

Original article

Infant frequent wheezing correlated to Clara cell protein 10 (CC10) polymorphism and concentration, but not allergy sensitization, in a perinatal cohort study

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Background: Wheezing episodes are common in young infants. However, the molecular mechanism of wheezing is unclear, and very few therapeutic regimens are effective.

Objective: This study investigated the genetic and environmental factors predisposing to infant wheezing in a birth cohort study.

Methods: A cohort of 1211 pregnant women was recruited for this study. Infant wheezing episodes during the first 18 months of life were correlated to parental atopic history, parental smoking, prematurity, CB IgE levels, and the sequence variant (G+38A) of the Clara cell protein 10 (CC10) gene encoding a secretory anti-inflammatory CC10 protein.

Results: Nine hundred eighty-three infants completed umbilical cord blood collection, and 813 infants completed the 18-month postnatal follow-up. Twenty-two percent of the infants experienced at least 1 wheezing episode, and 6.6% of the infants experienced frequent wheezing (≥ 3 episodes). Multivariate logistic regression showed that male sex and the CC10 G+38A polymorphism, but not prematurity, CB IgE level, passive smoking, or parental atopy, were predictors of frequent wheezing. Further studies found that infant frequent wheezing was significantly associated with the CC10 +38AA genotype and lower plasma CC10 levels at 18 months of age ($P = .046$), and infants with acute wheezing episodes had significantly lower CC10 levels than those without ($P = .023$). No association of wheezing episodes with allergic sensitization was observed in this cohort population.

Conclusion: Infant frequent wheezing is associated with the CC10 G+38A polymorphism and lower CC10 levels but not infant atopy.

Clinical implications: Lower CC10 expression, but not allergy sensitization, is involved in the pathogenesis of infant frequent wheezing. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Wheezing infants, cord blood IgE, gender, Clara cell 10-kd protein, polymorphism, asthma

A large population of young infants experience wheezing episodes,^{1,2} a significant debilitating problem for which very few therapeutic regimens are proved to be effective.³⁻⁵ The younger the child, the greater the possibility that a diagnosis other than atopy might explain the recurrent wheeze.¹ Also, the identity of the causative, genetic, and/or environmental factors underlying the susceptibility to wheezing disorders remains as yet to be defined.¹⁻⁶ Although family history of atopy associated with early wheezing episodes or recurrent chest infections in infancy was correlated with the development of childhood asthma,^{7,8} a recent study reported that infant wheezing should not be used as an indicator of the atopic phenotype.⁹ This suggests that the occurrence of frequent wheezing episodes in infants might not be simply related to the atopic status but rather that both genetic and environmental factors could predispose for wheezing episodes of infants through altered inflammatory responses.

Many genes have been implicated in the development of asthma,^{10,11} but very few studies have examined the genetic aspect of infant wheezing. Of interest, the sequence variant (G+38A; relative to the transcription initiation site) of the Clara cell 10-kd protein (CC10) gene encoding an anti-inflammatory CC10 protein has been shown to be associated with asthma.¹² CC10, also called CC16, uteroglobin, or secretoglobin 1A1, with primary expression in the uterus and nonciliated bronchiolar cells, has an anti-inflammatory effect on the urogenital and respiratory tracts.¹³⁻¹⁶ In a CC10-deficient mouse model, we have shown that deficiency of CC10 aggravates pulmonary allergic inflammation through augmentation of the TH2 response, and reconstitution of CC10 in CC10-deficient mice is able to reverse the altered phenotypes.^{17,18} As a

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Abbreviations used

CB: Cord blood
 CC10: Clara cell secretory 10-kd protein
 OR: Odds ratio

corollary, Shashikant et al¹⁹ also showed that administration of recombinant CC10 improved lung function and decreased pulmonary inflammation in a lamb model of respiratory distress syndrome. Moreover, clinical studies also showed that a significant decrease in blood CC10 levels was found in patients with transplantation-induced obliterative bronchiolitis,²⁰ and a significantly lower CC10 expression on the epithelium of the small airways was found in patients with chronic obstructive pulmonary disease,²¹ suggesting its potential involvement in the inflammatory disorders of the small airways.

In a previous study we showed that maternal atopy defined by maternal atopic history plus detectable specific IgE levels directed against 1 or more allergens of the common allergens in Taiwan was correlated with increased cord blood (CB) IgE levels and infant atopic eczema in a Chinese birth cohort population.²² To evaluate the effects of genetic and perinatal factors on frequent wheezing episodes of young infants, we sought to study this population for the association of infant wheezing episodes with perinatal factors, including parental atopy, sex, passive smoking, and prematurity, and with the polymorphism and plasma levels of CC10. We provided evidence herein that in this cohort population an increase in infant wheezing episodes is associated with the genetic polymorphism and reduced plasma levels of CC10 but not with infant atopy.

METHODS

Subjects and CB collection

A total of 1211 pregnant women were prenatally recruited for this study after informed consent was obtained. A research nurse was trained to explain the purpose of this study to eligible pregnant women as they visited our obstetric clinic. On recruitment, a questionnaire was applied to collect a history of family allergy and parental smoking. Blood samples from both parents were collected for measurement of allergen sensitization with detectable specific IgE levels.²² CB samples were collected between September 1999 and December 2004 at Chang Gung Memorial Hospital, Kaoshiung, Taiwan. Serum samples and blood cells in umbilical cord blood were separated by means of centrifugation at 800g for 15 minutes and subjected to measurement of CB IgE levels and collection of DNA samples. Nine hundred eighty-three (81.2%) newborns from the 1211 participating families completed CB collection for CB IgE detection and DNA extraction for determination of CC10 gene polymorphisms (see below). CB collection was not performed for 228 (18.8%) newborns because of drop-offs or emergency delivery. Of the 983 newborn infants, 813 returned for the 18-month follow-up visit, at which a questionnaire was used to assess wheezing episodes that were physician diagnosed and plasma samples were collected for measurement of plasma CC10 concentration from infants whose parents agreed to the blood test.

CB IgE detection and determination of parental atopic diseases

Increases of CB IgE levels have been correlated to atopic disease in childhood.^{23,24} In this study we also measured CB IgE levels by using a sensitive low-range IgE detection system (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden).^{22,24} Because distribution of the CB IgE levels in raw data or log-transformed data is not a normal distribution, we defined CB IgE increase at the cut-off value of 0.5 kU/L or greater (>80th percentile), as previously described.²²⁻²⁵ Paternal and maternal atopy were defined as clinical diagnosis of asthma, rhinitis, or atopic dermatitis, together with detectable serum specific IgE levels directed against 1 or more allergens of the common allergens in Taiwan, including *Dermatophagoides pteronyssinus*, cockroach, dog dander, seafood, or pollen mixture.²⁶ The serum specific IgE was detected by using the Pharmacia Cap system, as previously described.^{22,26}

DNA extraction and determination of CC10 polymorphisms

CB leukocytes were subjected to DNA extraction with 0.5% SDS lysing buffer, followed by proteinase K (1 mg/mL) digestion of nuclear protein for 4 hours at 60°C.^{25,26} Genomic DNA was harvested by means of standard phenol-chloroform extraction, followed by 70% alcohol precipitation. The CC10 G+38A polymorphism was studied by means of RFLP of PCR products. Genomic DNAs were amplified by the use of the following primers in a total of 40 cycles: forward primer, 5'-GCA GTA TCT TAT GTA GAG CCC T-3'; reverse primer, 5'-TCT CTG AGC ACT CAC CGG AGC T-3'. The PCR products were incubated with the *Sau96 I* restriction enzyme (New England Biolabs, Boston, Mass) at 37°C for 6 hours before being subjected to a 2% agarose gel electrophoresis analysis of RFLP. Three different visible products representing AA, AG, and GG genotypes, respectively, could be read on the gels.²⁵

Measurement of plasma CC10 concentrations and infant allergy sensitization

To prove that the CC10 G+38A polymorphism involved in recurrent wheezing of young infants is correlated to plasma CC10 levels but not allergy sensitization, we collected 222 plasma samples of 18-month-old infants in this cohort population whose parents agreed to the plasma collection at the 18-month follow-up visit. The plasma samples were collected after informed consent was obtained from the parents. To confirm that acute wheezing of infants was associated with CC10 plasma concentrations, we additionally included plasma samples from infants aged between 6 and 36 months with acute wheezing episodes (n = 49) and those without wheezing (n = 63) outside the cohort population as positive and negative controls. The plasma samples were collected after informed consent was obtained from the parents. The mean ages (mean ± SD) among the 18-month cohort follow-up infants (1.54 ± 0.08 years), the infants with acute wheezing episodes (1.59 ± 0.75 years), and the infants without acute wheezing episodes (1.58 ± 1.02 years) were not significantly different (*P* = .853). The plasma CC10 levels in the 3 groups of infants were measured by using ELISA at the manufacturer's recommended time. The CC10 ELISA assay kits were purchased from BioVendor Laboratory Inc (Modrice, Czech Republic). The plasma samples from 18-month-old infants in this cohort population were also subjected to detection of infant allergy sensitization, as determined by using the Pharmacia CAP assay of specific IgE (≥0.50 KU/L) directed against infant common allergens: milk, egg white, peanut, *Candida albicans*, or house dust mite.

Data analysis and statistics

The rates of ever wheezing (≥1 times) and frequent wheezing (≥3 times) episodes in the first 18 months of age were correlated with

parental atopy, increase of CB IgE levels, different sexes, genotypes, or alleles by using univariate analysis and multivariate logistic regression. Because 22% of infants had ever wheezed (≥ 1 times) during their first 18 months of life in this study and one third of wheezing infants had persistent wheezing in previous studies,⁶⁻⁸ we therefore defined frequent wheezing episodes as 3 or more times, which approximately accounted for one third of all wheezing infants (6.6% vs 22%). Univariate analysis was used to analyze the association of infant wheezing episodes with prenatal environmental factors and *CC10 G+38A* polymorphisms. In consideration of interaction among independent variables predictive of frequent wheezing episodes, a multivariate logistic regression model was used to explore the interaction.²⁷ CC10 plasma concentrations were presented as the mean $\pm$ SD and tested by using ANOVA and the Student *t* test. The study power estimation was originally set at 0.9, which was based on a 2-group χ^2 test with a 2-sided significance α level of .05 and the effect size at a 20% difference of the rates of frequent wheezing episodes between 2 groups. A *P* value or less than .05 was regarded as statistical significance. SPSS (version 13.0; SPSS, Inc, Chicago, Ill) software was used for statistical analysis.

RESULTS

Demographic data of subjects with and without completion of the 18-month follow-up

A total of 1211 pregnant women were initially recruited for this cohort study. Nine hundred eighty-three of the newborn infants completed CB collection, and 813 of the 983 newborn infants returned for the 18-month follow-up of wheezing episodes in our pediatric outpatient clinic. Demographic data between infants with and without completion of the perinatal follow-up (813 vs 398) revealed no significant differences in terms of maternal atopy, paternal atopy, passive smoking, or prematurity (Table I).

Correlation of ever wheezing to *CC10* polymorphisms and perinatal risk factors

Because many studies have shown that infants with 3 or more wheezing episodes tended to be atopic, we therefore stratified wheezing infants into those with at least 1 episode of wheezing (ever wheezing, ≥ 1 time) and those with 3 or more episodes of wheezing (frequent wheezing) for analysis. It was found that 22.0% of infants experienced at least 1 episode of wheezing during the first 18 months of life. In univariate analysis we found that male subjects had a significantly higher rate of ever wheezing (25.1% vs 18.4%, *P* = .023). Prematurity (<37 weeks of gestational age) revealed a trend toward association with ever wheezing (26.5% vs 21.7%, *P* = .432), although it did not reach statistical significance. Infants with the AA genotype had a higher rate of CB IgE increase than those with the AG or GG genotypes (27.8% vs 21.2%, *P* = .141), but this did not reach statistical significance. Maternal atopy, paternal atopy, prematurity, passive smoking, and CB IgE increase were not correlated with ever wheezing in infants (Table II). Multivariate logistic regression analysis also revealed that only male sex was significantly associated with infants ever wheezing (odds ratio [OR], 1.60; Table II).

TABLE I. Demographic data of infants with and without completion of umbilical cord blood collection and the 18-month follow-up

Perinatal factors	Completion (n = 813)	Loss of follow-up (n = 398)*	<i>P</i> value
Sex of infants (male)	54.0%	51.5%	.388
Preterm (<37 wk)	6.0%	5.8%	.851
Maternal atopy	24.5%	21.2%	.172
Paternal atopy	22.2%	25.0%	.248
Parental smoking†	34.1%	34.0%	.972

*Of 1211 participants, 228 infants were lost to CB collection, and 170 infants were lost to the 18-month follow-up. *P* values are analyzed by using the χ^2 test.

†In this cohort study only 4 of the mothers have a smoking habit, but the rate of paternal smoking (34.1%) is similar to that of other studies.

Correlation of frequent wheezing to *CC10* polymorphisms and perinatal risk factors

In analysis of frequent wheezing episodes (≥ 3 episodes), it was found that 6.6% of infants had experienced frequent wheezing episodes during the first 18 months of life. The *CC10 G+38A* polymorphism and male sex, but not maternal atopy, paternal atopy, prematurity, passive smoking, or CB IgE increase, were significantly correlated to infants with frequent wheezing (Table III). Infants with the AA genotype had a significantly higher rate of frequent wheezing than those with the AG or GG genotypes (12.4% vs 5.9%, *P* = .016). In multivariate analysis we also found that male sex revealed a significant correlation with frequent wheezing (≥ 3 times) in infants (OR, 3.48; *P* = .001), and the *CC10 +38G* allele appeared to be protective against frequent wheezing episodes (OR, 0.45; *P* = .007). When the interaction between sex and the *CC10 G+38A* polymorphism was considered, male infants with the AA genotype had the highest risk of frequent wheezing (OR, 3.80; *P* = .016; Fig 1).

Correlation of infant wheezing to plasma CC10 levels but not allergy sensitization

After identifying that the *CC10 G+38A* polymorphism was significantly associated with infant frequent wheezing, the relationship between infant frequent wheezing and plasma CC10 levels was examined. As shown in Fig 2, A, there was a significant difference among infants carrying different *CC10 G+38A* polymorphisms in this cohort study (*P* = .032), and infants with the AA genotype had the lowest CC10 concentration (3.12 ± 0.31 vs 3.19 ± 0.20 vs 3.96 ± 0.26 $\mu\text{g/L}$). Infants with frequent wheezing (≥ 3 times) carrying the AA genotype, which was significantly associated with a higher rate of frequent wheezing, had a significantly lower plasma CC10 concentration than those carrying the GA or GG genotypes (2.61 ± 0.40 vs 3.11 ± 0.39 vs 4.08 ± 0.71 $\mu\text{g/L}$, *P* = .046; Fig 2, B). In contrast, different frequencies of wheezing was not significantly associated with the allergy sensitization of detectable specific IgE directed against 1 or more allergens of the infant common allergens at the age of 18 months (*P* = .241; Fig 2, C). An additional independent

TABLE II. Association of ever wheezing (≥ 1 episodes) during the first 18 months of life with *CC10* polymorphism and perinatal factors

	Ever wheezing (≥ 1 episodes; ratio)	P value of UVA	P value of MVA*	OR (95% CI)
<i>CC10</i> +38A/G polymorphisms				
AA vs AG vs GG	27/97 vs 81/388 vs 71/328	.327	—	—
AA + AG vs GG	108/485 vs 71/328	.834	.839	.67~1.38
AG + GG vs AA	152/716 vs 27/97	.141	.159	.42~1.16
Perinatal factors				
Male vs female sex	110/439 vs 69/374	.023	.022	1.06~2.10
CB IgE levels	39/172 vs 140/641	.815	.929	.65~1.50
Preterm	13/49 vs 166/764	.432	.368	.38~1.43
Maternal atopy	46/204 vs 133/609	.832	.823	.71~1.55
Paternal atopy	33/181 vs 146/632	.163	.166	.49~1.13
Parental smoking	56/277 vs 123/536	.373	.323	.58~1.20

UVA, Univariate analysis; MVA, multivariate logistic regression analysis; OR, odds ratio.

*Variables included for the multivariate logistic regression analysis are *CC10* G+38A polymorphisms (A allele: AA + AG vs GG; G allele: GG + GA vs AA; not 3 genotypes) and perinatal factors.

TABLE III. Association of frequent wheezing (≥ 3 episodes) during the first 18 months of life with *CC10* polymorphism and perinatal factors

	Frequent wheezing (≥ 3 episodes; ratio)	P value of UVA	P value of MVA*	OR (95% CI)
<i>CC10</i> +38A/G polymorphisms				
AA vs AG vs GG	12/97 vs 17/388 vs 25/328	.012	—	—
AA + AG vs GG	29/485 vs 25/328	.356	.065	.29~1.04
AG + GG vs AA	42/716 vs 12/97	.016	.007	.15~.74
Perinatal factors				
Male vs female sex	41/439 vs 13/374	.001	.001	1.49~5.44
CB IgE levels	11/172 vs 43/641	.884	.825	.45~1.88
Preterm	3/49 vs 51/764	1.000	.898	.32~3.68
Maternal atopy	9/204 vs 45/609	.139	.189	.28~1.28
Paternal atopy	12/181 vs 42/632	.994	.956	.52~2.00
Parental smoking	18/277 vs 36/536	.906	.736	.50~1.64

UVA, Univariate analysis; MVA, multivariate logistic regression analysis; OR, odds ratio.

*Variables included for the multivariate logistic regression analysis are *CC10* G+38A polymorphisms (A allele: AA + AG vs GG; G allele: GG + GA vs AA; not 3 genotypes) and perinatal factors.

group of age-matched infants with ($n = 49$) and without ($n = 63$) acute wheezing episodes was included for comparison to confirm that the lower plasma CC10 concentration was associated with acute wheezing episodes. The results showed that similar to those found in this cohort study, infants with acute wheezing episodes had significantly lower CC10 plasma levels than nonwheezing infants (3.35 ± 0.24 vs 4.05 ± 0.23 $\mu\text{g/L}$, $P = .023$; Fig 2, D).

DISCUSSION

The prevalence of infant wheezing and childhood asthma has significantly increased in the past 2 decades.^{1,2,28,29} Infants of atopic parents who have early wheezing episodes or recurrent chest infections are often given diagnoses of infantile asthma.^{7,8} They might be treated with corticosteroids or an inhaled bronchodilator. Unfortunately, the use of corticosteroids or an inhaled

bronchodilator for wheezy infants has been proved useless in randomized clinical trials.³⁻⁵ In this perinatal cohort study we found that frequent wheezing episodes of infants were not correlated to parental atopy, CB IgE levels, or infant allergy sensitization but were significantly correlated to sex and the AA genotype of the *CC10* G+38A variant, a genotype with the lowest plasma CC10 concentration. This result is considered important because infants who wheeze at this age are unlikely to have aeroallergen sensitization by that time, and other genetic and environmental predispositions, such as *CC10* and sex, are likely involved. To our knowledge, this is the first study to show that the *CC10* G+38A polymorphism is associated with recurrent wheeze in infants, independent of atopic mechanism.

Wheeze is more common in boys for many reasons, most of which are not known at present. The presence of the *CC10* AA genotype might simply reduce the threshold for wheezing when other factors are present. Given the

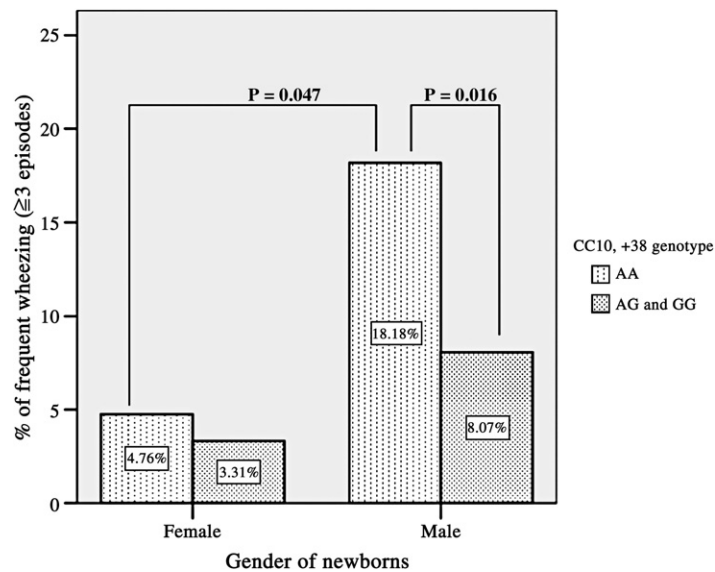


FIG 1. Interaction of male sex with the *CC10* +38AA genotype on frequent wheezing (≥ 3 episodes).

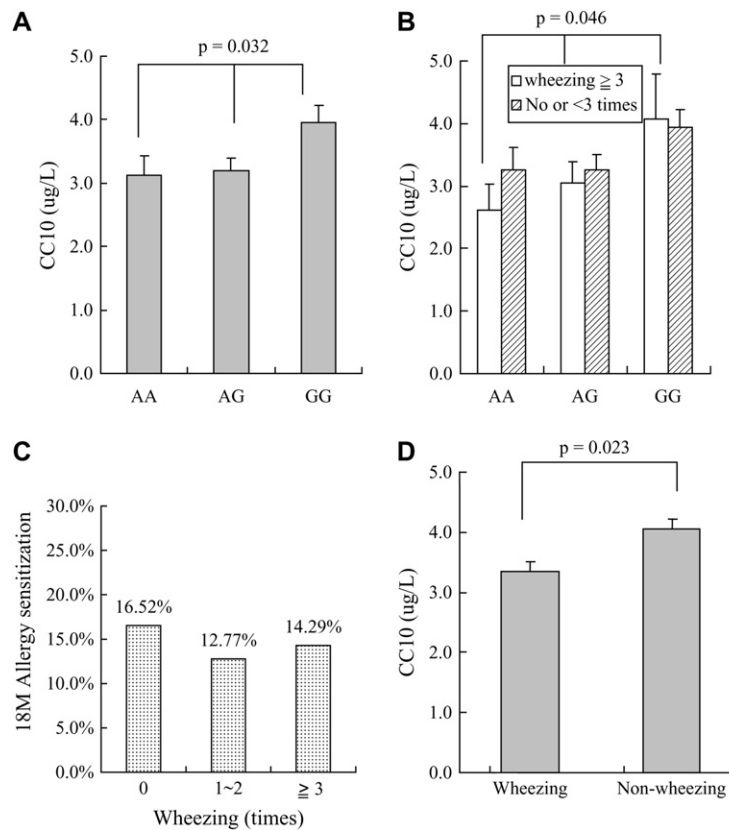


FIG 2. Infant wheezing associated with the *CC10* G+38A polymorphism and plasma concentration but not allergy sensitization at the age of 18 months. **A**, Infants with the *CC10* +38AA allele had lower CC10 levels than those with the *CC10* +38GG genotype at the age of 18 months ($n = 222$; $P = .032$, ANOVA). **B**, Infants with frequent wheezing (≥ 3 episodes) carrying the *CC10* +38AA genotype had the lowest plasma CC10 concentration ($P = .046$, ANOVA). **C**, Infants with different numbers of wheezing episodes (0, 1-2, or ≥ 3) were not significantly ($P = .241$, χ^2 test) associated with allergy sensitization of the 18-month (18M) infants. **D**, In an additional study with age-matched infants outside this cohort population, it was demonstrated that infants ($n = 49$) with acute wheezing episodes had significantly ($P = .023$, t test) lower CC10 concentrations than nonwheezing infants ($n = 63$).

information on sex, atopic character, *CC10* *G*+38A polymorphism, and plasma CC10 concentration, frequent wheezing in infants should be differentiated from atopic asthma in terms of diagnosis and treatment.

The Tucson Children's Respiratory Study has pointed out that infants with recurrent wheezing episodes can be classified into atopic wheezing, nonatopic wheezing, and transient wheezing categories while they grow up to childhood and early adult life.⁶ Another study from the Danish National Cohort has recently reported that infant wheezing should not be used as an indicator of the atopic phenotype.⁹ A couple of nonatopic factors have been implicated in infants with frequent wheezing episodes. Smaller caliber of airways has been attributed to the higher incidence of bronchiolitis in premature babies.³⁰ In this study we found that prematurity tended to predispose to a higher rate of wheezing episodes, but it did not reach a significant correlation to infant wheezing episodes. Passive smoking exposure has been implicated in the development of asthma and chronic obstructive pulmonary disease.^{31,32} In our analysis we did not find the association of passive (parental) smoking with frequent wheezing episodes in young infants. This could be because passive smoking is not a risk factor for infant wheezing, or it might be that Chinese parents usually do not smoke in front of their babies, although they have a similar prevalence (34.1%) of paternal smoking habit.

CC10, a protein secreted from nonciliated bronchiolar Clara cells and urogenital epithelium,^{13,14} has been implicated in fetal lung growth³³ and protection from infections and oxidation stress.³⁴ The sequence variant (*G*+38A) of the *CC10* gene was first shown to be associated with asthma in Australian children in 1998^{12,35} and later in adult Danes in 2005.³⁶ However, certain studies showed no significant association of the *CC10* *G*+38A polymorphism with asthma in British, Japanese, and German populations.³⁷⁻³⁹ A study with German children found that the *CC10* +38AA genotype was associated with airway hyperreactivity but not with childhood allergic asthma.⁴⁰ The study with adult Danes also reported an atopy-independent association between the *CC10* +38AA genotype and asthma prevalence.³⁷ These discrepant results might be due, in part, to the differences in the severity, age distribution, and inclusion of both atopic and nonatopic asthmatic populations in their studies. The present study on the frequent wheezing of infants younger than 18 months old indicates that the *CC10* +38AA genotype is associated with infant frequent wheezing, independent of allergy sensitization.

The mechanism by which the *CC10* +38AA genotype was associated with asthma or airway inflammation is related to its functional linkage to a lower plasma CC10 concentration.^{35,36} This is supported by the present study showing that infant frequent wheezing was associated with the *CC10* +38AA genotype and its lower plasma levels and by other studies showing a decrease of CC10 serum levels in patients with transplantation-related obliterative bronchiolitis.²⁰ In contrast, an increase in blood CC10 levels was also implicated in acute lung injury.⁴¹

Given the evidence that CC10 recombinant protein can correct pulmonary inflammatory insults of CC10-deficient animals,^{18,42} augment surfactant improvement of respiratory distress syndrome in infant animals,¹⁹ and improve pulmonary inflammatory indices in premature babies with respiratory distress syndrome,⁴³ it is likely that a decrease of CC10 secretion is involved in airway inflammation and airway hyperreactivity, and correction of CC10 expression improves airway inflammation and hyperreactivity. Whether the *CC10* polymorphism associated with a lower CC10 concentration is the real pathogenesis of infant frequent wheezing requires further confirmation by other cohort studies.

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