

Clara cell protein 16 (CC16) gene polymorphism influences the degree of airway responsiveness in asthmatic children

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Background: Several studies have indicated linkage of chromosome 11q12-13 to asthma and associated traits. Among other candidate genes, the Clara cell protein 16 (CC16) gene maps to this region. CC16 is expressed in the bronchial epithelium and exhibits potent anti-inflammatory properties. A single-nucleotide polymorphism (SNP) in the CC16 gene (A38G) was previously associated with asthma.

Objective: We evaluated the role of the CC16 SNP in pediatric asthma and asthma severity in 2 German study populations. **Methods:** The German Multicenter Allergy Study (MAS) cohort (n = 872, 94 asthmatic patients) and 112 allergic asthmatic children recruited in Freiburg, Germany, were included in the present study. Histamine provocations were performed at the age of 7 years in the MAS cohort to determine bronchial hyperreactivity; in the Freiburg study population a standardized exercise-induced decrease in FEV₁ was evaluated. For genotyping, melting-curve analysis and restriction enzyme digestion were applied.

Results: No association of the CC16*38A allele with asthma could be observed in either study population. However, in asthmatic subjects (MAS cohort) PC₂₀FEV₁ values were significantly lower in individuals homozygous or heterozygous for the CC16*38A allele compared with those in subjects with the CC16*38GG genotype ($P < .05$ and $P < .03$, respectively). Similarly, allergic asthmatic patients in the Freiburg cohort showed a significantly greater decrease in FEV₁ after exercise when

homozygous for the CC16*38A allele compared with that seen in asthmatic patients with the *38AG or *38GG genotype ($P < .04$ and $P = .006$, respectively).

Conclusion: We conclude that the CC16*A38G SNP influences bronchial hyperreactivity and might be a genetic determinant of asthma severity in German children. (J Allergy Clin Immunol 2003;111:515-9.)

Key words: Genetics, Clara cell protein, CC16, asthma, bronchial hyperreactivity

Genome-wide searches and candidate gene studies have indicated linkage of asthma and associated phenotypes to chromosome 11q12-13.^{1,2} Among other candidate genes, the Clara cell protein 16 (CC16) gene has been mapped to this region.

CC16 (synonyms: uteroglobin and CC10) is a 16-kd homodimeric protein primarily expressed in the respiratory tract but also in urogenital tissues. It exhibits several immunomodulatory and anti-inflammatory effects.³ CC16 suppresses both IFN- γ expression and its biologic activity⁴ and diminishes chemotaxis, as well as phagocytosis of neutrophils and macrophages. CC16 has the potential to inhibit phospholipase A2 and the phosphoinositol-specific phospholipase C.⁵ These proteins are key enzymes in arachidonic acid metabolism and therefore influence the synthesis of several mediators of the inflammatory cascade, such as leukotrienes and prostaglandins.

CC16 was evaluated as a biomarker of lung injury and correlated to lung function and lung diseases: Van Vyve et al⁶ demonstrated that CC16 protein is reduced in the bronchoalveolar lavage fluid of asthmatic individuals. CC16 protein has also been shown to be diminished in the serum of asthmatic patients.⁷ Functional genetic variants of CC16 that influence protein expression might therefore contribute to asthma or asthma severity. Laing et al^{8,9} identified a single-nucleotide polymorphism (SNP) in the CC16 gene (*A38G) and described an association of the *38AA genotype with asthma and lower CC16 serum levels in an Australian population of white ethnicity. To date, these observations have not been confirmed.

The objective of the present study was the evaluation of this SNP in 2 German pediatric study populations with regard to asthma and asthma severity.

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Abbreviations used

BHR: Bronchial hyperreactivity
CC16: Clara cell protein 16
MAS: Multicenter Allergy Study
SNP: Single-nucleotide polymorphism
SPT: Skin prick test

METHODS**The Multicenter Allergy Study cohort**

The German Multicenter Allergy Study (MAS) has been described in detail previously.¹⁰ Briefly, MAS initially comprised 1314 children of German descent born in the year 1990 who were followed up from birth to the age of 10 years. Yearly follow-up visits included standardized interviews, questionnaires, and physical examinations. Total and specific IgE antibodies against 9 common food and inhalant allergens (CAP-RAST FEIA; Pharmacia, Uppsala, Sweden) were determined at the ages of 1, 2, 3, 5, 7, and 10 years. Allergic sensitization was defined as specific IgE levels of 0.7 kU/L or greater against at least one of the tested allergens.

Genomic DNA from blood samples was obtained in 888 MAS children (DNA blood kit; Qiagen, Heidelberg, Germany).

Pulmonary function tests and histamine provocations were performed at the age of 7 years in 800 and 645 individuals, respectively. For histamine challenges, a validated reservoir method (Pari Provocation Test 2; Pari, Starnberg, Germany) was used, as described previously.¹¹ Briefly, 0.5 mg/mL (in asthmatic patients 0.125 mg/mL) to 8 mg/mL histamine was applied to children until a 20% decrease in FEV₁ was observed.

As previously reported, a high degree of airway responsiveness was found in the MAS cohort, even in nonasthmatic children; however, a PC₂₀FEV₁ value of 0.6 mg/mL histamine could discriminate between symptomatic and asymptomatic subjects.¹¹

Parents provided written informed consent. The study was approved by the local ethics committee.

Current asthma at age 7 years was defined when one or more wheezing episodes were reported during the previous 12 months.¹² Genomic DNA, as well as data on bronchial responsiveness to histamine, were available for 57 of 94 asthmatic MAS participants.

Nonatopic, nonasthmatic control subjects (n = 118) were also selected from the MAS cohort. They had never had symptoms of asthma, allergic rhinitis, or atopic dermatitis and IgE against any tested allergen below the detection limit (<0.35 kU/L) at the age of 7 and 10 years. For definition of these atopy-related phenotypes, see the study by Nickel et al.¹³

Freiburg asthma study population

Two hundred fifty children with suspected asthma (age range, 5–18 years; mean age, 10.8 years) from the southwestern part of Germany were evaluated at the Children's University Hospital in Freiburg, Germany, by using a standardized clinical protocol (questionnaires on clinical symptoms and medication, determination of total and specific IgE antibodies, skin prick tests [SPTs], pulmonary function tests, and determination of exercise-induced decrease in FEV₁). The participants were asked to discontinue early in advance any asthma or allergy medication before the clinical testing.

SPTs were performed by means of intracutaneous applications of 17 common allergens (house dust mites, different grass and tree pollen, *Aspergillus fumigatus*, *Alternaria alternata*, *Cladosporium herbarum*, and dog, cat, rabbit, duck, and horse dander), as well as positive (histamine) and negative controls. The diameters of the wheal responses were recorded after 15 minutes. SPT responses were regarded as positive when the wheal induced by the allergen was at

least half the size of the wheal induced by the positive control.

Specific IgE was detected by means of ELISAs against 2 mixtures of grass pollens, mite allergens (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, respectively), cat and dog dander, and hazel and birch pollens (Magic Lite; Chiron Diagnostics, Fernwald, Germany). The cutoff point for a positive test result was 1.43 ML units.¹⁴ Measurement of total serum IgE was carried out by using an enzyme allergosorbent test (Phadezym, Pharmacia; note that different cutoff points were used for defining the allergic phenotype in the MAS cohort and the Freiburg asthma population because of different IgE assays).

Pulmonary function tests were performed by using a standard protocol. In addition, the exercise-induced decrease in FEV₁ was measured to evaluate bronchial hyperreactivity (BHR). The probands performed physical exercise for 6 minutes under standardized conditions. The first spirometry and peak flow measurements were performed 2 to 3 minutes after exercise and the second 5 to 6 minutes after exercise.

After performing the tests, 112 of the 250 children were given a diagnosis of allergic asthma. The diagnosis was based on a clearcut history of asthmatic symptoms, the use of antiasthmatic medication, a positive SPT response, and specific serum IgE antibodies to at least one of the tested allergens.

The collection of serum and DNA material, as well as the experimental protocol, were approved by the Ethics Committee of the University of Freiburg. Parents provided written informed consent.

Genotyping

Genotyping of the CC16*A38G SNP was performed using RFLP, as described previously.⁸ PCR conditions were initial denaturation at 95°C for 12 minutes and 50 cycles at 95°C for 10 seconds, 52°C for 30 seconds, and 72°C for 40 seconds. The primer sequences were 5'-TCTGGGTGCTGCTGCCTA-3' and 5'-CTGCAGCAGAGAGCCAGTG-3'. We used 1.5 mmol/L MgCl₂ and 0.5 U of Amplitaq Gold in a 25-μL reaction volume.

Once LightCycler technology was available, the remaining samples were genotyped using melting-curve analysis. A pair of fluorescence resonance energy transfer probes were applied to the PCR product and analyzed with the LightCycler system (Roche Diagnostics, Mannheim, Germany). The donor probe was 5'-GGAACCAGAGACAGGCCAGAGCA-FL-3' (5,6-carboxyfluorescein attached to 3'-o-ribose), and the acceptor probe was 5'-CCCCCTCCTCCACCATGAACTCGC-ph-3' (LightCycler Red 705 attached to the 5' terminus, 3' phosphate; probes were designed by Olfert Landt, Tib Molbiol, Berlin, Germany). The donor probe was complementary to the mutant sequence. During melting-curve analysis, hybridization of the probe was more stable with the mutant compared with the wild-type allele, resulting in different melting temperatures for each allele (Fig 1).

Statistical analysis

The Pearson χ^2 test and the nonparametric Mann-Whitney *U* test were applied for statistical analysis. *P* values of less than .05 were considered statistically significant.

RESULTS

The allele frequencies for CC16*A38G were consistent with previous reports from white individuals (MAS cohort: CC16*38A frequency of 0.34 and *38G frequency of 0.66; Freiburg allergic asthmatic patients: CC16*38A frequency of 0.31 and CC16*38G frequency of 0.69).⁸ CC16 genotypes were in Hardy-Weinberg equilibrium in both populations. No significant differ-

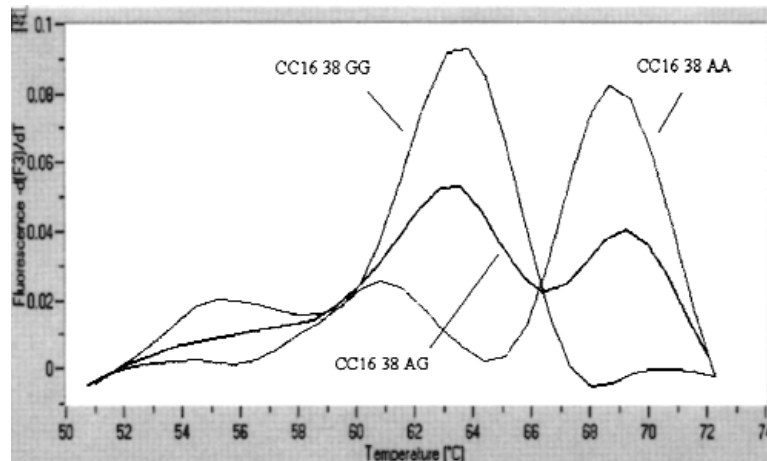


FIG 1. *CC16* genotyping by means of melting-curve analysis. A pair of fluorescence resonance energy transfer probes were applied to the PCR product and analyzed with the LightCycler system. A curve with one maximum represents a homozygous individual, peak temperature depending on sequence and fluorescence resonance energy transfer probes. A curve with 2 maxima characterizes a heterozygous subject.

TABLE I. *CC16* genotype frequencies in 2 pediatric, white asthmatic study populations and control subjects

Phenotype	n	Genotype		
		<i>CC16*38AA</i>	<i>CC16*38AG</i>	<i>CC16*38GG</i>
MAS asthmatic patients	84	0.07	0.51	0.42
Freiburg allergic asthmatic patients	104	0.08	0.46	0.46
MAS control subjects	118	0.10	0.42	0.48

No significant differences were observed.

ence in genotype distributions or allele frequencies could be observed when asthmatic patients from either study population were compared with nonasthmatic, nonatopic control subjects (Table I). However, in asthmatic MAS participants $PC_{20}FEV_1$ values differed significantly between *CC16* genotypes: the lowest values were observed in children homozygous for the *38A allele, followed by heterozygous subjects. The highest $PC_{20}FEV_1$ values and therefore the lowest degree of BHR among asthmatic patients was observed in subjects homozygous for the wild-type (*38G) allele ($P < .03$ and $P < .05$ compared with individuals with the *CC16*38AG* or *38AA genotypes, respectively; Fig 2). All children with the *CC16*38AA* genotype were sensitized to one or more inhalant allergens (specific IgE, ≥ 0.7 kU/L). Forty-one of the asthmatic patients genotyped in the MAS cohort and 55 subjects of the Freiburg asthmatic population were sensitized against house dust mite. However, differences in bronchial responsiveness were not more pronounced when the analysis was restricted to the house dust mite-sensitized subjects (data not shown).

In nonasthmatic MAS children $PC_{20}FEV_1$ values did not differ significantly between *CC16* genotypes. Furthermore, no other atopy-related phenotype (high total and specific IgE, allergic rhinitis, and atopic dermatitis) could be associated with a certain *CC16* genotype (data not shown).

The analysis of the Freiburg asthma population revealed similar results: the greatest decrease in exercise-induced FEV_1 was observed in asthmatic patients homozygous for the *CC16*38A* allele, which differed significantly from subjects heterozygous and homozygous for the wild-type allele ($P < .04$ and $P = .006$, respectively; Fig 3).

DISCUSSION

Because of its expression pattern and function, *CC16* is an intriguing candidate gene for chronic inflammatory lung diseases, such as asthma. We tested the role of the *CC16*A38G* SNP in asthma and BHR in 2 well-characterized German pediatric populations. We did not observe an association of the *CC16*38A* allele with asthma per se (as described by Laing et al⁸); however, the degree of BHR among asthmatic patients differed significantly between *CC16* genotypes in both German study populations. The highest degree of BHR was observed in children with the *CC16*38AA* genotype. We could not test for any other markers for asthma severity because most asthmatic children from the MAS cohort are only mildly affected, an asthma severity score has not been assessed in the Freiburg asthma population, and prescribed anti-asthma medication in the study population varies widely and does not necessarily reflect severity of disease.

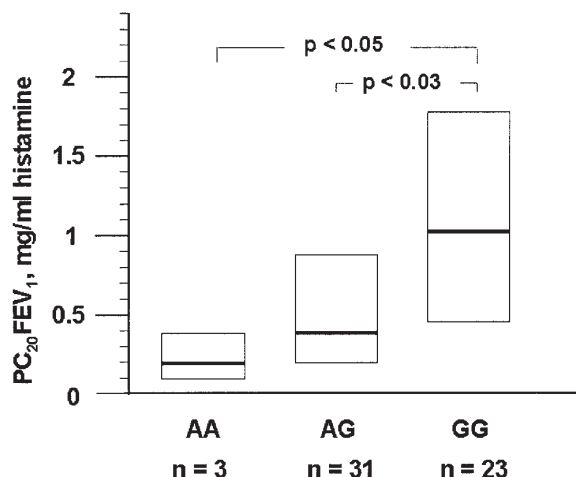


FIG 2. Median (with 25th-75th percentiles) PC₂₀FEV₁ values in asthmatic children with different *CC16* genotypes at age 7 years. Lowest PC₂₀FEV₁ values were observed in individuals with the *CC16**38AA genotype. All individuals homozygous for the *A allele were sensitized against at least one inhalant allergen.

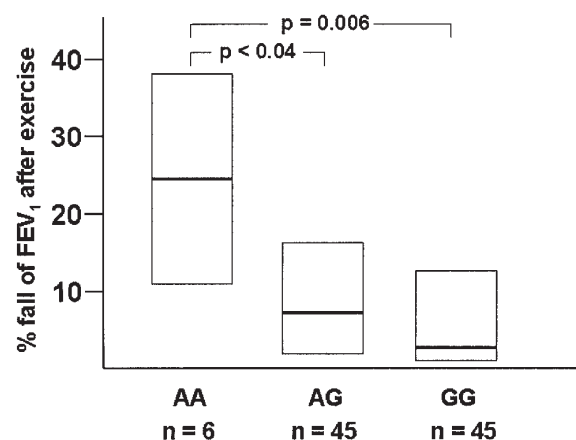


FIG 3. Exercise-induced decrease in FEV₁ (median with 25th-75th percentile) was determined in 96 of 112 allergic asthmatic patients (Freiburg). Values are stratified for *CC16**38AA, *38AG, and *38GG genotypes. Asthmatic children homozygous for the *38A allele showed a significantly greater decrease in FEV₁ after exercise compared with individuals with the *CC16**36AG or *36GG genotype.

Laing et al⁸ observed an association with the *CC16**38A allele and asthma (an effect on asthma severity was not described) in an Australian study population. These discrepancies with regard to the present study results might be explained by differences of study populations (ethnicity, age of subjects, and asthma severity) and environmental factors.

Functionality of this SNP has not yet been demonstrated in vitro. However, the location in a nontranslated part of exon 1 corresponds to a region in the rat *CC16* gene that exhibits the function of a minimal promoter

that is crucial for cell type-specific gene expression in the bronchiolar epithelium.^{15,16} Apart from an association with asthma, Laing et al⁹ could also demonstrate significantly lower CC16 protein serum levels in individuals with the *38AA genotype compared with those in heterozygous or homozygous subjects with the *38GG genotype. Taken together, these results suggest functionality of the *CC16**A38G SNP.

Before the genetic studies, CC16 protein levels were evaluated in chronic inflammatory lung diseases: decreased CC16 protein levels in bronchoalveolar lavage fluid were not only observed in asthma⁶ but also in chronic obstructive pulmonary disease.¹⁷

The anti-inflammatory properties of *CC16* have also been demonstrated in animal studies. *CC16* deficiency renders mice more susceptible to oxidative stress.^{18,19} Also, a higher degree of eosinophilic infiltration after challenge with ovalbumin, as well as a marked increase of T_H2 cytokine and eotaxin expression in lung tissue, were recently described in *CC16*-deficient mice.²⁰ In conclusion, reduced pulmonary CC16 protein levels might also result in an increased T_H2 cytokine production in human airways. Therefore we assume that a genetic variant leading to a decreased CC16 protein expression might significantly affect asthma severity, particularly in allergic asthmatic patients. However, because of the limited number of *CC16**A homozygous individuals in our study, this hypothesis has to be tested in larger populations.

Because CC16 protein is primarily expressed in the lung, its anti-inflammatory effects are likely to be most pronounced in the airways. This hypothesis is supported by the present study, which related the *CC16* genotype to the degree of BHR in asthmatic patients but not to any other atopy-related phenotype.

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