Analysis of 927T > C CYSLTR1 and –444A > C LTC4S polymorphisms in children with asthma

S. Arriba-Méndez*, C. Sanz*, M. Isidoro-García†, M. Pascual*, C. Ávila*, I. Dávila* and F. Lorente*


ABSTRACT

Introduction: The cysteinyl leukotrienes (Cys-LTs) are potent inflammatory mediators in asthma. It has been suggested that the different response of patients to Cys-LTs inhibitors could be due to the presence of polymorphisms in the genes implicated in this pathway.

Methods: In this study, polymorphisms 927T > C CYSLTR1 and –444A > C LTC4S were analysed in a Spanish population of 188 individuals (109 asthmatic children and 79 controls). Standardised history, skin prick tests and lung function measurements were performed in all patients. Genotypes were determined by sequencing after PCR amplification.

Results: Differences were observed in 927T > C CYSLTR1, regarding the severity of asthma in males. A greater presence of allele C in the population with persistent asthma versus the control group (Fisher’s p-value = 0.001; Monte Carlo p-value = 0.003; OR: 12.35; 95 %CI: 2.18-70.00) was observed. Differences were also detected in the combined study of both polymorphisms, among controls and asthmatic patients (Monte Carlo p-value = 0.0002). In the group of males with asthma, an increase of AC variant (–444A LTC4S and 927C CYSLTR1) and a reduction in the AT genetic combination were detected.

Conclusions: The combined study of polymorphisms in genes of the leukotriene pathway could explain the differences observed in the studies reported on polymorphism –444A < C LTC4S individually analysed.

Key words: Leukotriene. Gene. Receptor.

INTRODUCTION

Asthma is a chronic inflammatory disease characterised by reversible airflow obstruction, bronchial hyperresponsiveness and cellular inflammation. Its prevalence varies from 2-32 %, and the disease constitutes an important cause of hospital admission in the paediatric population. Environmental and genetic factors have been implicated in the pathogenesis of asthma. The cysteinyl leukotrienes (Cys-LTs) are potent inflammatory mediators derived from the oxidative metabolism of arachidonic acid through the 5-lipoxygenase (5-LO) enzyme pathway. Leukotriene C4 synthase (LTC4S) is a key enzyme in the formation of Cys-LTs, since it transforms leukotriene A4 (LTA4) into leukotriene C4 (LTC4) through the addition of a glutathione group in position C-6.
LTC4S is located fundamentally in the cell membrane of eosinophils, mast cells and macrophages of the lung alveoli, although other cells can also produce Cys-LTs. LTC4S gene is found on chromosome 5 (5q35) and it is expressed mainly in eosinophils of asthmatic patients. Among the genetic variants described for LTC4S, polymorphism –444 A > C is located in the promoter region of the gene. This change would affect the binding sites of the transcription factors, modifying the expression of LTC4S. An increase in the prevalence of the C allelic variant has been reported in adult patients with severe asthma, although other studies have failed to confirm this association.

At least two types of receptors for Cys-LT (CysLTR1 and CysLTR2) have been identified. The type 1 receptor predominates at bronchial level. The gene encoding for this receptor, CYSLTR1, is located on Xq13.2-21.1 and it is expressed in the spleen, leukocytes, pulmonary macrophages, and smooth muscle of the respiratory tract. Selective CysLTR1 antagonists have been developed. Montelukast, zafirlukast and pranlukast are commercially available for the treatment of asthma.

Subjects

A total of 188 non-consanguineous Caucasian patients were recruited in the Allergy Department of Salamanca University Hospital (Spain): 109 children with asthma between 6 and 16 years of age, and 79 controls. The study complied with the recommendations of the Hospital Ethics Committee. Data were collected on children diagnosed with asthma in the Paediatric Allergology clinic, presenting at least two symptoms of asthma (cough, wheezing and dyspnoea) in the last 12 months, and three asthma crises as documented in the case history, in the absence of other pulmonary disease.

Lung function was assessed in all patients by spirometry, following the indications of the ATS. The severity of asthma was classified into four groups according to the applicable Spanish consensus guidelines. Thus, 41.8% of patients had infrequent episodic asthma, 23.6% of them had frequent episodic asthma, 26.4% of them had moderate persistent asthma and 8.2% of patients had severe persistent asthma. We also documented other disorders associated to asthma, such as allergic rhinitis or atopic dermatitis. In the case of rhinitis, the diagnosis was based on the criteria of the American Academy of Allergy, Asthma and Immunology, and on the Consensus Conference of the American Academy of Dermatology in the case of atopic dermatitis.

Skin prick tests were performed according to the recommendations of the European Academy of Allergology and Clinical Immunology (EAACI), using a battery of aeroallergens previously described (ALK-Abello, Madrid, Spain). Antihistamines were discontinued before skin testing according to published guidelines. Saline was used as negative control and histamine 10 mg/ml was used as positive control. Skin tests were considered positive if at least one allergen elicited a wheal reaction of more than 3 mm in diameter after subtraction of the negative control. Total serum IgE levels were measured by a fluoroenzyme immunoassay with the Pharmacia Cap System (Pharmacia, Uppsala, Sweden), following the instructions of the manufacturer.

A population of children without asthma could be used as controls, however, to confirm lack of development of asthma and allergy during childhood, a population of adult controls was strictly selected. Healthy controls meeting the following criteria were included: (i) Absence of clinical symptoms compatible with bronchial asthma or other respiratory diseases; (ii) Absence of chronic skin diseases; (iii) No symptoms or history of allergy; (iv) Absence of first-degree relatives with asthma or atopy; and (v) Negative skin prick tests to the same battery of common aeroallergens (< 1 mm wheal greater than saline).

Genotype analysis

For the genotype study, genomic DNA purification from total blood was performed according to the previously described procedure. The fragments comprising the SNP’s studied were amplified by Polymerase Chain Reaction (PCR). The sequences of the upstream and downstream primers employed in these amplifications were 5’-CTCCATTCTGAAG-CAA-3’ and 5’-AGACCGCTCACCATT-3’ (for –444 A > C of LTC4S and 927 T > C of CYSLTR1) and asthma in a paediatric population of patients.
All fragments were visualised using ethidium bromide on a 2% agarose gel. To clean up the PCR products, GENECLEAN Turbo kit (Q-BIOgene) was used. The amplicons were sequenced in a 3100 Genetic Analyzer (Applied Biosystems) using the primers previously employed in PCR amplification. Chromas 2.3 (Technelysium Pty. Ltd. 1998-2004) was used to align and view the resulting chromatograms. The Genebank accession numbers for the reference genomic sequences used for CYSLTR1 and LTC4S alignments were AY242130 and U62025 respectively. The analysis was performed blindly with respect to case-control status.

Statistical analysis

Since the CYSLTR1 gene is located in the X chromosome, males and females were analysed separately. For the comparison of categorical variables, contingency tables with the chi-squared test, the Fisher and Pearson exact tests, and the odds ratio (OR) (95% confidence interval) were used. Likewise, the Monte Carlo simulation was applied. For the comparison of categorical and continuous variables the analysis of variance (ANOVA) was used. Statistical significance was accepted for $p < 0.001$ considering Bonferroni correction for multiple comparisons. Hardy-Weinberg equilibrium was assessed by chi-square test ($\chi^2$) (Pearson p-value). Total IgE levels were expressed as $\log_{10}$.

The data were analysed using the SPSS version 12.0 statistical package (Chicago, Illinois, USA). The SHEsis software platform\(^2\), was used to define haplotypes, considering the different distribution of haplotypes according to sex. In the analysis of the allelic combinations only genetic variants appearing in over 1% of the patients and controls were considered. The statistical power\(^2\) and the false-positive report probability (FPRP)\(^\text{24}\) were calculated to control the study.

RESULTS

Differences were observed in the case of polymorphism 927T > C CYSLTR1, regarding the severity of asthma in males. A greater presence of allele C in the population with persistent asthma versus the control group (Fisher’s $p$-value = 0.001; Monte Carlo $p$-value (after $10^4$ simulations) = 0.003; OR: 12.35; 95% CI: 2.18-70.00) and versus episodic asthma (Fisher’s $p$-value = 0.001; Monte Carlo $p$-value = 0.002; OR: 8.43; 95% CI: 2.05-34.65) was observed (Table I). No differences were detected in the female population.

In the study of the interaction of the two genes, differences were observed among controls and patients with asthma considered globally (Monte Carlo $p$-value = 0.0002). In the group of males with asthma, a clear increase of AC variant (–444A LTC4S and 927C CYSLTR1) and a reduction in the AT genetic combination was detected (Table II).

### Table I

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Controls (n = 158)</th>
<th>Asthma (n = 218)</th>
</tr>
</thead>
<tbody>
<tr>
<td>–444 A &gt; C LTC4S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allele A</td>
<td>0.70</td>
<td>0.79</td>
</tr>
<tr>
<td>Allele C</td>
<td>0.30</td>
<td>0.21</td>
</tr>
<tr>
<td>927T &gt; C CYSLTR1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allele T</td>
<td>0.92</td>
<td>0.74</td>
</tr>
<tr>
<td>Allele C</td>
<td>0.08</td>
<td>0.26</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allele T</td>
<td>0.79</td>
<td>0.77</td>
</tr>
<tr>
<td>Allele C</td>
<td>0.21</td>
<td>0.23</td>
</tr>
</tbody>
</table>

### Table II

<table>
<thead>
<tr>
<th>Combinations(^a)</th>
<th>Males</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Controls (n = 24)</td>
<td>Asthma (n = 64)</td>
</tr>
<tr>
<td>AC</td>
<td>0.00</td>
<td>0.26(^b)</td>
</tr>
<tr>
<td>AT</td>
<td>0.67</td>
<td>0.46</td>
</tr>
<tr>
<td>CC</td>
<td>0.08</td>
<td>0.00</td>
</tr>
<tr>
<td>CT</td>
<td>0.04</td>
<td>0.28</td>
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</table>

<table>
<thead>
<tr>
<th>Combinations(^a)</th>
<th>Females</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls (n = 55)</td>
<td>Asthma (n = 45)</td>
</tr>
<tr>
<td>AC</td>
<td>0.11</td>
<td>0.22</td>
</tr>
<tr>
<td>AT</td>
<td>0.56</td>
<td>0.55</td>
</tr>
<tr>
<td>CC</td>
<td>0.10(^d)</td>
<td>0.00</td>
</tr>
<tr>
<td>CT</td>
<td>0.23</td>
<td>0.22</td>
</tr>
</tbody>
</table>

\(^a\) The order of the SNPs in the combinations was –444A > C and 927T > C.
\(^b\) Fisher’s $p$-value = 0.006; Monte Carlo $p$-value (after $10^4$ simulations) = 0.003.
\(^c\) Fisher’s $p$-value = 0.0007; Monte Carlo $p$-value (after $10^4$ simulations) = 0.0006.

\(^d\) Fisher’s $p$-value = 0.007; Monte Carlo $p$-value (after $10^4$ simulations) = 0.008.
The AC variant was observed with greater frequency in the males with persistent asthma. In the present study, the association between the AC allele and persistent asthma was found to be statistically significant (Monte Carlo p-value < 0.0001). In contrast, the AT combination was not associated with persistent asthma. A separate analysis of this combination against the other polymorphisms confirmed these results in both situations (Monte Carlo p-value = 0.001, OR: 25.87, 95% CI: 2.82-237.59 for persistent asthma) (Monte Carlo p-value = 0.001, OR: 23, 95% CI: 2.54-208.62 for atopic dermatitis).

DISCUSSION

In the present study an association between allele C of 927T > C CYSLTR1 polymorphism and the asthmatic phenotype was detected in the group of male children. Choi et al. had previously described this polymorphism suggesting that although it constitutes a synonymous change, this SNP may affect the efficiency of CYSLTR1 gene transcription or translation, or may be in tight linkage disequilibrium with another polymorphism(s) in functionally important genomic elements of the CYSLTR1 gene.

No association between –444A > C LTC4S polymorphism and the asthmatic phenotype was observed. The LTC4S gene is located on chromosome 5q in a region associated with many other genes linked with asthma and atopy. The literature contains different studies on the possible association of polymorphism –444A > C LTC4S and the asthmatic phenotype, with controversial results. Studies combining several polymorphisms in genes encoding for proteins involved in signals transmission in the same pathway are important, since the analysis of a single polymorphism usually yields different results in different populations – probably because the interrelations with other polymorphisms in the “architecture” of the genome are obliterated. In the present study the allelic combination of two polymorphisms was analysed. In this population the AC allelic combination (–444A LTC4S and 927C CYSLTR1) seems to appear more frequently in males with asthma, particularly persistent asthma, and in asthmatic patients with atopic dermatitis. In contrast, the AT combination appears more frequently in controls. We observed no differences in the female population.

We have previously identified an association between atopic dermatitis and the C allele of polymorphism 927T > C in male children. The leukotrienes have been implicated in the pathogenesis of atopy, fundamentally in those presentations associated to allergenic triggering factors. An increase in urinary LTE4, proportional to the severity of atopic dermatitis, has been reported. The studies conducted to date on treatment with antileukotrienes for atopic dermatitis have yielded irregular results, although patients with improved response to such therapy also presented associated respiratory disease. It is therefore postulated that treatment response is in the case of atopic dermatitis associated to rhinitis or allergic asthma.

To the best of our knowledge, this is the first study of the combination of these two polymorphisms in a paediatric population. In a previous study Park et al. analysed the polymorphism –444A < C of the LTC4S gene, along with other polymorphisms that affect the cysteinyl leukotriene receptor (in this case the type 2 receptor). The authors concluded that the associations with asthma induced by aspirin are found to be more intense when combining the analysis of both polymorphisms. Likewise, in our study, the combination of A allele of –444A > C LTC4S with the polymorphism of the receptor seems to increase the association. Although one limitation of the study could be the sample size, considering the frequencies detected in this population, the statistical power exceeds 70% with an alpha error < 0.05. In addition, the false positive report probability (FPRP) would be less than 7% for a pre-test probability of 10%.

Many factors may influence positive results in genetic association studies; and consequently, the results from these studies must be considered with caution. However, this study clearly highlights the limitation of analysing only one polymorphism in association studies. Further studies are needed to independently confirm these associations of multiple polymorphisms that could be of interest in the diagnosis and management of asthma in childhood.

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REFERENCES