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Association of an intragenic microsatellite marker in the *CC16* gene with asthma in the Indian population

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Abstract The gene for *Clara cell secretory protein* (*CC16*) is an ideal candidate for investigating genetic predisposition to asthma because of its role in the airway as an anti-inflammatory molecule, differences in its levels between asthmatics and healthy controls, and its genetic location (11q13). We investigated the association of an SNP (A38G) and an intragenic repeat polymorphism in the *CC16* gene with asthma and its associated traits, such as total serum IgE levels, in a case control as well as in a family based study design. A significant association was observed for the microsatellite repeat at the level of alleles and genotypes with asthma ($P < 0.05$) in both the study designs. However, no association was observed for the A38G SNP with asthma. When haplotypes were constructed for these two loci and compared, the haplotype A₁₈ was found at higher frequency in patients (OR = 1.59, 95%CI = 1.08, 2.33, $P = 0.016$). Also, in the family based design, a biased transmission was observed for haplotypes from parents to affected offspring ($P = 0.003$). Individually, haplotype A₁₈ showed preferential transmission (82.6%) to affected offspring ($P = 0.001$), thereby confirming the case-control results. In summary, this is the first study identifying the *CC16* gene to be associated with asthma in the Indian population.

Keywords Asthma · Chromosome 11q13 · *CC16* · Genetic association studies · Haplotypes · Indian population · SNPs · Serum IgE

Introduction

Asthma is a chronic airway disease affecting 15–18% of the world's population (Smith 2000). It has been esti-

mated that atopic asthma accounts for up to 11–12%, with a loss of man-days due to asthma and airway disorders to be 34% in Indian population (Ghosh et al. 2003; Smith 2000). Both genetic and environmental factors have been implicated in the causation of the disease (Ghosh et al. 2003). Amongst the various genes implicated, the human *Clara cell secretory gene* (*CC16*) encoding the 16 kDa protein is an important potential candidate gene for asthma because of its expression, anti-inflammatory function and genetic location (Mansur et al. 2002). The CC16 protein is primarily expressed in the respiratory tract (7% of the total protein content in the bronchoalveolar lavage fluid) by nonciliated bronchiolar secretory (Clara) cells, which play important immunomodulatory and anti-inflammatory roles in airway inflammation (Bernard et al. 1992; Broekaert and Bernard 2000). Even micromolar concentrations of CC16 have been shown to inhibit chemotaxis of neutrophils and monocytes (Laing et al. 1998). The protein is known to control the proliferation and migration of fibroblasts in the lungs (Lesur et al. 1995). In addition, it also inhibits the activity of secreted and intracellular phospholipase A₂, thereby limiting the synthesis of prostaglandins and leukotrienes (Levin et al. 1986). Interferon- γ (IFN- γ), a Th1 cytokine, has been found to increase the expression of CC16 mRNA in the mouse lung and the CC16 protein levels in cultured human airway epithelial cells (Magdaleno et al. 1998; Yao et al. 1998). Moreover, mice deficient in CC16 expression were found to exhibit a higher susceptibility to oxidant-induced lung injury and an excessive inflammatory response (Harrod et al. 1998; Johnston et al. 1997; Mango et al. 1998). All this evidence suggest that CC16 plays a protective role in the lung, and it is possible that low activity variants of CC16 may be associated with inflammatory lung disorders such as asthma. This view gains support from the observation that asthmatic individuals demonstrate significantly decreased levels of CC16 protein in their bronchoalveolar lavage fluid (BALF) (Van Vyve et al. 1995) and serum when compared to normal controls (Shijubo et al. 1999).

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The gene for CC16 in humans has been localized to chromosome 11q12-13, a region shown to be highly linked to asthma and atopy (Hay et al. 1995). An adenine to guanine substitution has been detected at position 38 (A38G) downstream from the exon 1 transcription initiation site (Laing et al. 1998). The first intron also contains a polymorphic microsatellite at +2,921 to +3,110 (Mao et al. 1998). Polymorphisms within the gene, which either alter the production of CC16 or its activity, may therefore predispose an individual towards an increased risk of developing asthma. The A38G polymorphism has earlier been shown to be associated with altered plasma levels of CC16 (Laing et al. 2000); however, the mechanism for this modulation remains unclear. In a case-control study carried out in the Western Australian population, it was observed that the AA homozygotes were at 6.9-fold greater risk and AG heterozygotes were at 4.9-fold greater risk of developing asthma (Laing et al. 1998). A follow-up study showed that the asthmatics had lower plasma levels of CC16 compared to normal controls (Laing et al. 2000). This polymorphism has also been associated with an increased risk of physician-diagnosed asthma in a population of Australian children (Laing et al. 1998). A study carried out in the German population showed that PC(20) FEV(1) values were significantly lower in individuals homozygous or heterozygous for the *CC16**38A allele compared with those in subjects with the *CC16**38GG genotype (Sengler et al. 2003). In contrast to these results, studies on populations of Japanese and British adults (Gao et al. 1998), North American children, North European Caucasians (Laing et al. 2000), and Chinese (Gui et al. 2003) failed to replicate these associations. Also, no association has been reported between asthma and an intragenic microsatellite repeat polymorphism in *CC16* in studies carried out in different populations (Mao et al. 1998).

Here, we have conducted a case control as well as a family based study to identify the genetic association of *CC16* locus with asthma in the Indian population. We have investigated the A38G and the intragenic microsatellite repeat polymorphism in the study population and have attempted to evaluate the association between these markers individually and at the haplotype level to asthma status and the quantitative traits associated with asthma such as total serum IgE levels. Our results show that the *CC16* gene is associated with asthma in the Indian population.

Materials and methods

Subjects

Two hundred and fifty-nine unrelated asthmatic patients (28.2 ± 14.82 years) were recruited from various collaborating hospitals and institutions from northern India after taking written informed consent. For the family based association study, 106 trios (proband and parents)

[proband mean age = 14.5 ± 9.7 years, gender ratio (male versus female) = 58:42] were also recruited. The ethics committees of the participating centers and hospitals approved the study. Subjects were clinically characterized by completing a validated respiratory questionnaire designed as per the National Asthma Education and Prevention Program (Expert Panel Report-2) guidelines (Nagarkatti et al. 2004). These included patient details of the present and the past history of physician-diagnosed asthma, history of atopic symptoms, family history, the geographical region of origin, and migration status. Clinical tests performed to validate the asthma phenotype were PFT [FEV₁, bronchial reversibility (>15%) test using β_2 -agonist inhaler (albuterol/salbutamol)], skin-prick test (SPT, wheal reaction >3 mm diameter) to a panel of 15 local environmental allergens, and total serum IgE. Only individuals with a family history of asthma/atopy were included in the study. Healthy volunteers/normal controls ($N=251$, 26.82 ± 10.42 years) were selected on the basis of the criteria of having no symptoms or history of allergic diseases (Table 1). Total serum IgE levels were determined for all the control samples recruited for the study; however, SPT was performed wherever consent was obtained. Individuals who were active smokers in the past 2 years or suffering from parasitic/helminthic infestations (as determined from the clinical histories) were excluded. The genetic homogeneity between patients and controls was confirmed by genotyping loci as yet unlinked to asthma or related atopic disorders (Sharma et al. 2004). The panel of unlinked markers used were: D20S117, D6S1574, D20S196, D6S470, D12S368, D16S404, D6S446, D16S3136, D6S441, D8S264, D8S258, D8S1771, D8S285, D8S260, D8S270, D8S1784, D8S514, D8S284, D8S272, D5S406, D5S416, D5S419, D5S426, D5S418, D5S407, D5S647, D5S424, D5S641, D5S428, D5S2027, D5S471, D5S2115, D5S436, D5S422, D5S408, D6S281, D6S308, D6S264, and D6S287. These markers were tested for differences in allele frequencies in patients and controls.

Total IgE estimation

Sera were separated from blood, and total IgE levels were estimated by using the human IgE quantification kits (Bethyl Laboratories Inc., Montgomery, TX, USA), as described earlier (Nagarkatti et al. 2002).

PCR amplification and genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the modified salting out procedure (Nagarkatti et al. 2004) and genotyped for polymorphisms in the *CC16* gene. All subjects recruited for the study were genotyped for the A38G polymorphism by amplifying exon 1 using primers and PCR conditions, as described (Gao et al. 1998). Amplified DNA fragments were genotyped by restriction digestion with *Sau96I*

Table 1 Demographic profile of patient and control groups. *ND* not done

	Patients	Controls
Native place ^a	North India	North India
Mean age (standard deviation)	28.2 (± 14.82 years)	26.82 (± 10.42 years)
Gender ratio (M:F)	0.55:0.45	0.58:0.42
Familial history of asthma/atopy ^b	All	None
Smoking history ^c	None	None
Percent reversibility from baseline FEV1 (after β_2 -agonist usage)	> 15%	ND
Log mean total serum IgE	2.93 ± 0.83 IU/ml	2.42 ± 0.83 IU/ml
Self-reported history of allergies	All	None

^aPatient and control samples were recruited from the states of Delhi, Haryana, Punjab, and Rajasthan

^bControl individuals were subjected to a questionnaire so as to eliminate all individuals having atopic disorders or family history

of atopic disorders. Only those patients who had at least one first-degree relative affected with atopy and/or asthma were included
^cPatients and controls known to be active smokers in the past 2 years or were suffering from parasitic infections were excluded

(1 U/15 μ l reaction, New England Biolabs, Beverly, MA, USA), as per the manufacturer's instructions. PCR products from individuals with AA genotypes were refractory to digestion with *Sau96I*, while DNA from homozygotes for the G allele (GG genotype) was completely digested into two fragments. The accuracy of the RFLP (restriction fragment length polymorphism) genotyping was confirmed by direct sequencing of the DNA samples ($N=20$) for all three respective genotypes.

Genotyping for the polymorphic microsatellite repeat in the intron was performed using oligonucleotide primer pairs 5'-TTTGGCTGGTACAATCTCAATGG-3' and 5'-TTCTGGGCACCCGTAACCTA-3'. PCR was carried out in a total volume of 15 μ l containing 25 ng of genomic DNA, 1.25 pmol of each primer, 1.5 mM MgCl₂, 0.25 mM of each dNTP, and 0.03 U/ μ l of Taq DNA polymerase (Bangalore Genie, India). The forward primer was labeled with fluorescein. PCR products were loaded with internal standard ladder (ROX-labelled) in each lane on an ABI Prism 3100 Automated Sequencer and analyzed using GeneMapper Ver3.5 (Applied Biosystems, Foster City, CA, USA).

Statistical methods

Kolmogorov-Smirnov (KS) test was used to test the association of the microsatellite allele size distribution with disease (Nagarkatti et al. 2002). This test assesses whether or not the largest difference in the cumulative frequencies in the two distributions has been caused by a chance fluctuation and tries to determine if two datasets differ significantly (Maalej et al. 2004). It has the advantage of making no assumption about the distribution of data. Thus, it is nonparametric and distribution free. Chi-square test and Fisher's exact test (FET) were used to test the genotypic and allelic association of the polymorphisms with asthma. Likelihood ratio (LR) tests and odds ratios (OR) were calculated (<http://home.clara.net/sisa/>). Hardy-Weinberg equilibrium (HWE) for patients as well as controls was calculated

using De Finetti program (<http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>). ANOVA was used to assess the significance of association between polymorphisms of the *CC16* gene and quantitative phenotypes such as total serum IgE levels. Haplotypes were generated in patients and unrelated controls using PHASE version 2.1 (Stephens and Donnelly 2003). The association of haplotypes was tested with asthma status and total serum IgE levels.

For the family based association study, extended transmission disequilibrium testing (E-TDT) was used to detect preferential transmission of allelic and haplotypic combinations to the affected offspring (Sham and Kurtis 1995). The association of A38G SNP with asthma was studied using TDT/STDT Program 1.1 (<http://genomics.med.upenn.edu/spielman/TDT.htm>) (Spielman et al. 1993). The genotype data in each trio were verified for Mendelian inheritance prior to the analysis.

Results

Total serum IgE levels in patients and healthy volunteers

The mean logarithmic total serum IgE levels were 2.93 ± 0.83 IU/ml and 2.42 ± 0.83 IU/ml for patients and controls, respectively, and were found to be significantly different from each other ($P < 0.0001$). There was no significant difference between cases and controls with respect to age or male-to-female ratio (Table 1).

Association analysis with asthma and total serum IgE levels

For the A38G SNP, the distribution of *CC16**A and G alleles were not found to be significantly different between cases and controls (FET $P=0.30$). Also, the distribution of AA, AG, or GG genotypes was concordant in cases and controls ($P=0.88$) (Table 2). The observed

genotype counts for the markers did not deviate significantly from those expected according to the Hardy–Weinberg equilibrium ($P > 0.20$). Also, none of the alleles/genotypes for this locus was associated with log total serum IgE levels ($P > 0.05$).

However, for the microsatellite repeat in intron 1, a significant difference in the allele count distribution was observed between control and patient groups (KS $\chi^2 = 8.58$, $df = 2$, $P = 0.014$) (Table 2). The heterozygosity index for this repeat polymorphism is 0.6. An examination of allele counts showed that the largest difference between patients and controls was for the 18 repeat. The odds ratio for patients having 18 alleles rather than any other allele (non-18) compared to controls was 1.49 with Wald's 95% CI = (1.14, 1.97); LR $\chi^2 = 8.31$; ($P < 0.0039$). The genotype 18/18 was also found to be marginally associated with asthma [OR = 1.59, 95% CI = (0.99, 2.53); $P = 0.051$]. Further, the 16/17 genotype was found to be overrepresented in controls compared to patients (relative frequency 0.23 versus 0.14). The odds ratio for patients having the 16/17 genotype compared to controls was 0.54 with Wald's 95% CI = (0.34, 0.85) ($P = 0.0075$). One-way ANOVA showed a significant association between the genotypes for this locus and serum IgE levels (F ratio = 2.44, $df = 7$, $P = 0.019$) (Table 3). Hence, the microsatellite repeat in intron 1 of the *CC16* gene is strongly associated with asthma.

Further, to confirm the results obtained from our case-control study, we performed a family based study

Table 2 Allele and genotype frequencies for polymorphisms studied in the *CC16* gene

	Number (%) ^a		<i>P</i> value	
	Patients	Controls		
A38G				
Alleles				
A	262 (50.58)	263 (52.39)	0.0039	
G	256 (49.42)	239 (47.61)		
Genotypes				
AA	72 (27.80)	74 (29.48)		
AG	119 (45.95)	115 (45.82)		
GG	68 (26.25)	62 (24.70)	0.0075	
Microsatellite				
Alleles				
16	71 (13.71)	89 (17.73)		
17	269 (51.93)	288 (57.37)		
18 ^b	168 (32.43)	122 (24.30)		
19	10 (1.93)	3 (0.60)	0.05	
Genotypes				
16/16	5 (1.93)	5 (1.99)		
16/17 ^b	37 (14.29)	59 (23.51)		
16/18	23 (8.88)	20 (7.97)		
17/17	96 (37.07)	99 (39.44)	0.0075	
17/18	37 (14.29)	30 (11.95)		
17/19	5 (1.93)	1 (0.40)		
18/18 ^b	53 (20.46)	35 (13.94)		
18/19	2 (0.77)	2 (0.80)		
19/19	1 (0.39)	0 (0.00)	0.05	

^aNumbers in parentheses indicate the frequency (%)

^b P value < 0.05 when compared to the control group

using 106 trios. The genotypic distribution followed HWE in the parents recruited for this study ($P = 0.20$). The TDT/STDT program showed equal transmission of the *CC16**A allele to that of the G allele (53 transmitted versus 53 nontransmitted) ($P = 1.0$). On the other hand, when the transmission frequency of the microsatellite repeat in intron 1 was analyzed using E-TDT software, a biased transmission was observed for the alleles from parents to their affected offspring (allele-wise TDT $\chi^2 = 9.35$, $df = 3$, $P = 0.02$ and genotype-wise TDT $\chi^2 = 10.50$, $df = 5$, $P = 0.06$, respectively). Individually, significance was also observed for allele 18 where the transmission was observed in 66.7% of cases (TDT $\chi^2 = 6.12$, $df = 1$, $P = 0.01$). The results were statistically significant, even after Bonferroni correction ($\alpha = 0.05$ /number of tests performed = 0.01).

Association of *CC16* haplotypes with asthma and total serum IgE levels

The association of the A38G polymorphism and the microsatellite repeat in intron 1 at the haplotype level was investigated using PHASE in the case-control study (Stephens and Donnelly 2003). Phase probabilities of each site were calculated for each individual by this software. Individuals with phase probabilities less than 80% were excluded from the analysis. A total of six haplotypes were obtained (Fig. 1). It was observed that the haplotype A_18 was found to be present at a higher frequency in patients compared to controls (0.15 versus 0.098; $N_P = 76/518$ and $N_C = 49/502$). The odds in favor of patients rather than controls having the haplotype A_18 was 1.59 with 95% CI = (1.08, 2.33). The corresponding likelihood χ^2 showed P value less than 0.016 (LR $\chi^2 = 5.76$). On comparing the different haplotypic combinations with respect to the log total serum IgE levels, a marginally significant difference was obtained (F ratio = 2.08, $df = 5$, $P = 0.066$). The log total IgE levels corresponding to the respective haplotypes are given in Table 3. Thus, the two-locus haplotype A_18 is associated with asthma and its associated trait serum IgE.

Table 3 Mean log total serum IgE values in patients and controls according to genotype/haplotype. IgE values are given as mean $\pm$ SEM. Numbers in parenthesis indicate the number of chromosomes used for analysis

Genotype	Mean log total IgE values (IU/ml) ^a	Haplotype	Mean log total IgE values (IU/ml) ^b
16/16	2.58 $\pm$ 0.30 (10)	A_16	2.55 $\pm$ 0.07 (105)
16/17	2.51 $\pm$ 0.08 (130)	A_17	2.67 $\pm$ 0.04 (285)
16/18	2.93 $\pm$ 0.13 (52)	A_18	2.48 $\pm$ 0.08 (82)
17/17	2.64 $\pm$ 0.05 (310)	G_17	2.59 $\pm$ 0.06 (148)
17/18	2.78 $\pm$ 0.10 (98)	G_18	2.69 $\pm$ 0.06 (116)
17/19	3.16 $\pm$ 0.30 (10)	G_19	3.02 $\pm$ 0.23 (9)
18/18	2.51 $\pm$ 0.09 (124)		
18/19	1.88 $\pm$ 0.48 (4)		

^a F ratio = 2.44; $df = 7$; $P = 0.019$

^b F ratio = 2.08; $df = 5$; $P = 0.066$

Fig. 1 The frequency distribution of the two-locus haplotypes of the *CC16* gene in patients and controls. The haplotypes were plotted on the X-axis and their respective relative frequencies (%) on the Y-axis

The haplotypes for the two loci were constructed by following the inheritance of alleles in trios. The software E-TDT was used to assess the transmission of haplotypes in the trios. A total of eight haplotypes were obtained (Fig. 1); however, two haplotypes (G_16 and A_24) had counts ≤ 2 . These haplotypes were also not observed in the case-control study.

A biased transmission was observed for haplotypes from parents to their affected offspring (haplotype-wise TDT $\chi^2 = 21.63$, $df = 7$, $P = 0.003$). Individually, the A_18 haplotype was transmitted preferentially to the affected offspring (19 of 23 times, 82.6% transmitted) (TDT $\chi^2 = 9.78$, $df = 1$, $P = 0.001$). The difference in transmission was found to be statistically significant, even after Bonferroni correction ($\alpha = 0.006$).

Discussion

This is the first study showing a positive association between the microsatellite repeat polymorphism in the *CC16* gene and asthma/total serum IgE levels in both case-control and family based study. Earlier studies carried out in the Caucasian and Japanese (Mao et al. 1998) population, however, failed to detect any association for this repeat with asthma. We also show here an association of haplotypes in this gene with asthma.

A few earlier studies performed in other populations showed a significant association of A38G (exonic) polymorphism with asthma (Laing et al. 1998, 2000). We have observed no association between the A38G polymorphism and asthma/log IgE in the Indian population. The study conducted in the Australian population suggested that this polymorphism is located within a region corresponding to the rat minimal promoter and therefore may influence levels of CC16 expression (Laing et al. 1998). However, three other studies carried out in different populations (Gao et al. 1998; Gui et al. 2003; Mansur et al. 2002) found no association between this *CC16* polymorphism and asthma. Our study supports

the latter observation. These differences in results obtained from studies carried out in different populations could be due to variation on phenotype definitions, ethnicity, and environmental exposure. One possible reason for the discrepancy in results obtained by Laing et al. (1998) from others may be that their group examined asthma in children, and it is likely that the phenotypes that constitute asthma in that study group may be different from those present in the adult population. Also, the replication of association or linkage in complex disorders has proven controversial, as the power to detect an effect is limited (Suarez and Hampe 1994). Further, as multiple genes interact to express the phenotype in complex heterogeneous traits like asthma, association will normally be observed in samples that contain an exceptionally high proportion of individuals influenced by that particular gene. However, as many studies have been unable to replicate association with this SNP, it might be possible that the cause of association observed in the first study may be due to the phenomenon of linkage disequilibrium.

To observe the combined effect of the intragenic microsatellite repeat and the A38G SNP, we constructed haplotypes using the Bayesian method for inferring haplotypes in the case-control setup (Stephens and Donnelly 2003). The inferred haplotype A_18 was found to be significantly associated with asthma ($P = 0.01$). Our family based study also corroborated this result. Interestingly, it may be noted that the strength of association of this haplotype was weaker than the repeat allele 18 alone ($P = 0.01$ versus $P = 0.004$) in the case-control setup. This suggests that the combined effect of both loci taken together is weaker and the intragenic microsatellite repeat plays a bigger role compared to the SNP. Also, no linkage disequilibrium was detected between the A allele of the A38G SNP and the 18 allele of the microsatellite repeat ($D' = 0.11$). However, in the family based analysis, a stronger association was observed for the A_18 haplotype when compared to allele 18 alone ($P = 0.001$ versus $P = 0.01$). This difference in the P values may be due to the fact that the A_18 haplotype in the *CC16* gene is playing an important role in these families, leading to an increased susceptibility to asthma.

Though the functional role of this intronic microsatellite repeat is not yet known, it is possible that the polymorphic nature of these sequences can lead to variable effects on expression, splicing, and stability of the mRNA encoding the CC16 protein. It is known that variable repeat lengths can form secondary structures and contribute to phenotype diversity, as it was observed for the *CFTR* gene, where a dinucleotide repeat in intron 8 affects splicing (Hefferon et al. 2004). Also, the CA repeat polymorphism in the *IFN- γ* gene contributes to the variable levels of IFN- γ protein in vitro (Pravica et al. 1999).

Since asthma is a complex disorder caused by the interaction of many genes each having only a small effect, we have used a case-control study for finding any

association with one of the promising loci (Sharma et al. 2004). However, a case-control study has a higher chance of finding a false-positive association than a family-based study. To circumvent this problem, the results from a well-characterized (age- and gender-matched) case-control cohort have been confirmed in a trio-based family study. We also ensured genetic homogeneity between patients and controls by genotyping markers as yet unlinked to asthma. We found no significant difference in the allele counts in patients and controls used for this study ($P > 0.05$, data not shown).

It is important to note that the human *CC16* gene maps to an important genetic region (11q12-13), which has been linked to asthma and its related phenotypes in studies carried out in various populations (Blumenthal et al. 2004; Huang et al. 2003; Simon Thomas et al. 2000; Young et al. 1992). In addition to *CC16*, several other important candidate genes for asthma, such as *Fcε R1β*, *CD20*, and *Glutathione S-transferase pi (GSTP1)* are also localized in this region (Mansur et al. 2002). Earlier association studies suggested that the *CC16* gene is not contributing towards the association of the 11q13 region to asthma (Gao et al. 1998; Gui et al. 2003; Mao et al. 1998). In contrast, our results suggest that *CC16* is an important candidate gene in this region to be considered for genetic studies. We have recently reported a strong association between the polymorphisms of *Fcε R1β* and asthma in two independent cohorts from the Indian population (Sharma et al. 2004). Therefore, it would be interesting to investigate the gene-gene interactions for various important genes present in this locus.

In summary, using both case-control and family based strategies, we report here the association of an intragenic repeat polymorphism in the *CC16* gene with asthma.

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