

Original article

MCP-1 gene regulatory region polymorphism in Chinese children with mild, moderate and near-fatal asthma

Background: A polymorphism in the monocyte chemoattractant protein 1 (MCP-1) gene regulatory region has been associated with asthma in Caucasians. This polymorphism is possibly endemic to the Asian region, but its impact on Asian populations is unclear. In addition, the relationship of this marker with life-threatening asthma has not been clarified. The aim of this study was to test the genetic association between the MCP-1 –2518A/G polymorphism and asthma/atopy in a cohort of Chinese children, with particular emphasis on those patients who had experienced life-threatening asthma attacks.

Methods: Forty-eight children with near-fatal asthma, 134 mild-to-moderate asthmatics, 69 allergic-disorder cases without asthma, and 107 nonasthmatic, nonatopic control children were genotyped by a polymerase chain reaction-based assay.

Results: Comparison of the four groups of children ($n = 358$) revealed no detectable differences in genotype or allele frequencies of the MCP-1 –2518A/G polymorphism. There was no evidence of association between the polymorphism and any of the outcomes of interest including clinical severity, blood eosinophil count, atopy, total serum IgE levels, and degree of bronchial hyper-responsiveness.

Conclusion: These results suggest that the MCP-1 –2518A/G polymorphism is not a risk factor for near-fatal asthma. Furthermore, this polymorphism seems to play no role in the development of asthma or atopy in Chinese subjects, possibly as a result of the genetic heterogeneity between Asian and Caucasian populations with respect to regulation of MCP-1 expression. Our results underscore the necessity of accounting for ethnic background in the investigation of asthma-predisposition genes.

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Asthma is a chronic inflammatory disorder of the airways that is increasing in prevalence and can result in significant morbidity and mortality (1). There has been recognition of a group of asthmatic patients who suffer from severe and life-threatening asthmatic attacks and, in these individuals, high doses of steroids, frequent hospitalization and occasional intubation and ventilation are required. Thus, the identification of these at-risk individuals, preferably in early childhood, remains an important challenge. Little data are available, however, on possible genetic markers of fatal or near-fatal asthma.

Abbreviations: MCP-1, monocyte chemoattractant protein 1; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; BHR, bronchial hyper-responsiveness; PC₂₀, provocation concentration causing a 20% fall in FEV₁; FEV₁, forced expiratory volume in 1 s.

Several candidate genes have been implicated in the pathogenesis of asthma, one of which is the monocyte chemoattractant protein 1 (MCP-1) gene. The MCP-1 gene lies on chromosome 17q11.2 (2), a region for which linkage to asthma or atopy has been demonstrated in several independent studies (3–5). MCP-1, one of the CC chemokines, appears to play a significant role in asthma pathogenesis because of its ability to attract monocytes, eosinophils, and activate mast cells and basophils, inducing leukotriene C₄ release into the airway, which directly induces airway hyper-responsiveness (6, 7). MCP-1 also can drive undifferentiated T-lymphocyte populations towards IL-4-producing Th2-type cells (8). In addition, it has been demonstrated that MCP-1 neutralization drastically diminishes bronchial hyper-responsiveness (BHR) and airway inflammation in a murine model (9). Furthermore, increased MCP-1 expression has been demonstrated in the bronchial

epithelium of asthmatic patients (6), and comparison of the same asthmatic patients showed marked elevation of sputum MCP-1 levels preceding exacerbation of acute asthma attacks in comparison to the asymptomatic state (10).

Human MCP-1 gene transcription appears to be under the control of two distinct areas of the 5'-flanking region of the gene: proximal and distal regulatory regions (11). The distal regulatory region is located 1.8–2.7 kb upstream of the transcriptional start site. This segment contains two nuclear factor- κ B binding motifs that are essential for cytokine induction of MCP-1 expression. A polymorphism at position –2518 (A to G) relative to the major transcription start site of the MCP-1 gene has been found to increase transcriptional activity of this region and subsequent MCP-1 production in response to an inflammatory stimulus (11). It is quite likely, therefore, that this polymorphism is a possible locus involved in asthma susceptibility or modulation of its severity. In a recent study, Szalai et al. showed that the MCP-1 –2518G allele is associated with increased susceptibility to asthma in Hungarian children (12). They also demonstrated that the MCP-1 –2518G allele is associated with asthma severity and increased eosinophil levels. It has been suggested, however, that there are significant interethnic differences in the allele frequencies for asthma-predisposition genotypes between Asian and Caucasian populations (13). With regard to the MCP-1 –2518A/G polymorphism, the prevalence of –2518G allele was estimated to be 47% in a small group of Asians (11) and up to 65% in a Japanese population (14), strikingly higher than analogous figures reported for studies of Caucasian populations (21.1–29%) (11, 12, 15, 16). Given that this functional polymorphism alters the expression of MCP-1, and that it is possibly endemic to the Asian region, it may have a more substantial impact on Asian populations. Further, there is evidence for a genetic contribution to risk for fatal or near-fatal asthma attacks (17). Recently, it has also been demonstrated that the levels of MCP-1 are increased in the broncho-alveolar lavage fluid of ventilated subjects with status asthmatics compared to mild-to-moderate asthmatics (18), suggesting that this CC chemokine may be an important factor in life-threatening asthma attacks.

Although Szalai et al. found an association of MCP-1 –2518 genotypes with asthma (12), it has not been clarified whether this polymorphism plays any role in life-threatening asthma. In addition, their findings have not been confirmed independently. In this study, we hypothesized that MCP-1 –2518A/G polymorphism is associated with asthma/atopy susceptibility and is a risk factor for life-threatening asthma attacks. This hypothesis was tested by investigating the frequency of this polymorphism among Chinese children with near-fatal asthma, mild-to-moderate asthma, allergic disorder but without asthma, and nonasthmatic, nonatopic controls.

Materials and methods

Study subjects

This study was conducted at Chang Gung Children's Hospital, a tertiary teaching medical center in northern Taiwan. A total of 358 unrelated Chinese children (age, 7.4 ± 3.5 years) were enrolled and divided into four groups: 1) those who had experienced near-fatal asthma attacks ($n = 48$); 2) mild-to-moderate asthmatics ($n = 134$); 3) atopic nonasthmatics ($n = 69$); and 4) normal controls ($n = 107$).

The first group consisted of subjects who either had histories of hospital admission requiring intubation and ventilation for acute exacerbation of asthma symptoms, or had developed hypercapnic respiratory failure during an acute asthmatic episode with a $\text{PaCO}_2 > 45$ mmHg. At the end of the study period, there had been no asthma fatalities at our hospital involving individuals aged 16 years or less during the previous 10 years. Thus, no asthma fatalities were included in this cohort. The second study group consisted of subjects with mild-to-moderate asthma. These subjects had never experienced a fatal or near-fatal asthma episode and were not taking oral steroids. The diagnosis and classification of the clinical severity of asthma was made according to the Global Initiative for Asthma guidelines (19). These mild-to-moderate asthmatics were further classified into mild and moderate asthma subgroups according to the clinical features before treatment and lung function over 12 months. The inclusion criteria for the mild-asthma subgroup included a symptom frequency of less than once a day, nocturnal symptoms less than once a week, brief exacerbations, no symptoms and normal peak expiratory flow between exacerbations, a percent-predicted forced expiratory volume in 1 s (FEV_1) $\geq 80\%$, and an FEV_1 variability $\leq 30\%$. The inclusion criteria for the moderate-asthma subgroup included daily or continual symptoms, frequent exacerbations affecting activity and sleep, frequent nocturnal symptoms, and a percent-predicted $\text{FEV}_1 < 80\%$, and an FEV_1 variability $> 30\%$. This classification of mild-to-moderate asthmatics was performed without prior knowledge of the subject's genotypes. The third study group consisted of atopic nonasthmatic subjects who had a specialist-physician allergy diagnosis according to the following criteria: atopy; clinical signs of severe allergy (including allergic rhinitis, atopic dermatitis, food allergy and urticaria); and no symptoms or history of asthma. The fourth study group consisted of age-comparable normal controls and these subjects were selected from children attending the hospital for treatment of nonallergic and nonimmunological diseases who met the following criteria: no symptoms or history of allergic diseases; and total serum IgE levels below the general population mean for their ages. Informed consent was obtained from the parents or guardians of each subject before commencement and the study was preapproved by the hospital ethics committee.

Genotyping

Genomic DNA was extracted from whole blood using an Easy Pure Genomic DNA Purification Kit (Biomart Scientific Co., Taipei, Taiwan). A polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method, as described by Szalai et al. (20), was used to genotype patients and controls for the target polymorphisms. Briefly, the MCP-1 –2518 genotype was determined using a *PvuII* site introduced with a mismatch into the PCR primer next to the G/A transition. Amplification with the primers, MCP417S: 5'-TCT CTC ACG CCA GCA CTG ACC-3' and MCP650AS: 5'-GAG TGT TCA CAT AGG CTT CTG-3', generated a 234 bp product. Digestion with *PvuII* yields 159 and 75 bp fragments when G is at position –2518. Genomic DNA

(100 ng) was amplified in a 25 µl PCR reaction under the following cycling conditions: denaturation at 94°C for 5 min followed by 40 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 45 s, with a final extension at 72°C for 10 min. Five microliters of the PCR products were digested with 3 U of *PvuII* (Roche Diagnostics, Mannheim, Germany) at 37°C overnight. The digestion product was analyzed on a 3% agarose gel stained with ethidium bromide. Both the direct sequencing reactions, using the ABI prism 377 automated DNA sequencer (Applied Biosystems, Foster City, CA) and restriction enzyme digestion, resulted in identical genotypes for the initial 15 samples, all other samples being subsequently genotyped by PCR-RFLP.

Total and allergen-specific IgE, blood eosinophil counts, and bronchial hyper-responsiveness

Peripheral venous blood was collected from patients and control subjects for measurement of serum total and specific IgE levels in response to common aeroallergens using fluorescent enzyme immunoassay (AutoCAP System, Pharmacia Diagnostics AB, Uppsala, Sweden). Total serum IgE levels were defined as high when they exceeded the general population mean for their respective ages; specific IgE was considered positive in those detectable allergen-specific IgE (≥ 0.35 kU/l). Children were considered atopic if they had at least one positive result for allergen-specific IgE to aeroallergens common to Taiwan, including *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cockroach, cat and dog dander, and *Candida albicans*. The absolute peripheral-blood eosinophil counts were measured using a Sysmex SE-9000 Automated Hematology Analyzer (TOA Medical Electronics Co., Kobe, Japan). Bronchial challenge tests were performed in accordance with a method modified from our previous reports (21, 22). Baseline lung function was measured and a dose of saline was used as the control. Baseline lung function was measured again and used as the reference value. Methacholine was administered using an aerosol provocation system, with a fixed output (APS Jaeger) in doubled doses (0.075, 0.156, 0.312, 0.625, 1.25, 2.5, 5.0, 10.0, and 25.0 mg/ml). Lung function was measured 1 min after each dose, at an interval of about 5 min. At each dose level, the subject performed five successive full-capacity inspirations, with nebulization triggered by inspiration. Each inhalation was 0.5 s in duration. An inhalation unit was defined as one inhalation of 1 mg/ml methacholine. The provocation concentration causing a 20% fall in FEV₁ (PC₂₀) was interpolated, using a provocative concentration of methacholine to produce a 20% fall in FEV₁ on the concentration–FEV₁ response curve. The challenge was terminated prior to completion of the dose schedule if FEV₁ declined by 20% or more from the reference value after saline inhalation. To aid recovery from induced bronchoconstriction, fenoterol was administered via inhalation when necessary. The methacholine challenge was performed at the beginning of the study in asthmatic patients who had no symptoms for at least 2 weeks previously.

Statistical analysis

A statistical power analysis indicated that our study design could detect a difference in allele frequencies of 0.2 with 95% power. Data analysis was performed using the SPSS statistical package (SPSS 10.0 for Windows, SPSS Inc., Chicago, IL). The genotype frequencies for the different groups were compared using the Pearson's chi-squared test. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated as a measure of the association between each genotype and asthma. The genotype distributions for the MCP-1 –2518 polymorphism tested in this study population were found to be in

Hardy–Weinberg equilibrium. Blood eosinophil counts, total serum IgE levels, and PC₂₀ values between different groups were compared using Mann–Whitney's *U*-test or the Kruskal–Wallis test, as appropriate. *P* values of <0.05 were considered statistically significant.

Results

Subject characteristics

The salient demographic and asthma-related clinical characteristics for the four study groups are shown in Table 1. No significant difference was found between the mean ages or sex ratios of patients in the near-fatal asthma, mild-to-moderate asthma, and atopic-nonasthmatic groups compared with nonasthmatic–nonatopic controls ($P > 0.1$ in all cases).

Association of MCP-1 –2518 genotype with asthma and atopy

The genotype frequency of the MCP-1 –2518A/G polymorphism in asthmatic, atopic–nonasthmatic and nonasthmatic–nonatopic subjects is shown in Table 2. The frequency of the MCP-1 –2518G allele in control subjects was 51.4%. Contrary to our hypotheses, the frequency of the MCP-1 –2518G allele and the distribution of genotypes did not differ between asthmatic children and nonasthmatic–nonatopic controls (Table 2). Although the frequency of the MCP-1 –2518G allele in atopic–nonasthmatics (60.9%) was higher than in nonasthmatic–nonatopic controls (51.4%), this difference was not significant ($P = 0.102$). These results indicate that the MCP-1 –2518G allele was not associated with asthma or atopy susceptibility in this pediatric population.

Association of MCP-1 –2518 genotypes with asthma severity

When the mild-to-moderate asthmatic group was subdivided into two subgroups according to asthma severity, 50 children (37.3%) were classified as moderate asthmatics and 84 (62.7%) as mild asthmatics. Comparison of the MCP-1 –2518G allele frequency between these two subgroups revealed no significant difference (50 and 55.4%, respectively; $P = 0.469$; Table 3). Paradoxically, the frequency of the MCP-1 –2518G allele in the near-fatal asthmatics (45.8%) was slightly lower, although this

Table 1. Salient demographic and asthma-related clinical characteristics of the study subjects

	Near-fatal asthma	Mild-to-moderate asthma	Atopic nonasthmatics	Healthy controls
Number	48	134	69	107
Age (years)	5.9 ± 4.0	7.8 ± 2.8	8.1 ± 3.9	7.3 ± 3.6
Gender (male/female)	28/20	81/53	37/32	51/56
Total serum IgE (kU/l)	821 ± 665	671 ± 582	876 ± 1278	45 ± 31
Atopy: no. (%)	44 (91.7)	117 (87.3)	69 (100)	0 (0)

Table 2. The genotype frequency of the MCP-1 –2518A/G polymorphism and allele frequencies in asthmatic, atopic nonasthmatic, and healthy control children

Groups	<i>n</i>	Genotypes [<i>n</i> (%)]			G allele frequencies (%)	OR (95% CI)	<i>P</i> -values
		A/A	A/G	G/G			
Asthmatics	182	42 (23.1)	93 (51.1)	47 (25.8)	51.4	0.999 (0.713–1.400)	1.000
Atopic nonasthmatics	69	13 (18.8)	28 (40.6)	28 (40.6)	60.9	1.471 (0.952–2.271)	0.102
Healthy controls	107	30 (28.0)	44 (41.1)	33 (30.8)	51.4	1.0	–

OR, odds ratio, reference group (healthy controls) designated with an OR of 1.0; CI, confidence interval.

Table 3. Genotype and allele frequencies for MCP-1 –2518 A/G polymorphism in children with different asthma severity

Groups	<i>n</i>	Genotypes [<i>n</i> (%)]			G allele frequencies {%}	OR (95% CI)	<i>P</i> -values
		A/A	A/G	G/G			
Near-fatal asthma	48	16 (33.3)	20 (41.7)	12 (25.0)	45.8	0.800 (0.494–1.296)	0.433
Moderate asthma	50	13 (26.0)	24 (48.0)	13 (26.0)	50	0.945 (0.588–1.520)	0.912
Mild asthma	84	13 (15.5)	49 (58.3)	22 (26.2)	55.4	1.172 (0.782–1.759)	0.506
Healthy controls	107	30 (28.0)	44 (41.1)	33 (30.8)	51.4	–	–

OR, odds ratio, reference group (healthy controls) designated with an OR of 1.0; CI, confidence interval.

difference was not significant compared to their moderate (50%; $P = 0.659$) and mild (55.4%; $P = 0.173$) asthmatic counterparts. This allele frequency was also lower in near-fatal asthmatics compared to nonasthmatic–nonatopic controls [45.8 vs 51.4%; OR = 0.800 (0.494–1.296); $P = 0.433$].

Association of MCP-1 –2518 genotypes with blood eosinophil counts, total serum IgE, specific IgE levels, and bronchial hyper-responsiveness

In the subset of 61 asthmatic children with available blood eosinophil counts, a statistically significant difference in mean eosinophil counts was observed between the moderate-asthma and mild-asthma subgroups (0.57 ± 0.31 vs $0.36 \pm 0.24 \times 10^6/\text{ml}$; $P = 0.004$). However, no significant difference was found in mean eosinophil counts between asthmatic children carrying the MCP-1 –2518G allele ($0.44 \pm 0.26 \times 10^6/\text{ml}$) and those homozygous for the –2518A allele ($0.46 \pm 0.36 \times 10^6/\text{ml}$; $P = 0.809$; Table 4). In all 182 asthmatic children, comparison of carriers of the –2518G allele and those homozygous for the –2518A variant revealed no significant differences in the percentage of atopic subjects (87.1 vs 92.9%; $P = 0.459$) or in total serum IgE concentrations (654.2 ± 570.3 vs 814.8 ± 665.9 kU/l; $P = 0.263$; Table 4). Results of methacholine-challenge tests performed on 35 asthmatic children who had been symptom-free for at least 2 weeks indicate that three did not have a positive BHR (FEV_1 had not decreased by 20% with a methacholine concentration > 8 mg/ml). No significant difference was found in the mean PC_{20} level of carriers of the –2518G allele compared to those homozygous for the –2518A allele (1.84 ± 2.24 vs 3.10 ± 3.24 mg/ml; $P = 0.346$; Table 4).

Table 4. Association of MCP-1 –2518 genotypes with blood eosinophil counts, atopy, total serum IgE levels, and bronchial hyper-responsiveness

	MCP-1 –2518		<i>P</i> -values
	A/A	A/G + G/G	
Blood eosinophil ($10^6/\text{ml}$) (<i>n</i>)	0.46 ± 0.36 (19)	0.44 ± 0.26 (42)	0.809
Atopy: no. (%)	39 (92.9)	122 (87.1)	0.459
Total serum IgE (kU/l) (<i>n</i>)	814.8 ± 665.9 (42)	654.2 ± 570.3 (140)	0.263
PC_{20} (mg/ml) (<i>n</i>)	3.11 ± 3.24 (12)	1.84 ± 2.24 (20)	0.346

Discussion

This study is the first to investigate the potential role of MCP-1 –2518A/G polymorphism in the pathogenesis of life-threatening asthma, and the first to examine its prevalence in an ethnic Chinese population. Asthma currently affects 100 million people worldwide, with a rising prevalence particularly among the young in the Western world (23). Similarly, in Taiwan, an Asia-Pacific country, our previous studies have revealed that current asthma prevalence in children has increased eightfold during the past two to three decades (from 1.3% in 1974, to 5.1% in 1985, 5.8% in 1991, and to 10.8% in 1994) (24, 25). During the period 2001–2002, we estimated that 10.1% of Taiwanese school children aged 6–7 years experienced current wheezing, according to written responses of parents to questionnaires from the International Study of Asthma and Allergies in Childhood (unpublished observation). In a recent study, we also demonstrated that the severity of childhood asthma seems to have increased in Taiwan, as reflected by a significant upward trend in hospital admission rates (25). The identification of asthma patients at risk for more severe or even life-threatening attacks remains an important

issue. Our recent study demonstrated that the RANTES – 28C/G polymorphism is a genetic risk factor for life-threatening asthma attacks in Chinese children (26). We also demonstrated that it would be advantageous to include asthmatic children with a history of near-fatal attacks in genetic studies of asthma (26). Identification of more analogous genetic markers may increase the feasibility of genotyping asthmatic subjects in early childhood for risk of life-threatening episodes of asthma. Furthermore, the identification of specific polymorphisms may facilitate the development of effective therapies, which target these abnormalities. However, our current work does not contribute to this course of action. In our cohort of Chinese children, the presence of the MCP-1 –2518G allele was not a risk factor for near-fatal asthma.

In a recent study, Szalai et al. (12) found an association between the MCP-1 –2518G allele and asthma susceptibility as well as asthma severity in a cohort of Hungarian children. They also demonstrated that the MCP-1 –2518 genotype was correlated with circulating eosinophil levels. Although the results of Szalai et al. (12) are encouraging, reproducibility is critical in gene association studies and confirmatory data from different ethnic populations obtained based on rigorous scientific designs are needed. This study failed to detect an association between the MCP-1 –2518A/G polymorphism and asthma. In this cohort of Chinese children, no detectable association was found between the MCP-1 –2518G allele and asthma or allergy in general. There are several possible explanations for this discrepancy. The absence of these associations might be attributable to the genetic heterogeneity in the pathogenesis of asthma. Different risk alleles may play different roles in different populations. Alternatively, the effects of the MCP-1 genotype in Chinese may be overshadowed by as yet unidentified genetic or environmental factors.

In comparison to the previous study assessing the role of the MCP-1 –2518A/G polymorphism in asthma, this study had several novel aspects. First, Szalai et al.'s study (12) reported this relationship in a Hungarian Caucasian population, whereas this investigation focused on ethnic Chinese children. In addition, it appears that Asia may be an area endemic for the MCP-1 –2518A/G polymorphism (11, 14). Further confirmation of this genetic heterogeneity is required because of the clinical consequences of this ethnic variation. Second, in the previous investigation by

Szalai et al. (12), the most severe cases were not included, while our sample included 48 asthmatic children with a history of near-fatal attacks. Third, the genotype and allele frequencies in this study showed that the MCP-1 –2518G allele was fairly common among this ethnic Chinese population from Taiwan. In this study, the prevalence of the G allele was 51.4% among Chinese children, which is in good correlation with data previously reported for a small group of Asians (47%) (11), and similar to that found previously in Japanese subjects (65%) (14), but significantly higher than in studies of Caucasian populations (21.1–29%; $P < 0.01$ in all cases) (11, 12, 15, 16). This suggests a significant ethnic variation exists in the prevalence of the G allele. The distinct differences in the allele frequencies of the MCP-1 polymorphism between Asian and Caucasian populations are important for several reasons. First, these findings suggest a genetic heterogeneity between Asian and Caucasian populations with respect to regulation of MCP-1 expression, supporting the proposition that Asia is an endemic area for the MCP-1 –2518A/G polymorphism. Furthermore, these differences underscore the necessity of accounting for ethnic background in the investigation of genes, which predispose to the development of asthma.

In conclusion, this study has demonstrated that the presence of the MCP-1 –2518G allele is not a risk factor for near-fatal asthma. Additionally, this study found no association of the –2518A/G polymorphism in the distal gene regulatory region of MCP-1 with asthma susceptibility or severity, in contrast to Szalai et al.'s findings in a Hungarian population. It is noteworthy that the high prevalence of the –2518G allele in these ethnic Chinese subjects from Taiwan suggests a potentially important ethnic variation in the regulation of MCP-1 production. This variation may contribute to the pathogenesis of asthma and must be considered in gene-association studies in different ethnic populations.

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