

A Common Exonic Variant of *Interleukin21* Confers Susceptibility to Atopic Asthma

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Key Words

Asthma · Haplotype · IL-21 level · Microsatellite repeat marker · Single nucleotide polymorphisms

Abstract

Background: Interleukin (IL)-21, an IL-2 family multifunctional cytokine, is produced by activated CD4⁺ T cells and is known to potentially affect growth, survival and function of numerous immune cells. As IL-21 regulates IgE production, a key mediator of various allergic disorders and asthma, it is a prime candidate gene for studying atopic asthma. **Methods:** In atopic asthma, analyses of four single nucleotide polymorphisms (SNP; C1455T, G1472T, C5250T and C8381T), a tetranucleotide microsatellite repeat (GAAT)_n and their haplotypes were performed, and serum total IgE (TslgE) was determined in ethnically matched unrelated patients (n = 255), unrelated controls (n = 245) and nuclear families (n = 140). Correlation between an exonic SNP C5250T in the asthmatics with serum IL-21 levels was also made. **Results:** In both the case-control and family study groups, the exon-3 polymorphism C5250T of the *IL21* gene was significantly associated with atopic asthma and TslgE. The C5250T polymorphism was found to affect the concentration of serum IL-21 levels in atopic asthmatics. Also, this observation was supported by the structural alteration in *IL21* mRNA as predicted by *mfold* software. Further, our haplotypic studies indicated that while minor haplotypes 4_C_T_C_C and two locus haplotype T_C_C were associated with asthma in the case-control

cohort, none of the major haplotypes was found to be associated with either asthma or TslgE levels. **Conclusion:** Our study provides evidence that *IL21* is associated with atopic asthma, TslgE and serum IL-21 levels. Thus, it may initiate further research to elucidate the role of the *IL21* gene in asthma pathogenesis.

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Introduction

Interleukin (IL)-21 is a newly described cytokine produced by CD4⁺ T cells. It is probably the last member of the IL-2 family to be identified and is known to selectively modify both humoral and cell-mediated immune responses through interaction with various cell types including T, B, natural killer (NK) and dendritic cells [1]. Similar to the other IL-2 family members, IL-21 binds to γ_c receptor along with its specific receptor IL-21R α [2, 3]. IL-21 functions by activating the janus kinases JAK1 and JAK3, which in turn leads to the activation of the signal transducers and activators of transcription STAT1 and STAT3 [4, 5]. It can enhance the clonal expansion of antigen-activated naive T cells in a manner similar to IL-2 [6, 7]. Recently IL-21 has been reported to potentially induce the T_H17 cell differentiation and Foxp3 suppression [8, 9]. It has also been shown to down-regulate IgE production from IL-4-stimulated B cells by the inhibition of germ line C (epsilon) transcription [10] and to indirectly

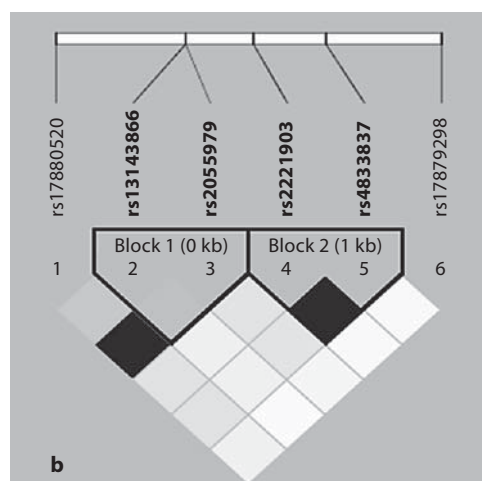
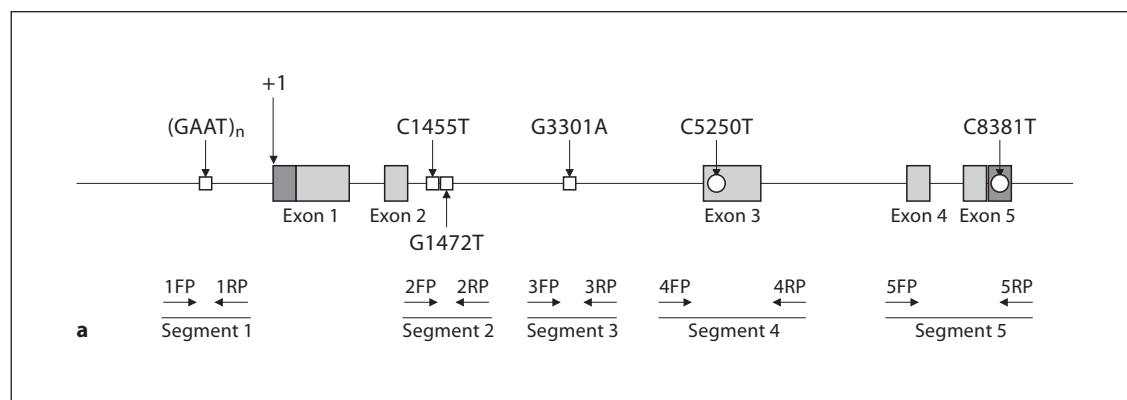
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SNP	NCBI rs ID	Position	ObsHET	PredHET	MAF
(GAAT) _n	rs17880520	-1183	0.483	0.498	0.468
C1455T	rs13143866	+1455	0.276	0.267	0.158
G1472T	rs2055979	+1472	0.479	0.5	0.488
G3301A	rs2221903	+3301	0.306	0.346	0.222
C5250T	rs4833837	+5250	0.306	0.346	0.222
C8381T	rs17879298	+8381	0.046	0.052	0.027

c

Fig. 1. Gene map, LD plot and SNP information in *IL21*. **a** Gene map and SNPs in *IL21* on chromosome 4q26–q27. Coding exons are marked by shaded blocks. The first base of the transcriptional site was denoted as nucleotide +1. SNPs studied in the Indian population are marked on the map. **b** LD map of *IL21*. LD was calculated using Haploview. Black box indicated strong evidence of LD between the polymorphisms ($r^2 = 1$), while white boxes showed no or little LD between pairs ($r^2 = 0$). $0 < r^2 < 1$ are represented by grey boxes. **c** General information about the SNPs from the sequencing cohort. MAF = Minor allele frequency.

inhibit IL-4-induced IgE production via induction of interferon (IFN)- γ production from T and NK cells [11, 12]. Moreover, IL-21 is known to induce the production of IgG₁ and IgG₃ in naive surface IgG⁺ B lymphocytes by Ig isotype class switch [13]. As IgE plays a key role in atopic asthma, all these findings make *IL21* a primary candidate gene for atopic asthma.

The gene for IL-21 is present on chromosome region 4q26–q27 in humans and is only 180 kb away from the *IL2* gene. It is a 8432-bp-long gene encoding for a 162-amino-acid polypeptide precursor forming a fully processed mature protein of 133 amino acids having a molecular weight of 15 kDa [1]. It consists of five exons of which all are coding exons (fig. 1a). Although a promoter

polymorphism (T–83C) of the *IL21 receptor* (*IL21R*) gene has been found to be associated with IFN- γ -mediated inhibition of human IgE synthesis [14] and elevated IgE levels in female human subjects [15], *IL21* gene polymorphisms have not been studied in either atopic asthma or any asthma-associated phenotype yet.

Here, we have performed both case-control and family-based studies to establish the association of *IL21* with atopic asthma. We report, for the first time, the significant association of an exonic variant of *IL21* with atopic asthma and serum total IgE levels in an Indian population. Moreover, the exonic polymorphism was significantly correlated with serum IL-21 levels in asthmatic patients.

Table 1. Characteristics of the patient and control groups (case-control study) and the probands (family-based study)

Characteristics	Patients (n = 255)	Controls (n = 245)	Probands (n = 140)
Native place ^a	North/North-West India	North/North-West India	North/North-West India
Mean age, years	28 ± 13	25.6 ± 9	14.6 ± 8.2
Sex, females/males	47/53	42/58	41/59
Familial history of asthma/atopy ^b	all	none	all
Smoking history ^c	none	none	none
FEV ₁ , % of predicted	77.6 ± 15.8	96.2 ± 15.3	73.1 ± 14.8
Reversibility from baseline FEV ₁ (after β ₂ -agonist usage), %	>15	ND	>15
log ₁₀ TsIgE (mean), IU/ml	3.02 ± 0.73	2.1 ± 0.73	2.83 ± 0.55
Self-reported history of allergies	all	none	all

FEV₁ = Forced expiratory volume in 1 second; ND = the test was not done.

^a Patients and controls were recruited from Delhi, Lucknow, Jaipur, Shimla, Chandigarh and Mumbai.

^b Control individuals were recruited with the help of a questionnaire to eliminate all individuals having atopic disorders or a family history of atopic disorders.

^c Patients and controls known to have experienced smoking in the past or suffering from parasitic infections were excluded from the study.

Methods

Study Population

Unrelated asthmatic probands (n = 255) and normal controls (n = 245) were recruited from various collaborating hospitals of Northern and North-Western India after obtaining ethical approval from the review board of each hospital (table 1). The study consisted of a homogeneous population of Indo-Aryan origin. A standard questionnaire was filled to recruit the subjects and to obtain their clinical details [16]; environmental history and migration status was also recorded. The participants provided a written informed consent for performing skin prick test (SPT) and drawing blood samples. Patients (mean age 28 ± 13 years) were diagnosed for asthma on the basis of the National Asthma Education and Prevention Program (Expert Panel Report-2) guidelines [17] and were examined for self-reported history of breathlessness and wheezing.

A total of 140 asthmatic families (3–12 individuals/family) were recruited by a group of common physicians in the follow-up family visits of the probands recognized by a primary physician. Asthma diagnosis was also made as discussed above for all the family members after receiving their consent. A total of 625 individuals were recruited for the family-based association studies.

Phenotypic Data

The clinical tests done included pulmonary function tests [forced expiratory volume in 1 s and bronchial reversibility (>15%) using β₂-agonist inhaler (albuterol/salbutamol)] and SPT (wheal reaction >3 mm in diameter) to a panel of 16 local environmental allergens (house dust mites, *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 and house dust). Healthy volunteers (referred to as normal controls; mean age 25.6 ± 9 years) were selected on

the basis of the criteria of having no self-reported history of allergic diseases or asthma. All individuals having a history of smoking and parasitic/helminthic infestations in the past 3 years were excluded from the study. SPTs and pulmonary function tests were done for all probands and their family members. Pulmonary function tests were performed at three different time points. Diurnal variations in peak flow over 1–2 weeks were noted when symptoms were reported, but lung functions were normal (table 1). These tests were also performed for the unrelated controls except for the 3% of individuals who did not consent. Asthma diagnosis was defined as a positive response to all of the following questions on the questionnaire: 'Have you ever had attacks of breathlessness at rest with wheezing?' 'Have you ever had an asthma attack?' 'Have you ever used any medicine to treat asthma, or any breathing problems?' The control subjects answered no to all of these questions. Serum total IgE (TsIgE) levels were assessed for all individuals using ELISA as described [18] except for few individuals (<10%) for whom sera were not available. Specific serum IgE was estimated for six common allergens [house dust mite (0.27), cockroach male (0.144), mosquito (0.23), moth (0.03), grain dust rice (0.05) and hay dust (0.04); the cut-off optical density values for each allergen tested are shown in parentheses] using the method described by Voller et al. [19] with slight modifications. Atopy was defined as a dichotomous variable, having wheal reaction equal to or greater than histamine for at least one allergen, or high specific IgE to at least one allergen, where SPT was not done.

Genotyping of Single Nucleotide Polymorphisms and Microsatellite Repeat

DNA was isolated from blood using the modified salting out method [20]. On the basis of existing HapMap data, functional relevance (in promoter or exonic regions of the gene) and the existing pattern of linkage disequilibria (LDs), four single nucleotide polymorphisms (SNPs) and a tetranucleotide microsatellite

Table 2. *IL21* polymorphism/repeat locations, genotyping primers and PCR cycling conditions

SNP/Repeat	Location	Direction	PCR primer	Cycling conditions	Extension primer
(GAAT) _n	promoter	FWD REV	5'-GGCAACCCTTAGTCCACAT-3' 5'-TCCTCCTTTCTTCTGCTTCTCT-3'	59°C; 30 cycles	
C1455T	intron	FWD REV	5'-GAACCCAAACACTCTCA-3' 5'-GTTACACTATTACTCACTTAT-3'	57°C; 35 cycles	5'-TGCCTAATTATCAAAACATA-3'
G1472T	intron	FWD REV	5'-GAACCCAAACACTCTCA-3' 5'-GTTACACTATTACTCACTTAT-3'	57°C; 35 cycles	5'-AAAACATACATTTCTAGCATCTCAT-3'
G3301A	intron	FWD REV	5'-GGCTGCCCTTTGATGTGC-3' 5'-AACCGGCTTAACTTACTGGAAAAA-3'	57°C; 35 cycles	5'-CAATGGGGTTTTGTTTTTCTT-3'
C5250T	exon	FWD REV	5'-CCCAAAGATTAAGTAGGAGGTGA-3' 5'-AAGGCATAACTATAGGTAAGGAAG-3'	57°C; 35 cycles	5'-GAGTGGTCAGCTTTTTCCTG-3'
C8381T	3'UTR	FWD REV	5'-GCCCTTCATGTGATTCTTAT-3' 5'-ATTTTAGCCTTCTCCTTCAAC-3'	57°C; 35 cycles	5'-TGCAGTTGGACACTATGTTA-3'

FWD = Forward; REV = reverse.

repeat located 1183 bp upstream of the gene were selected for the genetic studies in such a way that the whole *IL21* gene was mapped (fig. 1b). The genomic DNA of 50 individuals (25 adult asthmatic patients and 25 normal controls) was sequenced using the primer sets detailed in table 2 by the dideoxy chain termination method using the BigDye Terminator Cycle Sequencing Kit on an ABI 3130xl sequencer (Applied Biosystems, Foster City, Calif., USA), and the presence of the SNPs and the microsatellite repeat was validated in our study population. The four polymorphisms were studied using the SNaPshot ddNTP Primer Extension Kit (Applied Biosystems) according to the manufacturer's instructions. These samples were subsequently electrophoresed using the ABI Prism 3130xl Genetic Analyzer. The results were analyzed using ABI Prism GeneMapper v3.7 (Applied Biosystems). Genotyping for the microsatellite (GAAT)_n repeat in the promoter was performed by PCR using a labeled oligonucleotide primer pair as described previously [21]. The primers and PCR conditions for SNP and microsatellite analysis are described in table 2.

Serum IL-21 Level Determination

IL-21 in serum was determined by ELISA according to an online protocol (<http://www.capralogics.com/IL21ELISA.htm>). Recombinant human IL-21 (part: PHCO215) was procured from BioSource (Camarillo, Calif., USA).

Prediction of the Secondary Structure and Stability of IL21 mRNA

The RNA-folding software RNA *mfold* (version 3.2) [22] was used to compare the secondary structure and folding energy of *IL21* mRNA containing either of the exon-3 variants, 5250T or 5250C.

Data Analysis

LDs were evaluated using Haploview [23]. The Hardy-Weinberg equilibrium (HWE) was assessed using FINETTI (<http://ihg.gsfc.de/linkage/download/finetti.zip>). Association analysis, based on the case-control design, was performed using the Armitage trend test, following the guidelines given by Sasieni [24] as implemented in the program FINETTI. The Armitage trend test based

on the genotypes remains valid even if HWE does not hold. Multiple logistic regression analysis was performed to see the effect of age and sex, if any. Haplotypes of each individual were inferred using the algorithm developed by Stephens et al. [25] (PHASE version 2.1). Differences in haplotype frequencies in cases and controls were compared using a Monte Carlo approach as implemented in CLUMP 2.3 [26]. Odds ratios (OR) were calculated for the haplotype whose distribution was significantly different in the two study groups. For family-based association test (FBAT) studies, additive and recessive models of FBAT (<http://www.biostat.harvard.edu/~fbat/fbat.htm>) with the *-o* option were used. The *-o* option generates an offset value based on minimizing the variance in the test statistics. FBAT analysis extends the methodology of the transmission disequilibrium test to evaluate nuclear families including both affected and unaffected offspring. It conditions on the observed traits and parental genotypes, and, where parental data are missing, the offspring genotype configuration to specify the distribution of a score statistic. The conditional distribution is used to calculate the mean and variance of each family's contribution to a general score statistic. The haplotypic analysis of the family samples was done using HBAT. ANOVA was used to test the effect of *IL21* gene variations on the log-transformed values of TsIgE and serum IL-21 levels.

Results

Using the available HapMap data, three tagged SNPs of the human *IL21* gene were selected for the genetic association study with asthma and related phenotypes in the Indian population. Additionally, three exonic SNPs and a tetranucleotide microsatellite repeat present in the promoter of the *IL21* gene were genotyped in the case-control cohort and asthmatic families, as detailed above. To verify the SNPs and repetitive sequences in the Indian population, we sequenced the DNA samples from 50 ran-

Table 3. Genotype frequencies, log₁₀TsIgE levels and serum IL-21 levels with respect to (GAAT)_n repeat, C1455T, G1472T, C5250T and C8381T polymorphisms in the *IL21* gene

Genotypes	Frequencies of different genotypes in case-control studies				log ₁₀ TsIgE levels with respect to different genotypes ^a			Serum IL-21 levels with respect to the C5250T genotype ^b		
	patients	controls	common OR	p value	log ₁₀ TsIgE levels ± SE	individuals n	overall p value	serum IL-21 levels ± SE	individuals n	overall, p value
(GAAT) _n										
4_4	53 (21.81)	69 (28.99)			3.03 ± 0.09	33				
4_6	122 (50.21)	115 (48.32)	1.28	0.054	2.98 ± 0.05	87	0.62			
6_6	68 (27.98)	54 (22.69)			2.92 ± 0.07	46				
C1455T										
CC	166 (68.31)	171 (70.37)			2.95 ± 0.05	113				
CT	65 (26.75)	67 (27.57)	1.28	0.31	2.86 ± 0.08	45	0.57			
TT	12 (4.94)	5 (2.06)			2.80 ± 0.20	8				
G1472T										
GG	74 (29.37)	60 (24.79)			2.87 ± 0.07	53				
GT	128 (50.79)	116 (47.93)	0.78	0.06	2.94 ± 0.06	92	0.17			
TT	50 (19.84)	66 (27.27)			3.12 ± 0.10	30				
C5250T										
CC	9 (3.59)	17 (6.94)			2.64 ± 0.26	6		30.21 ± 3.6	6	
CT	65 (25.9)	75 (30.61)	1.43	0.02	2.76 ± 0.10	42	0.003	23.78 ± 2.9	11	0.006
TT	177 (70.52)	153 (62.45)			3.12 ± 0.05	121		16.49 ± 2.1	21	
C8381T										
CC	231 (92.03)	229 (95.02)			2.94 ± 0.04	159				
CT	20 (7.97)	11 (4.56)	1.6	0.27	2.89 ± 0.16	12	0.75			
TT	0 (0.00)	1 (0.41)								

Figures in parentheses are percentages.

^a Analysis with log₁₀TsIgE levels was done in asthmatics.

^b Analysis with serum IL-21 levels was done in asthmatics for the C5250T SNP only. Unit of IL-21: pg/ml.

domly selected individuals and calculated the LD among the identified polymorphic *IL21* variants (fig. 1). Two LD blocks were identified where the G3301A and C5250T SNPs were found to be in complete LD ($D' = 1$, $r^2 = 1$); therefore, the G3301A SNP was exempted from further analysis. Also, since the previously reported exonic SNP G8395A (rs17886348) was found to be non-polymorphic, it was not genotyped further. Thus, a total of four SNPs, viz C1455T, G1472T, C5250T and C8381T, and a (GAAT)_n microsatellite repeat were genotyped from patients and controls as well as all the recruited family members of the asthmatic probands to establish the association of the *IL21* gene with asthma.

Association of IL21 Gene Polymorphisms with Atopic Asthma and TsIgE

The HWE was calculated for all of the four SNPs and the promoter microsatellite repeat. All polymorphisms and the microsatellite marker were found to follow HWE ($p > 0.05$; data not shown), implying the absence of detectable selection differentials among individuals with or without mutation in our population. To find an associa-

Table 4. Multiple logistic regression analysis of the *IL21* polymorphisms for risk of asthma

Variable	Adult cohort		
	LR χ^2	d.f.	p value
Model 1			
(GAAT) _n	0.68	2	0.71
C1455T	2.1	2	0.34
G1472T	1.86	2	0.39
C5250T	8.45	2	0.01
C8381T	0.46	1	0.49
Age	14.68	1	0.0001
Sex	4.12	1	0.04
Model 2			
(GAAT) _n	0.52	2	0.77
C1455T	3.34	2	0.18
G1472T	1.9	2	0.38
C5250T	8.28	2	0.01
C8381T	2.22	2	0.32

Model 1 = There was controlling for age and sex; model 2 = there was no controlling for age and sex. LR = likelihood ratio.

Table 5. FBAT for the (GAAT)_n repeat and the C1455T, G1472T, C5250T and C8381T polymorphisms studied in the *IL21* gene with atopic asthma

Allele	Atopic asthma							
	additive model				recessive model			
	families	S	E(S)	p value	families	S	E(S)	p value
(GAAT) _n								
4	134	-6.819	-2.773	0.22	91	-1.852	-0.267	0.46
6	134	3.622	-0.424		91	3.312	0.75	0.25
C1455T								
C	74	-0.727	-0.636	0.97	22	1.37	0.154	0.25
T	74	0	-0.091		72	1.865	0.537	0.52
G1472T								
G	132	3.427	0.782	0.44	89	1.993	0.727	0.59
T	132	-5.835	-3.189		87	-2.236	-0.616	0.45
C5250T								
C	59	-5.535	-0.012	0.014	56	-6.7	-0.263	0.0007
T	59	4.233	-1.291		20	-0.991	-0.11	0.37
C8381T								
C	19	-1.935	-1.371	0.59	0	NA	NA	NA
T	19	0.194	-0.371		19	0.286	-0.315	0.56

The null hypothesis of no association and no linkage was tested at the maximum offset values using the -o option of FBAT. S = Observed transmissions of the allele or genotype to affected offspring; E(S) = expected transmission of the allele or genotype under mendelian inheritance; NA = not assessed.

tion of these SNPs and the GAAT repeat with asthma, the Armitage trend test was performed. The genotype frequencies are depicted in table 3. The SNPs C1455T, G1472T and C8381T were not found to be associated with atopic asthma in our case-control studies (table 3). Only two alleles were obtained for the GAAT repeat and they were also not found to be associated with asthma (table 3). Interestingly, the exonic synonymous polymorphism, C5250T, was found to be associated with atopic asthma ($p = 0.02$, common OR = 1.43 with 95% confidence interval). Also, using multiple logistic regression analysis, we confirmed the association of the C5250T polymorphisms with atopic asthma and found that the association was not influenced by sex and/or age (table 4).

The results were further confirmed in independent family samples by FBAT analysis, with a significant association being observed for the exonic synonymous polymorphism C5250T when analyzed by recessive ($p = 0.0007$ for asthma) and additive model ($p = 0.014$ for asthma; table 5). None of the other SNPs or the microsatellite repeat showed any association with asthma in family studies.

TsIgE levels were measured by ELISA, and the TsIgE values were converted to log₁₀ scale for analysis and were found to follow the normal distribution in patients and

controls. As expected, there was a highly significant difference between the log₁₀TsIgE values between the patients (3.0 ± 0.7) and controls (2.1 ± 0.7 ; $F = 144.7$, d.f. = 1, $p < 0.0001$). Using ANOVA, the genetic effects of *IL21* polymorphisms were tested on the log₁₀TsIgE levels in the atopic asthmatic patient cohort and no significant association was detected with respect to SNPs, viz C1455T, G1472T and C8381T, and the (GAAT)_n microsatellite repeat. Nevertheless, a strong association was observed for the exonic polymorphism C5250G ($F = 5.76$, d.f. = 2, $p = 0.003$ using ANOVA; table 3). However, when analyzed in a control cohort, no significant association was observed for any of the SNPs or the promoter microsatellite repeat ($p > 0.05$ for all SNPs).

Association of IL21 Haplotypes with Asthma and TsIgE Levels

To study the combined effect of the four SNPs and the microsatellite marker, haplotypes were constructed in 255 cases and 245 controls using PHASE version 2.1. However, only 4 haplotypes were present at frequencies $>5\%$ in the population. When we analyzed the haplotype frequency difference in the two groups (adult patients vs. normal controls) using the Monte Carlo test with one

Table 6. Results of the haplotype frequency estimation for the atopic asthma and control populations obtained with PHASE version 2.1 and observed and expected scores of allele transmission in FBAT

Subject No.	Haplotypes	Case-control studies			Family-based studies				
		frequency		global p value (d.f.)	frequency	S (n = 140)	E(S) (n = 140)	global χ^2	global p value (d.f.)
		controls (n = 245)	patients (n = 255)						
1	4_C_T_T_C	45.1	42.9	0.001 (13)	0.439	137.916	141.011	6.23	0.39 (6)
2	6_C_G_T_C	11.02	14.9		0.158	61.916	60.067		
3	6_T_G_T_C	14.29	15.69		0.149	54	48.998		
4	6_C_G_C_C	16.94	15.29		0.126	43	44.388		
5	4_C_G_T_C	2.45	2.55		0.039	14.084	14.795		
6	6_C_G_T_T	2.45	4.12		0.031	14	11.25		
7	6_C_T_T_C	1.22	1.18		0.018	NA	NA		
8	6_T_T_T_C	0.2	1.18		0.009	NA	NA		
9	4_T_T_T_C	0	0		0.008	NA	NA		
10	4_C_T_C_C	4.69	0.2		0.008	NA	NA		
11	4_C_G_C_C	0.2	0.59		0.005	NA	NA		
12	6_T_G_C_C	0.41	0.59		0.003	NA	NA		
13	6_C_T_C_C	0	0		0.002	NA	NA		
14	4_T_G_T_C	0.7	0.59		0.002	NA	NA		
15	6_C_T_T_T	0.2	0		0.002	NA	NA		
16	4_T_T_C_C	0	0		0	NA	NA		
17	4_T_G_C_C	0.82	0.59		0	NA	NA		
18	T_T	46.94	45.1	0.00003 (3)	0.47	-3.745	-2.315	6.57	0.08 (3)
19	G_T	30.82	38.24		0.384	2.614	-2.593		
20	G_C	17.96	16.47		0.132	-2.336	0.6		
21	T_C	4.29	0.2		0.014	NA	NA		

The haplotype involves polymorphisms -1183(GAAT)_n, C1455T, G1472T, C5250T and C8381T. S = Observed transmissions of a haplotype to affected offspring; E(S) = expected transmission of a haplotype under mendelian inheritance.

million simulations, a significant association was observed [normal χ^2 (T1) was 33.74, d.f. = 13, $p = 0.001$, and after collapsing columns with small expected values together (T2) χ^2 (table 6) was 28.78, d.f. = 7, $p = 0.0001$]. Interestingly, none of the major haplotypes showed any association with atopic asthma. The overall significance was the result of a minor haplotype 4_C_T_C_C ($\chi^2 = 26.36$, $p = 0.0000$; OR = 0.04; 95% confidence interval: $0.0054 < \text{OR} < 0.2966$). In the family studies also, none of the haplotypes was found to be overtransmitted to asthmatic offspring ($p > 0.05$; table 6). Moreover, we also constructed two locus haplotypes using the common SNPs G1472T and C5250T. In the case-control study population, the observed significance [χ^2 (T1 & T2) 23.47, d.f. = 3, $p = 0.00003$] was due to a minor haplotype T_C, while in the family studies, none of the haplotypes was found to be overtransmitted to asthmatic offspring ($p > 0.05$; table 6).

Furthermore, when we analyzed haplotypes of the asthmatic patients with respect to $\log_{10}$ TsIgE, none of the haplotypes was found to be associated with TsIgE levels (overall F-ratio = 0.33, d.f. = 11, $p = 0.97$). Similar results were obtained for the control individuals (overall F-ratio = 0.52, d.f. = 12, $p = 0.90$).

Functional Correlation of C5250T Polymorphisms of the IL21 Gene with IL-21 Serum Levels

To find any functional correlation of the exonic synonymous polymorphism C5250T with the level of IL-21 expression, the concentration of IL-21 was determined in serum samples of 40 unrelated patients using ELISA. The C5250T polymorphism was found to affect serum IL-21 levels. The presence of the C allele was significantly associated with higher concentrations of serum IL-21 levels (F-ratio = 15.06, d.f. = 2, $p = 0.0002$) and this effect seemed to be dose dependent as the CT heterozygotes had higher

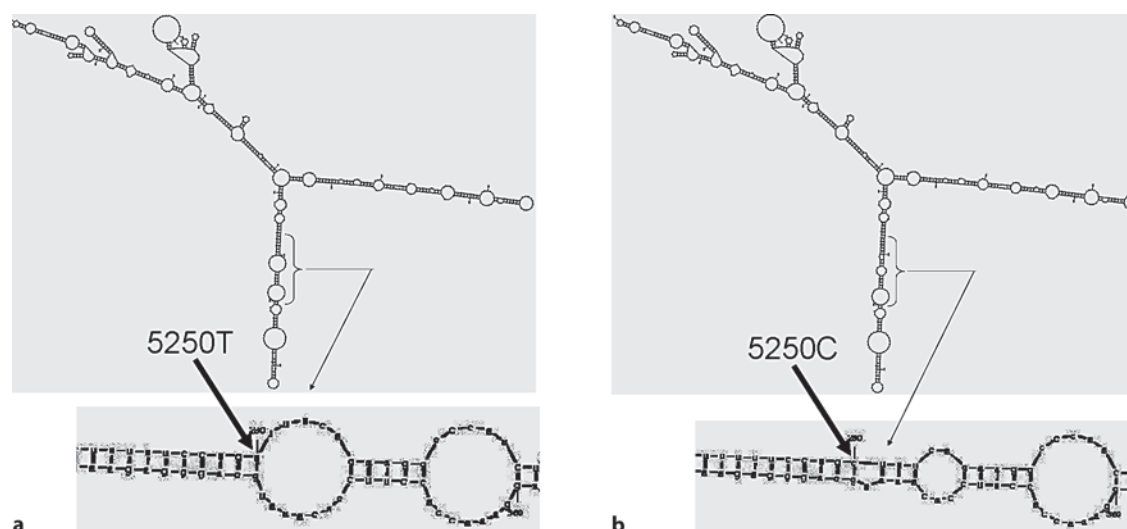


Fig. 2. Folding of RNA structure containing 5250T (**a**) or 5250C SNP (**b**) on exon 3 of the human *IL21* gene, as predicted by *mfold*. *mfold* software predicts secondary structures of nucleotides based on the minimal energy of folding. The result shows that the C5250T SNP affects the secondary structure of *IL21* mRNA, as reflected by the less negative folding energy (ΔG) of the *IL21* 5250T RNA. **a** $\Delta G = -160$ kcal. **b** $\Delta G = -162$ kcal.

IL-21 levels than the TT but lower than the CC homozygotes (F-ratio = 5.83, d.f. = 2, $p = 0.006$; table 3).

Assessment of the Secondary Structure of *IL21* mRNA Containing C5250T Variants

To assess the effect of C5250T SNP on *IL21* mRNA stability, the structure and folding of *IL21* mRNA containing the two exonic variants were evaluated. An altered folding was observed on comparing the mRNAs containing C or T variants (fig. 2). The mRNA stability of the 5250T variant was predicted to be less ($\Delta G = -160$ kcal/mol/base) than the 5250C variant ($\Delta G = -162$ kcal/mol/base), as measured by the free folding energy [Gibbs free energy (ΔG)]. ΔG is the energy required to convert the secondary structure of RNA into its denatured state. Therefore, the lower the ΔG value the higher is the stability of a molecule.

Discussion

To the best of our knowledge, this is the first study to investigate the potential role of *IL21* gene polymorphisms with atopic asthma and its associated phenotypes. Our results regarding the synonymous exonic SNP C5250T seem to be important: we have observed that the TT genotype of C5250T SNP is associated with atopic asthma,

TsIgE and serum IL-21 levels for the first time. Although the HapMap data did not indicate any other SNP to be in LD with C5250T, we observed that the intronic SNP G3301A was in complete LD with C5250T SNP in our study population. Thus, the resulting significance may be due to an effect of the G3301A SNP or a combination of both these SNPs. Also, the possibility of the presence of any other functional polymorphisms, for example in the *IL2* gene, in LD with the C5250T SNP cannot be ignored. Although the association of C5250T SNP with IL-21 levels in the asthmatic patients is quite significant, its causal relationship with atopic asthma remains to be elucidated. It is known that synonymous exonic SNPs in a gene can lead to its altered mRNA stability and hence altered protein expression [27, 28]. It is thus possible that the T allele of the C5250T SNP might cause potential structural changes in the *IL21* mRNA that may alter the expression of the *IL21* gene. In fact, using the mRNA folding software *mfold*, we observed that mRNA derived from the mutant 5250T allele is less stable than that of the 5250C allele.

For further understanding of the contribution of the SNPs and the promoter microsatellite repeat in *IL21* towards asthma, we constructed five and two locus haplotypes, and found a significant difference in the haplotype frequency in the case-control cohort but no such association was observed in the family samples. On further anal-

ysis it was observed that the association was due to minor haplotypes 4_C_T_C_C and T_C whose frequency was <5% in both the case and the control groups. Furthermore, due to the low frequency of 4_C_T_C_C and T_C haplotypes, they were not considered for analysis by HBAT in the family-based studies. Although the frequencies of 4_C_T_C_C and T_C are low in our population, it could be significantly higher in other ethnic populations, which remains to be tested in the future.

Asthma is a complex disorder caused by the interaction of multiple genes, each contributing to only a small effect. In our case-control study, even minor contributions of the effector genes were detected because of its ample power. However, case-control studies have always been questioned with respect to the problem of population stratification, resulting in false-positive associations [29]. On the other hand, due to small effects of individual genes, a family-based study might fail to detect significant associations and hence provide false-negative results in complex genetic disorders such as asthma [30]. To circumvent this problem, genetic homogeneity between the patients and controls from both groups was confirmed by genotyping various loci that are not linked to asthma or related atopic disorders to date ($p \geq 0.05$, 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). Moreover, our results from a well-characterized case-control cohort have been further con-

firmed in a family-based study. Also, our careful recruitment of age-, sex- and ethnicity-matched patients and controls, along with multiple logistic regression analysis for age and sex, may rule out any error due to stratification or an inherent statistical bias.

In summary, our results establish for the first time a significant association of the *IL21* gene with atopic asthma. We report that the T allele of the C5250T SNP is a low IL-21-producing allele in atopic asthmatics, and this synonymous exonic variant is associated with atopic asthma and TsIgE levels. Further studies to elucidate the functional significance of this polymorphism and the intronic polymorphism G3301A, which is in complete LD with the C5250T SNP would be valuable in elucidating the role of the *IL21* gene in the pathogenesis of asthma. Moreover, a gene-gene interaction study of these SNPs with the promoter SNP T-83C of the *IL21R* gene, which has recently been found to affect the IFN- γ -mediated inhibition of human IgE synthesis, will also be highly interesting. Thus, our results may spark further research on the exact role of *IL21* in the pathogenesis of asthma.

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