Articles

Association and preferential transmission of the CCR2V64I polymorphism with absence of asthma in high-risk families

Saleh A. Al-Abdulhadi, MSc, PhD, Mohammed W. Al-Rabia, MSc, PhD.

ABSTRACT

الأهداف: لاستكشاف العلاقة المحتملة بين التغير في التركيب الوراثي للجين CCR2V64I، داء الربو، والأعراض الجانبية كالتغيرات الكيميائية أو الوظيفية دون الحساسية المفرطة.

الطريقة: أُجراء هذه الدراسة خلال الفترة ما بين سبتمبر 2004م وحتى ديسمبر 2006م، مستشفى ابردين الملكي للأطفال – كلية الطب – جامعة ابردين – المملكة المتحدة. تم تحديد عدد 154 من الأسر التي يوجد لديها طفل واحد على الأقل مصاب بالربو (العدد الإجمالي من الأفراد 598 يشمل الأولاد والوالدين). تم تحليل التغير في التركيب الوراثي للجين CCR2V64I لمعرفة ارتباطه بداء الربو، أو وظيفة الرئة FEV1% المتوقع، الاستجابة القصبية المفرطة BHR للميثاكولين، مستوى انتجين E في الدم s-IgE، واختبار الحساسية (اختبار وخز ايجابي للجلد ضد عناصر التحسس الشائعة) في 154 من العائلات المصابة بداء الربو.

النتائج: أظهرت تحليلات PTD والتحليلات الإحصائية بالمقارنة بين الأفراد الغير مصابين والأفراد المصابين أن التغير في التركيب الوراثي للجين CCR2V64I مرتبطة بصورة ملحوظة بغياب داء الربو، متخذين قياس FEV1% كمعيار لتحديد الحالة فقط 83%، بغض النظر عن مستوى s-IgE أو وجود أي تحسس من اختبار الوخز بالإبر.

خاتمة: قمنا بتحديد العلاقات أو الارتباطات بين التغير في التركيب الوراثي للجين CCR2V64I والوقاية من الربو، مستخدمين مستوى FEV1 وغياب BHR بين العائلات الأكثر عرضة للإصابة بداء الربو في حالة وجود وعدم وجود الحساسية، والتي أثبتت علاقة ودور مهم للمستقبل CCR2 في تنظيم التهاب المجري الهوائي وليس الحساسية.

Objectives: To explore a possible association between the major functional CCR2V64I polymorphism and asthma and related phenotypes independent of atopy.

Methods: We conducted this study in the Royal Aberdeen Children's Hospital, University of Aberdeen Medical School, United Kingdom from September 2004 to December 2006. One hundred and fifty-four unrelated nuclear families (598 individuals including children and parents) were identified from the local Grampian population. The major functional polymorphism CCR2V64I was analyzed for associations with asthma, lung function (forced expiratory volume% [FEV1%] of predicted), bronchial hyperresponsiveness (BHR) to methacholine, total serum-immunoglobulin E (s-IgE) and allergic sensitization (positive skin prick test to common allergens) in 154 asthmatic families.

Results: Pedigree disequilibrium test and case control analyses showed that the CCR2V64I polymorphism was significantly associated with the absence of asthma FEV1%, predicted above the population median of 83%, but not with s-IgE levels or specific sensitization.

Conclusion: We identified associations between the V-64I CCR2 polymorphism and protection against asthma, higher FEV1, and absence of BHR in families at high risk of asthma and atopy, suggesting an important role for the CCR2 receptor in modulating airway inflammation independent of atopy.

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From the Department of Child Health (Al-Abdulhadi), Royal Aberdeen Children's Hospital, University of Aberdeen, Medical School, Westburn Road, Aberdeen, Scotland, United Kingdom, and the Department of Hematology and Immunology (Al-Rabia), College of Medicine, Umm Al-Qura University, Makkah, Kingdom of Saudi Arabia.

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Address correspondence and reprint request to: Dr. Saleh A. Al-Abdulhadi, Department of Pathology and Laboratory Medicine, Saad Specialist Hospital, Al-Khobar, Kingdom of Saudi Arabia. Tel. +966 (3) 8012676. Fax. +966 (3) 8344686. E-mail: dr.salehalabdulhadi@gmail.com

Although the increase in childhood asthma and related atopic diseases in developed and developing economies implicate environmental factors associated with affluence; significant genetic contributions are also supported by familial and twin studies[1] and the identification of associated chromosomal regions[2] and candidate genes.[3,4] Among these chromosomal regions 3q21.3 which harbors a number of chemokine receptor genes, has been identified as a region of interest.[5] Chemokine receptors also have biological plausibility, as chemokines are involved in the airway inflammation underlying the expression of asthma.[6] Among the chemokine receptors, chemokine receptor-2, a G-protein-coupled receptor mediating intracellular calcium flux and binding of the CC subfamily,[7] is clustered with other chemokine receptors CCR3, and CCR5 (<300kb span) on chromosome 3q21.3.[5] The chemokine-2 receptor gene (CMKBR2) has 4 identified single nucleotide polymorphism (SNP's) in exon 3 of which V64I and its underlying G→A non-synonymous mutation, is the most common with heterozygosity of 25.6%, and an allelic frequency variant from10-15% in the Caucasian populations,[8] and which encodes 2 isoforms of receptor for monocyte chemo-attractant protein-1 (MCP1).[9] Monocyte chemo-attractant protein-1 is the major ligand for CCR2 and once bound is an attractant for monocytes basophils and neutrophils.[9,10] This process has been observed in rheumatoid arthritis[11] and the inflammatory component of atherosclerosis.[12] The receptor encoded by this gene reduces dependent calcium mobilization and inhibition of adenyl cyclase,[13] and the G→A mutation appears to result in a reduced binding of MCP1αβ,[14] which in turn may reduce local recruitment of monocytes.[7] The G→A polymorphism has also been shown to be protective against HIV-1 infection when co-associated with the CCR5Δ32 polymorphism.[8,15,16] We hypothesized that the G→A 64 allele would not only be relevant to HIV infection and to inflammatory processes underlying rheumatoid arthritis and atherosclerosis, but also to that component of asthma due to non-allergic airway inflammation. We therefore sought to test the hypothesis that individuals from families at high risk of asthma carrying the mutant G→A allele would exhibit lower risks of asthma and that this allele would be preferentially transmitted to such asymptomatic individuals independent of atopy.

Methods. This study was conducted in the Royal Aberdeen Children's Hospital, University of Aberdeen Medical School, United Kingdom from September 2004 to December 2006. The patients attending the allergy clinic was chosen from several centers in the United Kingdom. We identified 154 unrelated nuclear families (598 individuals including children and parents) from our local Grampian population. One hundred and five of these families were identified from a local network of general practices (primary care centers), as families containing at least 2 children and young adults between 8 and 24 years, with a physician diagnosis of asthma (PDA), with wheeze reported in the past 12 months, and 49 families identified through parent probands who was studied as children in our community in the mid 1960's.[17,18] All 154 families were white Caucasian, and were drawn from a population in which the UK 2001 census showed that 84% were born in Scotland, 12% from other parts of the UK, and only 4% out of UK. Atopy was defined as at least one positive skin prick test (SPT) with a wheal size of ≥3 mm among 5 inhalant allergens (cat, dog, house dust mite, grass, and alternaria), referenced to negative control and/or total serum IgE levels ≥120 IU/ml. Bronchial hyper-responsiveness (BHR) was assessed by methacholine challenge,[19] only omitting this test in subjects with a baseline FEV1 below 70% predicted. Predicted FEV1 values for adults ≥18 years were from Crapo et al.[20] and for children <18 years from Wang et al[21] study. Baseline FEV1 was categorized as above, or below the median whole population percentage predicted value. Deoxyribonucleic acid was isolated from ethylene-diamine-tetra-acetic acid (EDTA) anti-coagulated whole blood using the phenol-chloroform method. Ethical approval for all studies was awarded by the Grampian Research Ethics Committee.

The diluted genomic DNA samples were pooled in groups: random even samples group (100 cases), random odd samples group (100 cases), and 200 cases (combined groups). The exact protocol was repeated for controls, and called pre-polymerase chain reaction (PCR) pools. The poolings were carried out by hand. Each pre-PCR pool was amplified by PCR for all CCR2 candidate SNPs at 3 replicates, using standard PCR condition as follows: 50 μl total volume containing 100 ng of genomic DNA; 10 × PCR buffer; 25 mM magnesium chloride; 10 mM deoxyribonucleotide triphosphates; 10 pmol/μl of each primers (**Table 1**), and 5 U of *Taq* DNA polymerase. Polymerase chain reaction conditions was 40 cycles of 95°C for 45 seconds, 57°C for 45 seconds, and 72°C for 1.5 minutes. The PCR products as called post-PCR pool was checked for clearly scored product using 1.5% agarose gel electrophoresis stained with ethidium bromide. Then 30 microliters of each post-PCR pools product were used to determine the allelic frequencies using pyrosequencing™ technology (PSQ).[22] Pyrosequencing™ technology reactions were performed on all post-PCR pools in 3 replicates. The PCR products were prepared for PSQ using a PSQ96 sample prep tool, and PSQ reactions were performed using the PSQt96 SNP reagent kit, both steps according

to the manufacturer's instructions. Each of the SNPs Sequencing primers were listed on **Table 1**. Each PSQ96 plate contained one negative control (no template) and one positive control per genotype. The program PSQt96 evaluation allelic quantification (AQ) software was used, to obtain the ratio of one allele peak height to the sum of height of both allele peaks. To allow the conversion of this peak height ratio into allele frequency for the DNA pools, a standard curve based on the individual samples was made. The ratio of one allele peak height to the sum of height of both allele peaks was plotted against the sample frequency. The equation of the linear regression best-fit line was determined for each of the SNPs, and used to convert the allele peak height ratios to allele frequencies in the DNA pools. For each post-PCR pool, the mean value of the 3 replicates was used in the statistical analyses of accuracy. Primers and probes were designed by assay-by-design (Applied Biosystem), and probe lengths were adjusted such that both probes had approximately the same melting temperature (67°C). The probe melting temperature was 7-8°C above the primer melting temperature of 60°C. The 2-fluorogenic allele-specific probes one match the wild-type sequence, and one matches the mutant sequence. Each probe is labeled at the 5´end with a fluorescent reporter dye, and at the 3´ end with the non-fluorescent quencher dye (NFQ). The reporter dyes in this study were VIC™ for the wild-type sequence, and 6-carboxyflurescein for the mutant sequence. TaqMan universal master mix (Applied Biosystem) was used at final concentration of 1× and a volume of 25µl along with 50 ng of genomic DNA, forward and reverse, wild-type probe and mutant probe (**Table 1**). Optical plates (Applied Biosystem) were thermal cycled in the Prism 7700 Sequence Detection System (SDS) for real-time detection and end-point analysis. Polymerase chain reactions products were then cleaned, using the ExoSAP ITTM kit (USB Corporation, Cleveland, Ohio, USA), and sequenced using the DYEnamic ET dye terminator cycle sequencing kit (Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, UK). Deoxyribonucleic acid sequencing was performed in 96-well plates, which were run on a MEGABACE-1000 automated DNA sequencer (Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, UK). Direct sequencing was performed in random samples of each genotype and in all cases were identical to the genotypes inferred from TaqMan Assay.

In order to assess familial transmission of the -64I allele, the pedigree disequilibrium test (PDT) was used as this test has advantages over the classical transmission disequilibrium test (TDT).[23,24] Transmission disequilibrium test is not a valid test of association if related nuclear families and/or siblings from larger pedigrees are used.[23,24,25] Pedigree disequilibrium test overcomes this problem by treating the triads (parent-child trios) as the independent entities, whereas TDT treats the contribution from heterozygous parents to an affected child as independent,[23,24] and is therefore open to confounding by phenocopies or heterogeneity. The PDT test is based on markers with 2 alleles M1 (the common allele), and M2 (the affected allele), and

Table 1 - Oligonucleotide probe and primers sequence of CCR2-SNPs for PCR, TaqMan Assay, and pyrosequencing™ technology (PSQ).

Assay	Descriptions	5'-Sequence-3'
SNP1,2,3[a]	PCR-Forward primer[b]	Biotin-CGGTGCTCCCTGTCATAAAT[c]
	PCR-Reverse primer[b]	AGCCCACAATGGGAGAGTAA
	V64I-PSQ-Sequencing primer	TTTTGCAGTTTATTAAGATGA
	V52V-PSQ-Sequencing primer	CCCACAAAACCAAAGATG
	L45V-PSQ-Sequencing primer	AGTAGAGCGGAGGCA
SNP 4[a]	PCR-Forward primer	Biotin-GAGGCATAGGGCAGTGAGAG[c]
	PCR-Reverse primer	CTGAACTTCTCCCCAACGAA
	N260N-PSQ-Sequencing primer	TGTTCAGGAGAATGACAA
CCR2V64I	TaqMan-Forward primer	CGGTGCTCCCTGTCATAAATTTGA
	TaqMan-Reverse primer	GTCAGTCAAGCACTTCAGCTTTT
	TaqMan-Probe 1	VIC-ACATGCTGGTCATCCT-NFQ[d]
	TaqMan-Probe 2	FAM-ACATGCTGGTCGTCCT-NFQ[d]

[a]SNP - single nucleotide polymorphism 1: V64I, 2: V52V, 3: L45V, 4: N260N.
[b]PCR - polymerase chain reaction primers were used for SNP1, 2, and 3 as they are only span <20bp from each other.
[c]Forward sequences were labelled with biotinylated probe at 5' position. [d]Probes were labelled with reporter dye VIC™ for allele 1 and 6-carbo-. xyfluorescein (FAM) for allele 2. Non-fluorescent quencher dye (NFQ) were used for both sequences.

Table 2 - Population characteristics of unrelated parents and children.

Parameters	Unrelated children n (%)	Unrelated parents n (%)
Gender		
Male	84 (56)	149 (49.1)
Female	66 (44)	154 (50.9)
Cigarette smokers		
Yes	12 (8)	122 (40.3)
No	138 (92)	181 (59.7)
Asthma status (PDA)		
Yes	83 (55.3)	93 (30.7)
No	67 (44.7)	210 (69.3)
Atopy status		
Yes (SPT ≥ wheal 3mm)	112 (74.7)	210 (69.3)
No (SPT wheal 0-2 mm)	38 (25.3)	93 (30.7)
s-IgE ≤120 IU/ml	41 (27.3)	207 (68.3)
s-IgE >120 IU/ml	109 (72.7)	96 (31.7)
Diagnostic categories		
Asthmatic/atopic	70 (46.7)	68 (22.5)
Asthmatic/not atopic	13 (8.7)	11 (3.6)
Not asthmatic/atopic	29 (19.3)	128 (42.2)
Not asthmatic/not atopic	38 (25.3)	96 (31.7)

PDA - Physician diagnosed asthma, SPT - Skin prick test, s-IgE - serum IgE >120 iu/m

pairs of these alleles (M1M2) or (M2M2) transmitted from heterozygote parents to an affected child, and pair of alleles (M1M1) not transmitted. The analysis is performed as in any family-based test, by the McNemar test.[25] We selected families with at least one parent heterozygote for the mutant allele. The test was applied separately for PDA, BHR, percentage predicted FEV-1 below the population median, atopy defined as a least one SPT at least 3mm greater than negative control, and for serum-IgE greater than 120 IU/ml. Of the 598, only 453 subjects contributed to the case control study, as participants had to be unrelated to each other. None of the parents were related thus, providing 210 individuals including 114 cases and 96 controls from the 105 families identified through child siblings pairs, and 98 individuals from the 49 families identified from the population followed from childhood, a grand total of 308 subjects. For the case control study in the younger generation, we selected only the first identified child within each of the 105 siblings pair families, and the 49 families based on long term follow up of their parents. Combining these 2 sources produced a grand total of 154 younger generation subjects. Allele frequencies for the wild type (wt), and the mutant

Table 3 - Allelic quantification (AQ).

Chemokines	SNPs	Location	Wild type allele	Function	Observed cases PDT	Observed controls PDT	Expected PDT
CCR2/SNP1	C/G	Coding Exon 3	V45L	Non-synonymous	0.0020	0.0031	0.005
CCR2/SNP2	G/T	Coding Exon 3	V52V	Synonymous	0.0016	0.0022	0.004
CCR2/SNP3	A/G	Coding Exon 3	I64V	Non-synonymous	0.063	0.210	0.152
CCR2/SNP4	T/C	Coding Exon 3	N260N	Synonymous	0.070	0.063	0.141

SNP - single nucleotide polymorphism, PDT - pedigree disequilibrium test

Table 4 - Results of PDT analysis for V64I allele for different disease phenotypes.

Phenotype	Informative pedigrees	Parent genotype		Children		Allele transmission				Z-score	PDT
						Expected		Observed			
		AG AA	GG	Affected	Unaffected	A (%)	G (%)	A (%)	G (%)		
PDA	50	65	35	102	8	66.7	33.3	87.5	12.5	5.618	0.0001
SPT	47	61	33	94	16	64.9	35.1	56.3	43.7	2.916	0.0863
s-IgE	47	61	33	91	19	64.9	35.1	62.5	37.5	2.654	0.0672
FEV1%	50	65	35	63	47	66.7	33.3	75.0	25.0	3.986	0.001
BHR	50	65	35	98	12	66.7	33.3	81.3	18.7	4.265	0.0001

The table evaluates only alleles that were transmitted from heterozygous parents (G/A) to unaffected children.
PDA - physician diagnosed asthma, SPT - skin prick test, s-IgE - serum IgE >120 iu/ml, FEV1% - forced expiratory volume% (<83.5% predicted), BHR - bronchial hyperresponsiveness (PC 20 <8 mg/ml methacholine), PDT - pedigree disequilibrium test

Table 5 - CCR2V64I genotypes and allele frequencies in unrelated parents and unrelated children.

Patient group	Genotypes n (%)			Alleles n (%)		PDT	Odds ratio	95% confidence interval
	GG	AG	AA	G	A			
Unrelated parents								
Non asthmatics/non-atopic	68 (70.9)	27 (28.1)	1 (1.0)	163 (84.9)	29 (15.1)	0.001	6.253	1.978-19.761
Non-asthmatic/atopic	92 (71.9)	34 (26.5)	2 (1.6)	218 (85.2)	38 (14.8)	0.001	5.937	1.894-18.604
Asthmatic/non-atopic	9 (81.8)	1 (9.1)	1 (9.1)	19 (86.4)	3 (13.6)	0.475	2.200	0.253-19.138
Asthmatics/atopic	64 (94.1)	3 (4.5)	1 (1.4)	131 (96.3)	5 (3.7)			
Unrelated children								
Non asthmatics/non-atopic	26 (68.4)	10 (26.3)	2 (5.3)	62 (81.6)	14 (18.4)	0.001	12.938	4.906-34.117
Non-asthmatic/atopic	26 (89.7)	2 (6.9)	1 (3.4)	54 (93.1)	4 (6.9)	0.065	3.080	0.892-10.637
Asthmatic/non-atopic	11 (84.6)	1 (7.7)	1 (7.7)	23 (88.5)	3 (11.5)	0.738	1.438	0.175-11.784
Asthmatics/atopic	67 (95.7)	2 (2.8)	1 (1.4)	136 (97.1)	4 (2.8)			

Cross tabulation analysis. Reference category (asthmatic, atopic), G versus A allele, PDT - pedigree disequilibrium test

allele (-64I) were compared in all diagnostic categories, atopic asthmatic, atopic not asthmatic and not atopic asthmatic referenced to not atopic not asthmatic for both parent and child generations. Genotype and allele frequencies among different groups were tested by Chi-square, odds ratios, and 95% confidence interval (CI) with adjustment for age and gender. Hardy-Weinberg equilibrium was tested in a contingency table of observed genotype frequencies. Association analyses were carried out using SPSS version 11 (SPSS Inc. Chicago, IL) with Chi-square tests applied to 2 × 2 genotype/phenotype tables and PDT with version 3.12.

Results. Of the 154 unrelated children probands (age 8-24 years [median 14 years]) 150 had complete data, and of 308 parents (age 34-61 years [median 43 years]) 303 had complete data (**Table 2**). Of the whole population (parents and children), 211 were defined as asthmatic atopic subjects: 61 were non-atopic asthmatics, 171 were atopic without asthma, and 155 had neither atopy nor asthma (**Table 2**). The overall mutant allele frequency was 19.5%.

Deoxyribonucleic acid pooling and allelic quantification (AQ). **Table 3** shows the output of the entire 4 candidate SNPs with their genetic variant, location, observed allelic frequency by using pyrosequencing-allele quantification, and expected frequencies throughout the National Center for Biotechnology Information and literatures. Out of the 4 SNPs only one have shows highly frequent in this population. However, our observed frequencies were similar, but not identical to our expected value. This experiment suggested that SNP-V64I is more frequent, and so it was essential to confirm, and evaluate this finding by farther genotyping and analyses.

Family based association test (FBATs). From the 154 families, 49 contained 182 child parent trios with at least one heterozygous parent, and were therefore eligible for FBATs with PDT. In these informative families, the mutant (G→A allele) was preferentially transmitted to non-asthmatic non-atopic children (transmitted n=15 not transmitted n=167, p=0.0001) **Table 4**.

Case-control analysis. The A allele was significantly associated with non-asthmatic non-atopic unrelated children (n=150, p=0.001, OR=12.938, 95% CI=4.9-34.1), and marginal not significant with atopy alone (p=0.065), but not significant with asthmatic non-atopic (p=0.738) (**Table 5**). In 303 unrelated parents, the presence of the A allele was significantly associated with non-asthmatic non-atopic (p=0.001, OR=6.253, 95% CI=1.9-19.7), and non-asthmatic atopic (p=0.001, OR=5.9, 95% CI=1.8-18.6), but not with asthmatic non-atopic (p=0.475) (**Table 5**). There were no significant association between the AG genotype and positive skin test in both unrelated children (p=0.738), and unrelated parents (p=0.879), nor with elevated serum IgE with both children (p=0.558) and parents (p=0.8) (**Table 6**). Highly significant associations between AG genotype were seen for a negative BHR (>8 mg/ml) in both children (p=0.001, OR=3.741, 95% CI= 1.7-7.9) and parents (p=0.001, OR=3.335, 95% CI=1.7=6.3) in contrast to those with bronchial hyperreactivity (≤8 mg/ml, BHR) (**Table 6**). In the whole population (parents and children), the median FEV1 was 83.5% predicted, and when unrelated child probands were categorized above and below this median value, a non-significant trend for better baseline lung function was observed with the AG genotype in unrelated children (p=0.072), but with a significant association in unrelated parents (p=001) (**Table 6**).

Table 6 - CCR2V64I genotype and allele frequencies according to asthma related phenotypes.

Patient's groups	Genotypes n (%)			PDT	Odds ratio	95% confidence interval	Alleles n (%)	
	GG	AG	AA				G	A
Unrelated parents SPT								
SPT				0.738	1.094	0.648-1.848		
Negative	71 (76.3)	20 (21.5)	2 (2.1)				162 (87.1)	24 (12.9)
Positive	162 (77.1)	45 (21.4)	3 (1.4)				369 (87.8)	51 (12.2)
s-IgE level				0.80	1.068	0.640-1.784		
IgE <120 IU/L	162 (78.3)	43 (20.7)	2 (1.0)				367 (88.7)	47 (11.3)
IgE >120 IU/L	71 (73.9)	22 (22.9)	3 (3.1)				164 (85.4)	28 (14.6)
BHR				0.001	3.335	1.740-6.390		
>8mg/ml	113 (66.5)	52 (30.6)	5 (2.9)				278 (81.8)	62 (18.2)
≤8mg/ml	120 (90.2)	13 (9.8)	0 (0.0)				253 (95.1)	13 (4.9)
FEV1%				0.001	4.538	2.309-8.919		
>83% predicted	90 (63.4)	49 (34.5)	3 (2.1)				229 (80.6)	55 (19.4)
≤83% predicted	143 (88.8)	16 (9.9)	2 (1.3)				302 (93.8)	20 (6.2)
Unrelated children								
SPT				0.879	1.069	0.456-2.503		
Negative	32 (84.2)	4 (10.5)	2 (5.3)				68 (89.5)	8 (10.5)
Positive	98 (87.5)	11 (9.8)	3 (2.7)				207 (92.4)	17 (7.5)
s-IgE level				0.556	1.259	0.590-2.686		
IgE <120 IU/L	35 (85.4)	5 (12.2)	1 (2.4)				75 (91.5)	7 (8.5)
IgE >120 IU/L	95 (87.1)	10 (9.2)	4 (3.7)				200 (91.7)	18 (8.3)
BHR				0.001	3.741	1.771-7.903		
>8mg/ml	34 (72.3)	10 (21.3)	3 (6.4)				78 (82.9)	19 (16.1)
≤8mg/ml	96 (93.3)	5 (4.8)	2 (1.9)				197 (95.6)	9 (4.4)
FEV1%				0.072	2.432	0.908-6.515		
>83% predicted	74 (82.2)	12 (13.3)	4 (4.4)				160 (88.9)	20 (11.1)
≤83% predicted	56 (93.3)	3 (5.0)	1 (1.6)				115 (95.8)	5 (4.2)

Cross-tabulation analysis. Reference genotype GG. PDT - pedigree disequilibrium test
SPT - skin prick test, s-IgE - serum IgE >120 iu/ml, FEV1 - <83.5% predicted, BHR - PC 20 <8mg/ml methacholine

Discussion. The CCR2V64I G→A polymorphism was transmitted preferentially from parents to asymptomatic non-asthmatic children. In our study, we confirmed that in unrelated parents, this polymorphism is significantly associated with absence of asthma with/or without co-existent atopy. The inheritance of asthma and atopy is likely to be complex and can be confounded by relatively small contributions from a number of different genes, by partial penetrance, asymptomatic carriers who have not yet been exposed to the relevant environmental hazard, and by disease phenocopies. These factors are particularly relevant to the condition of variable airflow obstruction referred to as asthma. All of these factors are likely to be operating, and thereby decrease the power of linkage/association analysis due to the inherent assumption that the inheritance of asthma and atopy follow simple mendelian inheritance. We have successfully show that the CCR2V64I polymorphism was not only transmitted preferentially to unaffected children, but that in their unrelated parents the allele was associated with unaffected individuals' independent atopic status with non-responsive airway (BHR ≥8 mg/ml), and with baseline FEV1 above the population median (**Table 4**). The frequency of individual genotypes and the -64 A allele was not significantly associated with positive SPT nor with serum IgE suggesting that CCR2 does not contribute to atopy defined by SPT, or IgE in these families at high risk families. Multivariate analyses also confirmed the association with asthma, independent of atopy. In unrelated children, similar association was seen, although the high prevalence of atopy in the selected population (75%) resulted in too few non-atopic asthmatic in order to be confident that the protection in there present was independent of atopy (**Tables 3 & 4**). However, highly significant association between the mutant allele and asthma characterized by BHR was observed, and also with basline FEV1 in unrelated parents, whereas in children the association was only with BHR (**Table 4**). Our data appears to

confirm the association of the -64 A allele with asthma protection, but this hypothesis is less/or not protective against atopy. The role of the chemokine receptors in the pathogenesis infectious and respiratory diseases suggests that CCR2 is a good candidate for asthma.[26] Point mutation in CCR2 reported to be associated with HIV-1 protection and/or delay of disease progression[27] suggested that this allele maybe associated with asthma protection. Low allele and genotype frequencies of CCR2V64I polymorphism with HIV-1 affected patients.[28,29] Monocyte chemoattractant protein (MCP1) is a chemokine ligand to CCR2, which is produced by endothelial cells, smooth muscle cells, and macrophages in response to a variety of mediators.[30] It may be involved in inflammatory processes in disorders including rheumatoid arthritis, alveolities, and tumor infiltration.[11] We have supported the evidence that this association is a real familial transmission, and by confirming that the association is predominantly towards non-asthmatic only and non-asthmatic atopic. Family based association test enable robust methods for confirming or refuting the contribution of genetic candidates to complex diseases such as asthma, as they effectively eliminate the confounding factor. Pedigree disequilibrium test has showed its advantage including more informative data and maybe better account for mixed genetic models. The CCR2 appeared to be protective against asthma in both children and adults. However, this association hypothesis was in children than in adults, and that might be because asthma in adults is more complicated, in which it involved much more biological mechanism linked to other diseases, such as atopy. Case-control study in Korean population suggested that this point mutation is more common in asthmatic patients, especially among atopic subjects.[31] This finding supports our hypothesis, but with possible limitations on our case-control study, for our population this may require reconfirmation by using larger case control study.

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