

ORIGINAL ARTICLE

The CC16 A38G polymorphism is associated with the development of asthma in children with allergic rhinitis

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Summary

Background Although asthma and allergic rhinitis (AR) are considered to be one syndrome, many questions remain unanswered. Why do some AR patients develop asthma but others do not, and which factors play a role in the development of asthma that have so far not been clearly elucidated.

Objective We hypothesize that children with AR who have the Clara cell secretory protein (CC16, *secretoglobin 1A1*) 38A/38A genotype (rs3741240) have an increased likelihood of developing asthma.

Methods The study sample included 117 children, with AR, but no asthma diagnosed within the following 5 years, as the control group. Cases group ($n = 202$) included age- and gender-matched children with AR first, and asthma developed 3–5 years later, as the study group. The CC16 genotype was determined by PCR and *Sau96I* restriction digestion of PCR products. The serum CC16 levels were measured by ELISA. Total serum IgE, allergen specific IgE, eosinophil count and pulmonary function were also measured.

Results In children with rhinitis who develop asthma, the frequencies of the AA genotype were significantly higher than those who did not develop asthma [odds ratio (OR) = 2.527; 95% confidence interval (CI) = 1.571–4.065; $P < 0.01$]. Serum CC16 levels in the children with rhinitis who develop asthma and carry the AA genotype were significantly lower than those who carry the non-AA genotype and those who did not develop asthma.

Conclusions and Clinical Relevance Results of this study suggest that CC16 38A/38A genotype plays a role in the development of early asthma in children with AR. Early identification of rhinitis children at risk may assist in designing preventative approach to asthma development.

Keywords allergic rhinitis, asthma, atopy, CC10, CC16, CCSP, childhood, polymorphism, secretoglobin 1 A1, uteroglobin

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Introduction

Asthma and allergic rhinitis (AR) are currently thought of as one syndrome occurring in two different parts of the respiratory tract [1]. AR often precedes the development of asthma, and it has been suggested that rhinitis might be a risk factor for asthma [2]. However, why do some AR patients develop asthma symptoms but others do not? The factors that play a role in the development of asthma in AR patients have so far not been clearly elucidated. The development of asthma in children with AR causes a

greater likelihood of poor health in the child as well as increased financial burdens to the child's parents and healthcare provider. Determining factors associated with the development of asthma, could predict the high-risk patients and allow interventions to be identified that prevent the development of asthma.

Allergic disease is a complex genetic disorder. Family history of asthma was more common in children who had comorbid AR [3]. In our study, the common 289 bp insertion/deletion polymorphism (rs 4646994) in intron 16 of the gene for angiotensin-converting enzyme (ACE)

might play a role in the development of the asthma phenotype in children with AR [4]. These findings suggest that a genetic predisposition may have some impact on the comorbidity of AR and asthma. Clara cell secretory protein (CC16), also known as secretoglobin 1A1 (SCGB1A1), CC10, CCSP and uteroglobin, primarily expressed in the respiratory tract [5], is a potent anti-inflammatory agent [6] and the protects airway from inflammation. CC16 levels are reduced in the bronchoalveolar lavage of patients with asthma [7]. There is a single nucleotide polymorphism, the A38G polymorphism (rs3741240) [8], and the A allele is associated with decreased transcriptional activity of the gene [9]. The AA genotype is associated with allergy and asthma [8, 10, 11]. Subjects with the AA genotype had significantly lower serum CC16 levels than those with the GG and AG genotypes [11]. It is reasonable to suspect that children with AR who have the CC16 38A/38A genotype (and lower serum CC16 levels) have the most potent bronchial inflammation and thus, an increased likelihood of developing asthma.

Materials and methods

Study population

Control group. The AR-only group. Two hundred and seventeen unrelated children aged 8–12 years, with AR were recruited from the paediatric outpatient clinics of a university teaching hospital in Taichung, Taiwan. No asthma symptoms/signs (S/S), no airway hyper-responsiveness [abnormal forced expiratory volume in 1 s (FEV1) and 15% or greater reversibility of FEV1 after bronchodilator treatment] were found. The diagnosis of AR by two immunologists was based on clinical grounds, as the occurrence of AR S/S including sneezing, watery rhinorrhoea and blocked or itchy nose in the absence of a common cold. According to the new Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines [12], the severity of rhinitis symptoms were classified as moderate/severe persistent. These children had a history of AR for at least 5 years.

Case group. The AR-developing-asthma group. Two hundred and two, age- and gender-matched children were recruited from the same hospital. These children had AR for at least 5 years. Asthma had been noted about 3–5 years after the onset of AR. Asthma was diagnosed by two immunologists and fit the criteria of the American Thoracic Society [13]. The diagnosis of asthma was confirmed by a clear clinical history with clinical symptoms (episodic wheezing, chest tightness and dyspnoea), abnormal FEV1, 15% or greater reversibility of FEV1 after bronchodilator treatment and reversibility of the wheezing and dyspnoea after bronchodilator treatment. The severity of rhinitis

symptoms was classified as moderate/severe persistent, according to ARIA guidelines [12].

All children were atopic as defined by having allergic S/S, a positive family history and at least one allergen-specific IgE of >0.35 kU/L (described below). Children with rhinitis or asthma caused by aetiologies other than allergy were excluded.

We included only the pre-pubertal children because of the natural outgrowth of asthma often seen in adolescents and this may have confounded the analysis.

Total and allergen-specific immunoglobulin E and blood eosinophil count

Total serum IgE levels were tested by a fluorescence enzyme immunoassay using a commercially available immunoCAP system, (Phadia, Uppsala, Sweden) to determine the allergen sensitivity type. Concentrations of specific IgE reactions to common aeroallergens in Taiwan, including dust mite: *Blomia tropicalis* (Bt), *Dermatophagoides pteronyssinus* (Dp), *D. farinae* (Df); cockroach: *Blattella germanica* (Bg), dog dander and cat dander were tested by a fluorescence enzyme immunoassay using the commercially available immunoCAP system. Allergen-specific IgE of >0.35 kU/L was labelled as allergen sensitivity.

A peripheral venous blood sample was collected and the eosinophils were counted in a Fuchs Rosenthal chamber after staining with eosinophil staining solution.

Determination of the CC16 genotype

Genomic DNA was extracted from the peripheral blood using standard procedures [14]. The exon 1 of the CC16 gene was amplified by polymerase chain reaction (PCR) as described previously [8]. PCR products were digested using the restriction enzyme *Sau* 96 I, which recognizes the 38G allele. After restriction enzyme digestion, PCR products were separated by 3% agarose gel electrophoresis, and identified with ethidium bromide staining. Subjects were then classified according to the nucleotide at position 38, downstream from the exon 1 transcription initiation site of the CC16 gene, and grouped according to whether they had the AA, AG, or GG genotype.

Determination of the serum CC16 concentration

According to the proportion of AA and non-AA genotype in both groups, we randomly selected 176 plasma samples from the two groups. Peripheral blood samples were gathered in recent 1 year when the conditions of the children were stable. The serum CC16 levels in the two groups were measured using a commercially available ELISA kit (BioVendor Laboratory Inc., Modrice, Czech Republic).

Pulmonary function tests

In both groups, a flow volume curve was performed to determine the degree of airway obstruction when there was no acute exacerbation of asthma or rhinitis S/S. We acquired the regular lung function data every 3 months and irregular lung function data when clinical condition changes. We randomly selected one of the lung function data in recent 1 year. The FEV1 and the forced vital capacity (FVC) were measured, and the FEV1/FVC ratio was calculated.

Statistical analysis

The frequencies of the CC16 genotypes in children from both groups were compared using the two-tailed χ^2 test. The χ^2 test was used for differences in prevalence of allergen sensitivity type between different groups and different genotypes. As the distributions of the serum total IgE levels and eosinophil counts were skewed, they were log-transformed to approximate a normal distribution. Independent *t*-test was used to study the relationship between the different phenotypes, genotypes and Log IgE levels, Log eosinophil count and serum CC16 levels.

Results

Demography

There was no difference in the age and gender distribution between the two groups (Table 1). Total serum IgE level, blood eosinophil count, serum CC16 levels and allergen-specific IgE (allergen sensitivity type) did not differ between the two groups. The FEV1/FVC values differed between the two groups.

Frequencies of alleles and genotypes

The result is shown in Table 2. In the control group (AR only), the frequencies of the AA, AG and GG genotypes were 15.2%, 37.8% and 47.0%, respectively. The distributions of the A and G alleles were 0.34 and 0.66. In the case group (AR developing asthma), the respective frequencies of the AA, AG and GG genotypes were 31.2%, 38.6% and 30.2%. The distributions of the A and G alleles were 0.51 and 0.49. The result indicated that AA genotype and A allele was more frequent in the case group than in the control group.

In AR children, those with AA genotype were 2.527 times more likely to have early onset asthma [odds ratio (OR) = 2.527; 95% confidence interval (CI) = 1.571–4.065; $P < 0.01$] than those with AG and GG genotypes (defined as non-AA) (Table 2).

Table 1. Demographic data and other parameter between control and case group

Variable	Mean $\pm$ SD		<i>P</i> value
	AR*	AR+AS [†]	
Age (years)	10.53 $\pm$ 0.20	10.73 $\pm$ 0.19	0.155
Gender	<i>n</i> (%)	<i>n</i> (%)	
Male	143 (65.9%)	139 (68.8%)	0.534
Female	74 (34.1%)	63 (31.2%)	
Log IgE (kU/L)	2.43 $\pm$ 0.07	2.49 $\pm$ 0.09	0.272
Log eosinophil (/ μ L)	2.48 $\pm$ 0.05	2.53 $\pm$ 0.06	0.195
Allergen specific IgE	<i>n</i> (%) [‡]	<i>n</i> (%) [‡]	
> 0.35 kU/L			
Bt	132 (60.8%)	133 (65.8%)	0.168
Dp	140 (64.5%)	136 (67.3%)	0.308
Df	139 (64.1%)	134 (66.3%)	0.349
Bg	49 (22.6%)	47 (23.3%)	0.479
Dog or cat	27 (12.4%)	30 (14.9%)	0.282
FEV1 (%)	91.6 $\pm$ 8.2	80.3 $\pm$ 13.6	< 0.01
FVC (%)	98.2 $\pm$ 9.8	95.1 $\pm$ 14.4	0.014
FEV1/FVC (%)	83.4 $\pm$ 5.0	74.4 $\pm$ 12.7	< 0.01

*AR: control group, allergic rhinitis children who did not develop early asthma.

[†]AR+AS: case group, allergic rhinitis children who develop early asthma.

[‡]Number/percent of allergen sensitivity (> 0.35 kU/L) in each group.

Bt, *Blomia tropicalis*; Dp, *Dermatophagoides pteronyssinus*; Df, *D. farinae*; Bg, *Blatella germanica*.

Table 2. Genotype frequencies of the CC16 gene polymorphism

Variable	AR*	AR+AS [†]	<i>P</i> value
CC 16			
AA	33 (15.2%)	63 (31.2%)	< 0.01
AG	82 (37.8%)	78 (38.6%)	
GG	102 (47.0%)	61 (30.2%)	
AA	33 (15.2%)	63 (31.2%)	< 0.01
Non-AA	184 (84.8%)	139 (68.8%)	
A	148 (34.1%)	204 (50.5%)	< 0.01
G	286 (65.9%)	200 (49.5%)	

*AR: control group, allergic rhinitis children who did not develop early asthma.

[†]AR+AS: case group, allergic rhinitis children who develop early asthma.

Serum total immunoglobulin E, allergen-specific immunoglobulin E and eosinophil count

No association was found between the CC16 genotype, eosinophil count, serum total IgE and allergen-specific IgE in different groups (Table 3). Total serum log IgE levels ($P = 0.272$) and Log eosinophil counts ($P = 0.195$) did not differ between the two groups. The distribution of allergen-specific IgE positivity in the two groups did not differ ($P > 0.05$). This means that subjects in both groups had abnormal systemic immunologic changes and that these

Table 3. The association between CC16 A38G polymorphism and parameters in different groups

	AR*		AR+AS†	
	AA	Non-AA	AA	Non-AA
Log IgE (kU/L)	2.51±0.15	2.41±0.08	2.50±0.12	2.48±0.10
Log eosinophil (/μL)	2.51±0.13	2.48±0.06	2.54±0.07	2.52±0.06
Allergen specific IgE >0.35 kU/L	n (%)	n (%)	n (%)	n (%)
Bt	22 (66.7%)	110 (59.8%)	38 (60.3%)	95 (68.3%)
Dp	21 (63.6%)	119 (64.7%)	43 (68.3%)	93 (66.9%)
Df	22 (66.7%)	117 (63.6%)	40 (63.5%)	94 (67.6%)
Bg	7 (21.2%)	42 (22.8%)	17 (27.0%)	30 (21.6%)
Dog or cat	5 (15.2%)	22 (12.0%)	9 (14.3%)	21 (15.1%)

*AR: control group, allergic rhinitis children who did not develop early asthma.

†AR+AS: case group, allergic rhinitis children who develop early asthma.

There is no significant association between different genotypes and parameters in different groups (all *P* value > 0.05).

Bt, *Blomia tropicalis*; Dp, *Dermatophagoides pteronyssinus*; Df, *D. farinae*; Bg, *Blatella germanica*.

changes were of the same magnitude, and the allergen sensitivity type of the two groups was almost the same.

Pulmonary function tests

The FEV1/FVC ratios were statistically different between control and case groups (Table 1). The FEV1/FVC ratios were not statistically different within the two groups when genotype groups were compared (AA and non-AA genotypes) (Table 3).

Serum CC16 concentration

In the control group (AR only), the mean serum CC16 level was 11.08±0.82 ng/mL. In the case group (AR developing asthma), the mean serum CC16 level was 10.51±0.85 ng/mL (*P*=0.350). Thus, we did not find an association between serum CC16 levels and asthma development in AR children.

In the control group, the serum CC16 levels in AA and non-AA genotype subjects were 10.92±1.47 and 11.10±0.94 ng/mL, respectively (Fig. 1). In the case group, the serum CC16 levels in AA and non-AA genotype subjects were 9.05±1.24 and 11.23±1.08 ng/mL, respectively. In the case group, those carrying the AA genotype had significantly lower serum CC16 levels than those carrying the non-AA genotype (*P*=0.016). Children in the case group carrying the AA genotype have significantly lower serum CC16 level than subjects in the control group with different genotypes (AA genotype, *P*=0.049, non-AA genotype, *P*=0.033; respectively). In contrast, there were no associations with regard to serum CC16 levels and different genotypes in the control group (*P*=0.781).

Discussion

Asthma and AR are currently thought of as a single common pathway. However, not all patients with rhinitis

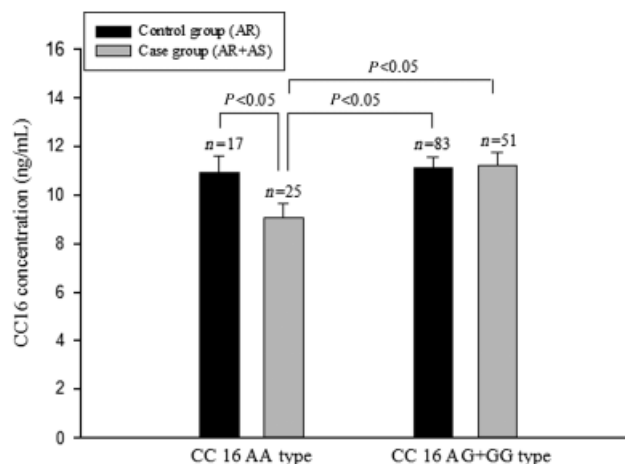


Fig. 1. Serum CC16 level in different genotype and phenotype. Serum CC16 levels were analysed by CC16 ELISA kits as described in Materials and methods. In case group [allergic rhinitis (AR) children who develop early asthma], serum CC16 level is significantly lower in subjects with the AA genotype than those with the non-AA genotype. In control group (AR children who did not develop early asthma), serum CC16 level is not significantly lower in subjects with the AA genotype than those with the non-AA genotype.

develop asthma. The prevalence of asthma in patients with rhinitis varies from 10% to 40% [15, 16]. Some factors must promote the development of asthma in patients with AR. However, the factors have so far not been clearly elucidated. In order to investigate this, we recruited children who had only AR for at least 5 years. We also recruited children with AR, who then developed the asthma phenotype 3–5 years after the onset of AR. We hypothesized that the frequency of the AA genotype might be higher and serum CC16 levels might be lower in rhinitis subjects who develop asthma than in those who did not. Then, we compared the association of the CC16 A38G polymorphism and serum CC16 levels between different groups.

Factors other than CC16 polymorphism and serum CC16 levels that may contribute to the development of different phenotypes must be excluded. Among adults, nasal symptoms are associated with chronic bronchitis, emphysema and asthma [17]. This suggests a potential confounding effect of chronic obstructive pulmonary disease. Therefore, we chose children as our study group to eliminate the confounder.

In patients with AR, naso-bronchial reflex, mouth breathing and pulmonary aspiration of nasal contents [18] may contribute to the development of asthma. In subjects with asthma preceding or both starting in the same year, the confounding factors from rhinitis mentioned above are not seen, or the duration is too short. In our study, the subjects of the case group had rhinitis preceding asthma for 3–5 years. In the control group, rhinitis was diagnosed for at least 5 years. Thus, the factors of asthma development from rhinitis mentioned above should be found in both groups; furthermore, the bias would decrease.

Under-diagnosis of asthma is a feature in many patients with rhinitis. One study [19] showed that 30% of rhinitis patients without an asthma diagnosis might be considered to be asthmatics. Defining AR in children is difficult because of the high frequency of upper respiratory infections. In our study, the diagnoses of asthma and AR were made by two immunologists according to specific criteria as well as pulmonary function tests. Therefore, misdiagnosis and selection bias were minimized.

Studies of asthma and AR have been mostly based on questionnaire responses from the patients or their parents [2, 19, 20]. Evaluation of symptoms with questionnaire is prone to recall bias. There is still no standardized questionnaire to evaluate for the relationship between AR and asthma. In our study, subjects were selected randomly, data were gathered from previous records and the diagnoses were made by two immunologists, rather than from questionnaire. Therefore, the recall bias and demerit of questionnaire were excluded.

Recently, the ARIA guidelines classified rhinitis according to both its duration (intermittent and persistent) and severity (mild and moderate/severe) [12]. Studies demonstrate that both the duration and the severity of nasal symptoms affected the risk of having a diagnosis of asthma [2]. To exclude severity as a confounding factor, the rhinitis symptoms of subjects in our study were all moderate/severe persistent. The duration between first rhinitis diagnosis and first asthma diagnosis was approximately 3–5 years.

Studies [21] indicated that both AR and positive skin test responses increased the risk of development of asthma. Boulet et al. [22] demonstrated that sensitization to indoor allergens was frequently associated with the presence of asthma, whereas sensitization to pollen alone was associated with the presence of rhinitis. The study results

lead to the question of whether atopic status or allergen type was acting as a confounder. In our study, subjects in the control group and the case group, the allergen sensitivity type were the same (Table 1). Thus, the confounder was excluded. One study [23] indicated that different environments appear to exert an effect via opposite alleles of CC16 gene, and then may generate different phenotypes of allergic disease. In our study, subjects were from the same area (Taichung, Taiwan), with the same environment. Thus, the confounder of different environments was excluded.

Peripheral blood eosinophil counts and total IgE levels may reflect severity [24], hyper-responsiveness [25] and inflammation of airway [26]. In our study, serum total IgE levels and eosinophil counts did not differ between the two groups (Table 1). From these data, we noted that subjects both groups had the same 'systemic immunologic changes' and the same levels of 'airway inflammation'.

With the same systemic immunologic changes, the same allergen sensitivity in both groups, and excluding other confounding factors and bias that may have contributed to the development of the asthma phenotype, we then compared the association of the CC16 genotypes and serum CC16 levels with different phenotypes. In our study results (Table 2), we found that in the case group (AR developing asthma), the frequency of the AA genotype was higher than in the control group (AR only). AR children with AA genotype were 2.527-folds more likely to develop asthma than those with AA and AG genotypes. Now, we have shown that the CC16 A38G polymorphism is associated with the development of early asthma in children with AR.

With regard to the association between CC16 level, different genotypes and study groups, we had some findings. The case group had more AA genotypes or A alleles than controls; cases with the AA genotype have significantly lower serum CC16 levels than (1) the cases with non-AA genotypes and (2) all the controls. Thus, there seems to be a genotype-dependent difference in CC16 levels between cases and controls. CC16 genotype is already known to alter gene expression levels of the protein [11], and therefore, the genotype would be expected to be associated with CC16 levels measured by ELISA in this study. It would have been expected that this association would be evident in the controls as well as the cases, but there are reasons why this may not have occurred, such as, (1) it may be that the differences in CC16 levels for children with different genotypes are smaller in control children and thus require larger numbers of children to reach statistical significance and/or (2) it may be that the contribution of the SNP to protein levels is only clear when the gene expression is stimulated, rather than constitutive. We could not conclude that this study provided clear evidence that genotype altered levels in the cases and caused the development of asthma, as the

blood was taken in the last year of the study, after asthma had developed in the children. However, the association of CC16 genotype with circulating levels evident at the end of the study may have been present and contributed to the development of asthma in the cases earlier in life

Whether the A38G polymorphism had a functional role in the development of the early asthma phenotype in children with AR, or was just in linkage disequilibrium with another aetiologically important mutation remains to be elucidated. However, our results showed that in children with AR who develop early asthma (case group) with the AA genotype had significantly lower serum CC16 levels than those with the non-AA genotypes as well as children with rhinitis who did not develop asthma regardless of genotype (Fig. 1). The results support our hypothesis that A38G genotype may play a functional role in the development of early asthma phenotype in children with AR by way of CC16 level, rather than in linkage disequilibrium with another aetiologically important mutation.

There were some drawbacks of our study. Firstly, our study is a case-control study, and can only find the association, rather than the causation. A cohort study is a better method to study the aetiologies that cause the asthma phenotype development in AR patients. We aim to complete a cohort study in the future. Secondly, our study focused on those with rhinitis preceding asthma. A future study of adults with asthma preceding rhinitis and starting in the same year will be necessary.

Why some AR patients develop asthma and others do not is still not fully understood. In our study, we first demonstrated that a genetic factor, the AA genotype of the CC16 A38G polymorphism, is associated with a higher risk of early asthma in children with AR. Although serum CC16 cannot be used for prediction, we can speculate that in rhinitis patients, those with both the AA genotype and lower CC16 levels have the highest risk for the development of asthma (Fig. 1). Further study is necessary.

It is not accurate to say that the polymorphism is the major cause of early asthma development in children with AR as there must be other factors that play a role in the development of asthma. The magnitude of the effect of CC16 gene to asthma pre-disposition is currently unknown [27]. Whether the CC16 polymorphism or serum levels are involved in the pathogenesis of the development early asthma in children with AR requires further confirmation by other cohort studies.

Clinical relevance

In the future, we may be able to use the CC16 A38G polymorphism to predict the high-risk patients and intervene to prevent the development of asthma in children with rhinitis. Our research also points out a new way to explore the underlying mechanisms, genetic role and aggravating factors for asthma development in rhinitis children.

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