

Association of *IL4* Gene Polymorphisms with Asthma in North Indians

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Key Words

5q31–33 · Asthma · CA repeat polymorphism · *IL4* · Indian population

Abstract

Background: Asthma is a complex airway disorder, and a number of genetic loci have been found to be associated with asthma. The 5q31–33 region is one of the most important loci linked to asthma and atopic disorders. However, association studies with candidate genes in this region, such as *IL4*, were inconclusive, as both positive and negative results were obtained in several populations studied. The aim of our case-control study was to determine the association between *IL4* and asthma in North Indians. **Patients and Methods:** Polymorphisms in the promoter and a dinucleotide repeat in the 2nd intron in *IL4* were genotyped by sequencing and GeneScan analysis, respectively, in ethnically matched, unrelated patients (n = 171) and controls (n = 128), following the guidelines of the American Thoracic Society. **Results:** The proximal promoter region of the *IL4* gene was found to be invariant. Previously reported polymorphisms, –590 C/T and +33 C/T, were found to be absent in our population. The χ^2 test using only large expected cell counts (more than 5% of the sample size) showed a sig-

nificant association between allele size and disease status ($\chi^2 = 38.08$, d.f. = 6, $p < 0.05$). In addition, a significant difference was observed for the allele and genotype frequencies ($p < 0.0005$ and $p = 0.0009$, respectively) in the patient and the control groups using the Fisher-Freeman-Halton test. **Conclusion:** Our studies indicate that the promoter of the *IL4* gene is invariant in our population. The case-control studies on the CA repeat polymorphism in the 2nd intron of the *IL4* gene have shown interesting results and indicate the need for further family-based studies.

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Introduction

Asthma is a complex airway disorder with both environmental and genetic factors playing a role in the pathogenesis of the disease [1] and affects approximately 10–15% of the population worldwide [2]. Clinically, asthma presents as reversible obstruction of bronchioles and is usually associated with high levels of total serum IgE, also referred to as atopy [3]. The T-helper-type-2 cells (Th₂), which release interleukins 4, 5 and 13, are responsible for the deviation of the immune system towards atopicity [4, 5]. A significant role for these cytokines in the pathogene-

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1018–2438/04/1343–0206\$21.00/0

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Table 1. Demographic profile of the patient and control groups

	Patients (n = 171)	Controls (n = 128)
Native place	Northern India	Northern India
Mean age, years	29.57 ± 12	27.3 ± 8.5
Sex ratio, males:females	0.61:0.39	0.55:0.45
History of asthma	all	none
Familial history of asthma/atopy	all	none
Mean log ₁₀ tSIgE, IU/ml	2.44 (n = 122)	2.22 (n = 88)
95% confidence interval	2.62–2.65	2.34–2.01
Self-reported history of allergy	all	none

Patients and controls were recruited from the states of Delhi, Haryana, Punjab and Rajasthan.

Controls were required to complete a questionnaire in order to eliminate all individuals having atopic disorders or a family history of atopic disorders. n = Number of individuals per group.

sis of asthma is evident, as they have been shown to play a critical role in IgE synthesis, eosinophil recruitment and bronchial hyperresponsiveness, respectively [4, 5]. Flow-cytometric studies in the bronchiolar lavage fluid of patients with acute asthma have shown significant numbers of T cells positive for these cytokines [6].

IL-4 is one of the major cytokines involved in the pathogenesis of asthma. Upon engagement of IL-4 with its receptor complex, signaling events lead to the phosphorylation, dimerization and nuclear localization of the signal transducer and activator of transcription 6 (STAT6) protein. This activated form of STAT6 binds to the promoter elements and causes the transcription of the *Cε* gene [7, 8]. Thus IL-4 regulates the IgE levels through STAT6 [9]. The importance of the *IL4* gene product in the development of asthma and atopy is underlined by the observations that antibodies against the protein were able to abrogate the clinical and biological effects of IL-4 in asthmatic individuals and animal models of atopy [10, 11].

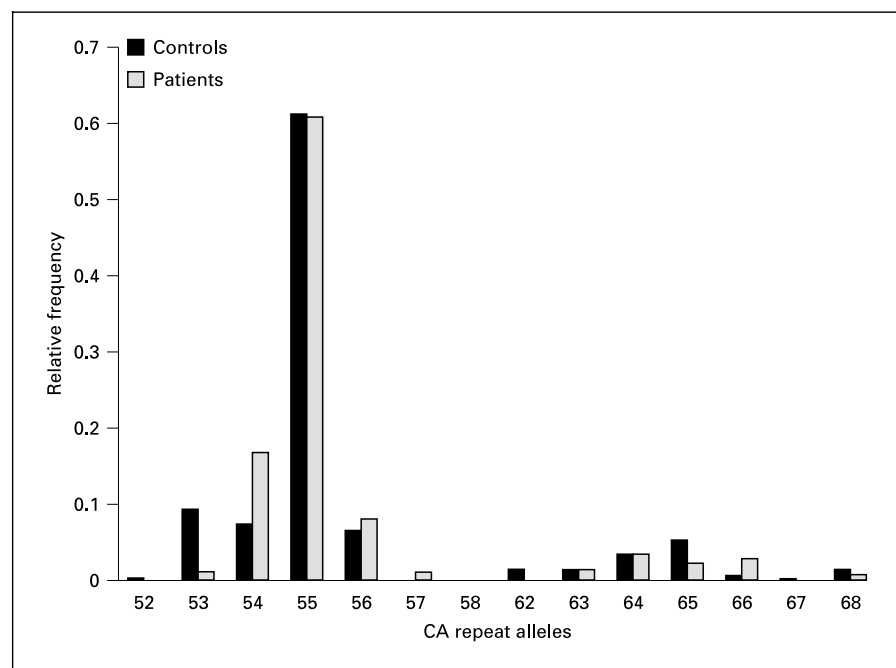
Genetic studies carried out in asthmatic cohorts recruited from various populations have indicated regions of chromosomes such as 5q31–33, 11p13 and 12q13–24 to be linked to asthma [12–15]. Of these loci, the 5q31–33 region is one of the most important candidate loci as it harbors the Th₂ cytokine gene cluster, including the *IL4* gene [12]. However, a study in Australians suggested that the 5q31–33 region was not linked to asthma [16]. Two C/T polymorphisms, at the –590 and the +33 position, and a dinucleotide repeat in the 2nd intron of this gene have been associated with asthma in various populations [17–20]. On the other hand, studies from other populations found no such associations [21–24]. We have carried

out a case-control study to determine the association of the known promoter and intronic polymorphisms in the *IL4* gene with asthma and associated subphenotypes in North Indians. The χ^2 test showed a significant association between allele sizes and disease status. In addition, significant differences were observed for the allele and genotype frequencies ($p < 0.0005$ and $p = 0.0009$, respectively) in the patient and control groups using the Fisher-Freeman-Halton test. To our knowledge, this is the first study on the 5q31–33 region reported from the Indian subcontinent.

Patients and Methods

Unrelated, asthmatic probands (n = 171) and normal controls (n = 128) were recruited from Northern India and clinically assessed based on the guidelines of the American Thoracic Society (table 1). Standard questionnaires were used for recruiting both patients and controls across all the regional centers to maintain uniformity. These included detailed clinical and environmental history. Asthma phenotype was established using a pulmonary function test (forced expiratory volume in 1 s, bronchial reversibility test $\geq 15\%$ increase in forced expiratory volume in 1 s or forced vital capacity) using a β_2 -agonist inhaler [25]. During the interviews, the region of origin and the migration status of the probands and unrelated controls were also obtained [26]. The demographic profile and a description of the patient and control groups is presented in table 1. The human ethics committees of all participating centers approved the study. Written informed consent was obtained from all the individuals including controls. Exclusion criteria were applied as previously stated [26]. Briefly, individuals who had a prior history of smoking and/or parasitic infestation and samples of controls with a history of asthma/allergy were excluded from the analysis. DNA isolation, serum separation and total IgE measurements were performed as described [26].

Fig. 1. The frequency distribution of alleles at the *IL4* 2nd intron CA repeat locus. The repeat size was plotted on the x-axis and their respective relative frequencies on the y-axis. The alleles were denoted by the number of repeat elements at this locus, i.e. (CA)_n. Alleles were defined by the values of (n).



For the identification of promoter polymorphisms, 5'-AGGA-GGGCTGATTTGTAAGTTGGT-3' and 5'-AGGATGGGCCCT-GTTCTATGG-3' primers (*IL4* accession No. AF375790) were used for PCR (2,524-bp product, including -1,063 bp of the promoter, 5'-UTR, and exon 1 of the gene). Products were sequenced for 35 individuals (15 patients and 20 controls) using the forward and reverse primers and the internal sequencing primer, 5'-AACTCA-TTTTCCTCGGTTTC-3', according to the manufacturer's instructions (Cycle Sequencing kit, ABI-Prism 377; Automated Sequencer PE Biosystems, USA). The CA repeat region was amplified using Tet-labeled forward primer (152- to 172-bp product) as reported elsewhere and was loaded with an internal standard ladder ABI-550 TAMRA [12]. Gels were analyzed using the GeneScan version 2.1 and Genotyper version 3.1. Random samples of homozygous individuals ($n = 10$) were sequenced to confirm the number of repeats. Allele sizes were calculated as described previously [26].

Statistical analysis was performed to test for differences in the allelic distribution. The Kolmogorov-Smirnov test was used to test for equality of allelic distributions in patients and controls ($N_C = 256$, $N_P = 342$), assuming rank ordering of alleles based on repeat sizes. Due to a large number of cells (6 of the alleles) having small expected counts (less than 5% of the sample size), an overall χ^2 test for association was not done. However, the χ^2 test was carried out after excluding alleles with low cell counts (d.f. = 6). The StatXAct program (version 4) was used to calculate the Fisher-Freeman-Halton exact test with the Monte Carlo method of repeated sampling. ANOVA was carried out to test the association of IgE values ($N_C = 88$, $N_P = 111$). The alleles have been denoted as X_N (X = the CA repeat size), and the genotypes denoted as X/Y (X and Y are CA repeat sizes).

Results

As promoter and 5'-UTR polymorphisms are important in gene regulation, we analyzed these regions of the *IL4* gene by sequencing a total of 35 individuals (15 patients and 20 unrelated controls). The sequencing results showed the proximal promoter of the *IL4* gene to be invariant. Additionally, no polymorphisms were detected in the 5'-UTR and exon 1 of the gene.

Further, to determine the association between the *IL4* gene and asthma in the Indian population, we amplified and genotyped the CA repeat in the 2nd intron in 342 patient chromosomes and 256 control chromosomes. The heterozygosity index and the polymorphic information content of the CA repeat polymorphism was found to be 0.52 and 0.35, respectively. Previous studies had reported 7 alleles at this locus [12]; however, in our population we found a total of 11 alleles. The frequencies of the alleles were found to be consistent with the Hardy-Weinberg equilibrium conditions in the control group.

To test for the difference in the distribution of the alleles at the *IL4* 2nd intron locus between patient and control groups, we carried out a Kolmogorov-Smirnov test (fig. 1). The χ^2 value of the largest difference in cumulative frequency for the distributions was 0.58 ($p = 0.75$). Thus the difference between the allelic distributions of the patients and the controls was not significant. However,

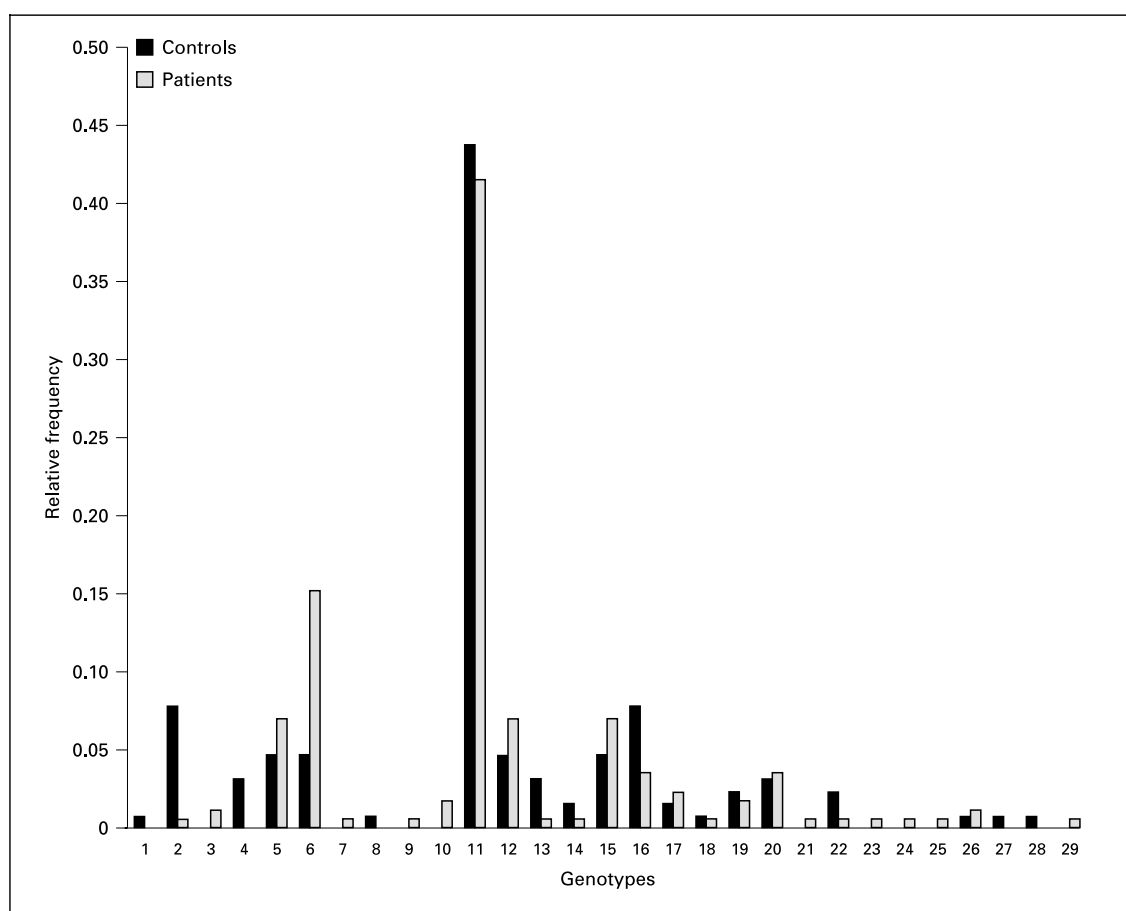


Fig. 2. The frequency distribution of genotypes at the *IL4* 2nd intron CA repeat locus. The genotypes were plotted on the x-axis and their respective relative frequencies on the y-axis. The genotypes are denoted as: 1 (52/55), 2 (53/53), 3 (53/54), 4 (53/55), 5 (54/54), 6 (54/55), 7 (54/56), 8 (54/64), 9 (54/65), 10 (54/66), 11 (55/55), 12 (55/56), 13 (55/62), 14 (55/63), 15 (55/64), 16 (55/65), 17 (55/66), 18 (55/67), 19 (55/68), 20 (56/56), 21 (56/57), 22 (56/65), 23 (56/66), 24 (57/57), 25 (57/58), 26 (63/63), 27 (64/64), 28 (65/68), 29 (66/66). The genotypes were denoted as X/Y, X and Y being repeat sizes.

allele 54 was found to be more predominant in the patient group with a relative frequency of 0.16 compared to 0.07 in the control group (fig. 1). The alleles with low expected cell counts (less than 5% of the total sample sizes) were excluded and the χ^2 test was also carried out. This gave a highly significant p value ($\chi^2 = 38.08$, d.f. = 6, $p < 0.05$). The largest difference was observed for alleles 53, 54 and 65. A significant difference (Fisher statistic = 47.64, $p < 0.0005$) was also observed in the distribution of the alleles between both groups using the Fisher-Freeman-Halton test (p value according to the Monte Carlo simulation of 10,000 tables).

To determine if there exists a genotypic association, we carried out the Fisher-Freeman-Halton test, and a highly significant difference was observed (Fisher statistic =

46.65, $p = 0.0009$). The 54/55 genotype showed the maximum difference in the relative frequency in the patient and the control groups (0.15 vs. 0.05; fig. 2).

As shown in table 2, we found a significant difference between the log-transformed total serum IgE ($\log_{10}$ tsIgE) levels in the patient and control groups ($t = 2.01$, d.f. = 208, SE of difference = 0.11, $p = 0.04$). Thus, in order to determine the association of the alleles and genotypes with tsIgE levels, we performed ANOVA. A one-way ANOVA of tsIgE levels against genotypes with at least 3 individuals revealed no significant difference in the control group [$F = 1.11$, d.f. = (7, 70), $p = 0.57$]. A similar analysis carried out for the patient group also found no significant difference between the genotypes and the tsIgE levels [$F = 0.32$, d.f. = (7, 108), $p = 0.91$].

Table 2. Mean log₁₀ tsIgE levels (in IU/ml) for controls and patients tabulated according to the genotypes

Genotype**	Controls		Patients	
	mean	95% CI	mean	95% CI
53/53	2.20 (n = 10)	1.84–2.55	–	
53/55	2.60 (n = 4)	2.21–3.00	–	
54/54	2.62 (n = 6)	2.12–3.11	3.08 (n = 9)	2.53–3.63
54/55	2.22 (n = 5)	1.66–2.77	2.13 (n = 16)	1.74–2.52
54/66	–		2.24 (n = 3)	1.05–3.44
55/55	2.13 (n = 33)	2.00–2.30	2.74 (n = 53)	2.46–3.01
55/56	2.40 (n = 4)	2.00–3.00	1.92 (n = 11)	1.56–2.28
55/64	–		1.86 (n = 8)	1.58–2.14
55/65	2.07 (n = 6)	1.58–2.55	1.85 (n = 4)	1.37–2.32
56/56	–		1.81 (n = 5)	0.94–2.68
56/65	2.13 (n = 3)	1.60–2.65	–	

CI = Confidence interval.

Discussion

We have sequenced the proximal promoter and the 5'-UTR of the *IL4* gene and found it to be invariant in the Indian population. Both the –590 C/T and the +33 C/T polymorphism were not detected in the samples sequenced. Previous studies on the association of *IL4* gene polymorphisms with asthma or related phenotypes have been inconclusive as both positive and negative results were reported [17–24]. Studies in UK and Australian populations on the –590 C/T polymorphism showed no association with asthma [21]. This was also observed in Kuwaiti Arabs and Japanese [22, 23]. However, in another study in a Japanese cohort, the –590 C/T polymorphism was associated with asthma but not with total serum IgE levels [17]. This polymorphism was also associated with life-threatening asthma in another study [18]. Promoter polymorphisms may be biologically functional if they induce a mutation in a transcription factor binding site [27]. However, no interaction of a transcription factor binding to the –590 or a nearby site has been demonstrated so far. Thus, the involvement of this site in the regulation of the *IL4* gene is purely speculative. The 5'-UTR polymorphism, +33 C/T, has been associated with total serum IgE levels in a Japanese population [28]. However, this observation has not been repeated in other populations [29]. Also, studies on other Th₂-mediated diseases such as atopic dermatitis have not found an association with polymorphisms in the *IL4* gene [29]. Recently, however, it has been reported that haplotypes of single nucleotide polymorphisms in the 5'-region of *IL4* and the *IL4*-*IL13* intergenic region are associated with asthma [30].

The dinucleotide repeat in the 2nd intron of the *IL4* gene was found to be polymorphic in our population (heterozygosity index = 0.52). To test for differences in the allelic distribution between patients and controls, the Kolmogorov-Smirnov test was used. The advantage of this test is that, in analysis where cell counts fall below 5, statistical differences are calculated based on the cumulative frequency, and thus it is more powerful and robust than the Pearson χ^2 test. The overall distribution of the alleles was not found to be significantly different between the patients and the controls ($p < 0.75$; fig. 1). However, when we deleted the cells with expected counts less than 5% of the sample size, we found a significant difference in the allelic distribution between the patients and the controls. Nevertheless, the procedure of ignoring some of the data is not considered to be good practice [31]. However, using the Fisher-Freeman-Halton exact test with the Monte Carlo method of repeated sampling, a highly significant difference was obtained (Fisher statistic = 47.64, $p < 0.0005$).

It is important to note that the 54/55 genotype showed the maximum difference in the relative frequency in the patient and control groups (0.15 vs. 0.05). Allele 54 (designated A1) has also been shown to be associated with asthma in a Tunisian population [32]. Further, in our study, a highly significant difference was obtained in the overall genotype distribution in the two groups using the Fisher-Freeman-Halton test (Fisher statistic = 46.65, $p = 0.0009$).

We observed a significant difference in the tsIgE levels between the patient and the control groups. As *IL-4* is needed for class switching and the production of IgE, we

expected to observe an association of the genotypes at the CA repeat locus with total serum IgE levels. However, the genotypes in both groups were not associated with total serum IgE levels. One of the possible reasons for this observation could be the lack of a functional role of the CA repeat locus in the regulation *IL4* gene expression. This remains to be tested in the future.

The differences in the results in our study and those of others demonstrating no association with asthma may be attributed to population differences and differences in the study design. We have used a case-control design to address the question of the association of the *IL4* gene with asthma. To minimize the errors caused by population stratification, we have recruited patients and controls from the same geographical area and ethnic background (table 1) [26]. Additionally, we have recruited only those patients who have at least one first-degree relative affected with atopy and/or asthma [26]. As asthma is a complex disorder believed to be caused by the interaction of many genes, each contributing a small effect, a family-based study may fail to detect a significant association even when it exists [33]. On the other hand, a case-control design has a higher probability of detecting false-positive associations. In terms of costs and labor, however, a case-control design is a better option as leads obtained from these can be further validated using a well-controlled family-based study. The sample sizes used to screen polymorphisms in the promoter and the 5'-UTR were sufficient to detect those which may have been present even at a relative frequency of 1.4% in the population studied. As our results have been obtained from a previously untapped genetic pool, it leads more weight to similar stud-

ies that have reported an association of the *IL4* gene with asthma and atopic diseases in other populations. The results thus suggest that the *IL4* gene is a susceptibility or modifier locus for the development of asthma. In addition, it is also possible that other as yet unidentified polymorphisms in the *IL4* gene or polymorphisms in other genes such as *IL4 receptor* and *STAT6*, which are in the same signaling cascade, may be associated with asthma and atopy. Indeed, we have identified novel polymorphisms in the *IL4RA* and the *STAT6* gene [34]. It would be interesting to see the genetic association of these polymorphisms in combination with those present at the *IL4* locus or other loci in linkage disequilibrium with it.

Our results on the *IL4* promoter and the 2nd intron CA repeat indicate the need for further studies on the 5q31–33 chromosomal region, as it is possible that other candidate genes, such as *IL13*, *IL5* and *IL9*, in the 5q31–33 region may also be involved in the susceptibility to asthma in the Indian population.

Acknowledgments

We are grateful for the help provided by Drs. C.B. Rao, P.V. Niphadkar, A.B. Singh and Harish K. Pemde, Ms. Sweetly Sharma and Shilpy Sharma, and Prof. S.K. Bhramachari. We thank Dr. Michael Kundi for helping us in statistical analysis, and the Council for Scientific and Industrial Research and the Department of Biotechnology, Government of India, for financial assistance. R.N. acknowledges the fellowship granted by the University Grants Commission, Government of India. We also express our thanks to the Functional Genomics Unit of IGIB, Delhi, for the assistance in sequencing and genotyping.

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