Original Article

# Analysis of *927T>C CYSLTRI and –444A>C LTC4S* Polymorphisms in Patients with Asthma

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**Abstract.** *Background:* The cysteinyl leukotrienes (cys-LTs) are proinflammatory mediators synthesized through the 5-lipoxygenase pathway of arachidonic acid metabolism. Cys-LTs exert their biological action by binding two types of G-protein-coupled seven transmembrane receptors, *CYSLTR1* and *CYSLTR2*. The contribution of the cys-LT receptors to bronchial asthma has been established by the therapeutic efficacy of biosynthetic inhibitors and selective CYSLTR1 blockers.
Objective: The present study was designed to analyse two different polymorphisms *927T>C CYSLTR1* and *-444A>C LTC4S*, and to determine whether there is an association between these polymorphisms and the asthma phenotype in a Spanish population.
*Methods:* Both single nucleotide polymorphisms (SNPs) were analysed in 208 individuals (130 asthmatic subjects and 78 controls). A standardized history, physical examination, skin prick tests and lung function measurement were taken from all patients. Genotypes were determined by direct sequencing after polymerase chain reaction (PCR) amplification.
*Results:* In the group of male patients, the C allele of *927T>C CYSLTR1* was more common among patients with asthma than controls. No association was detected between the *-444A>C LTC4S* polymorphism and the asthma phenotype. The combination of *927T CYSLTR1* and *-444A LTC4S* was less common in male patients with asthma than in controls (Fisher´s *P*-value=.039; Monte Carlo *P*-value (after 104 simulations)=.045 and the combination of *927C CYSLTR1* and *-444A LTC4S* was slightly more frequent in patients with asthma. No differences were observed in the female group.
*Conclusions:* The results suggest a certain trend of associations that could help to explain some controversial results in association studies of these genes from the leukotriene pathway, when considered individually. Further studies are needed to confirm such an association.

**Key words:** Asthma. Gene. *CYSLT. LTC4*. Leukotrienes. Polymorphism.

**Resumen.** *Introducción:* Los leucotrienos cisteinílicos (cys-LTs) son mediadores proinflamatorios sintetizados a partir del metabolismo del ácido araquidónico a través de la vía de la 5 lipooxigenasa. Los leucotrienos cisteinílicos ejercen su acción biológica mediante la unión a dos tipos de receptores (*CYSLTR1 y CYSLTR2*), acoplados a proteínas G. La contribución de los receptores de cys-LT en el asma bronquial se constata por la eficacia terapéutica de los inhibidores de su síntesis y de los antagonistas selectivos de estos receptores.
*Objetivo:* Analizar los polimorfismos *927T > C CYSLTR1* y *-444A > C LTC4S* para determinar la posible existencia de una relación con el fenotipo de asma en una población española.
*Métodos:* Se analizaron ambos polimorfismos en una población de 208 individuos (130 asmáticos y 78 controles). En todos los pacientes se realizó una historia clínica, exploración clínica y pruebas complementarias, incluyendo pruebas cutáneas y evaluación de la función pulmonar. Los genotipos se determinaron mediante secuenciación directa tras amplificación por PCR.
*Resultados:* La combinación de los alelos *927T CYSLTR1* y *-444A LTC4S* se detectó con menor frecuencia en los varones con asma respecto a los controles [p de Fisher = 0,039; p de Monte Carlo (tras 104 simulaciones)=0,045]. La combinación de los alelos *927C CYSLTR1* y *-444A LTC4S* se observó con una frecuencia ligeramente superior en los pacientes con asma. No se observaron diferencias en el grupo de las mujeres.

*Conclusiones:* Los resultados muestran cierta tendencia de asociación que podría contribuir a explicar la controversia que se observa en los estudios de asociación de estos genes de la vía de los leucotrienos, individualmente considerados; para confirmar esta asociación se necesitarán más estudios.

**Palabras clave:** Asma. Gen. CYSLT. LTC4. Leucotrienos. Polimorfismos.

## Introduction

Asthma is a chronic inflammatory disorder of the airways characterized by respiratory symptoms such as dyspnea, wheezing, cough, chest tightness and sputum production. Activation of the arachidonic acid cascade leads to the production of LTs, which are believed to play an important role in bronchoconstriction, edema formation, and increase in mucus production associated with the symptoms of asthma[1-3].

The cysteinyl leukotrienes (Cys-LTs) are lipid mediators, de novo generated from membrane-associated arachidonic acid [1, 4, 5]. Their biosynthesis involves the sequential action of a series of enzymes that constitute the 5-lipoxygenase pathway. Under the action of the 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 [6, 7]. After carrier-mediated export [8, 9], the sequential cleavage of glutamic acid and glycine from the glutathione moiety of LTC4 yields LTD4 and LTE4 [10, 11], respectively.

*LTC4S* is the rate-limiting enzyme for the synthesis of cysteinyl LTs. The expression of *LTC4S* mRNA has also been shown to be higher in blood eosinophils from asthmatic patients than in control subjects [12]. The *-444A>C* polymorphism was identified in the LTC4S gene promoter region. An association between the polymorphic -444C allele and susceptibility to asthma and/or asthma severity has been described [13-15].

Pharmacological studies have determined that cys-LTs activate at least two types of receptors, designated CYSLTR1 and CYSLTR2 [16, 17]. CYSLTR1-selective antagonists, such as montelukast, zafirlukast and pranlukast are important in the treatment of asthma [18-20]. CYSLTR1 is expressed in airway smooth muscle cells, tissue macrophages, monocytes, and eosinophils [21]. The biological actions of Cys-LTs probably occur because of binding to their receptors, CYSLTR1 and CYSLTR2, on the surface of target cells [22]. Activation of CYSLTR1 by LTD4 results in proliferation and contraction of smooth muscle, edema and eosinophil migration to the lung [23, 24]. In this study, we analyzed the polymorphisms *-444A>C* of LTC4S and *927T>C* of *CYSLTR1* and their combinations in a population of patients with asthma.

## Materials and Methods

### Study Populations

A total of 208 unrelated Caucasian individuals were included in this study. One hundred and thirty asthmatic patients and seventy-eight normal healthy controls were enrolled from the Allergy Department of the Salamanca University Hospital. The study was carried out after approval from the Hospital Ethical Committee and all the procedures followed its recommendations, including

*Table 1.* Demographic characteristics of subjects.

| Characteristic | Controls | Patients | *P*-value |
|---|---|---|---|
| No of subjects | 78 | 130 | |
| Age + SD (y) | 40.9±18 | 32.2±16.9 | 0.001 |
| Sex (No)<br>Female<br>Male | <br>53<br>25 | <br>77<br>53 | <br>0.24 |
| Log IgE | | | |
| Geometric mean | 1.52 | 2.30 | <0.0001 |
| 95% confidence interval | (1.37-1.64) | (2.19-2.41) | |

SD: Standard Deviation

 

obtaining informed written consent in all cases. Healthy individuals were enrolled as controls when meeting all the following criteria: no symptoms or history of asthma or other pulmonary diseases; no symptoms or history of allergy; negative skin prick tests to a battery of common aeroallergens (< 1 mm wheal greater than saline); absence of first degree relatives with a history of asthma or atopy. In order to permit a longer period for an asthma diagnosis to be made, control individuals were older. Patients with asthma were included in the study if they fulfilled the following criteria: at least two symptoms consistent with asthma (cough, wheeze and dyspnea); either bronchial hyperreactivity (BHR), defined by a positive methacholine test or a positive bronchodilator test; and absence of other pulmonary disorders. Lung function was measured by spirometry following ATS criteria [25]. The clinical characteristics of patients are shown in Table 1.

Skin tests were considered positive if at least one wheal reaction of more than 3 mm in diameter after subtraction of negative control was observed in a battery of common aeroallergens, as previously described [26] following the EAACI (The European Academy of Allergology and Clinical Immunology) recommendations [27]. Total IgE values were determined by a fluorenzymeimmunoassay (Pharmacia Cap System; Pharmacia, Uppsala, Sweden), following the manufacturer's instructions.

## Genotype Analysis

For the genotype study, genomic DNA extraction from total blood was performed with the *DANAPURE "SSS" DNA* Extracción Kit (Genedan, S.L, Spain). Polymerase chain reaction (PCR) amplifications of the corresponding fragments from the LTC4S promoter region and the *CYSLTR1* gene were performed in a MWG-BIOTHECH thermal cycler. For each SNP a pair of upstream and downstream primers was used to amplify the genomic region surrounding the SNP of interest: 5´-CTCCATTCTGAAGCCAAA-3' and 5'-AGACCGCCTCACCACTT-3' (for *-444A>C LTC4S*); and 5´-AAATCATGTTTTGGTCTTGC-3' and 5'-ATTTTCATTGGTTTGACTG-3' (for 927T>C CYSLTR1). The reaction mixtures were performed in 25 $\mu$l final volume reactions including 20 ng of genomic DNA, 1.25 pmol of each primer and 12.5 $\mu$l of a commercial PCR Master Mix (Promega, Madison, USA) containing Taq polimerase, dNTPs and MgCl2. Cycling conditions included one cycle of 95°C for 5 minutes; 40 cycles at 94#gC for 1 minute, 60°C for 1 minute and 72°C for 1 minute; and a final additional extension period at 72°C for 10 minutes. To avoid contaminations, negative controls without genomic DNA were included in each PCR reaction. Laboratory procedures were performed following the European Molecular Genetics Quality Network best practice guidelines.

Amplicons were visualised on a 2% agarose gel using ethidium bromide. GENECLEAN Turbo kit (Q-BIOgene, CA, USA) was used for the clean-up of the PCR products. To characterize and confirm polymorphisms, the clean PCR products were sequenced in a 3100 Genetic Analyzer (Applied Biosystems, CA, USA) using the primers previously employed in PCR amplification. Control and patients were not genotyped in separate batches. 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 blind with respect to case-control status.

## Statistical Analysis

For the 927T>C CYSLTR1 polymorphism, allele frequencies were analyzed taking into account that males provide only one copy of the X chromosome. Hardy-Weinberg equilibrium was estimated by Chi-square test (Pearson´s p value). For comparison of the distribution of categorical variables, $\chi^2$ test and Fisher exact test on contingency tables were applied. Monte Carlo simulation was provided when required. ANOVA was used to compare continuous variables across the levels of each genotype. IgE levels were transformed to log10 values to provide a normal distribution for statistical analysis. A *P*-value of less than .05 was considered statistically significant. The *927T>C CYSLTR1* and -444A>C LTC4S combination analysis was simultaneously performed considering all combinations with a frequency of more than 1 percent among either patients or controls. Monte Carlo simulation test, normal $\chi^2$ test and odds ratio test were used. Statistical power [28] and FPRP [29] were calculated when required. All statistical analyses were performed using SPSS version 12.0 (Chicago, Illinois, USA), and SHEsis software [30].

# Results

Phenotypic characteristics of our population are shown in Table 1. Logically, IgE levels were significantly lower in the group of controls than in patients, due to selection criteria. The distribution by sex was similar in both groups. However, results for male and female subjects were calculated separately because of the genetic location of *CYSLTR1* gene in chromosome X.

Genotype frequencies did not differ significantly from Hardy-Weinberg equilibrium in the female group. Genotype and allele frequencies of *-444A>C LTC4S* and *927T>C CYSLTR1* polymorphisms are shown in Table 2.

No association between the -444A>C LTC4S SNP and the asthma phenotype was detected in the population studied. However, for CYSLTR1 clear differences were observed in the allele distribution between genders. These differences are due to the fact that males provide only one copy of the X chromosome. Therefore, they are effectively haploid for that chromosome. Thus, haploid phenotype

*Table 2.* Genotype and allele frequencies of *927T>C CYSLTRI* and *–444A>T LTC4S* polymorphisms.

| SNP | | Genotype | | | Alelle | |
|---|---|---|---|---|---|---|
| | | Controls | Asthma | | Controls | Asthma |
| ***927T>C*** | | | | | | |
| Males | T | 0.92 (23) | 0.77 (41) | T | 0.92 | 0.77 |
| | C | 0.08 (2) | 0.23 (12) | C | 0.08 | 0.23 |
| Females | TT | 0.64 (34) | 0.62 (48) | T | 0.78 | 0.77 |
| | TC | 0.28 (15) | 0.30 (23) | | | |
| | CC | 0.08 (4) | 0.08 (6) | C | 0.22 | 0.23 |
| ***-444A>C*** | | | | | | |
| Males | AA | 0.60 (15) | 0.44 (23) | A | 0.76 | 0.66 |
| | AC | 0.32 (8) | 0.45 (24) | | | |
| | CC | 0.08 (2) | 0.11 (6) | C | 0.24 | 0.34 |
| Females | AA | 0.44 (23) | 0.52 (40) | A | 0.67 | 0.73 |
| | AC | 0.47 (25) | 0.42 (32) | | | |
| | CC | 0.09 (5) | 0.06 (5) | C | 0.33 | 0.27 |

reflects the presence of whatever allele is present. In the group of male individuals, the C allele of 927T>C CYSLTR1 was more common among patients with asthma (23%) than controls (8%), while in females, there were no differences in C allele distribution between patients with asthma (23%) and controls (22%) (Table 2).

The frecuencies of allele combinations of both polymorphims are shown in Table 3.

In the male group, we detected differences in the global distribution of *927T>C CYSLTR/-444A>C LTC4S* combinations between the group of patients with asthma and the group of controls. The combination of both the T allele of *927T>C CYSLTR1* and the A allele of *-444A>C LTC4S* was slightly more common in controls than in patients with asthma, Fisher´s *P*-value = .039; Monte Carlo *P*-value (after 104 simulations) = .045; OR, 0.37; 95% CI, 0.14-0.99. In addition, the CA combination (927C CYSLTR1 allele and -444A LTC4S allele) seems to be slightly more common in patients with asthma than in controls.

In the group of female subjects, no significant

*Table 3.* Genetic combination of *927>C CYSLTRI* and *–444A>T LTC4S* in controls and patients with asthma

| | Genotype | | | Alelle | |
|---|---|---|---|---|---|
| | Controls | Asthma | | Controls | Asthma |
| Male | n = 25 | n = 53 | | n = 25 | n = 53 |
| TAA | 0.60 | 0.36 | *TA | 0.88 | 0.67 |
| TAC | 0.28 | 0.34 | TC | 0.04 | 0.10 |
| TCC | 0.04 | 0.07 | CA | 0.00 | 0.09 |
| CAA | 0.00 | 0.08 | CC | 0.08 | 0.14 |
| CAC | 0.04 | 0.11 | | | |
| CCC | 0.04 | 0.04 | | | |
| Females | n = 53 | n = 77 | | n = 53 | n = 77 |
| TTAA | 0.28 | 0.31 | TA | 0.55 | 0.54 |
| TTAC | 0.30 | 0.26 | TC | 0.23 | 0.23 |
| TTCC | 0.06 | 0.05 | CA | 0.12 | 0.19 |
| TCAA | 0.15 | 0.16 | CC | 0.10 | 0.04 |
| TCAC | 0.13 | 0.13 | | | |
| TCCC | 0.00 | 0.01 | | | |
| CCAA | 0.00 | 0.05 | | | |
| CCAC | 0.04 | 0.03 | | | |
| CCCC | 0.04 | 0.00 | | | |

*The order of the SNPs in the combinations was 927T>C and -444A>C

 

differences were observed between controls and patients with respect to the 927T>C SNP. In this group, the overall distribution of the SNPs combinations was not significantly associated with asthma.

## Discussion

In the present study, due to the genetic location of the *CYSLTR1* gene in chromosome X, we separately analyzed the male and female populations, and obtained different results in both groups. We detected an increase of the *C* ALLELE of *927T>C SNP* in the group of male individuals with asthma. Choi et al, previously described this polymorphism without detecting any association with the asthma phenotype, but in their study individuals were not distributed by sex. Although this is a synonymous change, it has been suggested that this SNP may affect the efficiency of *CYSLTR1* gene transcription or translation, or alternatively be in tight linkage disequilibrium with (an) other polymorphism(s) in functionally important genomic elements of the *CYSLTR1* gene [31]. In this sense, we previously identified a significant association between atopic dermatitis and the C allele of the *927T>C* polymorphism in a group of male children.

$LTC_4$ synthase is the terminal enzyme in the generation of cys-LTs. $LTC_4$ synthase metabolizes $LTA_4$ to $LTC_4$ through a glutathione transferase. The gene encoding $LTC_4$ synthase resides on human chromosome 5q in a region associated with many other genes linked with asthma and atopy [32]. The cysteinyl leukotrienes (cysLTs)-leukotriene C4 (LTC4), LTD4, and LTE4 have long been implicated in the pathogenesis of asthma. The *-444A>C LTC4S* polymorphism has been found to create an additional response element for histone H4 transcription factor-2 and to increase the transcription rate or the gene in vitro and in vivo in a Polish population [12, 33]. The variant LTC4 synthase genotypes are reported to be highly prevalent in patients with aspirin intolerant asthma [33], and severe asthma [12, 13], although these results are still controversial [14,15, 34-36]. We found no association between the *-444A>C LTC4S SNP* and the asthma phenotype in our population.

In the present study, we analyzed two different polymorphisms in two genes that are implicated in the action of leukotrienes. Consequently, we decided to analyze the effects of the allelic and genomic combinations of both polymorphisms because it has been suggested that although most identified polymorphisms have a small influence on multifunctional diseases, combinations of genetic polymorphisms have larger functional effects than individual variants, and thus might better explain susceptibility to asthma [37].

We identified a slight increase of the SNP combinations in the male population with asthma. Particularly, we identified two specific combinations CA (*927C CYSLTR1* allele and *-444A LTC4S allele*) and TA (927T CYSLTR1 allele and *-444A LTC4S allele*) that showed differences in their distribution among patients with asthma and controls. The CA combination was more common in patients with asthma but given its odds ratio (OR), the results should not be considered significant. The TA combination was more frequent among controls. In the group of female subjects, no association between overall distribution of the SNP combinations and asthma was observed. Data from epidemiological studies consistently reveal that asthma in children is more frequent in boys than in girls [38, 39]. As age increases, however, the difference between the genders narrows.

As positive results in genetic association studies for detection of genetic variants contributing to complex outcomes may be influenced by several factors, special caution must be taken when approaching these studies. Great care must be taken in phenotype classification and application of quality control in the performance of laboratory procedures, [40, 41] although true heterogeneity in gene-disease associations may be found. Our study was performed applying restrictive criteria to the asthma phenotype classification. In addition, controls' and patients' genotype was determined double blinded in mixed batches, and SNPs were closely scrutinized to ensure that they were genotyped correctly [42]. Finally, the European Molecular Genetics Quality Network (EMQN) good practice guidelines were followed in all measures to guarantee the quality of laboratory procedures.

Furthermore, this association could be affected by type I error. Multiple comparisons and the sample size are important aspects that must be considered. Apart from the restrictive criteria for inclusion, distribution of patients according to sex further limits the sample size of this study. Since this is the first study of this genetic combination, there are no previous data on its frequency, thus we could not reliably calculate the statistical power [28]. Taking as expected frequency the frequency detected in our population, the statistical power would be lower than 70% with an alpha error of less than .05. In addition, the False Positive Report Probability (FRPR) [29] could be calculated. Since there are no previous data, if we consider the present OR, the FPRP would be more than 70% for a pre-test probability of 1%. This is a clear example of how a statistical significance lower than .05 is insufficient to confirm a genetic association. Nevertheless, the results suggest a certain trend of associations that could help to explain some controversial results in association studies of these genes from the leukotriene pathway, when considered individually. To the best of our knowledge, this is the first study of the *927T>C CYSLTR1/-444A>T LTC4S* combination. Further studies are needed to confirm associations and to characterize variants that might be of potential interest in the evaluation of the diagnosis, prognosis and treatment of an illness as frequent as asthma.

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