

Letter to the Editor

Monocyte chemotactic protein (MCP3) promoter polymorphism is associated with atopic asthma in the Indian population

To the Editor:

Monocyte chemotactic protein (MCP3) interacts with several CC chemokine receptors expressed on various leukocyte subpopulations, thus activating crucial effector cells involved in asthma pathogenesis. *MCP3* mRNA expression was found to be significantly increased in the bronchial mucosa of atopic and nonatopic asthmatic subjects. Additionally, MCP3 levels were significantly increased in bronchoalveolar lavage fluid of asthmatic subjects and associated with increased accumulation of bronchial mucosal eosinophils.¹

MCP3 is located on the 17q11-21 chromosomal region (see Fig E1, A, in this article's Online Repository at www.jacionline.org), which harbors many other important chemokines, including *RANTES*, macrophage inflammatory protein 1 α , macrophage inflammatory protein 1 β , eotaxin, *MCP1*, and inducible nitric oxide synthase.² Previous studies from our laboratory established the association of inducible nitric oxide synthase³ and eotaxin gene variants⁴ with asthma and related phenotypes, demonstrating the importance of the 17q region in asthma predisposition in the Indian population. Although a functional promoter polymorphism in *MCP1* was correlated with asthma and blood eosinophil levels, a genetic study on the *MCP3* gene in a Korean population did not demonstrate any significant association with asthma.⁵ However, no such studies have yet been undertaken in an ethnically divergent Indian population. Our previous results on the 17q region prompted us to undertake a comprehensive association and functional study of genetic variations in *MCP1* and *MCP3* genes with asthma in case-control and family-based study designs.

Unrelated asthmatic patients (n = 235) were recruited from various collaborating hospitals in northern and northwestern India (Table I). For family-based studies, a total of 230 families with asthmatic probands were recruited. Patients were recruited and examined for asthma, as detailed earlier.⁴ Asthma in the recruited study population was defined based on clinical history and validated by interview questions. Fifteen common environmental allergens were used for the skin prick tests, and all patients had positive responses to at least 1 allergen. Age- and sex-matched healthy control subjects (n = 239) were recruited as previously described.⁴

Genomic DNA was isolated from peripheral blood leukocytes by using a modified salting-out procedure. The complete *MCP3* gene, including the approximately 1.5-kb promoter region, was sequenced by using 5 sets of primers (see Table E1 in this article's Online Repository at www.jacionline.org). *MCP3*–1382T/C, *MCP3*+563C/T, and *MCP1*–2518A/G single nucleotide polymorphisms (SNPs) were genotyped by using SNaPshot (Applied Biosystems, Foster City, Calif), as previously described.⁴ A microsatellite repeat (M1) within the *MCP3* promoter was identified by using the RepeatMasker software and validated by means of PCR with a 6-FAM-labeled forward primer and a non-labeled reverse primer, as previously described.⁴ A *P* value of less than .05/4 = 0.013 is considered significant to account for multiple testing.

By using the Armitage trend test for association, the genotypic distribution of the *MCP1*–2518A/G SNP was significantly different in patients and control subjects ($\chi^2 = 18.91$; $P = .00001$; common odds ratio [OR], 0.515), in whom GG was found to be the risk genotype. However, no significant transmission distortion was observed in family-based association test (FBAT) analysis. The genotypic frequency for the *MCP3*–1382T/C SNP was found to be significantly different in patients and control subjects ($\chi^2 = 10.42$; $P = .0021$; common OR, 2.09). Also, in FBAT analysis evidence of association was observed with asthma when analyzed by using a dominant model ($P = .038$) and an additive model ($P = .039$). We have also tested the null hypothesis of “no linkage and no association” and found a similar level of association for the *MCP3*–1382T/C SNP with asthma ($P = .024$, additive model; $P = .032$, dominant model). A significant association was also observed with the atopic phenotype ($P = .005$, additive model; $P = .008$, dominant model). However, the *MCP3*+563C/T SNP was not associated with asthma in case-control or FBAT analyses (Table II).

Seven alleles were observed for the repeat in the *MCP3* promoter (heterozygosity index = 0.68). A statistically significant difference was observed in the case-control analysis by using CLUMP22 with 1 million simulations (normal χ^2 [T1] = 28.07, $df = 6$, $P = .00009$; χ^2 from table after collapsing columns with small expected values together [T2] = 26.75, $df = 3$, $P = 7 \times 10^{-6}$). The allele with 15 CA repeats was found to be most significantly different when compared between patients and control subjects (χ^2 [T3] = 25.84, $df = 1$, $P = .0000$). Although no significant association was observed in FBAT analysis, a highly noticeable trend was observed with total serum IgE levels, where subjects with a lower repeat size were associated with higher mean IgE levels (F ratio = 4.38, $df = 3$, $P = .0045$).

Haplotypes were constructed with PHASE-2.1 to study the combined effect of the 3 SNPs and the microsatellite repeat. Only 5 haplotypes were present at frequencies of greater than 5% in the population. When we analyzed the haplotype frequency difference in the 2 groups (patients vs healthy control subjects) using the Monte Carlo test with one million simulations, a significant association was observed (normal χ^2 [T1] = 45.2, $df = 22$, $P = .002$; χ^2 from table after collapsing columns with small expected values together [T2] = 40.11, $df = 9$, $P < .0001$). The overall significance was the result of a major haplotype, G_T_15_C ($\chi^2 = 22.33$, $P < .0001$; OR, 2.06; 95% CI, 1.52–2.78). The linkage disequilibrium (LD) table (see Fig E1, B) shows a strong LD (D') between the CA repeat and –1382 T/C, and thus it is expected that the association of the 15 CA repeat is mostly driven by the –1382 T/C SNP.

To investigate any functional correlation between the *MCP3*–1382T/C SNP and its plasma levels, we measured the MCP3 levels in the plasma (MCP3 DuoSet ELISA; R&D Systems, Minneapolis, Minn) of 140 unrelated patients, but levels were detected only in 31 subjects (62 alleles). We found that the T allele was associated with higher mean plasma MCP3 levels (n = 56, 184.36 ± 15.96 pg/mL) compared with the C allele (n = 6, 68.8 ± 15.47 pg/mL). This difference was statistically significant (F ratio = 4.98, $df = 1$, $P = .029$). We could not detect MCP3 levels in patients homozygous for the CC genotype. Those with the CT genotype were found to have lower mean MCP3 levels

TABLE I. Characteristics of the patient and control groups (case-control study)* and the probands (family-based study)

Characteristics	Patients (n = 235)	Control subjects (n = 239)	Probands (n = 230)
Native place*	North/northwest India	North/northwest India	North/northwest India
Mean (SD) age (y)	34.4 (12.5)	35.0 (10.6)	15.6 (11.2)
Sex ratio (F/M)	48/52	47/53	44/56
Familial history of asthma/atopy†	All	None	All
Smoking history‡	None	None	None
Reversibility (%) from baseline FEV ₁ (after β_2 -agonist use)	>15%	ND	>15%
Log ₁₀ mean (SD) serum total IgE (IU/mL)	2.84 (0.49)	2.30 (0.54)	2.84 (0.63)
Atopy/self-reported history of allergies	All	None	All§

Parentheses contain the values for SDs.

ND, Not done.

*Patients and control subjects were recruited from Delhi, Lucknow (Uttar Pradesh), Jaipur (Rajasthan), Shimla (Himachal Pradesh), Chandigarh (Punjab), Mumbai, and Guwahati (Assam).

†Control subjects were subjected to a questionnaire so as to eliminate all those having atopic disorders or a family history of atopic disorders.

‡Patients and control subjects known to have experienced smoking in the past or having parasitic infections were excluded from the study.

§Eighty-six percent of the probands were found to have positive skin prick test responses or had high specific serum IgE levels. Fifteen common environmental allergens (house dust mite, *Amaranthus spinosus*, *Brassica campestris*, *Cynodon dactylon*, *Parthenium hysterophorus*, *Prosopis juliflora*, *Ricinus communis*, *Alternaria tenuis*, *Aspergillus fumigatus*, cockroach, mosquito, moth, grain dust rice, hay dust, and house dust) were used for the skin prick tests.

TABLE II. Genotype frequencies and results of association analysis with respect to *MCP1-MCP3* gene-cluster SNPs

Genotypes	Patients	Control subjects	Control frequency from the HapMap data	Common OR	P value
<i>MCP1</i> –2518A/G (rs1024611)*					
AA	88 (38.1)	130 (55.32)	55.7	0.515	.00001
GA	112 (48.48)	94 (40.0)	37.5		
GG	31 (13.42)	11 (4.68)	6.8		
AA vs GA + GG	143 (61.9)	105 (44.68)		0.49 (0.343-0.719)	.00019
<i>MCP3</i> –1382T/C (rs3091318)†					
CC	1 (0.43)	3 (1.26)	0.2	2.09	.0021
CT	26 (11.06)	51 (21.34)	15.3		
TT	209 (88.51)	185 (77.41)	83.1		
TT vs CT + CC	27 (11.49)	54 (22.60)		2.26 (1.36-3.73)	.001
<i>MCP3</i> +563C/T (rs3091321)†					
TT	1 (0.44)	3 (1.30)	10	0.90	.717
CT	53 (23.14)	53 (22.94)	30		
CC	175 (76.42)	175 (75.76)	60		

Numbers in parentheses indicate frequencies (percentages). Values in boldface indicate a statistically significant association.

*Control frequency from the HapMap database is from Gujarati Indians in Houston (n = 88).

†Control frequency from the HapMap database is from Utah residents with northern and western European ancestry from the CEPH collection (CEU, n = 50 and 59) because the frequency for the Indian population is not available in the HapMap database.

when compared with subjects with TT genotypes (F ratio = 6.01, *df* = 1, *P* = .02).

An electrophoretic mobility shift assay was performed, as previously described⁶ with appropriate modifications, to further investigate the functional significance of the *MCP3*–1382T/C SNP. Briefly, 20 μ g of nuclear extract from uninduced and LPS-induced THP-1 cells were incubated with ³²P end-labeled double-stranded oligonucleotides representing C and T variants. For the supershift assay, 2 μ g of rabbit anti-YY-1 antibody, 2 μ g of rabbit anti-ETS antibody, or 2 μ g rabbit antihuman IgG (nonspecific antibody control) was added to the subsequent reactions. With LPS-induced nuclear extracts, 2 major nucleoprotein complexes were observed in the presence of the T probe (Fig 1, A) whereas both of them were absent when incubated with the C probe. Competition assays with unlabeled specific

oligonucleotides and an irrelevant competitor evidenced the DNA-protein complexes (I and II) to be specific because unlabeled specific oligonucleotides were able to chase the band in a concentration-dependent manner, whereas the unlabeled C oligonucleotide, as well as an irrelevant oligonucleotide, did not chase the specific nucleoprotein complex.

TESS software (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>) predicted that the T-to-C change at the –1382 position leads to loss of the YY-1 transcription factor binding site. To confirm this prediction, we performed competition assays with unlabeled oligonucleotides specific to CCAAT enhancer-binding protein (CEBP), GATA, Ying-Yang 1 (YY-1), pre B-cell leukemia transcription factor (PBX), and erythroblast transformation specific (ETS). We observed that complex I was completely abolished in competition assays with unlabeled oligonucleotide for YY-1.

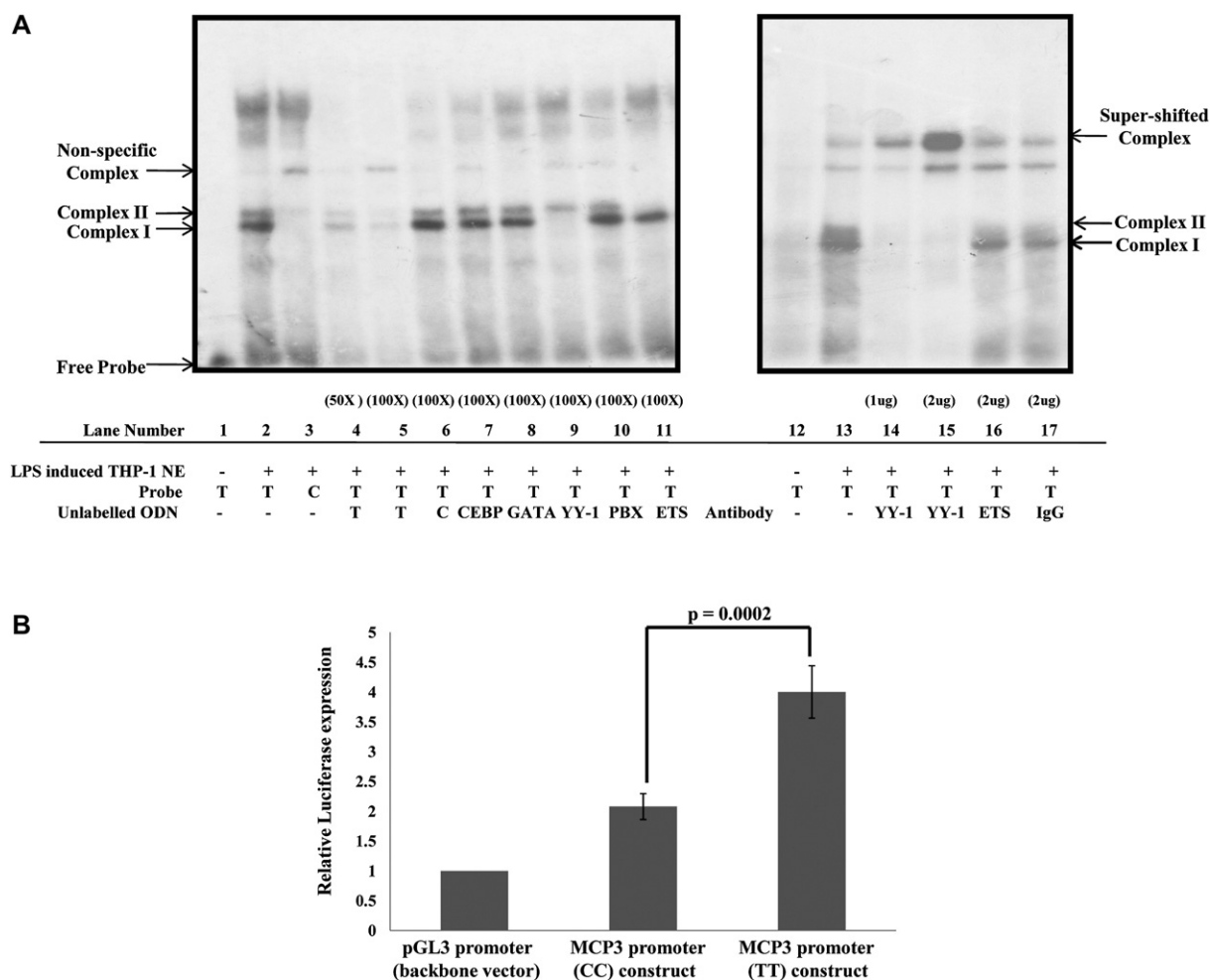


FIG 1. A, Analysis of differential binding of nuclear proteins to C and T alleles of the *MCP3*-1382T/C polymorphism by using the electrophoretic mobility shift assay. *Lanes 1 and 12* represent free probe. *Lanes 2, 3, and 13* represent complexes with T/C probe. *Lanes 4 to 6 and 7 to 11* represent competition assays. *Lanes 14 to 17* represent supershift assays. *CEBP*, CCAAT enhancer-binding protein; *ETS*, erythroblast transformation specific; *PBX*, pre B-cell leukemia transcription factor; *YY-1*, Ying-Yang 1; **B**, Relative transcriptional activities for *MCP3*-1382T/C reporter constructs measured by using transient transfection assays. The figure represents data from 3 independent experiments.

To further confirm this binding, we performed a supershift assay and observed that the addition of antibodies to YY-1 resulted in a supershift of both the specific bands. This change in electrophoretic mobility appears to be specific to YY-1 antibody because ETS antibody, as well as nonspecific anti-IgG, did not induce a supershift.

Reporter constructs were generated by subcloning 1.276-kb promoter segments upstream of the luciferase gene in the reporter plasmid pGL3-Promoter (Promega, Madison, Wis) to confirm the functional effect of the *MCP3*-1382T/C SNP. Promoter DNA was PCR amplified from the genomic DNA of subjects homozygous either for the C or T allele of the *MCP3*-1382T/C SNP (see Table E2 in this article's Online Repository at www.jacionline.org). PCR fragments were gel purified and subjected to TA ligation in pDRIVE cloning vector (Qiagen, Hilden, Germany). Clones were screened, subjected to restriction digestion (*KpnI* and *SacI*), and ligated into the pGL3-Promoter vector. After validation by means of restriction digestion and sequencing, these constructs were subjected to transient transfection assays. Briefly, 3 μ g of *MCP3* constructs was cotransfected with 50 ng

of Renilla luciferase normalization plasmid in 1×10^6 THP-1 cells by using Lipofectamine LTX (Invitrogen, Carlsbad, Calif), per the manufacturer's protocol. The same amount of pGL3-Promoter vector cotransfected with Renilla luciferase plasmid was used as a transfection control and assayed for relative luciferase expression by using firefly and Renilla luciferase assay kits (Promega). We observed a 2-fold decrease in luciferase expression for the construct with the CC variant compared with the construct with the TT variant ($P = .0002$), which further confirms a functional association of promoter SNPs with *MCP3* gene expression (Fig 1, B).

In summary, we established, for the first time, a significant association of *MCP3* variants with atopic asthma. We further demonstrated that the *MCP3*-1382T/C variant alters the binding of YY-1 transcription factor, thus providing evidence that promoter polymorphisms might modulate *MCP3* gene expression. Additionally, *MCP3* (chromosome 19; 32597240-32599256) is located close to the ORM1-like 3 (*ORMDL3*) gene (chromosome 19; 38077296-38083854; within the 7MB region), which is present on chromosome 17q21. In a recent genome-wide association

study of asthma, an SNP in *ORMDL3* was shown to associate with the risk of childhood asthma,⁷ replicated,⁸ and extended to a 380-kb region, including the gasdermin B (*GSDMB*) gene in other independent studies.⁹ The association of the *MCP3* gene could be explained by long-range LD with *ORMDL3/GSDMB*, or this might be an independent loci associated with risk of asthma. Thus, our study might initiate further confirmatory investigations to elucidate the exact role of *MCP3* and its genetic variants in asthma pathogenesis.

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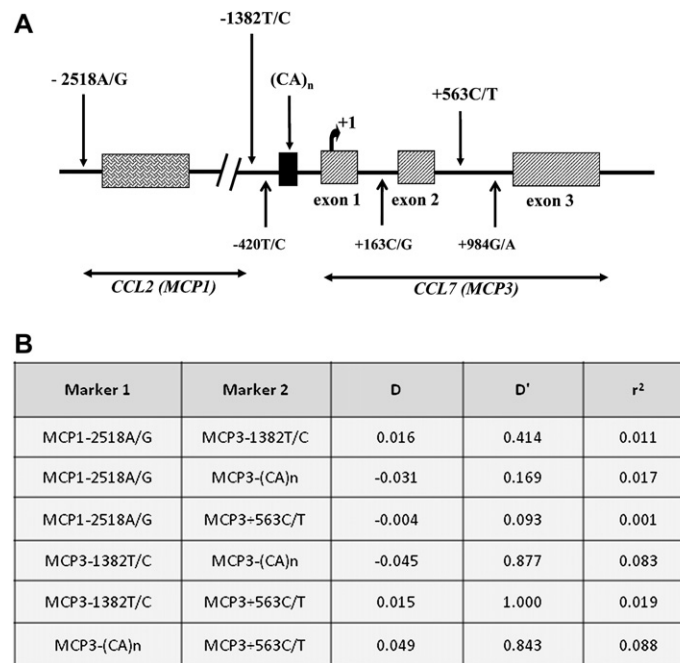


FIG E1. A, Schematic representation of the *MCP1-MCP3* gene cluster on chromosome 17q21.2. The SNPs and microsatellite repeat (M1) studied in our population are marked in boldface. **B**, Pairwise LD for 2-way comparisons among the polymorphisms investigated in the *MCP3* gene, as calculated with EMLD software.

TABLE E1. Primers and PCR conditions used for sequencing/genotyping the *MCP1* and *MCP3* polymorphisms

Primer name	Polymorphism	Primer sequence	Annealing temperature (°C)	Size of PCR product (bp)
(CA) _n _FP	Microsatellite	5' CTGTGCAGTTCCGATAGC 3'	58	298-308
(CA) _n _RP	Microsatellite	5' ACAAGGCCTTCATTCAGTG 3'		
MCP3_SET1_FP	−1382T/C	5' ATAGGGGTAAGATAAAACGACATAAA 3'	Touchdown 68-58	789
MCP3_SET1_RP	−1382T/C	5' ACAAGGCCTTCATTCAGTG 3'		
MCP3_SET2_FP	−420T/C	5' AACCCAGAAGATTGTTATTGTTTCA 3'	Touchdown 55-60	467
MCP3_SET2_RP	−420T/C	5' CGAGGCTAGCCTGTTTTTCA 3'		
MCP3_SET3_FP	+163C/G	5' GACCCTACCTCACCTACCACTC 3'	60	663
MCP3_SET3_RP	+163C/G	5' CACACCCATCCCCAGAAG 3'		
MCP3_SET4_FP	+563C/T, +984G/A	5' CTTCTGGGGATGGGTGTGC 3'	Touchdown 68-58	979
MCP3_SET4_RP	+563C/T, +984G/A	5' GGTTACGCTGGGAGTGTG 3'		
MCP3_SET5_FP	Exon 3	5' GTCCCCTTCCTTTGTTTTATCTTC 3'	61	903
MCP3_SET5_RP	Exon 3	5' GGGGGTGGGTTCATCTGG 3'		
MCP1_SET1_FP	−2518A/G	5' TCTCTCACGCCAGCACTGACC 3'	58	234
MCP1_SET1_RP	−2518A/G	5' GAGTGTTCACATAGGCTTCTG 3'		
−1382T/C_SS	−1382T/C	5' TATAATGCTCAATCCCCCA 3'	55	20
+563C/T_SS	+563C/T	5' CCATAGTTGGGTACCCTTG 3'	55	20

TABLE E2. Primers for amplification and subcloning of the *MCP3* promoter region

Primer name	Primer sequence	Annealing temperature (°C)	Length of PCR product (bp)
MCP3_cloning_FP	5' ATAGGGGTAAGATAAAACGACA 3'	60	1276
MCP3_cloning_RP	5' CGAGGCTAGCCTGTTTTTCA 3'		
pGL3_FP	5' TTCCCAGTCACGACGTTGTAAAA 3'	62	1506
pGL3_RP	5' GAAAAAGTTGCGCGGAGGAGTT 3'		