



Genetic contribution of chemokine receptor 2 (CCR2) polymorphisms towards increased serum total IgE levels in Indian asthmatics

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ABSTRACT

The chemokine (C–C motif) receptors (CCR) 2 and 5 are members of a large family of G protein-coupled receptors, playing important roles in asthma pathogenesis. Using standard sequencing techniques, a total of 15 single nucleotide and 8 insertion/deletion polymorphisms (DIPs) (5 novels) were identified in and around these two genes. None of the studied polymorphisms ($N = 7$, selected on the basis on linkage disequilibrium) was associated with asthma in a case ($N = 315$) – control ($N = 337$) study and showed no evidence for non-random transmission to individuals with asthma/atopy in Indian pedigrees ($n = 235$). However, multilocus haplotype analysis based on simulations yielded a $P = 0.00005$ in the case–control study and a $P = 0.03$ for the family-based association studies. Furthermore, rs3918356 and rs743660 polymorphisms in CCR2 were found to be associated with total serum IgE levels in both the study designs. Thus, our study supports a significant role for chemokine receptor polymorphisms in genetic susceptibility to asthma.

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Background

The chemokine (C–C motif) receptors; CCR2 (MIM#601267) and 5 (MIM#601373); are members of a large family of G protein-coupled seven-transmembrane domain receptors, playing important roles in asthma (NM#600807) pathogenesis [1,2]. CCR5 is expressed mainly on the T helper (Th) cells producing type 1 cytokines such as interferon- γ and tumor necrosis factor as well as on a major subset of CD8⁺ cytotoxic T cells, [3] and binds the pro-inflammatory chemokines CCL5 (regulated on activation, T-cell expressed and secreted), CCL3, CCL4 and CCL2 [4], while CCR2 is expressed on monocytes, basophils and eosinophils. The primary agonist of CCR2 is MCP-1 (CCL2), although other lesser agonists of this receptor include CCL8, CCL7, CCL13, and CCL12 [1,5]. CCR5 plays a key role in the distribution of CD45RO⁺ T cells and contributes to the generation of a T helper (Th) 1 immune response. Since Th2 immune responses are critical for the inflammatory reaction, it is hypothesized that the presence of CCR5 may delay the development of Th-2 mediated diseases like asthma. Evidently, CCR5 knockout mice demonstrated a shift towards type2-cytokine activation in a model of experimental colitis [6]. In contrary, an impressive accumulation of CCR5-positive T cells and macrophages is found in the lungs of the asthmatics after allergen challenge [2]. Also, the airways hyper responsiveness (AHR) was found to be

significantly lower in *A. fumigatus*-sensitized CCR5^{−/−} mice [7]. While, in a similar model, CCR2-deficient animals exhibited an increase in eosinophils and lymphocytes in the airways, serum IgE, Th2 cytokines and Th2-induced chemokines, AHR, and fibrosis in comparison to wild type mice [8]. However, neutralization of CCR2 using specific anti-CCR2 mAb leads to the reduction of antigen-induced bronchial HR and attenuates macrophage and eosinophil accumulation in the BAL of a primate model of asthma [9]. Thus, the role for both the chemokine receptors in asthma pathogenesis remains controversial.

Few genetic epidemiology studies have also documented a role for chemokine receptors and their ligands in the pathogenesis of asthma. The presence of the 64I allele of rs1799864: A>G (V64I) in CCR2 conferred significantly lower risk for the development of asthma in a Korean population [10]. A 32-bp deletion in CCR5 ($\Delta 32$), which causes loss of CCR5 cell surface receptors, has been associated with asthma and atopy in some studies [11,12], but not in others [13,14]. Recently, we have shown that the frequency of heterozygous mutant is much higher in patient group, in comparison to the control group ($P = 0.0089$), thus shown a protective role for the deletion mutation [15]. However, because of its low frequency in our population, we were not able to establish the role of CCR5 in atopic asthma. This observation, in part, motivated further identification of polymorphisms in the CCR5 gene and its flanking regions including CCR2. Thus, we screened the complete gene-length of the CCR5 and its flanking regions, and also sequenced the CCR2 exonic region and performed the association

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studies on the selected polymorphisms with asthma and serum total IgE levels in case–control and family-based study designs.

Results

Resequencing of CCR2 and CCR5 gene and linkage disequilibrium

We sequenced all exons, introns and ≈ 1.8 kb of 5' flanking region of the human CCR5, and the exonic regions of CCR2 gene in 20 individuals irrespective of their disease status using the primers given in Table 1. Besides 32 bp deletion, we have observed seven insertion/deletion polymorphisms (DIPS) (five novel exonic variants) and 11 SNPs (1 novel) in CCR5 and four exonic variants in CCR2 in our sequencing panel. A "CAA" DIP ≈ 5 kb upstream of the translation start site of CCR2 was also identified using Repeatmasker and confirmed by sequencing (Fig. 1A). None of the other reported mutations was found to be polymorphic in our sequencing panel, thus pairwise LD among the 17 polymorphisms (except for the rare polymorphisms) was calculated. A pattern of strong linkage disequilibrium was observed and three haplotype blocks were identified (Fig. 1B). Depending upon their functional significance and to capture maximum alleles based on LD, four polymorphisms from block 1, two from block 2 and one from block 3 were chosen for further genotyping.

Association of CCR2 and 5 polymorphism with asthma and IgE

Allelic and genotypic frequencies for the five SNPs viz. rs1799864: A>G, rs1799865: C>T (VAL64ILE), rs743660: G>A of CCR2 and rs1799988: T>C and rs1800023: A>G of CCR5, and two DIPS viz. a -/CAA (rs3918356) 5082 bp upstream of CCR2 and -/CAA (rs41515644) in the 370 bp upstream of CCR5 translation start site were determined in all the recruited individuals (Table 2). Genotype frequencies of the polymorphisms in cases and controls are given in Table 3. All the polymorphisms were found to be in Hardy–Weinberg equilibrium in both groups, when analyzed separately

from distinct geographical areas and also from the combined meta-analysis (data not shown). None of the seven polymorphisms showed significant association with atopic asthma ($P > 0.007$) in any cohort. In the families, also, none of the alleles demonstrated significant transmission distortion. Among the West-Indian families, rs1800023 and rs41515644 polymorphisms demonstrated a weak evidence of transmission disequilibrium (unadjusted $P = 0.01$, using recessive model). This distortion was not statistically significant (particularly after correction for multiple testing) and was not observed North and North-east families (data not shown).

Total serum IgE levels were found to follow a log normal distribution. When the cases and controls were compared with respect to the log total serum IgE levels, a highly significant difference was obtained ($t = 7.4$; $df = 5.71$; $P < 0.0001$). Because increased serum total IgE level is one of the major characteristics of atopic asthma, the genetic effects of chemokine receptor gene polymorphisms were tested on serum total IgE levels. None of the CCR5 variant was associated with TslgE levels (Table 4). On the other hand a significant effect was observed at the genotypic level for the rs3918356 and rs743660 in the patients ($P = 0.0028$ and 0.0002 , respectively). The mean $\log_{10}$ TslgE levels in individuals with BB (with insertion) at rs3918356 and with AA at rs743660 genotype were highest, followed by individuals with rs3918356_AB/rs743660_GA and rs3918356_AA/rs743660_AA genotypes (Table 4). Similar results were obtained when QTDIT was used for family-based association test of genotypes with $\log_{10}$ TslgE (Table 4). The data was highly significant ($P = 4 \times 10^{-9}$ for rs3918356 and $P = 2 \times 10^{-10}$ for rs743660: G>A SNP) when analyzed including dominance effect in the orthogonal model of Abecasis et al. (Table 4) [16].

Haplotype association with asthma

The permutation test for significant differences in haplotypes frequency in case and control group using PHASE resulted in a P value of 0.001 (data not shown) from the North cohort and 0.01 in

Table 1
Primers and PCR conditions used for sequencing and genotyping the CCR2 and CCR5 polymorphisms.

Primer name	Segment /polymorphism	Primer sequence	Annealing temperature (°C)	Size of PCR product (bp)
CCR5_1FP	1	5'-TCCTATGGGGTGTCCGAATGT-3'	58	1278
CCR5_1RP		5'-GCAAACTAGAAAGCTGAAAAGGTAA-3'		
CCR5_2FP	2	5'-GCTCTGATCGCTGGGCTATTCTA-3'	58	1278
CCR5_2RP		5'-TTTGTTCTCTGCTCATCCACTAC-3'		
CCR5_3FP	3	5'-TCATTGCTCTCTGGATAGTAATT-3'	58	1266
CCR5_3RP		5'-CCAAGTCCAGAGAAAGGTGATA-3'		
CCR5_4FP	4	5'-TATTCTCTGGGTATGTATGACAACT-3'	58	1269
CCR5_4RP		5'-GAGGCGGGCTGCGAATT-3'		
CCR5_5FP	5	5'-TTCATGGAGGGCAACTAAAT-3'	58	1208
CCR5_5RP		5'-CCAGCCCAGGCTGTGTAT-3'		
CCR5_6FP	6	5'-ACGCTTCTGCAATGCTGTCTAT-3'	58	1070
CCR5_6RP		5'-CTCCCTCTTCCCATCCTACGA-3'		
CCR5_7FP	7	5'-GGCATTGCTCCGTCTAAGTCAT-3'	58	959
CCR5_7RP		5'-CCCCACCCCATTCAGTC-3'		
CCR5_8FP	8	5'-AACACAGGCAGCATTAGCA-3'	58	630
CCR5_8RP		5'-CTCACCCTCATATTGAGAGGC-3'		
CCR2_1FP	9	5'-CTTTTCCTGCTTGGCACT-3'	62	963
CCR2_1RP		5'-TTTATAAACCAGCCGAGACTTCT-3'		
CCR2_2FP	10	5'-CTTTTCCTGCTTGGCACT-3'	68	1168
CCR2_2RP		5'-AGTTCTGCTCTGTCCCACTTCTT-3'		
CCR5_SS1	rs179988: T>C	5'-TGAGAAAAGCCGTAATAAAT-3'	55	23
CCR5_SS2	rs1800023: A>G	5'-GAAGAAGTGTCTCTGATT-3'	50	20
CCR2_SS1	rs1799864: A>G	5'-TTTTCAGTTTATTAGATGAGGA-3'		
CCR2_SS2	rs1799865: C>T	5'-AAGGTGTTTCTGAGAGAAATGACAAT-3'	55	24
CCR2_SS4	rs743660: G>A	5'-CCAGTGGGAAGTCTTAATCAA-3'	55	23
CCR5_M1_FP	rs3918356: (-)>(CAA)	5'-AGCCCGGGCAACATGACAAAACC-3'	68	313
CCR5_M1_RP		5'-CATTTGATTGTTGTTGGCTCTAGCAGTGG-3'		
CCR2_M2_FP	rs41515644: (-)>(CAA)	5'-GTGTGGTGGCGCTGTAGTCC-3'	62	272
CCR2_M2_RP		5'-GAAAGCACCATCTCACCAATAAATCT-3'		

Ref Seq: CCR5 (accession no. NM_000579); CCR2 (accession no. NM_000647).

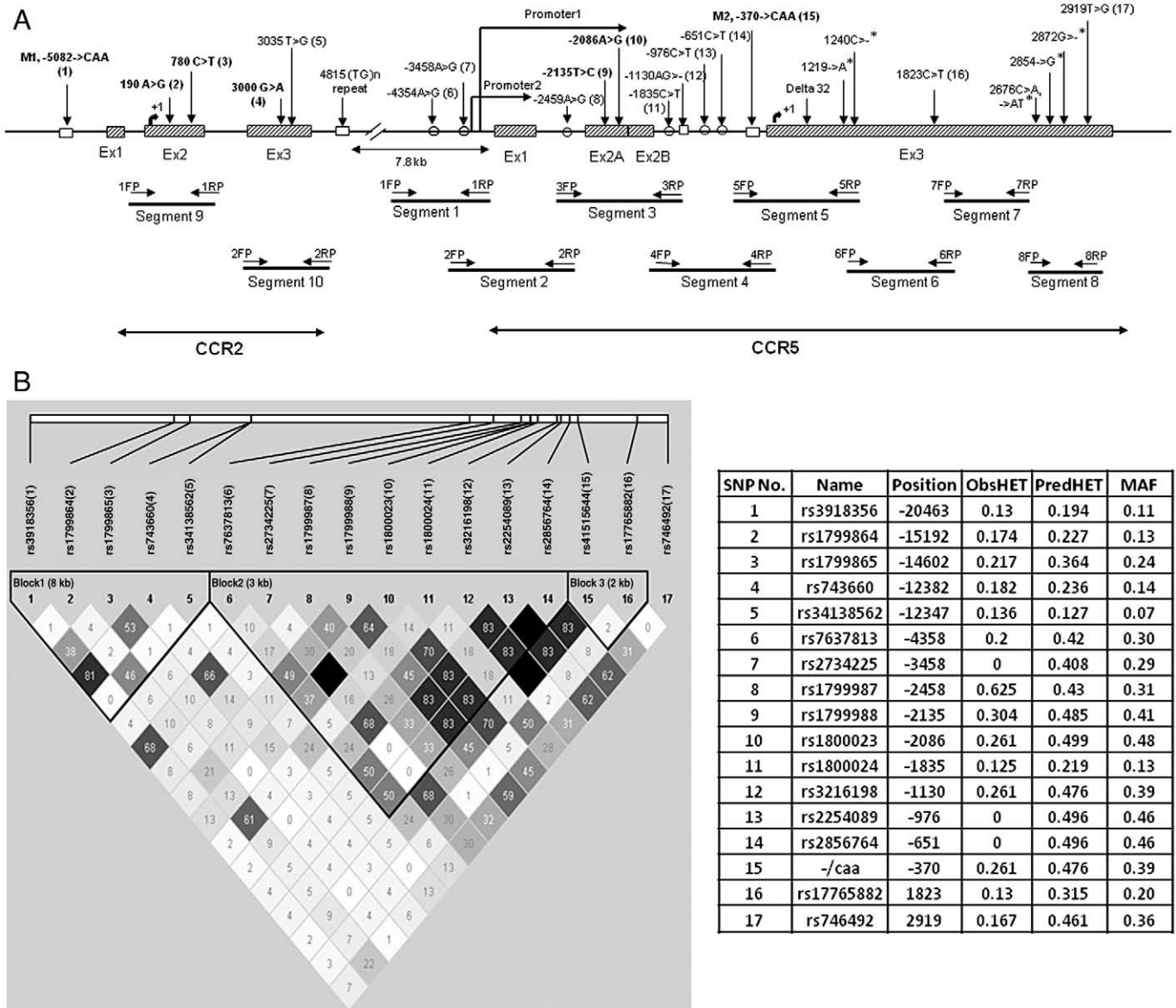


Fig. 1. (A) Genomic structure of chemokine receptors CCR2, 5 and flanking regions for the 24 polymorphisms (including $\Delta 32$) identified on the basis of NW_921651. The shaded boxes represent the exons. The translation start site is indicated by + 1. The SNPs genotyped in total study populations are represented in bold. The novel polymorphisms are represented by *. Numbers in () represents the corresponding rs IDs as given in panel B. Approximate positions of the sequencing primer pairs are shown in the lower half of the figure. (B) Haplotype-block structure in and around CCR2 and CCR5 genes and general information regarding the SNPs from the sequencing cohort. The haplotype blocks were generated by Solid Spine of LD of Haploview. Black boxes indicated strong evidence of linkage disequilibrium (LD) between the polymorphisms ($r^2 = 1$), while white boxes showed no or little LD between pairs ($r^2 = 0$). $0 < r^2 < 1$ is represented by grey boxes. MAF, minor allele frequency. Obs, observed; Pred, predicted; HET, heterozygosity.

the total cases and controls (Table 5). However, when the differences of the frequencies in the two groups were assessed using Monte Carlo test with one million simulations, highly significant association was observed with a normal χ^2 (T1) 67.6, $df = 37$; $P = 0.0016$ and χ^2 from 2×2 table clumped to produce maximum (T4) was 52.55, $P = 0.000003$ for the North population. This was also significant when all the samples from different geographical area were pooled to construct and analyze the haplotypic association with asthma (T1- $\chi^2 = 64.8$, $df = 40$; $P = 0.008$ and T4- $\chi^2 = 42.68$, $P = 0.00005$). The global test for the significance in the families using HBAT also showed a significant difference in the observed and expected transmission scores of the haplotypes (North families, $P = 0.049$ at $df = 8$; total families, $P = 0.03$ at $df = 10$), when families with less ambiguity about the parental haplotypes (maximum of 5) were utilized for the analysis.

Discussion

In this study, we examined the potential role of the regulatory and functional CCR5 and CCR2 polymorphisms in the asthma pathogenesis by an association study from a genetically untapped Indian population. Recently, a number of polymorphisms in the regulatory, promoter and exonic regions of the CCR5 and CCR2 genes that can affect transcription and function have been identified and associated with acceleration or protection with respect to HIV-1 progression to AIDS, sarcoidosis and rheumatoid arthritis [17,18,19,20]. For example, the homozygous allele combination 208G, 627C (rs1799988: T>C) and 676A (rs1800023: A>G) in the CCR5 promoter region has been linked to accelerated progression to AIDS in untreated individuals [17]. No study so far has been undertaken to find their association with asthma and associated phenotypes except for a study showing

Table 2

Demographic profile of the patient and the control groups in case–control study and the probands in family studies.

	Patients (N = 315)	Controls (N = 337)	Families (Probands = 235)
Native Place			
North India	227	270	151
North-west India	64	48	59
North-east India	24	19	25
Mean age (years)	37.13 ($\pm$ 15.7)	34.3 ($\pm$ 13.5)	15.5 ($\pm$ 10.6)
Sex ratio (M Vs F)	50:50	58:42	60:40
Familial history of asthma/atopy ^a	All	None	All
Smoking history ^b	None	None	None
% Reversibility from baseline FEV1 (after β_2 -agonist usage)	>15%	ND	>15%
Log ₁₀ -mean serum total IgE (IU/ml)	2.82 ($\pm$ 0.61)	2.41 ($\pm$ 0.71)	2.9 ($\pm$ 0.66)
Self reported history of allergies	All	None	All

Patients and controls were recruited from Delhi, Lucknow (UP), Mumbai, and Guwahati (Assam).

Parenthesis contains the values for standard deviation (SD). 'ND' denotes that the test is not done.

^a Control individuals were subjected to a questionnaire so as to eliminate all individuals having atopic disorders or family history of atopic disorders. Only those patients who have at least one first-degree relative affected with atopy and/or asthma were included for the study.

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

non-association of two exonic variants of *CCR5* with asthma [21] and the other demonstrating the significant association of 64I allele in *CCR2* with reduced risk of asthma [10].

Given the importance of chemokine receptors in allergic inflammation, we determined the prevalence and association of the various *CCR2* and *CCR5* polymorphisms with atopic asthma in Indian population in samples recruited from different geographical regions.

When we recruited our patients, there was no genetic map available for the Indian sub-population, thus we decided to undertake a separate analysis for cohorts recruited from different geographical regions. However, later we observed that the individuals recruited in our study, from West and North-east India were also of Indo-Aryan origin except for few (which were excluded from the analysis). Thus analysis of the pooled data from different geographical regions has also been done to enhance the power of statistical analysis of our study. Indeed, genetic homogeneity between the patients and controls from pooled group was confirmed by genotyping various loci, as yet unlinked to asthma or related atopic disorders; 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. These markers were analyzed for frequency distribution in cases and control using KS test ($P > 0.05$, data not shown). Additionally, we have validated our results in family-based studies. Although, our case–control cohort and familial cohorts are not completely independent (founders of the families are included in our case–control studies), it is worth to analyze them as two separate cohorts because; 1) the two study designs are entirely different. 2) Age difference i.e. probands of the familial cohort are younger than the cases/controls. Case–control study design has a higher chance than a family-based study, of finding a false-positive association. Since asthma is a complex disorder believed to be caused by the interaction of many genes, each having only a small effect, a case–control study, however, has provided us the directions for further familial studies.

Initially, we aimed to study *CCR5* and sequenced the complete *CCR5* gene and observed five novel polymorphisms in the 3' UTR of the gene. Four of them were present only in a single individual, who

Table 3

Genotype distributions of *CCR2* and *CCR5* polymorphisms in patients and controls from different geographical area of India.

Polymorphism (Genotype)	North		West		North-east		Total	
	Controls (N = 270)	Cases (N = 227)	Controls (N = 48)	Cases (N = 64)	Controls (N = 19)	Cases (N = 24)	Controls (N = 337)	Cases (N = 315)
rs3918356: (-)>(CAA)**								
AA	196 (73.41)	151 (68.33)	35 (72.92)	50 (79.37)	11 (61.11)	16 (69.57)	242 (72.67)	216 (70.59)
AB	61 (22.85)	64 (29.0)	12 (25.00)	12 (19.05)	7 (38.89)	5 (21.74)	80 (24.02)	81 (26.47)
BB	10 (3.75)	6 (2.71)	1 (2.08)	1 (1.59)	0 (0.00)	2 (8.70)	11 (3.30)	9 (2.94)
rs1799864: A>G								
GG	204 (76.98)	175 (77.78)	40 (83.33)	49 (76.56)	12 (66.67)	15 (62.5)	256 (77.34)	239 (76.36)
GA	57 (21.51)	45 (20.0)	8 (16.67)	15 (23.44)	5 (27.78)	8 (33.3)	70 (21.15)	68 (21.73)
AA	4 (1.51)	5 (2.22)	0 (0.00)	0 (0.00)	1 (5.56)	1 (4.17)	5 (1.51)	6 (1.92)
rs1799865: C>T								
CC	123 (46.42)	95 (42.22)	18 (37.50)	31 (48.44)	9 (50.0)	13 (54.17)	150 (45.32)	139 (44.41)
CT	120 (45.28)	103 (45.78)	29 (60.42)	27 (42.19)	6 (33.33)	9 (37.5)	155 (46.83)	139 (44.41)
TT	22 (8.3)	27 (12.0)	1 (2.08)	6 (9.38)	3 (16.67)	2 (8.33)	26 (7.85)	35 (11.18)
rs743660: G>A								
AA	6 (2.31)	4 (1.79)	1 (2.17)	0 (0.00)	1 (5.56)	1 (4.17)	8 (2.47)	5 (1.62)
AG	48 (18.46)	54 (24.11)	8 (17.39)	10 (16.67)	8 (44.44)	9 (37.5)	64 (19.75)	73 (23.70)
GG	206 (79.23)	166 (74.11)	37 (83.33)	50 (83.33)	9 (50.00)	14 (58.33)	252 (77.78)	230 (74.68)
rs1799988: T>C								
CC	48 (17.84)	35 (15.42)	5 (10.64)	8 (12.5)	4 (21.1)	4 (6.7)	57 (17.01)	47 (14.92)
CT	126 (46.84)	104 (45.81)	22 (46.81)	36 (56.25)	6 (32.0)	11 (45.8)	154 (45.97)	151 (47.94)
TT	95 (35.32)	88 (38.77)	20 (42.55)	20 (31.25)	9 (47.9)	9 (37.5)	124 (37.01)	117 (37.14)
rs1800023: A>G								
GG	61 (22.68)	52 (22.91)	14 (29.17)	10 (15.63)	9 (42.4)	8 (33.3)	84 (25.00)	70 (22.22)
AG	133 (49.44)	104 (45.81)	21 (43.75)	40 (62.50)	7 (36.8)	11 (45.8)	161 (47.92)	155 (49.21)
AA	75 (27.88)	71 (31.28)	13 (27.08)	14 (21.88)	3 (15.8)	5 (20.8)	91 (27.08)	90 (28.57)
rs41515644: (-)>(CAA)**								
AA	40 (14.81)	37 (16.44)	11 (22.92)	6 (9.52)	7 (36.8)	4 (17.4)	58 (17.21)	47 (15.11)
AB	127 (47.04)	113 (50.22)	17 (35.42)	36 (57.14)	7 (36.8)	14 (60.9)	151 (44.81)	163 (52.41)
BB	103 (38.15)	75 (33.33)	20 (41.67)	21 (33.33)	5 (26.3)	5 (21.7)	163 (37.98)	101 (32.48)

*Armitage trend test P value > 0.05 ; parenthesis contains frequency of each genotype.

** AA = -/-; AB = -/CAA; BB = CAA/CAA.

Table 4

Log₁₀ serum total IgE distribution in patients (case–control cohort) with respect to different CCR2 and CCR5 genotypes, and the results of Quantitative Trait Disequilibrium Test (QTDT) in asthmatic families of Indian origin.

Polymorphism (Genotype)	Distribution of log ₁₀ TslgE in patients from case–control cohort				P values obtained from QTDT analysis	
	No of Individuals	log ₁₀ TslgE	SE	P value	Orthogonal	Orthogonal dominance
rs3918356: (-)>(CAA)**						
AA	164	2.73	0.04	0.0028	0.0028	4.00E–10
AB	67	2.79	0.06			
BB	6	3.46	0.21			
rs1799864: A>G						
GG	178	2.76	0.04	0.11	0.96	0.55
GA	56	2.74	0.07			
AA	5	3.25	0.23			
rs1799865: C>T						
CC	104	2.69	0.05	0.007	0.088	0.03
CT	107	2.78	0.05			
TT	28	3.03	0.10			
rs743660: G>A						
AA	3	3.97	0.29	0.0002	0.021	2.00E–10
AG	59	2.79	0.07			
GG	177	2.74	0.04			
rs1799988: T>C						
CC	35	2.79	0.09	0.49	0.43	0.733
CT	117	2.73	0.05			
TT	87	2.81	0.06			
rs1800023: A>G						
GG	55	2.79	0.07	0.21	0.52	0.23
AG	116	2.71	0.05			
AA	68	2.85	0.06			
rs41515644: (-)>(CAA)**						
AA	37	2.83	0.09	0.14	0.4	0.6
AB	122	2.70	0.05			
AB	77	2.84	0.06			

ANOVA was used to analyze the association of log₁₀ TslgE with genotypes in asthmatic patients of case–control cohort.

Ref Seq: CCR5 (accession no. NM_000579); CCR2 (accession no. NM_000647).

** AA = -/-; AB = -/CAA; BB = CAA/CAA. Data in bold represents the significant association with serum IgE levels.

were non-asthmatic non-atopic controls. Thus, these mutations might not play a role in atopic disorders, but could be a contributor in protection against HIV susceptibility. The other novel mutation (2872G>del) was also present only in single patient in heterozygous form. Thus, these mutations were not included in LD calculations. Haplotype blocks were generated using Solid Spine of LD, an internally developed method of Haploview, which searches for a “spine” of strong LD running from one marker to another along the legs of the triangle in the LD chart. On the basis of these blocks, seven polymorphisms were genotyped in the two large cohorts of 662 individuals in case–control study and 822 in family-based studies. Our single-marker tests for association using the Armitage trend test did not yield significant results for any of the seven analyzed polymorphisms. However, we found a significant association of rs3918356 with total serum log₁₀IgE levels. This particular DIP was present ≈20.4 kb upstream of the CCR5 and in the promoter-regulatory region of CCR2. OVA-specific IgE has been shown to be significantly elevated in OVA-challenged CCR2^{-/-} mutants [9]. Thus, it is possible that this or any other SNP in LD may play a role in the regulation the CCR2 transcription. Thus, we also decided to completely sequence the exonic regions of CCR2 gene and found a significant association of rs743660: G>A SNP present in the 3' UTR of CCR2 gene to be associated with serum total IgE levels. Given the condition, occurrence of homozygotes for both SNP rs3918356 and rs743660 that were associated with increased levels of log-transformed TslgE is not frequent; only 6 subjects carry risk genotype for rs3918356 (2.5%) and 3 for rs743660 (1.2%), respectively. It would be important to revalidate our results in a relatively larger samples or other independent populations. Never-

theless, using mfold software we predicted very different secondary structures between the normal and variant fragments, with the two most stable predictions showing Gibbs free energy (ΔG) = -124.71 and -112.42 kcal/mol, respectively (Fig. 2). Thus we suspected that the rs743660: G>A substitution may affect the secondary structure of CCR2 mRNA and hypothesized a functional involvement of the A variant allele in reducing CCR2 mRNA stability and thus its levels [22,23]. This hypothesis is required to be further confirmed by reporter gene assays or correlation with the CCR2 expression levels.

Because haplotype analysis could be of higher informative value to embody the contribution of a gene to a specific disease [24], we also assessed the effects of haplotypes in our study population. Haplotypes of each individual in case–control study were inferred using PHASE, which uses a Bayesian approach incorporating a priori expectations of haplotypic structure from population genetics and coalescent theory to calculate the association statistics to compare the frequency in cases and controls. Although we have not observed any specific haplotype showing significant frequency difference between cases and control, we found significant results for the overall analysis using Monte Carlo simulations. An explanation for the fact that we obtained significant results only for the multilocus haplotype analyses could be that the combination of the information of all SNPs increases the sensitivity, and therefore the significance, as also found in other studies [25]. To further clarify the biological role of the two genes in asthma pathogenesis, we constructed four loci and three loci haplotypes for CCR2 and CCR5 respectively. There was no significant association for CCR2 haplotypes and asthma phenotypes, while for CCR5, when haplotype frequencies in the two groups were compared using Monte Carlo test with one million simulations, highly significant association was observed with a normal χ^2 (T1) 26.5, $df=7$; $P=0.00042$ and χ^2 from 2×2 table clumped to produce maximum χ^2 (T4) was 25.17, $P=0.000019$. Thus it is possible that CCR5 is playing more important role in asthma pathology.

In the present study, we have recruited only those patients who have at least one first-degree relative affected with atopy and/or asthma. As the case and control groups were age, sex, ethnically and geographically matched, it is very unlikely that the haplotypic

Table 5

Results of the haplotype frequency estimation (above 1%) for the atopic asthma and control populations obtained with PHASEv2.1 and “observed” and “expected” transmissions in the family-based studies using HBAT.

S. no.	Haplotypes ^a	Case–control studies ^b		Family-based studies ^c	
		Controls (N = 337)	Patients (N = 315)	Observed score (N = 236)	Expected score (N = 236)
1	1 C A G C C 1	2.37	1.11	0.032	16.2
2	1 C A G C C 2	3.86	2.06		
3	1 C A G C T 2	18.55	19.37	0.166	59.3
4	1 C A G T C 1	21.36	23.65	0.236	77.4
5	1 C A G T C 2	4.01	2.22	0.027	12
6	1 C A G T T 1	1.19	1.27	0.02	3.6
7	1 C A G T T 2	1.19	0.95	0.023	11
8	1 C G G T C 2	3.86	3.02	0.039	17
9	1 C G G T T 1	1.78	0.16		
10	1 C G G T T 2	12.31	15.56	0.127	49.9
11	1 T A G C T 2	8.75	11.11	0.099	41
12	1 T A G T T 2	1.04	0.16		
13	2 C A G C T 2	2.67	2.38	0.019	13
14	2 C G A T C 1	9.2	11.9	0.074	31.3
15	2 C G A T C 2	1.19	0		

The haplotype involves polymorphisms in an order rs3918356: (-)>(CAA), rs1799864: A>G (VAL64ILE), rs1799865: C>T, 3 rs743660: G>A of CCR2, and rs1799988: T>C, rs1800023: A>G, rs41515644: (-)>(CAA) of CCR5.

Ref Seq: CCR5 (accession no. NM_000579); CCR2 (accession no. NM_000647).

^a Alleles: 1, wild type; 2, insertion.

^b Chi-square from 2×2 table clumped to produce maximum (T4) was 42.68; $P=0.000051$.

^c Chi-squared test on 10 degrees of freedom = 19.79; $P=0.031$ (maximum allowable compatible mating haplotypes = 5).

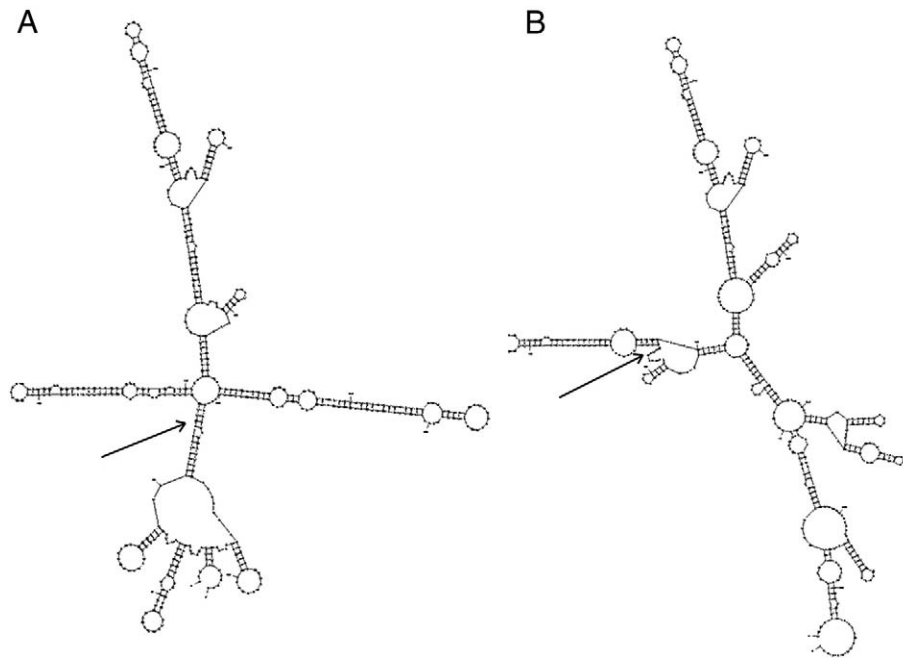


Fig. 2. RNA secondary structure prediction. To predict the possible conformation of the wild type (A) and the rs743660: G>A variant fragments (B) of CCR2, we used the mfold web server with default parameters and a folding temperature of 37 °C. The two most stable predictions obtained showed a ΔG of -124.71 and -112.42 kcal/mol, respectively. Arrows indicate the region in which the rs743660: G>A SNP is located.

association with asthma could be a false positive. Indeed, the lead from case–control data was proven by the transmission data from the familial studies also. Therefore, it is unlikely that our results are due to stratification or an inherent statistical bias. Our finding is therefore in accordance with our/others previous findings that *CCR2*/*CCR5* may have an immunoregulatory role in asthma.

In conclusion, the aim of our study was to search for polymorphisms in the *CCR2* and 5 genes and to investigate their association with atopic asthma. Altogether, based on haplotype analysis, we could show that the analyzed polymorphisms are associated with the asthmatic phenotype, while *CCR2* polymorphisms have a genetic contribution towards increased serum total IgE levels. Our study also indicates the need for further studies on the 3q21 chromosomal region, as it is possible that other candidate genes at this locus may be involved in the susceptibility to asthma in Indian population. Our study also have important implications in understanding the global distribution of *CCR2* and 5 polymorphisms and its possible impact on susceptibility towards various immunological diseases including AIDS.

Methods

Subjects

In a multicentre based asthma-genetic-study program, families ascertained through a total of 235 probands were recruited from various collaborating hospitals of India and segregated according to their geographical distribution (Table 2). Approval of the ethics committees of all the participating centres and hospitals was obtained. Written consents were obtained from all the participants for genetic studies, performing skin prick test and drawing blood samples. Study design was such that at least one atopic as well as atopic asthmatic probands along with both the parents were collected. Families were further extended wherever other members gave their consent to participate. Thus a total of 822 individuals were recruited with an average family size of 3.47 (3 to 8) individuals per family.

An independent cohort of 110 cases (from the above-mentioned hospitals) and 140 controls (from the general population) was also

recruited. A meta-analysis was performed including the disease status-confirmed founders of the above-mentioned families along with the case–control cohort to increase the power of the study. Non-asthmatic but atopic founders were however excluded from the meta-analysis.

Clinical evaluation of the phenotypes

Asthma, in the recruited study population was defined by clinical history and validated later by interview questions as described previously [26]. Medical data on the diagnosis of asthma and atopic diseases or other respiratory disorders, their duration, skin problems, types and doses of medications, and history of tobacco smoking were obtained by filling up detailed questionnaire. Details of environmental factors, family history of asthma/atopy, and the geographical region of origin and migration status were also enquired.

Patients and family members were diagnosed by physician for asthma on the basis of National Asthma Education and Prevention Program (Expert Panel Report-2) guidelines (<http://www.nhlbi.nih.gov/guidelines/asthma/asthgdln.pdf>) and were examined for a self-reported history of breathlessness and wheezing. All the probands met the following criteria: a positive skin prick test, a positive response to at least one of the two questions (Have you ever had attacks of breathlessness at rest with wheezing? Have you ever had an asthma attack?), associated with positive values for at least 2 out of the following three parameters: presence of BHR (defined as FEV1/FVC below 80% at the time of attack and FEV1 improvement by bronchodilator), hospitalization for asthma in life, or asthma therapy. The clinical parameters are summarized in Table 2.

Fifteen common environmental allergens with both negative and positive controls were used for the skin prick test [26]. Atopy was defined as a dichotomous variable, having wheal reaction equal to or greater than histamine for at least on allergen. Serum samples from both cohorts were analyzed for total IgE levels by using an enzyme immunoassay based on the sandwich technique (Bethyl Laboratories Inc., Montgomery, Texas, USA) except for few individuals (<5%) where sera were not available. All IgE values were converted to the log-natural scale for the analysis.

Healthy volunteers (referred to as normal controls) were recruited from the general population (case matched geographical area) who answered negatively to a screening questionnaire for respiratory symptoms and on the basis of the criteria of having no symptoms or history of allergic diseases. Individuals having a history of smoking and parasitic/helminthic infestations were excluded from the study. SPT and PFT were also performed, wherever consent was obtained.

Genomic DNA preparation

DNA was isolated from peripheral white blood cells using the modified salting out method and was stored at -20°C until further analysis.

Sequence analysis

To screen for sequence variation in the complete *CCR5* gene (accession no. NM_000579), 1.8-kb region of the *CCR5* promoter region, and *CCR2* (accession no. NM_000647) coding region, we divided the region into 10 segments and amplified each segment by PCR. PCR products were then sequenced on an ABI 3100 capillary sequencer (Applied Biosystems, Foster City, CA, USA) by Big Dye terminator kit V 3.1 using both forward and reverse primers (Table 1). Sequences were aligned and analyzed using SeqMan™ II. RNA secondary structures were predicted by using the mfold web server (<http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi>) setting default parameters and a folding temperature of 37°C using the last 500 bps of the *CCR2* mRNA.

SNP genotyping

The rs1799988: T>C and rs1800023: A>G polymorphisms in *CCR5* were investigated in the study population using primers used to amplify the 3rd segment and the primers up to penultimate base of the SNP as detailed in Table 1 (*CCR5_SS1* and *CCR5_SS2* resp.). Similarly, rs1799864: A>G (*CCR2_SS1*), rs1799865: C>T (*CCR2_SS2*) and rs743660: G>A (*CCR2_SS3*) in *CCR2* were genotyped using primers for 9th and 10th segments. All the SNPs were studied using SNaPshot ddNTP Primer Extension Kit (Applied Biosystems, Foster City, USA). 1 U 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).

Genotyping of repeat polymorphisms

Putative repetitive sequences in and around the two genes were identified using the RepeatMasker™ software (<http://ftp.genome.washington.edu/RM/RepeatMasker.html>). Two (CAA)_n trinucleotide repeats 20.4 kb (5 kb upstream of *CCR2*) and 370 bp upstream of the +1 of the *CCR5* were identified. Since, only two alleles were present for both of them, they are referred as DIPs in the manuscript. These DIPs were validated for distribution in our study population by means of a PCR using a 6-FAM-labelled forward primer and a non-labeled reverse primer (Table 1). Fragment lengths were determined using the GeneMapper Software version 3.7 (Applied Biosystems, Foster City, USA).

Statistical analysis

Linkage disequilibria between the polymorphisms identified by sequencing the genomic DNA of 20 individuals were evaluated using Haploview (<http://www.broad.mit.edu/mpg/haploview/index.php>) [27]. An association analysis, based on the case-control design, was performed for each polymorphism using the Armitage trend test,

following the guidelines given by Sasieni as implemented in the program FINETTI. The Armitage trend test based on the genotypes remains valid even if HWE does not hold [28]. We tested whether the genotype distribution was in HWE using FINETTI. For total serum IgE levels, linear trends were tested in ANOVA models of log-transformed values after making the distribution normal by excluding the extreme outliers using Shapiro–Wilk test using JMP® (SAS Institute Inc., Cary, N.C.). Haplotypes of each individual were inferred and analyzed using the algorithm developed by Stephens et al. (PHASE v 2.1) [29]. Differences in haplotype frequencies were also compared in the two groups using a Monte Carlo approach, by performing repeated simulations (1 million in this case) as implemented in CLUMP v 2.2 [30]. CLUMP is a program designed to assess the significance of the departure of observed values in a contingency table from the expected values conditional on the marginal totals. It works for any $2 \times N$ contingency table, especially where N is large and the table is sparse. The significance is assessed using a Monte Carlo approach, by performing repeated simulations to generate tables having the same marginal totals as the one under consideration, and counting the number of times that a chi-squared value associated with the real table is achieved by the randomly simulated data. Family data was analyzed for transmission of polymorphisms by the family-based association test (FBAT) (<http://biosun1.harvard.edu/~fbat>) using an additive and recessive genetic model to identify alleles with evidence for association with asthma. 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, conditions on 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. HBAT option was used for the haplotypic association studies. Total serum log₁₀ IgE levels were analyzed using the Quantitative TDT (<http://www.sph.umich.edu/80/csg/abecasis/QTDT>) [16]. The QTDT test statistics are obtained using likelihood-ratio tests, on the basis of the assumption of a normal distribution for the traits. An empirical P value based on the permutation of genotypes is obtained to protect against possible deviations from either normality or selection on the trait. We used orthogonal and orthogonal dominance model, the orthogonal model is the generalization of the Fulkner model, which allows for families of any size.

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