriene pathway genetics and pharmacogenetics in allergy. *Allergy* 2009;64:823–839.
- 10 Yoshikawa K, Matsui E, Kaneko H, Fukao T, Inoue R, et al: A novel single-nucleotide substitution, Glu 4 Lys, in the leukotriene C4 synthase gene associated with allergic diseases. *Int J Mol Med* 2005;16:827–831.
- 11 Sharma S, Ghosh B: Association of an intragenic microsatellite marker in the CC16 gene with asthma in the Indian population. *J Hum Genet* 2004;49:677–683.
- 12 Fan JB, Gunderson KL, Bibikova M, Yeakley JM, Chen J, et al: Illumina universal bead arrays. *Methods Enzymol* 2006;410:57–73.
- 13 Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, et al: PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007;81:559–575.
- 14 Barrett JC, Fry B, Maller J, Daly MJ: Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005;21:263–265.
- 15 Laird NM, Horvath S, Xu X: Implementing a unified approach to family-based tests of association. *Genet Epidemiol* 2000;19(suppl 1):S36–S42.
- 16 Rabinowitz D, Laird N: A unified approach to adjusting association tests for population admixture with arbitrary pedigree structure and arbitrary missing marker information. *Hum Hered* 2000;50:211–223.
- 17 Hallstrand TS, Henderson WR Jr: An update on the role of leukotrienes in asthma. *Curr Opin Allergy Clin Immunol* 2010;10:60–66.
- 18 Bizzintino JA, Khoo SK, Zhang G, Martin AC, Rueter K, et al: Leukotriene pathway polymorphisms are associated with altered cysteinyl leukotriene production in children with acute asthma. *Prostaglandins Leukot Essent Fatty Acids* 2009;81:9–15.
- 19 Sayers I, Barton S, Rorke S, Beghé B, Hayward B, et al: Allelic association and functional studies of promoter polymorphism in the leukotriene C4 synthase gene (LTC4S) in asthma. *Thorax* 2003;58:417–424.
- 20 Sayers I, Sampson AP, Ye S, Holgate ST: Promoter polymorphism influences the effect of dexamethasone on transcriptional activation of the LTC4 synthase gene. *Eur J Hum Genet* 2003;11:619–622.
- 21 Kumar A, Ghosh B: A single nucleotide polymorphism (A → G) in intron 3 of IFN γ gene is associated with asthma. *Genes Immun* 2008;9:294–301.
- 22 Howard TD, Koppelman GH, Xu J, Zheng SL, Postma DS, Meyers DA, Bleeker ER: Gene-gene interaction in asthma: IL4RA and IL13 in a Dutch population with asthma. *Am J Hum Genet* 2002;70:230–236.

The figure displays a genomic region on chromosome 1, specifically the area around 45220k. The top panel is a Manhattan plot showing the $-\log_{10}(p\text{-value})$ for various SNPs. The y-axis represents the $-\log_{10}(p\text{-value})$, and the x-axis represents the genomic position in kilobases (k). A significant peak is observed around 45220k, reaching a $-\log_{10}(p\text{-value})$ of approximately 12.5.

The bottom panel is a linkage disequilibrium (LD) heatmap showing the pairwise r^2 values for 22 SNPs. The color scale ranges from white (0) to red (1). The diagonal elements are all 1.0, indicating perfect self-LD. The off-diagonal elements show the degree of LD between pairs of SNPs. The heatmap is symmetric, with the lower triangle mirroring the upper triangle.

The SNPs included in the LD heatmap are:

- rs3824612
- rs3780894
- rs4948671
- rs11239505
- rs2029253
- rs7918542
- rs2115819
- rs10900213
- rs10900215
- rs11239523
- rs4948672
- rs12244801
- rs10900216
- rs7090328
- rs1565096
- rs3780908
- rs1487562
- rs12765320
- rs3780914
- rs10751382
- rs10751383

The LD heatmap shows that the SNPs are highly correlated, with many pairs having r^2 values close to 1.0. The highest LD is observed between rs3824612 and rs3780894, with an r^2 value of 1.0. Other high LD pairs include rs4948671 and rs11239505, and rs11239523 and rs4948672.

Supplementary Figure 1: Pair-wise linkage disequilibrium (LD) among the SNPs in *ALOX5*. The values in the boxes are Pearson's correlation (r^2) values. The LD plots have been constructed using Haploview (version 4.2) as described in Materials and Methods. At the top of each box is the structure of gene.

Supplementary Methods

Subjects

In the present study, asthmatic patients ($n=147$, 38.5 ± 15 years) and normal controls ($n=199$, 37.0 ± 13.5 years) were recruited from our collaborating centers following clinician's diagnosed asthma and a set of inclusion/exclusion criterion (**Supplementary Table 1**), after obtaining ethical clearance from our institute and the participating centers. Written consents were obtained from individuals for participating in the study, performing SPT (skin prick test) and obtaining blood samples. A standard questionnaire was filled by the candidates giving clinical details, migration status, environmental history, family history of diseases etc. The clinical test used for ascertaining asthma phenotypes were pulmonary function test (PFT) [FEV_1 , bronchial reversibility ($>15\%$) test using β_2 -agonist inhaler (albuterol/salbutamol)], skin prick test (wheal reaction $>3\text{mm}$ diameter) to a panel of 15 local environmental allergens and total serum IgE estimation as described earlier. Only individuals with self reported history of breathlessness and wheezing with positive family history were included in the study. Normal controls were recruited from general population taking details of migration status, ethnicity and no self reported history of asthma or allergic diseases. All individuals with smoking history and parasitic/helminthic infections in the past were excluded from the study. For the family based association studies 247 families were collected as ascertained through probands (15.6 ± 11.2 years) who were asthmatics along with both the parents affected/unaffected. Families were further extended wherever consents were obtained and a total of 1022 individuals were thus recruited with average family size of 3.94 (range from 3 to 12 members). The disease status was confirmed in a manner similar

to case-control study. The genetic homogeneity between patients and controls was confirmed by genotyping loci as yet unknown to be associated with asthma as described previously [E1].

Genotyping and data cleaning

DNA was isolated from peripheral white blood cells using the modified salting out method^{E1} and was stored at -20°C until further analysis. For *LTC4S* gene, PCR amplifications were done using primers and conditions as listed (**Supplementary Table 2**). Following successful PCR amplification, the rs3776944 and rs730012 SNPs were queried using SNaPShot ddNTP Primer Extension Kit (ABI) and primers and conditions as mentioned in (**Supplementary Table 2**). 1U of calf intestinal phosphatase (CIP) (New England Biolabs, Beverly, MA) was used to clean up the primer extension reaction. These samples were subsequently electrophoresed using the ABI Prism 3100 Genetic Analyzer as per the manufacturer's instructions. The results were analyzed using the program ABI Prism GeneMapper v3.7 (Applied Biosystems, Foster City, USA).

Polymorphisms in *ALOX5* and *CysLTR2* were genotyped using Illumina's Golden Gate Assay (Illumina Inc.) in accordance with manufacturer's protocol [E2]. Briefly, the OPA, querying a set of SNPs, is hybridized simultaneously to genomic DNA followed by allele specific primer extension and ligation. Subsequently, a set of fluorescently labeled universal primers (Cy3 and Cy5 labeled P1 and P2 respectively) were added and PCR was carried out, generating multiple labeled amplicons representing hundreds of different SNPs. These fluorescent products were then combined with beads on the Sentrix Array Matrix (SAM). The address sequences within the PCR amplicons hybridize to their related sequences on the beads, and the fluorescence on each bead is quantified resulting in a signal associated with a particular

address sequence. Each bead type is represented approximately 25 times on the array to improve the accuracy of the signal. After hybridization, the SAM was scanned using Beadstation 500 - Beadarray reader. The hybridization intensities from Beadarray reader were used for data processing, clustering and genotype calling using the genotyping module in the BeadStudio package v3. GenCall module of BeadStudio was used to generate genotype calls. The genotype clusters generated for each SNP locus by GenCall were edited manually after visual inspection of clusters on two-dimensional plot. All the genotype clusters were inspected and corrected manually; the threshold for GenTrain score of > 0.25 was set to call a SNP successfully genotyped [E2].

We retained markers for further analysis if the call rate was above 90%, maximum of one reproducibility error, maximum of five Mendelian errors and showed consistency with HWE at the level of $P > 0.001$.

Data/statistical analysis

Hardy-Weinberg equilibrium (HWE) for patients as well as controls was calculated using PLINK (<http://pngu.mgh.harvard.edu/~purcell/plink/>) [E3]. Pair-wise LD among the SNPs in the cases and control population was measured by complementary measures, Lewtonin's standardized LD coefficient (D') and Pearson's correlation (r^2) by using the software Haploview v 4.2 [E4]. Tag SNP selection was done using tagger as implemented in Haploview setting a threshold of $r^2 \geq 0.8$.

Case-control association analysis was performed for each polymorphism, using the Armitage trend test using PLINK [E3]. Odds ratios (OR) were also calculated using 2×2 contingency tables (<http://home.clara.net/sisa/>). Associations between serum IgE levels and

alleles of markers were analyzed in cases and controls using PLINK [E3]. Logistic regression analysis was performed to evaluate the effect of age and sex, if any, on the association of various genotypes on asthma or serum total IgE levels.

For the family based analysis (with binary trait asthma and $\log_{10}$ serum IgE levels), FBAT (Family Based Association Test) was used (<http://www.biostat.harvard.edu/~fbat>) since this method has several reported advantages and best suited for heterogeneous family structures [E5, E6]. We have also used TDT (transmission disequilibrium test) as implemented in PLINK [E3] (plink --tdt option) as a tool to validate observations made from FBAT analysis.

Haplotypic association analyses were performed using PLINK [E3] and HBAT (implemented in FBAT) [E5, E6]. PLINK uses standard Expectation-Maximization (E-M) algorithm to estimate haplotypes and then performs standard family based and population based (unrelated individuals) association testing. Since we have genotyped a number of markers in *ALOX5*, to avoid large number of haplotypes with frequency less than 0.05, we have used sliding window haplotypic association analysis [E7] (in case-control cohort) with window size of 5.

In families haplotype based TDT association test were performed using PLINK [E3] (plink --file mydata --hap myfile.hlist --hap-tdt) for regions (marker combination) identified as regions of highest significance in the case-control analysis. Furthermore, excessive transmissions of the multi-locus haplotypes were also tested using the HBAT as implemented in FBAT package [E5, E6], using the additive model with bi-allelic (individual haplotype based) and multi-allelic (global test of association) models. We also used the Monte Carlo permutation approach as implemented in FBAT (hbat --p option) to validate our observations.

Combining p values

We have performed family based and case-control studies (single gene or gene-wise analysis), where independent set of samples have been used i.e. individuals in families and case-control cohorts are non-overlapping. However, these are samples drawn from the same population. In each of the analyses (family based and case-control) p values have been obtained for all the association tests that we performed using appropriate methods for different types of samples. We used the Fisher's method or Fisher's combined probability test [E8] for combining the p values obtained from family based and case-control association analysis. Fisher's combined probability test is a technique for data fusion or meta-analysis [E8]. In its basic form, it is used to combine the results from several independent tests. Combining p-values from different tests, where same hypothesis is under testing, is an important method and has been suggested to provide higher strength towards decision-making [E9] as it is based on more information from both types of samples considered here. It is to be noted that only p values obtained in allelic association analysis have been combined since this was the screening step for identifying statistically significant associations.

Correction for multiple testing

We have performed Bonferroni corrections for multiple testing. For the single gene analysis a total of 19 SNPs were included in association analysis and therefore the SNPs showing a combined p value of 0.0026 were considered to be significantly associated with asthma or total serum IgE. In contrast, for previously reported asthma (or related phenotypes) associated polymorphisms (different study/population), we have relied on raw p values (non-

corrected/combined p values), since there is prior evidence of negating null hypothesis (H_0), and replication of the effect in a new population is being tested.

References

- E1 Kumar A, Ghosh B. A single nucleotide polymorphism (A --> G) in intron 3 of IFN-gamma gene is associated with asthma. *Genes Immun* 2008; 9 (4): 294-301.
- E2 Fan JB, Gunderson KL, Bibikova M, Yeakley JM, Chen J, Wickham Garcia E, Lebruska LL, Laurent M, Shen R, Barker D . Illumina universal bead arrays. *Methods Enzymol* 2006; 410: 57-73.
- E3 Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, Sham PC. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007; 81 (3): 559-75.
- E4 Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; 21(2): 263-5.
- E5 Laird NM, Horvath S, Xu X . Implementing a unified approach to family-based tests of association. *Genet Epidemiol* 2000; 19 Suppl 1: S36-42.
- E6 Rabinowitz D, Laird N . A unified approach to adjusting association tests for population admixture with arbitrary pedigree structure and arbitrary missing marker information. *Hum Hered* 2000; 50 (4): 211-23.
- E7 Li Y, Sung WK, Liu JJ. Association mapping via regularized regression analysis of single-nucleotide-polymorphism haplotypes in variable-sized sliding windows. *Am J Hum Genet* 2007; 80 (4): 705-15.
- E8 Fisher RA (1932). *Statistical Methods for Research Workers*. 4. London: Oliver and Boyd.
- E9 Lasky-Su J, Won S, Mick E, Anney RJ, Franke B, Neale B, Biederman J, Smalley SL, Loo SK, Todorov A, Faraone SV, Weiss ST, Lange C. On genome-wide association studies for family-based designs: an integrative analysis approach combining ascertained family samples with unselected controls. *Am J Hum Genet* 2010; 86(4): 573-80.

Supplementary Table 1: The inclusion and exclusion criteria for the recruitment of asthma patients and families

Inclusion criteria:

- Patients of six to thirty five years of age with recurrent symptoms of cough with expectoration,
- Clinical improvement with inhaled steroids,
- Reversibility of the bronchial obstruction after administration of β_2 -agonists (an increase in FEV1 greater than 15)
- Absolute eosinophil count > 500/cubic mm,
- Total serum IgE>250IU/ml,
- Positive skin-prick test (SPT) to common allergens (**Table 1**) in the study area,
- Positive family history of asthma as per questionnaire and verified by clinicians.

Exclusion criteria:

- Patients with smoking history,
- Pregnancy,
- Parasitic infestations,
- Patients with other possible causes of eosinophilia such as drugs, tropical pulmonary eosinophilia (TPE) etc.

All participants were required to sign informed consent form as approved by ethical committee at IGIB and the participating medical colleges/hospitals.

Supplementary Table 2: Details of Primers used for genotyping *LTC4S* polymorphisms

Primer Name	Primer Sequence	Annealing Temperature °C (T _m)	Product size	Fragment/Polymorphism
LTC4S_rs3776944FP	5' GCC ACC GCA AAC CCA CTC AA 3'	67-62*	385	rs3776944
LTC4S_rs3776944RP	5' GCT GCC CTC CCC ACA CTC 3'	67-62*	385	rs3776944
LTC4S_rs730012FP	5' AAG GAG AAA GAG CTT GTG GG 3'	57	161	rs730012
LTC4S_rs730012RP	5' ACT CCT CCA CCC ACC TTA TC 3'	57	161	rs730012
LTC4S_rs3776944SS	5' CTG GAG TTC TGG GTG TCT CCC T 3'	55	23	SNaPShot Primer
LTC4S_rs730012SS	5' ACA GCC TGG ATG GGG AC	55	18	SNaPShot Primer

Abbreviations: **FP**:-Forward primer; **RP**:-Reverse primer; **SS**:-Snapshot Primer

The last column represents the polymorphisms genotyped in the PCR amplified products.

SNaPShot Primers are primers designed one base before the polymorphic loci to be used for single base extension using SNaPShot ddNTP Primer Extension Kit (Applied Biosystems, Foster City, USA).

* This is annealing temperature for touchdown PCR. For the initial 10 cycles annealing temperature falls @-0.5°C to 62°C. subsequently 25 cycles of cycling reactions are performed with annealing temperature 62°C.

The PCR thermo-cycling conditions, generally involved an initial denaturation step at 94°C for 10 min, followed by 35 cycles of 30 sec denaturation at 94°C, 30 sec annealing at primer-pair specific temperature (T_m), extension at 72°C for 1 min per kb of the amplicon; followed by a final extension at 72°C for 10 min.

(Note:-The region containing the rs28365149 could not be PCR amplified using multiple primer sets (not shown))