A polymorphism of the CC16 gene is associated with an increased risk of asthma

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Abstract
Several quantitative traits associated with the asthma phenotype have been linked to markers on chromosome 11q13, although the gene responsible has yet to be well established. The gene for Clara cell secretory protein (CC16) is an ideal candidate for involvement in an inherited predisposition to asthma because of its chromosomal location, the role of the CC16 protein in controlling airway inflammation, and differences in levels of the protein between asthmatics and healthy controls. All three CC16 exons were screened in an unselected population of 266 subjects from 76 families and a cohort of 52 severely asthmatic children. A combination of single strand conformational polymorphism (SSCP) analysis, heteroduplex analysis, DNA sequencing, and restriction digestion was used. Mutation detection methods identified an adenine to guanine substitution in the CC16 gene at position 38 (A38G) downstream from the transcription initiation site within the non-coding region of exon 1. In the unselected population, 43.6% were homozygous for the polymorphic sequence (38GG) and 46.2% were heterozygous (38AG). All the asthmatic and unaffected children from both populations were selected for an unmatched case control analysis consisting of 67 asthmatic and 46 unaffected subjects. Those homozygous for the published sequence (38AA) had a 6.9-fold increased risk of developing asthma (p=0.049) and heterozygotes (38AG) a 4.2-fold increased risk (p=0.028). Modelling of genotype as a continuous covariate indicated evidence of a significant linear trend across the three genotypes (odds ratio=2.84 per unit increase in genotype code, p=0.018). These associations were independent of age, gender, and tobacco smoke exposure. These data and the known anti-inflammatory role of CC16 in the respiratory tract suggest that alteration to the gene at position 38 may contribute to asthma. (J Med Genet 1998;35:463-467)

Keywords: CC16; polymorphism; asthma

Mutation screening of asthma candidate genes aims to detect sequence variations that alter the concentration or activity of the protein coded by that gene, ultimately contributing to the development of clinical asthma. Clara cell secretory protein (CC16) is a 16 kDa protein secreted by the non-ciliated, non-mucous secretory cells of the tracheobronchial epithelium and urogenital tract. CC16 has until recently been known as CC10 (mostly because of initial evidence that it was a 10 kDa protein) or CCSP (Clara cell secretory protein). The gene for CC16 has many attributes that suggest it is a plausible asthma candidate gene. It is located on the long arm of chromosome 11 between markers D11S16 and D11S97, within a region linked to atopy and increased airway responsiveness. Several candidate genes have been identified within this region, including the gene for the lymphocyte surface marker CD20 and the beta chain of the high affinity IgE receptor (FceRI-β). Several polymorphisms have been identified within the FcεRI-β gene, although to date none has been proven to alter the expression or function of the protein.

Secondly, high levels of CC16 are produced in the airway. It is the most common protein in the bronchoalveolar lavage fluid (BALF) of healthy non-smokers, averaging 7% of the BALF total protein content. CC16 levels are significantly lower in the BALF of asthmatic patients. CC16 appears to function as an anti-inflammatory agent in both the respiratory and urogenital tracts and can react with transthyretin to form a complex capable of suppressing the antigenicity of foreign proteins. This protects embryonic cells and spermatozoa from the maternal immune defence system in the urogenital tract. Micromolar concentrations of CC16 inhibit both neutrophil and monocyte chemotaxis, so preventing infiltration of inflammatory cells. Moreover, CC16 acts to regulate inflammatory cell chemotaxis in the respiratory tract as shown by its control of fibroblast proliferation and migratory behaviour. CC16 is also a potent inhibitor of phospholipase A2 (PLA2) activity, which limits the metabolism of arachidonic acid and the synthesis of prostaglandin and leukotriene mediators, which have been implicated in the pathogenesis of several inflammatory lung diseases. A side effect of PLA2 inhibition by CC16 is the reduced stimulation of receptor coupled PLA2, which thus contributes to the inhibition of interferon gamma (IFNγ) production and activity.

The CC16 gene is 4995 bp in length and consists of a 5' untranslated region and three short exons which are 550, 118, 187, and 135 bp, respectively. A microsatellite marker has been identified within a 190 bp region of intron 1 (+2921 to +3110) and no association was
found with asthma or allergy. In addition, a polymorphic Alu insert was found in intron 2. Neither of the two polymorphic regions appear to affect splicing of the CC16 mRNA transcript.

The CC16 gene is an ideal candidate for an inherited predisposition for asthma owing to its chromosomal location, nature, and function. The aim of this study was to screen the exons of the CC16 gene for sequence variations and determine whether these were associated with clinical asthma.

**Methods**

**Subjects**

Data and DNA were collected from two populations in this study. The first consisted of 266 white subjects from 76 nuclear families living in Perth, Western Australia. Proband families from this population were gathered as part of a long term follow up study of a birth cohort recruited randomly from a suburban general hospital antenatal clinic. Families were contacted when the index child reached 6 years of age and were unselected with respect to asthma status. From this population 47 non-asthmatic controls and 15 affected subjects were identified for the case control study.

The second population comprised 52 children selected on the basis of symptomatic asthma, from a respiratory outpatient clinic at a tertiary children's hospital in Perth, Western Australia. Subjects were white and aged 6 to 12 years. All of these children were included in the case control study.

Parental informed consent was obtained for all subjects. An EDTA anticoagulated blood sample was collected by venipuncture and a modified American Thoracic Society questionnaire was completed by the mother of each participant. Data were collected from both populations by the same investigators using identical methods.

Spirometry was performed using a hand held spirometer (Welch Allyn model No 61000) calibrated before each use. Airway responsiveness to inhaled histamine was assessed using the method described by Yan et al. The cumulative provocative dose of histamine producing a 20% fall (PD20) in forced expiratory volume over 1 second (FEV1) below post-saline value was calculated from the log dose response curve. The maximal cumulative dose given was 7.8 μmol histamine.

Sixty-seven cases were initially selected from the asthmatic and unselected populations on the basis of current respiratory physician diagnosed asthma requiring the use of prophylactic medication. Additional secondary definitions of case status (that is, asthma) included: (1) a positive response to the question: “Has your child ever had asthma diagnosed by a doctor?” and at least a 20% reduction in FEV1 by completion of a histamine challenge (that is, exhibited bronchial hyper-responsiveness (BHR)); (2) a positive response to the question: “Does his/her chest ever sound wheezy or whistling occasionally apart from colds?” and BHR.

Seventy and 30 cases, respectively, were identified for each of these secondary definitions. Controls were identified from the unselected population and were defined as those children with no personal or family history of asthma or other atopic disease and less than a 20% reduction in FEV1, by completion of a histamine challenge. All cases and controls were less than 12 years of age and were genetically unrelated.

**Mutation Detection**

Genomic DNA was extracted using a standard phenol/chloroform procedure from peripheral blood mononuclear cells obtained through centrifugation of whole blood.

The CC16 nucleotide sequence was obtained from published data and was used to design appropriate primers for the amplification of all three exons by polymerase chain reaction (PCR): exon 1: 5' CAGTATCTATT-GTGAAGCCG 3' and 5' CCTGAGATGTTCC-CTAAGTCCAGG 3', exon 2: 5' CCTGGAAGCATGTTGGCTCTCTCCG 3' and 5' CCGGCCGGAAGCCACTCTTCTAACC 3', exon 3: 5' GCAATTCCATTCACTGTTGGATTGCGG 3' and 5' GAGATGCTTTGGGTGTTATGAGG 3'.

For the PCR amplification of each exon, 100 ng of genomic DNA was used as template in a 25 μl reaction mixture which contained the following reagents: 3 pmol each primer (Bresatec), 0.5 units of Taq DNA polymerase (Biotech International), 1 x reaction buffer (67 mmol/l Tris-HCl, pH 8.8, at 25°C, 16.6 mmol/l [NH4]2SO4, 0.45% (v/v) Triton X-100, 0.2 mg/ml gelatin), 1.5 mmol/l MgCl2, and 200 μmol/l of each deoxynucleotide triphosphate (Biotech International). Following an initial denaturation at 95°C for five minutes the reactions were cycled 35 times through a temperature profile of 95°C for one minute, 58°C for one minute, and 72°C for two minutes. A final extension was performed at 72°C for 10 minutes. The products of the reaction were visualised by flat bed agarose gel electrophoresis in a 2% agarose gel containing ethidium bromide (1.5 μg/ml). PCR yielded fragments containing exonic sequences 258, 275, and 191 bp, for exons 1, 2, and 3, respectively.

Single strand conformational polymorphism (SSCP) analysis was used to detect alterations in the sequence of individual PCR products. Approximately 40-100 ng of DNA was mixed in a half volume of 10 mmol/l EDTA/0.5 mol/l NaOH and an equal volume of formamide loading buffer (-95% (v/v) formamide, 5% (v/v) glycerol, 20 mmol/l EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol). The samples were boiled for five minutes to denature the DNA fragments, then snap chilled on ice. The denatured samples were loaded onto a 10% polyacrylamide gel using 1 × Tris-borate-EDTA (TBE, 0.9 mol/l Tris base, 0.9 mol/l boric acid, 0.025 mol/l EDTA, pH 8.3) as running buffer. Electrophoresis of the gel was done using a Hoefer mighty small gel electrophoresis system at 200 V and 4°C for 1.5 to 2.5 hours. DNA was visualised using a
Results

MUTATION ANALYSIS

SSCP and heteroduplex analysis of the regions amplified by PCR showed a variation between individual migration patterns for exon 1 only. Sequence analysis identified an adenine to guanine substitution at position 38, downstream from the exon 1 transcription initiation site (A38G).

Sau96I endonuclease was used to genotype subjects as it is a restriction enzyme that specifically identifies samples with the 38G sequence, resulting in a different banding pattern for the three genotypes at position 38 (fig 1). Twenty-seven of the 266 participants (10.2%) from the unselected population were homozygous for the published sequence (38AA), 123 (46.2%) were heterozygous (38AG), and 116 (43.6%) were homozygous for the polymorphism (38GG).

All samples were screened using SSCP analysis and approximately 30% were verified by heteroduplex analysis. Direct sequencing was done on 10% of samples and a number of these were used as controls for genotyping using restriction digestion. For any given sample, the genotype obtained was identical for each of the methods used.

ASSOCIATION ANALYSIS

In the case control analysis, using the primary definition of case status (current physician diagnosed moderate or severe asthma), 46 unaffected and 67 asthmatic subjects, representing a total of 92 and 134 alleles, respectively, were analysed for allele frequency, as well as association between case status and genotype at the CC16 locus.

The frequency of the 38A allele was significantly higher in asthmatic cases (58/134=43.3%) when compared to non-asthmatic controls (24/92=26.1%; χ²=6.947, p=0.008).

Using the primary definition of asthma and taking genotype 38GG as a baseline, an adjusted relative risk of asthma for genotype 38AA was 6.93 (95% confidence interval=1.01 to 47.43, p=0.049). The equivalent relative risk for genotype 38AG was 4.18 (95% confidence interval=1.17 to 14.97, p=0.028; χ²=6.712, p=0.03) for a test against the null hypothesis that there is no relationship between CC16 status (38AA + 38AG + 38GG) and the risk of clinical asthma. Modelling of genotype as a continuous covariate indicated evidence of a

Figure 1  Restriction digestion of DNA samples showed three alternate digest patterns identifying heterozygous and homozygous subjects for both the published and polymorphic sequence. The control sample in the first lane displays the original 258 bp fragment identifying a homozygote with the published DNA sequence, lacking the Sau96I site (38AA). Sample 1 was completely digested by Sau96I into two fragments, 130 and 128 bp in length, that migrated together as a single band. This corresponds to a homozygote with the 38G substitution (38GG). Samples 2 and 3 contain three fragments, 258, 130, and 128 bp in length that appear as two bands. This pattern describes a heterozygote (38AG). The last lane contains a PCR marker G3161 (Promega) with fragment lengths of 1000, 750, 500, 300, 150, and 50 bp in length.

DNA silver staining system (Promega) and the gels were dried under vacuum.

Heteroduplex analysis was another mutation screening method used in combination with SSCP analysis to maximise the detection of all potential polymorphisms and to confirm genotype.24 Approximately 40-100 ng of DNA was mixed with non-denaturing loading dye (30% (v/v) glycerol, 0.05% (w/v) bromphenol blue, 0.05% (w/v) xylene cyanol), boiled for five minutes, and snap chilled on ice. The samples were loaded onto a 10% polyacrylamide gel and run as for SSCP gels. DNA was again visualised by silver staining and the gels dried.

A 10% random sample of individual PCR products was directly sequenced using the Sequenase PCR product sequencing kit (United States Biochemical) and α-32P-dATP (Bresatec). The sequencing reactions were separated on a 6% polyacrylamide gel containing 7 mol/l urea and visualised by autoradiography of the dried gel.

GENOTYPE ANALYSIS

Subjects were genotyped by restriction digestion of PCR products with Sau96I endonuclease (Promega Corporation). Digested DNA samples were examined following electrophoresis on a 2% agarose gel containing ethidium bromide (1.5 μg/ml). DNA was visualised using a single intensity transilluminator (300 nm) and photographed with a polaroid land camera.

STATISTICS

An unmatched case control design was used to test for association between clinical asthma status and genotype at the CC16 exon 1 locus. Cases were initially defined using the primary definition of clinical asthma and confirmatory analyses were then conducted using the secondary definitions of asthma. Unconditional logistic regression was used to adjust for age, gender, and parental smoking status.

For analytical purposes, genotypes were coded as homozygous polymorphic sequence (38GG)=0, heterozygous (38AG)=1, homozygous published sequence (38AA)=2. Evidence for a linear (genetically additive) effect on phenotype across the three genotypes was sought by entering genotype as a continuous covariate in a logistic regression analysis. In other analyses the assumption of a linear effect was relaxed by entering genotype as a categorical factor. Analyses were conducted using Minitab version 11 software.
significant linear trend in the risk of asthma across the three genotypes for the primary and both secondary definitions of asthma (table 1).

**Discussion**

The A38G polymorphism was identified in exon 1 of the CC16 gene and was present at a higher frequency than the published sequence in the unselected population. This suggests that the 38G allele is more likely to be the wild type, and the published allele (38A) may have been of a single subject who by chance had the less common sequence. The less common 38A allele was shown to be associated with a significantly increased risk of developing asthma, regardless of how it was defined, suggesting that mutations in either the CC16 gene or a locus in linkage disequilibrium with it contribute to an inherited predisposition for asthma. The finding of such a high allele frequency for a mutation associated with asthma is somewhat unexpected given its deleterious effect. Considering epidemiological trends in the prevalence of asthma, it is possible that the mutation was in Hardy-Weinberg equilibrium at a time when environmental factors did not select against the altered gene function. However, recent exposure to modern environmental triggers might well produce a dramatic rise in asthma prevalence owing to the unmasking of a latent genetic susceptibility. As asthma is not usually fatal before reproductive age, negative selective pressure is unlikely. Alternatively, another hypothesis suggests that asthma heritability factors act to protect people from parasite infection in regions of endemic parasitisation, thus exerting a positive selective pressure.

Several domains of the CC16 gene are critical to expression of the protein. These may include the regulatory elements contained in the 5’ untranslated region of the gene and the intronic sequences. Sequence analysis of the human CC16 gene has already identified a microsatellite marker in intron 1 that was not found to be associated with asthma or allergy. These results differ somewhat from the association observed between A38G and asthma in this case control study. However, A38G is an exonic polymorphism and is situated some distance from the microsatellite marker in intron 1. Two polymorphic regions in relatively close proximity may not necessarily be in linkage disequilibrium, especially if one or both are ancient in origin. In addition, differences in the range of environmental triggers to which a population is exposed may result in a different group of genetically predisposed people developing asthma. This, combined with a variable definition of asthma, may result in inconsistent statistical associations between studies.

Studies of the rat CC16 promoter and exon 1 non-coding region have identified a number of important transcription factors involved in the regulation of CC16 protein expression (fig 2). Region I of the CC16 promoter (-132 to -76) contains at least three functional binding sites. These include an upstream hepatocyte nuclear factor-3 (HNF-3) site, and

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**Table 1** Association of CC16 allele frequency and genotype with three definitions of asthma

<table>
<thead>
<tr>
<th>Case definition</th>
<th>38A frequency</th>
<th>Odds ratio*</th>
<th>95% confidence interval</th>
<th>x², p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Respiratory physician diagnosed, current symptomatic asthma</td>
<td>58/134 (43.3%)</td>
<td>2.84</td>
<td>1.20 to 6.71</td>
<td>6.71 (p=0.035)</td>
</tr>
<tr>
<td>2 Dr diagnosed asthma ever+BHR;</td>
<td>60/140 (42.9%)</td>
<td>2.88</td>
<td>1.20 to 6.93</td>
<td>22.28 (p=0.000)</td>
</tr>
<tr>
<td>3 Current wheeze+BHR</td>
<td>22/60 (36.7%)</td>
<td>3.5</td>
<td>1.07 to 11.47</td>
<td>6.45 (p=0.039)</td>
</tr>
</tbody>
</table>

*Genotypes coded as a continuous covariate: 0=38GG, 1=38AG, 2=38AA; model adjusted for age and gender.

**Figure 2** (A) Diagram of the rat CC16 promoter containing three transcription factor binding regions (referred to as Region I, Region II, and TTF-1) and the minimal promoter region. Region I includes an upstream HNF-3 binding site and, downstream, overlapping AP-1 and HNF-3 binding sites. (B) Diagram of the human CC16 gene containing three exons and the locations of the A38G polymorphism and the microsatellite marker.
downstream an activator protein-1 (AP-1) and HNF-3 site which overlap. Activation of region II of the CC16 promoter (within -320 to -170), or some other unknown transcriptional element, appears to promote synergy between the HNF-3 sites in region I. However, in the absence of region II, CC16 region I activity is stimulated by HNF-3 alpha and inhibited by HNF-3 beta. Thyroid transcription factor-1 (TTF-1) also binds to the promoter region of the CC16 gene and its activity may influence gene expression.29 Downstream from region I and the TATA box, a minimal promoter was identified within a -50 to +58 segment and was shown to be important in the transcriptional regulation of the CC16 promoter. A region within the transcribed sequence of the insulin-like growth factor I gene has also been shown to alter expression levels.30 Experiments in region I of the rat CC16 promoter indicate that additional factors interacting via the minimal CC16 promoter region are essential in determining the cell specific CC16 gene expression in the bronchial epithelium.31 The A38G polymorphism is located within a region corresponding to the rat minimal promoter; therefore, although the significance of an alteration to the CC16 exon 1 non-coding region in humans is yet to be fully clarified, it may impact significantly upon protein expression levels.

In conclusion, this study identified a significant relationship between genotype differences at position 38 of exon 1 of the CC16 gene and the risk of childhood asthma defined three different ways. The polymorphism may alter CC16 protein expression, and therefore alter the control of airway inflammation. The CC16 gene could provide an alternative explanation for previous findings of linkage of the chromosome 11q13 region to atopy24 and increased airway responsiveness32 and it may be an important gene in determining the development of asthma.