ORIGINAL ARTICLE

# Association of Polymorphisms in the Mast Cell Chymase Gene Promoter Region (-1903 G/A) and (TG)n(GA)m Repeat Downstream of the Gene With Bronchial Asthma in Children

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## Abstract

*Background:* Mast cell chymase is a mediator of inflammation and remodeling in the asthmatic lung. Although various studies have examined the association between the -1903 G/A single nucleotide polymorphism (SNP) in the mast cell chymase gene (*CMA1*) and allergic phenotypes, the results have been inconsistent. A $(TG)_n(GA)_m$ repeat polymorphism 254 base pairs downstream of *CMA1* has been reported in adult asthmatics. We investigated the relationship between these *CMA1* genetic variants and childhood asthma in Egyptian children.
*Methods:* A case-control study was undertaken in 15 children (6-10 years old) with bronchial asthma enrolled consecutively during exacerbation and 15 age-matched and sex-matched nonasthmatic control subjects. Genotyping was performed by polymerase chain reaction (PCR) restriction fragment length polymorphism to search for polymorphisms in the *CMA1* gene promoter region (-1903 G/A) and PCR amplification followed by sequencing to detect the $(TG)_n(GA)_m$ repeat 254 base pairs downstream of the gene.
*Results:* Our data showed a positive association between the *CMA1* -1903 G/A SNP and asthma in children. The G allele was detected in 70% of patients while the A allele was more frequent in the controls (83.3%). Concerning the (TG)n(GA)m repeat, allele 39 was only present in asthmatics while allele 37 was more common in controls.
*Conclusion:* We report the association of the -1903 G/A *CMA1* SNP and $(TG)_n(GA)_m$ repeat polymorphism with bronchial asthma in a group of Egyptian children. These polymorphisms are possible determinants of asthma susceptibility and may be involved in regulating immunoglobulin E levels.

Key words: Alleles. Asthma. Children. Chymase gene. Immunoglobulin E. $(TG)_n(GA)_m$ repeat polymorphism. Restriction fragment length polymorphism. Single nucleotide polymorphism.

## Resumen

*Antecedentes:* La cimasa de los mastocitos es un mediador de la inflamación y de la modificación en los pulmones asmáticos. A pesar de que diversos estudios han examinado la relación entre el polimorfismo de un sólo nucleótido (PSN) G/A 1903 en el gen de la cimasa de los mastocitos (*CMA1*) y los fenotipos alérgicos, los resultados han sido poco congruentes. Se ha observado un repetido polimorfismo $(TG)_n(GA)_m$ de 254 pares de bases en dirección 3' del *CMA1* en los asmáticos adultos. Investigamos la relación entre estas variantes del *CMA1* y el asma infantil en niños egipcios.
*Métodos:* Se llevó a cabo un estudio caso-control con 15 niños (6-10 años de edad) con asma bronquial inscritos consecutivamente durante la exacerbación y 15 sujetos control no asmáticos de la misma edad y sexo. Se realizó un genotipado mediante la reacción en cadena de la polimerasa (RCP) del polimorfismo en la longitud de los fragmentos de restricción con el fin de hallar los polimorfismos de la región promotora del gen *CMA1* (-1903 G/A) y la amplificación RCP seguida de la secuenciación para detectar los 254 pares de bases $(TG)_n(GA)_m$ repetidas en dirección 3' del gen.
*Resultados:* Nuestros datos mostraron que existe una relación positiva entre el *CMA1*-1903 G/A SNP y el asma infantil. El alelo G se detectó en el 70 % de los pacientes, mientras que el alelo A fue más frecuente en los controles (83,3 %). En referencia a la repetición de (TG)n(GA)m, el alelo 39 sólo estuvo presente en los asmáticos, mientras que el alelo 37 fue más frecuente en los controles.
*Conclusión:* Informamos de que existe una relación entre el -1903 G/A *CMA1* SNP y del polimorfismo repetido $(TG)_n(GA)_m$ con el asma bronquial en un grupo de niños egipcios. Estos polimorfismos surgen como posibles determinantes de la susceptibilidad de padecer asma y pueden estar implicados en la regulación de las concentraciones de inmunoglobulina E.

Palabras clave: Alelos. Asma. Niños. Gen cimasa. Inmunoglobulina E. Polimorfismo repetido de $(TG)_n(GA)_m$. Polimorfismo en la longitud de los fragmentos de restricción. Polimorfismo de un sólo nucleótido.

## Introduction

Atopic disorders are the result of complex interactions between genetic and environmental factors. The use of single-nucleotide polymorphisms (SNPs) has become popular in the analysis of complex genetic disorders [1]. Atopic asthma, one of the leading causes of morbidity in children and young adults, presents a model for complex phenotypes amenable to SNP association analysis. The identification of variations in specific genes that are involved in mediating the expression of the atopic asthma phenotype could help to identify specific molecular pathways with new potential drug targets. Both physiologic/pharmacologic and genetic studies in animal models and human populations have identified a number of candidate genes and pathways that regulate immunoglobulin (Ig) E levels and airway hyperresponsiveness [2-4].

Given the key role of mast cell mediators in the allergic response, the gene for mast cell chymase (*CMA1*), located on the long arm of chromosome 14 (14q11.2), is an ideal candidate for investigating genetic predisposition to atopic asthma. Mast cell chymase is a potential mediator of inflammation and remodeling in the asthmatic lung [5]. Various studies have examined the association between the *CMA1* promoter region (-1903 G/A) SNP and allergic phenotypes, but inconsistent results have been obtained [6]. Evidence for linkage was detected between asthma-associated phenotypes and the *CMA1* locus in a genome scan of white families in the United States [7]. However, that study did not involve *CMA1* -1903 G/A SNP analysis. A novel complex repeat polymorphism ($[TG]_n[GA]_m$ 254 base pairs [bp] downstream of *CMA1*) was identified for the first time by Sharma and coworkers [6] and was found to be associated with adult asthma in an Indian population.

We sought to investigate the association of the -1903 SNP and the $(TG)_n(GA)_m$ repeat polymorphism 254 bp downstream of *CMA1* with childhood asthma, aiming to identify local data on possible genetic variants of *CMA1* in Egypt. These associations might pave the way for novel therapeutic interventions.

## Subjects and Methods

A case-control study was performed in 15 asthmatic and 15 clinically healthy children. The patients were enrolled consecutively from the Pediatric Allergy and Immunology Unit of the Ain Sham University Children's Hospital in Cairo, Egypt. They were recruited while consulting for exacerbation. Informed consent was obtained from the parents or caregivers of each subject prior to enrollment and the study protocol was approved by the ethics committee of the Department of Pediatrics, Ain Shams University.

### Study Population

The asthmatic children (13 male and 2 female) were unrelated. Their ages ranged from 6 to 10 years (median, 7; mean [SD], 7.7 [1.5] years). The diagnosis of asthma was established according to the criteria of the American Thoracic Society [8]. The time since asthma diagnosis ranged from 4 to 9 years (median, 6; mean [SD], 6.7 [1.6] years). The patients were sub-classified [9] into 4 children with mild intermittent, 1 with mild persistent, 8 with moderate persistent, and 2 with severe persistent asthma. They were enrolled during acute asthma exacerbation; 2 patients had mild, 9 had moderate, and 4 had severe acute asthma exacerbation. At enrollment, 11 children (73.3%) were receiving inhaled corticosteroids at low-to-medium dosage (fluticasone propionate, 100-250 µg/d) with or without other medications such as inhaled long-acting ß2 agonists or theophylline. Some of them received several doses of short-acting ß2 agonists before they came to the hospital. All of them had a positive family history of allergy and children with present or past history suggestive of atopic dermatitis were excluded from the study. The control subjects were 15 clinically healthy unrelated children recruited from the outpatient clinic of Ain Shams University Children's Hospital. Their sex and ages were matched to those of the patients. They had no personal or family history suggestive of allergy. Subjects (patients or controls) with parasitic infestations or any chronic illness were excluded from the study.

### Blood Sample Collection and Processing

Six milliliters of venous blood was aseptically collected in aliquots for various study tests. The part used for genetic studies was collected in a vacutainer cell preparation tube containing sodium citrate (Becton Dickinson, Franklin Lakes, New Jersey, USA). The tube was stored upright at room temperature and centrifuged within 2 hours at 2760 rpm for 20 minutes at room temperature (22°C). The peripheral blood mononuclear cells (PBMC) appeared as a whitish layer just under the plasma layer. Half of the plasma was aspirated without disturbing the cell layer. Then, the cell layer was collected with a Pasteur pipette and transferred to a 25-mL conical centrifuge tube. The volume was made up to 25 mL with phosphate buffered saline and then the samples were centrifuged for 15 minutes at 560 rpm to wash the cells; the supernatant was discarded. This washing step was repeated twice. Then, the pellet was resuspended and subsequently used for DNA extraction from PBMC.

*Polymerase Chain Reaction Amplification and Genotyping.* Genomic DNA was extracted from PBMC [6,10] using the Pure Script Total DNA isolation kit (Gentra, Minneapolis, Minnesota, USA).

*Detection of* CMA1 *-1903 G/A polymorphism.* We investigated the -1903 G/A polymorphism using polymerase chain reaction (PCR) restriction fragment length polymorphism-based genotyping according to the technique described by Sharma et al [6]. The primer pairs used were 5'-GGAAATGTGAGCAGATA GCGCAGTC-3' and 5'-AATCCGGAGCTGGAGAACTCTTG TC-3'. PCR amplifications were performed in a final volume of 20 µL containing 0.1 µM of each primer, 1.5 mM MgCl2, 200 µM dNTPs, 50 ng of DNA template, 1 U of Taq DNA polymerase, and 1X PCR buffer in a Gene Amp PCR system (PerkinElmer 9600 Thermocycler, Waltham, Massachusetts, USA). The amplified product was visualized at position 280 bp by electrophoresis using 2% agarose gels (Amersham Biosciences, Uppsala, Sweden), stained with ethidium bromide, and viewed with UV light. Amplified DNA fragments were genotyped by restriction digestion

 

with *Bst*XI (New England Biolabs, Beverly, Massachusetts, USA) according to the manufacturer's instructions. PCR products from individuals with the GG genotype (homozygotes for the G allele) were refractory to digestion with *Bst*XI, giving the same band at 280 bp, while DNA from homozygotes for the A allele (AA genotype) was completely digested into 2 smaller fragments. The heterozygous GA genotype gave 3 bands: 1 at 280 bp and 2 other lower bands.

*Detection of (TG)n(GA)m repeat polymorphism downstream of CMA1.* The (TG)n(GA)m repeat (Accession number BV210164) 254 bp downstream of *CMA1* was examined in the study population according to the method described by Sharma et al [6]. The PCR was carried out in a total volume of 15 µL containing 25 ng of genomic DNA, 4 pmol each of a forward primer (5'-ACAACCCTAAGCCTCCAGA-AGTAT-3') and a reverse primer (5'-TGATGATTAAGGCAAAGAAGGAT-3'), 1.5 mM MgCl2, 0.25 mM of each dNTP, 1 U of Taq DNA polymerase and 1X PCR buffer. DNA amplification was carried out in a Gene Amp PCR system (PerkinElmer 9600 Thermocycler, Waltham, Massachusetts, USA). The PCR product, after visualization on a 2% agarose gel, was sequenced using the ALF express AutoRead sequencing kit (Amersham Biosciences, Uppsala, Sweden). Eight microliters of each sequencing reaction product were loaded onto the sequencing gel. The DNA sequence was read using an ALF express sequencer and the resultant chromatogram was analyzed using the Repeat Masker server (http://repeatmasker.genome.washington.edu/).

*Total serum IgE.* Analysis of total serum was performed by enzyme-linked immunosorbent assay (IgE enzyme immunoassay, BioChek Inc, Foster City, California, USA). A serum IgE level was considered elevated if it exceeded the highest reference value for the subject's age [11]. Control subjects who had elevated serum total IgE levels were excluded from the study.

### Statistical Analysis

Statistical analysis was performed using the statistical software package SPSS version 10 for Windows (SPSS Inc, Chicago, Illinois, USA). The data were expressed as median and interquartile range (IQR). Data were analyzed using the Mann-Whitney, Wilcoxon signed rank, and Kruskal-Wallis tests. The Fisher exact and $\chi^2$ tests were used for comparison of categorical data. For all tests, *P* values less than .05 were considered significant.

## Results

### CMA1 -1903 G/A SNP Allele and Genotype Frequency Distribution

The A allele was more frequent in the control group (83.3%) and the G allele was more frequent in the asthmatic patients (70%). The GG genotype was significantly more frequent in patients (53.3%) compared to controls, while an increased frequency of the AA genotype was observed in controls (66.7%). The frequency of the GA genotype was similar in both groups. The difference in distribution of the 3 *CMA1* genotypes between patients and controls was statistically significant (Table 1).

Table 1. Frequency Distribution of *CMA1*-1903 G/A SNP Alleles and Genotypes

| Variables | | Patients | | Controls | | *P*[a] |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| Alleles | A | 9 | 30 | 25 | 83.3 | < .001 |
| | G | 21 | 70 | 5 | 16.7 | |
| Genotypes | AA | 2 | 13.3 | 10 | 66.7 | .001 |
| | GA | 5 | 33.3 | 5 | 33.3 | |
| | GG | 8 | 53.3 | 0 | 0 | |

Abbreviation: SNP: single nucleotide polymorphism.
[a] Fisher exact test

### Allele and Haplotype Frequency Distribution of the $(TG)_n(GA)_m$ Repeat Downstream of the CMA1 Gene

A significant difference in allele counts was encountered between the patients and controls. The largest difference was observed for allele 39, which was only found in the asthmatic patients, and allele 37, which was represented at a higher

Table 2. Frequency Distribution of the $(TG)_n$ $(GA)_m$ Repeat Alleles[a]

| $(TG)_n$ $(GA)_m$ Repeat Alleles | Patients | | Controls | | *P* |
|---|---|---|---|---|---|
| | No. | % | No. | % | |
| 37 | 2 | 13.3 | 6 | 40 | |
| 38 | 0 | 0 | 3 | 20 | |
| 39 | 6 | 40 | 0 | 0 | |
| 40 | 1 | 6.7 | 2 | 13.3 | |
| 43 | 4 | 26.7 | 0 | 0 | .02 |
| 44 | 0 | 0 | 1 | 6.7 | |
| 43 | 1 | 6.7 | 1 | 6.7 | |
| 46 | 1 | 6.7 | 2 | 13.3 | |
| Total | 15 | 100 | 15 | 100 | |

[a] Each sequence is repeated at least 5 times in the DNA molecule.

Table 3. Frequency Distribution of the $(TG)_n$ $(GA)_m$ Repeat Haplotypes[a]

| Haplotypes | Patients | | Controls | | *P* |
|---|---|---|---|---|---|
| | No. | % | No. | % | |
| A37(AA) | 1 | 6.7 | 6 | 40 | |
| A38(AA) | 0 | 0 | 3 | 10 | |
| A44(AA) | 0 | 0 | 1 | 6.7 | |
| A49(AA) | 1 | 6.7 | 0 | 0 | |
| G39(GG) | 6 | 40 | 0 | 0 | |
| G43(GG) | 1 | 6.7 | 0 | 0 | |
| G45(GG) | 1 | 6.7 | 0 | 0 | .013 |
| G37A37 | 1 | 6.7 | 0 | 0 | |
| G40A40 | 1 | 6.7 | 2 | 13.3 | |
| G43A43 | 3 | 20 | 0 | 0 | |
| G45A45 | 0 | 0 | 1 | 6.7 | |
| G46A46 | 0 | 0 | 2 | 13.2 | |

[a] Each sequence is repeated at least 5 times in the DNA molecule.

frequency among controls. On comparing the major haplotypes (frequency > 5%), the frequency of haplotype G39 was higher in the asthmatic patients, while haplotype A37 was found at a higher frequency in the control group. The differences were statistically significant (Tables 2 and 3).

### *Association of Total Serum IgE With the -1903 G/A SNP*

Total serum IgE levels were significantly higher in the asthmatic children (median [IQR], 160 [160.7] IU/mL) than in the healthy control group (9.2 [8.3] IU/mL). Total serum IgE levels were higher in patients with the genotype GG than in those with GA or AA genotypes (median values of 214.6, 130, and 34.5 IU/mL, respectively) (Figure).

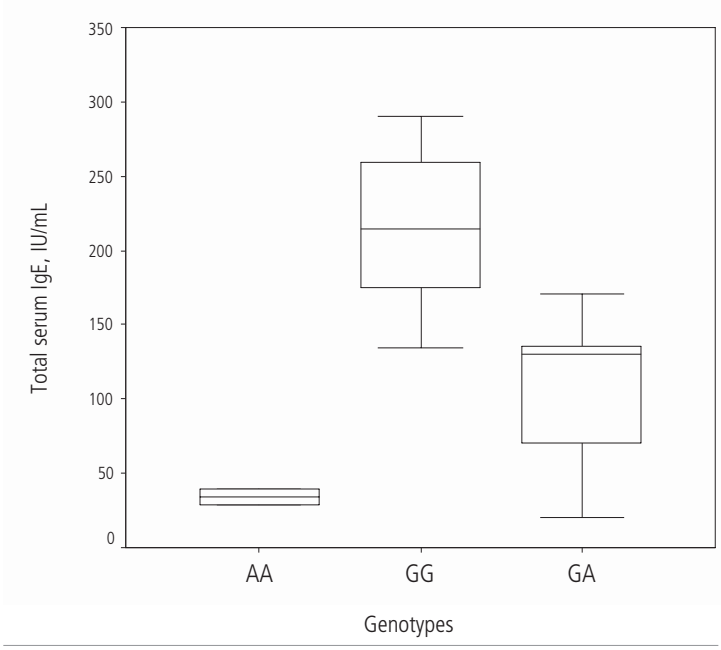


Figure. Relationship between *CMA1*-1903 G/A genotype frequency distribution and total serum immunoglobulin (Ig) E levels in asthmatic patients. Horizontal lines indicate median values, boxes enclose the interquartile range, and whiskers show ranges. Differences between the genotypes: Kruskal Wallis = 8.89, *P* = .012

## Discussion

Analysis of the -1903 G/A SNP results revealed that the frequency of the G allele was increased in asthmatic children whereas the A allele was more frequent among the control subjects. Also, 53.3% of the asthmatic children had the GG genotype, 33.3% had the GA genotype, and only 13.3% had the AA genotype. The frequency of the AA genotype was increased in the control children (66.7%) and none of them had the GG genotype. Our data demonstrate for the first time that there is a positive association between the -1903 G/A SNP and asthma in children. In contrast, Sharma and colleagues [6] found no significant difference in the distribution of the A and G alleles between asthmatics and controls and no significant association between -1903 G/A SNP genotypes and asthma in their study of an Indian population. The difference could be due to variations in phenotypic definition (eg, age) or ethnicity. It is also possible that the associations observed are due to another causal allele in linkage disequilibrium with the SNP studied [12]. Linkage disequilibrium may exist in the Egyptian population between this polymorphism and other functional variants of chymase that do not exist in the Indian or other populations where no association has been seen. Our observation is limited by the sample size.

Significant associations were previously reported between this SNP and atopic dermatitis. The 3 *CMA1* genotypes were associated with eczema but not with atopic asthma in adults [13] and schoolchildren [14] from Japan. Another study reported a link between the *CMA1* promoter polymorphism rs1800875 and atopic eczema but not with serum IgE levels, and this finding supported the hypothesis that *CMA1* serves as a candidate gene for atopic dermatitis [15]. Again, Tanaka and colleagues [16] found that *CMA1* genotypes were significantly associated with pure atopic eczema in patients who did not have a predisposition to atopic respiratory disease and whose serum IgE concentration was less than 500 IU/mL. The distribution of *CMA1* genotypes also differed significantly between patients with atopic eczema and those with concomitant bronchial asthma and a serum IgE concentration greater than 2000 IU/mL. They concluded that pure atopic eczema is associated with genetic variants of *CMA1* and that patients with atopic asthma are not genetically homogeneous and can be divided into at least 2 groups on the basis of the absence or presence of concomitant asthma. On the other hand, other studies carried out on atopic dermatitis and respiratory allergy failed to replicate these findings in white individuals from the United Kingdom [12] and in Japanese [17], Australian [18], and Italian populations [19]. In the white family cohort from the United Kingdom, there was a trend towards increased transmission of the -1903 G allele to sibs with reported asthma plus raised total IgE (age corrected), but it did not reach statistical significance [12].

Eight alleles were found for the $(TG)_n(GA)_m$ repeat 254 bp downstream of *CMA1* gene. A significant difference was obtained in the allele counts between patients and controls in the current study. The largest difference was observed for allele 39, which was only represented in the asthmatic children, and allele 37, which was more common in the controls. This novel genetic repeat was explored for the first time in 2005 in 2 large cohorts of adult asthmatics from northern and western India, and a positive association was reported [6]. Although no functional role had yet been assigned for this repeat, it might control the expression of *CMA1*, as had been shown by the results of earlier studies indicating that dinucleotide repeats were involved in gene regulation and were associated with various complex genetic disorders [6,20]. Alternatively, the association observed could be due to another causal allele in linkage disequilibrium with the complex repeat studied [6].

The distribution of the different haplotype combinations of the chymase gene was significantly different in patients and controls. We identified a major haplotype, G39 in patients and A37 in controls. A37 might prove to be protective against

 

asthma, as it was found in 40% of controls and in only 1 patient (6.7%). On the other hand, Sharma and coworkers [6] identified the G43 haplotype as a major haplotype in their control group and considered it protective. They noted that the distribution of the 43 allele for the $(TG)_n(GA)_m$ repeat was almost similar to the G43 haplotype. They suggested that the $(TG)_n(GA)_m$ repeat may play a more important role than the *CMA1* SNP.

The total serum IgE concentration was significantly higher in the asthmatic children with the GG homozygous genotype followed by the GA heterozygotes, while the AA homozygous genotype was linked to the lowest IgE expression. A study undertaken in a large cohort reported a similar association with total serum IgE in adult patients with atopic dermatitis, in which the GG genotype was associated with the highest IgE levels, followed by GA heterozygotes, while AA homozygotes had the lowest IgE expression [12]. In another study, the GG genotype was associated with the highest IgE levels but this was followed by the AA genotype and GA heterozygotes had the lowest IgE concentrations. The investigators stated that the reason for the difference was not clear. As IgE levels are controlled by many genes, it is possible that some other linked or unlinked loci contribute to the high IgE levels found in the GG homozygotes [6]. It is also possible that variability in sample phenotype or ethnicity may account for the difference.

In a previous study, an association between *CMA1* genotype and total serum IgE in patients with eczema was found in adults but not in the pediatric cohort [12]. The authors explained the finding by phenotypic variation between the pediatric and adult cohorts, age-related effects, or the possibility that chymase polymorphism exerts its effect in the context of chronic persistent eczema, rather than at its origin.

Our study has certain limitations. First, the sample size is too small to screen for genetic variants of a disease in the Egyptian population. Second, the study design lacks the inclusion of asthmatic and asymptomatic relatives of the patients, meaning that it was not possible to explore the familial expression of the associations found. The replication of the findings in independent Egyptian cohorts could strengthen our results. Further studies should include larger numbers of subjects and may use a well-controlled family-based design. Also, it would be interesting to investigate nucleotide variants affecting the transcription of *CMA1* or resulting in molecular variants in the gene product [17]. The detection of such functional variants will help to identify more precisely the relationship between *CMA1* polymorphisms and asthma.

The results of this pilot study show an association between the -1903 G/A SNP and $(TG)_n(GA)_m$ repeat polymorphism of *CMA1* and childhood bronchial asthma. The findings suggest that the alleles, genotypes, and haplotypes investigated may be important determinants of asthma susceptibility and might be involved in regulating IgE levels in atopic asthma. These observations might pave the way for future gene therapies targeted at reducing the ill effects of these polymorphisms.

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