Allelic association and functional studies of promoter polymorphism in the leukotriene C4 synthase gene (LTC4S) in asthma

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Cysteinyi leukotrienes (Cys-LTs) are critical mediators of airway narrowing and eosinophilia in bronchial asthma. They are generated by the sequential action of a series of enzymes that constitute the 5-lipoxygenase pathway. Under the action of cytosolic phospholipase A2, 5-lipoxygenase (5-LO) and 5-lipoxygenase activating protein (FLAP), membrane phospholipids release arachidonic acid which is converted to the unstable intermediate LTA4. By the action of the terminal enzyme, LTC4 synthase, LTA4 is conjugated with glutathione to form LTC4, the first member of the family of cysteinyl leukotrienes which also includes the extracellular metabolites LTD4 and LTE4.

The human LTC4 synthase gene (LTC4S) consists of five exons of 71–257 bp and four introns. The gene spans 2.51 kb and has been mapped to chromosome 5q35.4,5 5' extension analysis of KG-1 mRNA revealed three putative transcription initiation sites at positions –96, –69, and –66 relative to the initiation codon and, in the monocytic cell line THP-1, a single transcription initiation site was identified at position –78.5 Recently, there has been extensive interest in the elucidation of transcriptional regulation of this gene and multiple transcription factors have been reported to have a critical role, including the Inr (initiator like), Sp1, Sp3 and Kruppel-like transcription factors.6

LTC4S is a particularly strong candidate gene for a specific subgroup of patients with aspirin intolerant asthma (AIA).

Background: LTC4 synthase is essential for the production of cysteinyl leukotrienes (Cys-LT), critical mediators in asthma. We have identified a novel promoter polymorphism at position –1072 (G/A) and a –444 (A/C) polymorphism has previously been reported. The role of these polymorphisms in the genetic susceptibility to asthma was examined.

Methods: To test for genetic association with asthma phenotypes, 341 white families (two asthmatic siblings) and 184 non-asthmatic control subjects were genotyped. Genetic association was assessed using case control and transmission disequilibrium test (TDT) analyses. LTC4S promoter luciferase constructs and transiently transfected human Hela and KU812F cells were generated to determine the functional role of these polymorphisms on basal transcription.

Results: No associations were observed in case control analyses (–1072 A, q=0.09; –444 C, q=0.29); the TDT identified a borderline association between the –444 C allele and bronchial responsiveness to methacholine (p=0.065). Asthmatic children with the –444 C allele had a lower mean basal forced expiratory volume in 1 second (97.4 v 92.7% predicted, p=0.005). LTC4S promoter luciferase analyses provided no evidence for a functional role of either polymorphism in determining basal transcription.

Conclusion: This study does not support a role for these polymorphisms in genetic susceptibility to asthma but provides evidence to suggest a role in determining lung function parameters.

These patients experience Cys-LT dependent adverse respiratory reactions to aspirin and other cyclooxygenase (COX) inhibitors, and these are superimposed upon chronically increased Cys-LT production associated with persistent severe asthma.6 A fivefold overexpression of LTC4 synthase in bronchial biopsy specimens of patients with AIA compared with aspirin tolerant asthmatic (ATA) patients has been demonstrated, with no significant differences in other enzymes involved in the biosynthesis of Cys-LTs. A –444 A/C polymorphism was identified in the LTC4 synthase gene promoter region and was predicted to create an extra recognition site for the AP-2 transcription factor and lead to increased gene transcription.10 The presence of the C allele was identified as a risk factor for AIA patients compared with ATA or normal subjects (odds ratio 3.89).10 The role of the LTC4S promoter polymorphisms in AIA and ATA is unclear, with several studies indicating a functional role for the polymorphism in AIA11 12 while others question the significance of this polymorphism.13 14

In a preliminary study we examined the role of the –444 polymorphism in a small group of patients with severe persistent asthma and normal controls.15 The C allele was found to be more prevalent in patients with severe asthma than in normal subjects (p=0.04), and the presence of the C allele resulted in an approximate threefold increase in LTC4 production in isolated blood eosinophils stimulated with calcium ionophore (A23187) in the presence of indomethacin (p=0.04). The polymorphism was predictive of lung function response to treatment for 2 weeks with the Cys-LT receptor antagonist zafirlukast.16 We hypothesised that LTC4S polymorphisms may make subjects susceptible to asthma and/or predict asthma severity, irrespective of aspirin intolerance, based on putative upregulation of LTC4 synthase activity within eosinophils and other leucocytes in the airways.
To test this hypothesis we have identified a novel polymorph-
ism in the LTR4S promoter, completed a large scale
association study for asthma (irrespective of aspirin tolerance)
and atopy phenotypes, and determined the functionality of the
polymorphisms in basal transcription.

METHODS

Subjects and clinical assessment

Three hundred and forty one white families were recruited from
the Southampton area with at least two biological
siblings (age 5–21 years) with a current physician diagnosis of
asthma who were taking asthma medication on a regular
basis. Serum total IgE levels and specific IgE levels for grass,
house dust mite, cat, dog, Alternaria and tree allergens were
determined by RAST. Skin prick testing was also completed for
the same common allergens. At least 14 days after any respi-
atory tract infection, bronchodilator or antiallergic medi-
cation, baseline lung function tests (forced expiratory volume
in 1 second (FEV1), best of three values within 5%) were per-
formed using a Vitalograph dry wedge bellows spirometer.

An asthmatic sibling cohort (sibling cohort 1, n=341) of
mean (SD) age 13.0 (3.4) years, defined by doctor diagnosis,
current medication and questionnaire (see section on
transmission disequilibrium test below for stringent defini-
tion of asthma phenotypes), was used to explore the association
with asthma phenotypes using conventional statistical analysis.
Two further cohorts, one paediatric (sibling cohort 2) of mean
(SD) age 9.9 (3.1) years, composed of the full siblings of sib-
lings cohort 1, again with the stringent definition of asthma
(n=338), and an adult cohort composed of doctor diagnosed
asthmatic parents of mean (SD) age 40.2 (5.2) years (n=189)
were used as additional study groups. Non-asthmatic controls
of mean (SD) age 42.3 (10.6) years with no family history of
respiratory disease were recruited from the same Southamp-
ton area as the main study (n=184). DNA was extracted from
10 ml whole blood using a Genomic DNA Maxi-Prep kit (Qia-
gen, Crawley, UK). Ethical approval was obtained from the
Southampton and South West Hampshire joint ethics
committee.

Molecular methods

Using genomic DNA extracted from 30 asthmatic subjects, a
~1.4 kb fragment of the LTR4S promoter (corresponding to
-1430 to +116) was generated by polymerase chain reaction
(PCR). Jumpstart Taq (0.025 U/µl Sigma-Aldrich, Poole, UK)
was used for amplification of genomic DNA template (100 ng)
in the presence of standard PCR buffer, 2 mM MgCl2, 0.2 µM
each primer (forward 5′-CAGTCCGGCCAGTCTGCAGAG-3′,
reverse 5′-CAGGCAGGACCAGCTGCTGCGGAGC-3′), 0.2 mM dNTPs
(including fluorescent dCTP (R110) (ABI-Perkin Elmer,
Warrington, UK) 1:100 ratio of unlabelled dCTP) to give a final
reaction volume of 100 µl. Thermal cycling included a single
soak at 96°C for 10 minutes followed by 35 cycles at 94°C for 30
seconds, 65°C for 45 seconds, 72°C for 2 minutes, and finally
a 72°C soak for 7 minutes. Probe 1 was labelled with a 5′ phosphate and a 3′
biotin label (sequence 5′-AGGGAGACACCCAGAACTCC-3′;
single allele specific probes contained a 5′ fluorescein label and
differed only by a single nucleotide at the 3′ terminal
(5′-GGGGGCGCTCCAGGCGGGGC[A/G]).

The optical density (OD600) was measured using an ELISA plate reader (Titertek
Instruments Inc, AL, USA). In each plate an allele 1, allele 2
(PCR template) or no template was included. Typical positive
signals were 10× background control levels.

For total RNA isolation, 107 HeLa or KUB12F cells (in 10 ml
fresh media) were seeded into a 10 cm petri dish and grown
for 24 hours. Total RNA was isolated using Trizol reagent (Life
Technologies, Paisley, UK) as directed by the manufacturer
and resuspended in 20 µl DEPC treated water. cDNA was gen-
erated using the Omniscript reverse transcription kit (Qiagen)
as directed by the manufacturer using 2 µg RNA template.

GAPDH was used as a housekeeping gene PCR control. PCR
included 2 µl cDNA template (from the 20 µl RT-PCR reaction), Jumpstart Taq (0.025 U/µl)
standard PCR buffer, 1 mM MgCl2, 0.6 µM each primer (forward 5′-GTCCTGAC
TCAATGAGAATAC-3′, reverse 5′-AGGGATGCTGTTCTGGAGA
G-3′), and 0.2 mM dNTPs to give a final reaction volume
of 25 µl. Thermal cycling included a single soak at 96°C for 5
minutes followed by 30 cycles of 94°C for 30 seconds, 57°C for
30 seconds, 72°C for 30 seconds, and finally a 72°C soak for 7
minutes. The GAPDH PCR generated a 427 bp control
fragment. LTC4S PCR used 2 µl cDNA template (from the 20 µl
RT-PCR reaction), Jumpstart Taq (0.025 U/µl), standard PCR
buffer, 1.3 mM MgCl2, 0.2 µM each primer (forward 5′-
GTGCTGCGGCAGCCTGTTCTGCGGAG-3′; reverse 5′-GGGAGAGCTGA
GGGGAGACC-3′), 0.2 mM dNTPs and to give a final reaction
volume of 25 µl. Thermal cycling included a single soak at
96°C for 5 minutes followed by 30 cycles of 94°C for 30
seconds, 64°C for 30 seconds, 72°C for 30 seconds, and finally
a 72°C soak for 7 minutes. The LTC4S PCR generated a fragment of 155 bp; all fragments were visualised using ethid-
ium bromide on a 2% agarose gel.

1.3 kb fragments of the LTC4S promoter corresponding to
-1359 to −55 were generated by PCR using primers that engi-
neered MluI and BgIII restriction sites at the 5′ and 3′
of the product, respectively (forward 5′-CAGTCCGGCCAGTCT
GCAGAG-3′; reverse 5′-CAGTTCGTCGGAGGAGAGCTTAC
CTGCAGG-3′), 1.5 mM MgCl2, 0.2 µM each primer (forward 5′-
GTCCTGAC TCAATGAGAATAC-3′, reverse 5′-AGGGATGCTGTT
CTGGAGAG-3′), and 0.2 mM dNTPs to give a final reaction
volume of 25 µl. Thermal cycling included a single soak at
96°C for 5 minutes followed by 30 cycles of 94°C for 30
seconds, 64°C for 30 seconds, 72°C for 30 seconds, and finally
a 72°C soak for 7 minutes. The LTC4S PCR generated a fragment of 155 bp; all fragments were visualised using ethid-
ium bromide on a 2% agarose gel.
washed with D-PBS, and resuspended at a density of 10⁷ cells/ml in ice cold D-PBS. A water, pGL3basic (−) negative and pGL3-SV40 positive control was included. 10 µg pGL3 construct and 0.2 µg pRL-SV40 was combined with 0.8 ml cells. Cells were transiently transfected using electroporation (single pulse 300V, 500 µF) using a GenePulser (Biorad, Hemel Hempstead, UK). Each transfection was resuspended in 10 ml of the appropriate media and grown for 24 hours. Cells were harvested and luciferase activity determined using the dual luciferase assay (DLA; Promega) as described by the manufacturer using a dual injector Lucy 1 luminometer (LabTech, Andover, MA, USA). Luciferase activity was measured in triplicate and the mean value was used for analysis. Transfection efficiencies were normalised using co-transfection of pRL-SV40 and Renilla luciferase activity.

Transmission disequilibrium test/case control

Genetic association of the LTC4S polymorphisms with asthma and atopy phenotypes was evaluated using the transmission/disequilibrium test and case control analyses. This was implemented using STATA 6.0 (Stata Corporation, Texas, USA) and a TDT program written in STATA by David Clayton (MRC Biostatistics Unit, Cambridge). Results which showed a significance of 10% or less were repeated on TDT/STDT Biostatistics Unit, Cambridge). Results which showed a significance of 10% or less were repeated on TDT/STDT Program 1.1 (http://spielman07.med.upenn.edu/TDT.htm). Only the TDT section of this program was used as the data was presented as trios. Analysis was completed for the phenotypes: CRF asthma (Complete Report Form, positive questionnaire: “Have you ever had asthma?”; “Was this confirmed by a doctor?” and “Have you used any medicines to treat asthma, or any breathing problems, at any time in the last 12 months?”), total IgE, specific IgE, bronchial hyperresponsiveness to methacholine (PC₂₀), mean skin prick test (>3 mm) and atopy (defined by positive total IgE and/or specific IgE and/or mean skin prick test). Total IgE values were dichotomised using age related cut off values according to UK NEQAS, specific IgE >0.35 IU was classified as positive, and PC₂₀ was dichotomised using ATS criteria. PC₂₀ (mg/ml methacholine) was analysed for <4 mg/ml and ≤16 mg/ml. Both STATA and TDT/STDT Program 1.1 analysis used the first affected sibling where genotype data were available; p values of <0.05 were considered significant. Case control was completed using the χ² test to compare allele and genotype frequencies between CRF asthma groups and non-asthmatic control subjects with p values of <0.05 being considered significant.

Generation of phenotypic scores for genotype-phenotype analysis

Bronchial hyperresponsiveness (1/[least squares slope + 30])
To overcome the “censored” PC₂₀ value, the least squares slope developed by Chinn et al. to describe bronchial provocation data was used and an arbitrary constant of 30 added to avoid negative values.

Symptom score
Each family member completed a detailed questionnaire on asthma, atopy, and daily life events based on the MRC, ATS, IUALT and ISAAC questionnaires. The questionnaire was sent to a random selection of experienced professionals in the field of asthma and allergy to evaluate the questions as indicators of severity. These responses were formulated into a symptom severity score and a mean rank for each question was calculated. The reciprocal of the mean rank was taken to enable the “score” of 10 to reflect the most important. A score of 10 (arbitrary) was given to the highest reciprocal.

Treatment score
Medications used by subjects in the 12 months before the study were assigned a value according to the BTS guidelines. Inhaled steroids were converted to beclomethasone dipropionate equivalents—that is, fluticasone propionate dose was multiplied by two as it is twice as potent as beclomethasone. The following values were assigned: 1 = β₂ agonists (short acting); 2 = low dose inhaled steroids (<800 µg), Cromoglycates; 3 = high dose inhaled steroids (>800 µg); 4 = β₂ agonists (long acting), theophyllines, ipratropium bromide; 5 = oral steroids. This step up treatment approach enabled us to weight medications used by subjects accordingly and thereby reflect the severity of their asthma.

Atopy
Atopy was defined as either a positive skin prick test (>3 mm) or a raised specific IgE (>0.35 IU) to one or more common allergens.

Total IgE
Total IgE was adjusted for age using the number of SD away from the median for each age group. This value was then transformed using the natural logarithm to improve uniformity of variance.
Table 1 Hardy-Weinberg (H-W) equilibrium and allele frequencies

<table>
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<tr>
<th></th>
<th>aa</th>
<th>ac</th>
<th>cc</th>
<th>n</th>
<th>q</th>
<th>p value</th>
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<tr>
<td>–444</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>85</td>
<td>79</td>
<td>16</td>
<td>180</td>
<td>0.31</td>
<td>0.697</td>
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<td>Parents</td>
<td>330</td>
<td>256</td>
<td>59</td>
<td>645</td>
<td>0.29</td>
<td>0.360</td>
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<tr>
<td>Combined</td>
<td>415</td>
<td>335</td>
<td>75</td>
<td>825</td>
<td>0.29</td>
<td>0.533</td>
</tr>
<tr>
<td>–1072</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>143</td>
<td>31</td>
<td>0</td>
<td>174</td>
<td>0.09</td>
<td>0.197</td>
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<tr>
<td>Parents</td>
<td>519</td>
<td>103</td>
<td>3</td>
<td>625</td>
<td>0.09</td>
<td>0.379</td>
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<tr>
<td>Combined</td>
<td>662</td>
<td>134</td>
<td>3</td>
<td>799</td>
<td>0.09</td>
<td>0.166</td>
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Table 2 Phenotypic characteristics of study cohorts

<table>
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<tr>
<th></th>
<th>Pedigrees (n=1508)</th>
<th>Parents (n=681)</th>
<th>Non-asthma parents (n=492)</th>
<th>Asthma parents (n=189)</th>
<th>Sibling 1 (n=341)</th>
<th>Sibling 2 (n=338)</th>
<th>Controls (n=184)</th>
</tr>
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<tbody>
<tr>
<td>Age [years]</td>
<td>24.6</td>
<td>40.5</td>
<td>40.7</td>
<td>40.2</td>
<td>13.0</td>
<td>9.9</td>
<td>42.3</td>
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<td>Sex [% male]</td>
<td>51.8</td>
<td>49.9</td>
<td>51.0</td>
<td>47.1</td>
<td>56.9</td>
<td>53.6</td>
<td>47.0</td>
</tr>
<tr>
<td>Asthma [% doctor]</td>
<td>60.1</td>
<td>27.8</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Eczema [% questionnaire]</td>
<td>45.6</td>
<td>22.7</td>
<td>25.8</td>
<td>50.8</td>
<td>57.8</td>
<td>62.4</td>
<td>0</td>
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<tr>
<td>Hayfever [% questionnaire]</td>
<td>48.9</td>
<td>46.8</td>
<td>38.0</td>
<td>69.8</td>
<td>64.2</td>
<td>47.0</td>
<td>8.7</td>
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<tr>
<td>Symptom score</td>
<td>15.21</td>
<td>8.21</td>
<td>4.01</td>
<td>19.13</td>
<td>23.47</td>
<td>23.44</td>
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<td>Drug score B</td>
<td>1.90</td>
<td>0.65</td>
<td>0.08</td>
<td>2.20</td>
<td>3.40</td>
<td>3.45</td>
<td>ND</td>
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<tr>
<td>FEV1 [% predicted]</td>
<td>98.05</td>
<td>100.81</td>
<td>103.39</td>
<td>94.12</td>
<td>94.74</td>
<td>98.62</td>
<td>ND</td>
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<tr>
<td>BHR [1/L slope&gt;30]*1000</td>
<td>19.03</td>
<td>24.33</td>
<td>26.79</td>
<td>17.22</td>
<td>14.55</td>
<td>12.00</td>
<td>ND</td>
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<tr>
<td>log IgE [age corrected]</td>
<td>1.25</td>
<td>0.64</td>
<td>0.49</td>
<td>1.01</td>
<td>1.84</td>
<td>1.93</td>
<td>ND</td>
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</table>

FEV1=forced expiratory volume in 1 second; BHR=bronchial hyperresponsiveness.
Selection of an atopic asthmatic group from sibling cohort 1 did not maintain the correlation between the –444 genotype and BHR (p=0.07, ANOVA, n=231) but maintained the –444 correlation with FEV1 (p=0.025, ANOVA (CI –9.97 to –0.62), n=247). In non-atopic asthmatic subjects neither correlation was maintained. Further analysis of the –444 polymorphism in sibling cohort 1 with other phenotypes did not identify any significant correlation. Analysis of the –1072 polymorphism did not identify any significant genotype-phenotype correlation in this cohort (data not shown).

Analyses using sibling cohort 2 (n=338) and the adult asthmatic cohorts (n=189) with or without atopy did not identify any significant correlation between the presence of the –444 or –1072 alleles and the phenotypes described in table 2 (data not shown). Involvement of Cys-LT in inflammatory disease is not thought to be associated exclusively with atopy, and caution was therefore exercised when atopy was used as a variable due to a potential confounding influence.

Luciferase reporter assays
The potential functional role of LTC4S polymorphisms in transcription was explored using promoter analysis software (see methods). The LTC4S –444 A to C substitution is predicted to generate an H4TF-2 transcription factor binding site (GGTCC) and remove a glucocorticoid response element (GRE) (GGGACA), an NF-Elc (mnnnnTGATmnnnn), and an NF-E site (CTGTC) (Transfac/TFD). The –1072 G to A polymorphism is predicted to generate an additional LF-A1 (GGGCA) and alpha-INF-2 (AARKGA) site. Before promoter reporter studies the HeLa and KU812F cell lines were analysed for the presence of LTC4S mRNA using RT-PCR (fig 2B). A positive signal (155 bp) was observed for the KU812F cell line. Promoter fragments containing different haplotypes were constructed and cloned into the pGL3 basic luciferase vector (fig 2A); the A-C haplotype was engineered using standard molecular biology techniques due to the low abundance of this haplotype in the white population.

Table 3: Case control analysis (CRF asthma)

<table>
<thead>
<tr>
<th>Asthmatic cohort</th>
<th>Position</th>
<th>Allele (χ²)</th>
<th>Allele (p value)</th>
<th>Genotype (χ²)</th>
<th>Genotype (p value)</th>
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<tbody>
<tr>
<td>Sibling 1</td>
<td>–444</td>
<td>0.00</td>
<td>1.000</td>
<td>0.37</td>
<td>0.830</td>
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<td></td>
<td>–1072</td>
<td>0.33</td>
<td>0.567</td>
<td>3.38</td>
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<tr>
<td>Sibling 2</td>
<td>–444</td>
<td>0.83</td>
<td>0.362</td>
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<td>0.554</td>
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<tr>
<td></td>
<td>–1072</td>
<td>0.00</td>
<td>0.960</td>
<td>0.00</td>
<td>0.958</td>
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<tr>
<td>Parents</td>
<td>–444</td>
<td>0.46</td>
<td>0.500</td>
<td>0.83</td>
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<td></td>
<td>–1072</td>
<td>2.82</td>
<td>0.093</td>
<td>2.31</td>
<td>0.129</td>
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Table 4: Transmission disequilibrium test (TDT) analyses

<table>
<thead>
<tr>
<th>Variables</th>
<th>No of genotyped offspring</th>
<th>Allele</th>
<th>Observed</th>
<th>Expected</th>
<th>p value</th>
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<tr>
<td>CRF asthma</td>
<td>336</td>
<td>A</td>
<td>111</td>
<td>120.5</td>
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<td></td>
<td></td>
<td>C</td>
<td>130</td>
<td>120.5</td>
<td>0.567</td>
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<tr>
<td>–1072</td>
<td>313</td>
<td>G</td>
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<tr>
<td></td>
<td></td>
<td>A</td>
<td>40</td>
<td>43</td>
<td></td>
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<tr>
<td>Atopy + CRF</td>
<td>314</td>
<td>A</td>
<td>104</td>
<td>110.5</td>
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<td></td>
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<td>C</td>
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<td>110.5</td>
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<td></td>
<td></td>
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<td>SPT positive + CRF</td>
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<td>Total IgE + CRF</td>
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<td>Specific IgE + CRF</td>
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CRF=Complete Report Form; SPT=skin prick tests; PC20=concentration of methacholine needed to provoke a fall in forced expiratory volume in 1 second of 20% or more.
DISCUSSION

This is the most comprehensive investigation to date of the role of LTC4S promoter polymorphism in asthma susceptibility in a white population. We have identified a novel polymorphism in the LTC4S promoter (−1072G/A) in addition to a previously identified polymorphism (−444 A/C). A borderline association between the −444 C allele and BHR to methacholine (>4 mg/ml) was observed using TDT analysis. Using case control analyses, neither the −1072 nor −444 promoter polymorphisms constituted risk factors for the development of asthma. Functional analysis using in vitro reporter assay did not identify a role for these polymorphisms in determining basal LTC4S transcription levels.

The potential significance of LTC4 synthase as a regulator of the production of Cys-LTs in inflammatory disease was identified by an investigation of patients with AIA who constitute approximately 10% of asthmatic individuals. In the current study we used a comprehensive approach to clarify the role of LTC4S promoter polymorphism in asthma irrespective of aspirin tolerance. Case control analysis did not identify a statistical difference in the −444 C allele frequency between the control population and any asthmatic cohort (table 3). The case control findings for −444 polymorphism agree with studies in other populations including a Dutch population (asthmatic, q=0.28, non-asthmatic, q=0.32) and an American population (AIA, q=0.27; ATA, q=0.27, non-asthmatic, q=0.33). In our previous pilot study we identified a significant difference in the −444 C allele frequency between non-asthmatic controls (q=0.19, n=31) and severe asthmatics (q=0.28, n=23; p=0.065). This value is of borderline significance and may represent spurious results, although polymorphism within the LTC4S gene may potentially be predicted to alter lung function parameters. No significant association between the −444 polymorphism and BHR was reported in a study in a Dutch population.

The role of the LTC4S polymorphisms in determining asthma and atopy related scores was examined using conventional statistical analysis. Analysis of BHR in the sibling 1 cohort stratified by −444 genotype identified a significant difference between genotype groups. The finding that the wild type AA genotype group had a mean value of 15.28—intermediate to the two variant containing groups—and that the BHR was less severe in the presence of the variant allele does not agree with the original hypothesis. The absence of

**Figure 2** Functional analysis of polymorphic LTC4S promoter reporter constructs. (A) Schematic representation of the four engineered LTC4S promoter reporter haplotypes. (B) RT-PCR performed on HeLa and KU812F cell lines; LTC4 synthase mRNA was only detected in the KU812F cell line; GAPDH was included as a positive control 50 bp marker for sizing. (C) and (D) Data from transient co-transfection of HeLa and KU812F cells using LTC4S firefly luciferase constructs and the pRL-SV40 plasmid encoding for Renilla luciferase to normalise transfection efficiency. Data represent the normalised mean (SE) of eight independent transfections for each cell line quantified in triplicate. No statistical difference was observed when compared with the designated 100% luciferase activity wild type haplotype (G-A).
significant difference between the AA and AC/CC groups, the lack of expected correlation of severity with genotype frequency as shown recently in the positive association of GSTP1 genotypes with BHR, and the borderline significance (p=0.026) question this finding. However, the association between the –444 polymorphism and BHR identified in the TDT analyses suggests that the current finding deserves further investigation.

A significant correlation between reduced basal FEV1 and the presence of the –444 C allele was identified in the sibling 1 cohort (p=0.005). Further analysis suggested that the significant difference was due to differences between the AA and AC groups. Birth order has been implicated in allergic disease, which could possibly explain differences between the findings in the different sibling groups. However, the negative correlation in the adult cohort suggests that the initial finding may be unique to the individual paediatric cohort. This was explored further by comparing the asthma and atopy scores in the two sibling cohorts; no evidence for the uniqueness of the sibling 1 cohort was identified. The absence of a “dose effect” for the C allele make the significance of this finding difficult to interpret, unless a dominant mechanism was involved. This seems unlikely due to the location of the polymorphism in the gene that is, if the polymorphism was functional in determining transcription levels. One potential explanation for the positive association observed in the first affected sibling cohort with reduced basal FEV1, is the mean age of 13 years compared with 9.9 years in the second sibling cohort. The extent to which asthma is established will be dependent on age, so this may at least partly explain the differences between siblings. An examination of the effect of the –1072 genotype in determining asthma related phenotypes failed to support a role for this polymorphism in any analyses. One finding that has emerged from case control studies of the role of the –444 polymorphism in AIA is that the asthma severity of the population has a dramatic effect on the outcome of an association study, with more severe asthmatics showing a positive association between the –444 C allele and AIA. This was explored by selecting individuals of different asthma severity and testing for genotype-phenotype correlations but no further useful data were generated, which suggests that in our population asthma severity cannot explain the different associations observed.

The functional role of these LTC4S polymorphisms is uncertain, but both could potentially alter transcription factor binding (fig 1). RT-PCR analysis confirmed the presence of LTC4S mRNA in the human basophilic KU812F cell line but not in the HeLa epithelial cell line. This is in agreement with a recent study, although it has previously been shown that HeLa cells contain all the transcriptional machinery required for LTC4S transcription. Data generated in promoter reporter assays in the HeLa and KU812F cell lines show that no statistical difference in transcriptional activity results from the presence or absence of specific –444 and –1072 polymorphisms under basal conditions (fig 2). These data are supported by the finding that neither the –444 nor the –1072 polymorphisms are located in regions that have been reported to be critical for basal LTC4S transcription (fig 1). A recent analysis usingem a 550 bp fragment of the LTC4S promoter containing the –444 A or C alleles in the human monocytic cell line THP-1 also did not identify a role for the –444 polymorphism; however, Szczeiski and co-workers recently reported a functional role for the –444 polymorphism. In the latter study a 300 bp fragment of the LTC4S promoter containing the –444 polymorphism was cloned into a eukaryotic expression vector (pcDNA2.1) containing a truncated CMV promoter and β-Gal gene. On the basis of two experiments the authors concluded that the –444 C allele resulted in a 25% increase in transcriptional activity compared with the A allele.

The data presented in the current study for the human epithelial and basophilic cell lines, combined with data from the human monocytic cell line, provide strong evidence that, at the basal level, the –444 polymorphism does not alter LTC4S transcription. Both studies used established promoter reporter techniques and based their conclusions on at least seven experimental replicates. Further support for these conclusions comes from the finding that LTC4S mRNA levels from peripheral blood eosinophils isolated from asthmatic individuals with –444 A or C alleles showed no differences in abundance under basal conditions. Because of the novel nature of the –1072 polymorphism, this study is the first report of the functional role of this polymorphism; our results indicate that at the basal level it is not functional in either epithelial or basophilic cell lines.

We have previously postulated a functional role for the –444 C allele. The findings of this pilot study and others suggest that it might be productive to examine the functional role of LTC4S promoter polymorphism haplotypes under different “micro-environment” conditions including the presence/absence of different cytokines and growth factors. In addition, post transcriptional mechanisms regulating LTC4S activity are likely to be of importance in Cys-LT production.

In conclusion, we have identified a novel LTC4S promoter polymorphism at position –1072 and have completed a comprehensive analysis of the role of this and a previously reported polymorphism (–444) in the genetic susceptibility to asthma and atopic disease. Several approaches were used including genetic association analysis and conventional genotype-phenotype analysis and evidence to suggest a role for the –444 polymorphism in determining lung function parameters was found. Using promoter reporter technology, no functional role was identified for any combination of alleles in determining basal transcription levels. Because of the heterogeneous nature of asthma, these association studies need to be replicated in a number of cohorts of different ethnic backgrounds with asthma of different severity. However, the current investigation does not support an important role for these LTC4S promoter polymorphisms in asthma susceptibility in the white population. Such evidence does not preclude the likelihood that polymorphism in LTC4S may predict those asthmatic individuals in whom leukotrienes make a relatively large pathophysiological contribution, nor that knowledge of a patient’s genotype at these loci may provide useful information on his/her likely clinical response to leukotriene modifier therapy.
Inhaled corticosteroids and long acting β₂ agonists can help patients with COPD

This randomised, double blind, parallel group, placebo controlled study funded by GlaxoSmithKline assessed treatment with salmeterol, fluticasone, and the combination of these two drugs for 1 year in a total of 1465 patients of whom 1009 completed the study. Pre-bronchodilator FEV₁ was increased by all three treatments compared with placebo (p<0.0063) and by combined treatment with fluticasone and salmeterol compared with the individual drugs and placebo (p<0.0001). All three active treatments resulted in a reduction in the exacerbation frequency, although there was no significant difference between the treatment groups. However, combined treatment significantly reduced breathlessness and the use of reliever medication compared with the other groups. The combined treatment group showed a reduction in night time awakenings and an improvement in St George’s Respiratory Questionnaire scores that were significantly greater than in the placebo or salmeterol groups. The treatments were well tolerated with no differences in adverse events except for an increased frequency of oropharyngeal candidosis in the fluticasone (7%) and combination of two drugs previously shown to benefit lung function, symptoms, and health status results in a significant improvement in lung function and exacerbation frequency and thereby provides a promising treatment for selected patients with COPD.

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