

## ORIGINAL PAPER

# The cysteinyl-leukotriene type 1 receptor polymorphism 927T/C is associated with atopy severity but not with asthma

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## Clinical and Experimental Allergy

### Summary

**Background** The cysteinyl-leukotriene receptor type 1 (CysLT<sub>1</sub>) mediates the bronchoconstrictor and pro-inflammatory actions of cysteinyl-leukotrienes (LTC<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub>) in asthma and is the molecular target of the lukast class of oral anti-leukotriene drugs. We screened the *CYSLTR1* gene on chromosome Xq13–21 for coding region polymorphisms, and investigated their associations with allergy and asthma.

**Methods** Solid-phase chemical cleavage was used to screen polymorphisms in the coding region of *CYSLTR1*. A TaqMan allelic discrimination assay was used to genotype a 927T/C SNP and oligonucleotide ligation assays were used to genotype the previously reported 617T/C and 898G/A SNPs of *CYSLTR1* in 341 asthmatic families from the UK. Associations with asthma diagnosis, atopic status, serum-specific IgE and severity of allergy and asthma were examined. **Results** Family-based association tests showed that the 927 T allele was associated with atopy severity, especially in female subjects, but not with asthma diagnosis or severity, atopic status, bronchial hyper-responsiveness to methacholine or forced expiratory volume in 1 s.

**Conclusion** Mutation screening identified only one polymorphism, 927T/C, in the coding region of the CysLT<sub>1</sub> receptor. This polymorphism is predictive of atopy severity, but not associated with asthma.

**Keywords** asthma, atopy, genetic, leukotriene receptor, polymorphism

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### Introduction

The cysteinyl-leukotrienes (cys-LTs) (LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>) are lipid mediators generated in activated leucocytes from arachidonic acid via the 5-lipoxygenase/LTC<sub>4</sub> synthase pathway. Cys-LTs have significant pathophysiological activities in asthma, including bronchoconstriction, plasma exudation, mucus secretion and eosinophil recruitment [1].

Two human cys-LT receptors (CysLT<sub>1</sub> and CysLT<sub>2</sub>) have been defined pharmacologically [2] and more recently the genes encoding these receptors (*CYSLTR1* and *CYSLTR2*) have been cloned [3–6]. *CYSLTR1* has been mapped to chromosome Xq13–21 and encodes a seven-transmembrane-spanning G-protein-coupled receptor that mediates bronchoconstriction and many other activities of cys-LTs in asthma. The cloned cDNA is 1014 bp in size (Genbank accession number AF119711) and encodes 337 amino acids. New EST sequences deposited in Genbank extend the 5'UTR sequence of *CYSLTR1*. Alignment of this se-

quence with the human genomic DNA sequence suggests that *CYSLTR1* has three exons, the first two of which are transcribed but not translated. The coding region is in the third exon and is intronless. The structure of the *CYSLTR1* gene has been characterized recently, confirming that the cloned cDNA is from the dominant transcript and that its coding region is in the last exon and is intronless [7].

The CysLT<sub>1</sub> receptor is expressed on bronchial smooth muscle cells and macrophages in normal human lung, and on peripheral blood eosinophils, pregranulocytic CD34<sup>+</sup> cells and subsets of monocytes and lymphocytes [8]. Blockade of the CysLT<sub>1</sub> receptor by potent and selective antagonists such as montelukast and zafirlukast produces significant improvements in lung function and asthma control. These oral compounds entered clinical practice in 1997–1998 as the first entirely new class of anti-asthma drugs in many years [9]. Clinical responsiveness to leukotriene receptor antagonists varies between patients, but is relatively consistent in individual patients over time [10].

Coding polymorphism in the CysLT<sub>1</sub> receptor gene may predispose some individuals to an enhanced contribution of cys-LTs to asthma susceptibility and severity, with implications for the targeting of CysLT<sub>1</sub> receptor antagonists to those patients most likely to benefit.

The *CYSLTR2* gene has been mapped to the chromosome 13q14.2 and encodes a serpentine G-protein-coupled receptor of 346 amino acids. While CysLT<sub>1</sub> is a high-affinity receptor for LTD<sub>4</sub>, CysLT<sub>2</sub> is a somewhat lower-affinity receptor for LTC<sub>4</sub> and LTD<sub>4</sub>, with LTE<sub>4</sub> being much less active at both receptors. CysLT<sub>2</sub> receptors are found on some leucocyte subtypes, and also, unlike CysLT<sub>1</sub>, in the central nervous system and in the heart [5]. There is some evidence that genetic polymorphism in *CYSLTR2* plays a role in asthma susceptibility. A significant association of the *CYSLTR2* coding polymorphism 601 A/G with asthma was observed in populations from Denmark and Minnesota [11], and M202V (604 A/G) of *CYSLTR2* is associated with an atopic phenotype in a Tristan da Cunha population [12].

To test the hypothesis that polymorphism of *CYSLTR1* may increase susceptibility to asthma, we firstly screened the coding region of the *CYSLTR1* gene for the presence of novel polymorphisms. Secondly, two previously described polymorphisms (617T/G and 898G/A) (numbered from the ATG start codon) [13] that result in Ile206Ser and Gly300-Ser substitutions, respectively, were genotyped in a large family-based Caucasian cohort. Finally, an identified synonymous polymorphism (927 C/T) of the *CYSLTR1* coding region was genotyped in the same cohort.

## Materials and methods

### *Subjects and clinical assessment*

Three hundred and forty-one Caucasian affected sib-pair families were recruited from the Southampton area in the UK as described [14, 15]. Families had at least two biological siblings (aged 5–21 years) with a current physician diagnosis of asthma and were taking asthma medication on a regular basis. Serum total IgE levels were determined using a UniCAP system (Pharmacia Diagnostic, Milton Keynes, UK), and levels of specific IgE for grass, housedust mite, cat, dog, *Alternaria* and tree allergens were determined by a radioallergosorbent test. Skin prick tests (SPTs) were also performed for the same common allergens. Baseline lung function was assessed by forced expiratory volume in 1 s (FEV<sub>1</sub>, best of three values within 5%), performed at least 14 days after any respiratory tract infection. Bronchial responsiveness was measured in response to inhaled methacholine and the slope of the dose-response curve was used as a continuous variable (transformed to  $[1/(\text{LS slope} + 30)] \times 1000$  to improve normality). Subjects were defined as asthmatic if they had a physician's diagnosis of asthma, together with current asthma medication use. Atopy was defined as

either positive SPTs (>3 mm) or raised specific IgE (>0.35 IU) to one or more common allergens [14]. An atopy severity score was derived from the mean wheal diameters of each allergen and level of specific IgE response (magnitude of response), and the number of positive SPTs and specific IgE responses (range of response) principal component analysis. An asthma severity score was calculated by taking into account treatment, symptoms and bronchial hyper-reactivity (BHR) [14]. Ethical approval was obtained from the Local Research Ethics Committees of Southampton & South-West Hampshire and of Portsmouth and South-East Hampshire. Genomic DNA was extracted from 10 mL of blood using a genomic DNA MaxiPrep kit (Qiagen, Crawley, UK).

### *Mutation detection*

The coding region of *CYSLTR1* was screened for the presence of polymorphisms in 30 asthmatic individuals using solid-phase chemical cleavage of mismatches (CCM) as described [16]. Two sets of PCR products were generated using two sets of primers (one set biotinylated) on regions of interest; both probe and target amplicons were internally labelled using fluorescent dUTP. The primers (Table 1) were designed to span the coding region (genomic sequence supplied by Dr Jilly Evans, Merck & Co, West Point, PA, USA) to ensure that polymorphisms in the whole coding region could be detected by CCM. The size of the amplified fragment was 1595 bp. PCR cycling conditions were an initial 10 min at 96 °C, followed by 30 s at 94 °C, 30 s at 54 °C and 2 min at 72 °C for 36 cycles. After purification of the generated probe, it was mixed with target amplicon (molar ratio 1 : 20) to form a heteroduplex, and then modified by 4 M hydroxylamine (pH 6.0) and cleaved by 0.37 M piperidine. The products were mixed with GeneScan-2500 (ROX) size standard and loaded on a 12 cm 6% long ranger sequencing gel (6 M urea + 0.5 × TBE buffer) on the ABI Prism DNA sequencer equipped with GeneScan software. The gel was run at 750 V, 60 mA, 200 W and 51 °C for 5 h. The detected polymorphism was confirmed by DNA sequencing.

### *Genotyping*

The oligonucleotide ligation assay (OLA) was performed essentially as described [17] to genotype two previously identified polymorphisms in the *CYSLTR1* coding region [13]. PCR was performed using forward primer 5'-TGGA-TACCTGTAGGAT-3' and reverse primer 5'-ATGTATTTT-TATTGA-3'. The PCR conditions were the same as in the CCM. Twenty microlitres of PCR product was diluted in 50 µL of dilution buffer containing 0.1% Triton-X-100. Ten microlitres of the diluted PCR products were mixed with 10 µL of a solution containing 2 × ligase buffer (20 mM KCl, 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 40 mM Tris-HCl, 4 mM MgSO<sub>4</sub>, 0.2%

Table 1. Primer and probe sequences used in this study

| Application                            | Sequence (5' to 3')                      | Positions      |
|----------------------------------------|------------------------------------------|----------------|
| 1. CCM                                 |                                          |                |
| Forward primer                         | TGGATACCTGTAGGAT                         | – 220 to – 199 |
| Reverse primer                         | ATGTATTTTATTGA                           | 1355 – 1375    |
| 2. TaqMan allelic discrimination assay |                                          |                |
| Forward primer                         | AATTGTGCTTTGACCTCTCCTAT                  | 859–883        |
| Reverse primer                         | GGTACATAAGTCACGCTGGACAA                  | 940–962        |
| 927T probe                             | VIC-AGAATGCTTTCTA <u>A</u> ATGTAG-MGBNFQ | 920–939        |
| 927C probe                             | 6FAM-ATGCTTTCTG <u>A</u> ATGTAG-MGBNFQ   | 920–936        |
| 3. OLA                                 |                                          |                |
| 617 joining probe                      | P-TGTCTGTTACACAATGATCA-B                 | 618 – 637      |
| 617 T probe                            | F-ATCCCTTTTGTATTATAAT                    | 598–617        |
| 617 G probe                            | F-ATCCCTTTTGTATTATAAG                    | 598–617        |
| 898 joining probe                      | P-GTAACTTTAGGAAAAGGCTG-B                 | 899–918        |
| 898 G probe                            | F-CCTATATTTCTTTCTGGGG                    | 879–898        |
| 898 A probe                            | F-CCTATATTTCTTTCTGGGA                    | 879–898        |
| 4. Site-directed mutagenesis           |                                          |                |
| Primer A                               | CCACAAAAAGATGAGAAAAATAAT                 | 487–507        |
| Primer B1                              | CATTGTGTAACAGACACTTATAATAAC              | 610–636        |
| Primer B2                              | CCTAAAGTTACTCCAGAAAAG                    | 887–909        |
| Primer C1                              | TTTGTATTATAA <u>G</u> TGTCTGTTACA        | 604–623        |
| Primer C2                              | CTTTCTGGG <u>A</u> GTAACCTTAGG           | 887–909        |
| Primer D                               | CTTTCTGGGTACATAAGTCAC                    | 949–969        |

Positions listed are numbered relative to translation start site; the non-complementary bases are underlined. 6FAM and VIC, fluorescent reporter dyes; MGBNFQ, Minor groove binder/Non-fluorescent quencher; P, phosphate modified; B, biotin labelled; F, fluorescein labelled; OLA, Oligonucleotide ligation assay; CCM, chemical cleavage of mismatches.

Triton X-100, pH 8.8 and 2.5 U Taq DNA ligase) (New England Biolabs, Hitchin, UK), 1 pmol of allele-specific probe and 1 pmol of joining probe. Four separate OLA 50-cycle reactions were set up to detect 617T, 617G, 898G and 898A alleles, respectively, for each individual. Each cycle consisted of 93 °C for 1 min and 58 °C for 2 min for the 617T or 617G alleles, or 65 °C for 2 min for the 898G or 898A alleles. The primers and probes are listed in Table 1. Following ligation, reactions were stopped and products were transferred to a 96-well flat bottom streptavidin-coated microtitre plate that had been blocked with 0.5% BSA to reduce non-specific binding. This was then followed by two washes with NaOH buffer (0.01 M NaOH/0.05% Tween 20) and two washes with Tris buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20). Ligation products captured on the streptavidin plate were detected by the addition of horseradish peroxidase-labelled anti-fluorescein in 1 × phosphate-buffered saline with 0.5% BSA, followed by addition of TMB. Optical density was measured at 450 nm on an OD reader. OD > 0.15 was considered positive and < 0.15 was negative.

The 927T/C polymorphism was genotyped using a TaqMan allelic discrimination assay [18] developed for use on the 7900HT instrument (Applied Biosystems, Foster City, CA, USA). A pre-developed TaqMan assay reagent kit containing one pair of PCR primers and one pair of fluorescently labelled probes was used (Applied Biosys-

tems; <http://abassays.celera.com>). The primer and probe sequences are listed in Table 1. PCRs were performed in 5 µL reaction volumes in 384-well plates and contained 10 ng genomic DNA. The standard protocol provided with the kit was followed. Thermal cycler conditions were 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

### Statistical analyses

Family-based association tests (FBAT) using the additive model and bi-allelic tests [19] were used to investigate associations of the 927T/C polymorphism with asthma and atopy affection status and with related intermediate variables (FEV<sub>1</sub> % predicted, BHR, serum total IgE levels and asthma and atopy severity scores), using FBAT software available from <http://www.biostat.harvard.edu/~fbat.htm>. A disease severity analysis was performed using *t*-tests (SPSS software) to examine significant differences in the means of continuous atopy variables between single-gender genotype groups. A *P*-value of 0.05 or less was regarded as significant.

### Results

#### Polymorphisms within the coding region of *CYSLTR1*

A 927T/C polymorphism (numbered from ATG start codon) within codon 309 was identified using the CCM

assay and this SNP was confirmed by DNA sequencing. Fourteen out of 30 asthmatic samples contained this polymorphism, which does not modify the amino acid sequence of the CysLT<sub>1</sub> receptor protein. This polymorphism has subsequently been identified by other researchers [13]. The two amino acid-changing variants (617T/G or 898G/A) described previously [13] were not found using the CCM assay in any asthmatic or normal subject, indicating an allele frequency of these two polymorphisms  $\leq 3\%$  in the UK Caucasian population.

#### Genetic association studies

The 927T/C polymorphism was genotyped in the asthmatic cohort, with the allele frequency in the parents of 0.214 for the C allele. When the family-based association test [19] was used, the *CYSLTR1* 927T/C polymorphism did not show significant associations with asthma diagnosis, or atopic status, or with the continuous measures of lung function (BHR, FEV<sub>1</sub> % predicted) or with the asthma severity score (Table 2).

Two continuous atopy variables were also tested by FBAT, namely the atopy severity score and total serum IgE levels. As shown in Table 3, FBAT showed no significant association of 927T/C with total IgE, but a significant association was observed with the atopy severity score ( $P = 0.0148$ ); this association became more statistically significant when transmission to only female siblings was considered ( $P = 0.0087$ ), with no association being observed for male siblings ( $P = 0.2605$ ). No significant association was observed between the 927T/C polymorphism and total IgE or atopy severity score in male or female asthmatic siblings in the disease severity analyses ( $P > 0.05$ ).

As the two amino acid-changing variants (617T/G or 898G/A) may alter CysLT<sub>1</sub> interaction with its cys-LT agonists or with leukotriene antagonists, we used the OLA assay [17] to examine their frequency in 60 asthmatic families and in 90 non-asthmatic controls. Neither the 617T/G nor the 898G/A polymorphism was detected in any of these subjects. The frequency of these alleles in this representative subgroup (228 alleles) is  $\leq 0.004$  and therefore insufficient to justify genotyping the remaining 281 families. The results extend to well-defined asthmatic and normal populations the finding of a low frequency of 0.03 reported for these polymorphisms in immortalized lymphoblastoid cell lines (100 alleles) [13].

Validation of the OLA assay was required to draw firm conclusions regarding the frequency of these polymorphisms in the Caucasian population. The positive signal for the wild-type alleles (617T and 898G) at each locus was approximately five- to 10-fold greater than negative control, suggesting the OLA assay was valid for detecting these alleles (Fig. 1, upper panels). To confirm that the mutant alleles (617G and 898A) could also be detected, we

Table 2. FBAT results for *CYSLTR1*, asthma diagnosis and asthma phenotype

| Phenotype allele                          | Informative families | Allele frequency | FBAT <i>P</i> -value |
|-------------------------------------------|----------------------|------------------|----------------------|
| CRF asthma (dichotomous)                  |                      |                  |                      |
| Allele T                                  | 100                  | 0.808            | 0.4810               |
| Allele C                                  | 100                  | 0.192            | 0.4810               |
| Atopy (dichotomous)                       |                      |                  |                      |
| Allele T                                  | 99                   | 0.808            | 0.3232               |
| Allele C                                  | 99                   | 0.192            | 0.3232               |
| BHR (continuous)                          |                      |                  |                      |
| Allele T                                  | 99                   | 0.808            | 0.6873               |
| Allele C                                  | 99                   | 0.192            | 0.6873               |
| FEV <sub>1</sub> % predicted (continuous) |                      |                  |                      |
| Allele T                                  | 100                  | 0.808            | 0.9591               |
| Allele C                                  | 100                  | 0.192            | 0.9591               |
| Asthma severity score (continuous)        |                      |                  |                      |
| Allele T                                  | 99                   | 0.808            | 0.8046               |
| Allele C                                  | 99                   | 0.192            | 0.8046               |

BHR, bronchial hyper-reactivity; FEV<sub>1</sub>, forced expiratory volume in 1 s; FBAT, family-based association tests.

Table 3. FBAT Results for *CYSLTR1* and atopy variables, stratified by gender

| Phenotype (continuous) allele               | Informative families | Allele frequency | FBAT <i>P</i> -value |
|---------------------------------------------|----------------------|------------------|----------------------|
| Atopy severity score (all siblings)         |                      |                  |                      |
| Allele T                                    | 91                   | 0.808            | 0.0148*              |
| Allele C                                    | 91                   | 0.192            | 0.0148*              |
| Atopy severity score (female siblings only) |                      |                  |                      |
| Allele T                                    | 61                   | 0.807            | 0.0087*              |
| Allele C                                    | 61                   | 0.193            | 0.0087*              |
| Atopy severity score (male siblings only)   |                      |                  |                      |
| Allele T                                    | 65                   | 0.809            | 0.2605               |
| Allele C                                    | 65                   | 0.191            | 0.2605               |
| Total IgE (all siblings)                    |                      |                  |                      |
| Allele T                                    | 100                  | 0.808            | 0.3421               |
| Allele C                                    | 100                  | 0.192            | 0.3421               |
| Total IgE (female siblings only)            |                      |                  |                      |
| Allele T                                    | 72                   | 0.807            | 0.1785               |
| Allele C                                    | 72                   | 0.193            | 0.1785               |
| Total IgE (male siblings only)              |                      |                  |                      |
| Allele T                                    | 77                   | 0.809            | 0.8647               |
| Allele C                                    | 77                   | 0.191            | 0.8646               |

Three hundred and forty-one families tested for each phenotype, 240 families included at least one male sibling, 219 families included at least one female sibling.

\* $P$ -value  $< 0.05$ .

FBAT, family-based association tests.

used site-directed mutagenesis [20] with the primers shown in Table 1 to generate mutants containing the 617G or 898A allele. Each was blunt-cloned into the *Sma*I site of the plasmid vector pUC18. OLA analysis of the

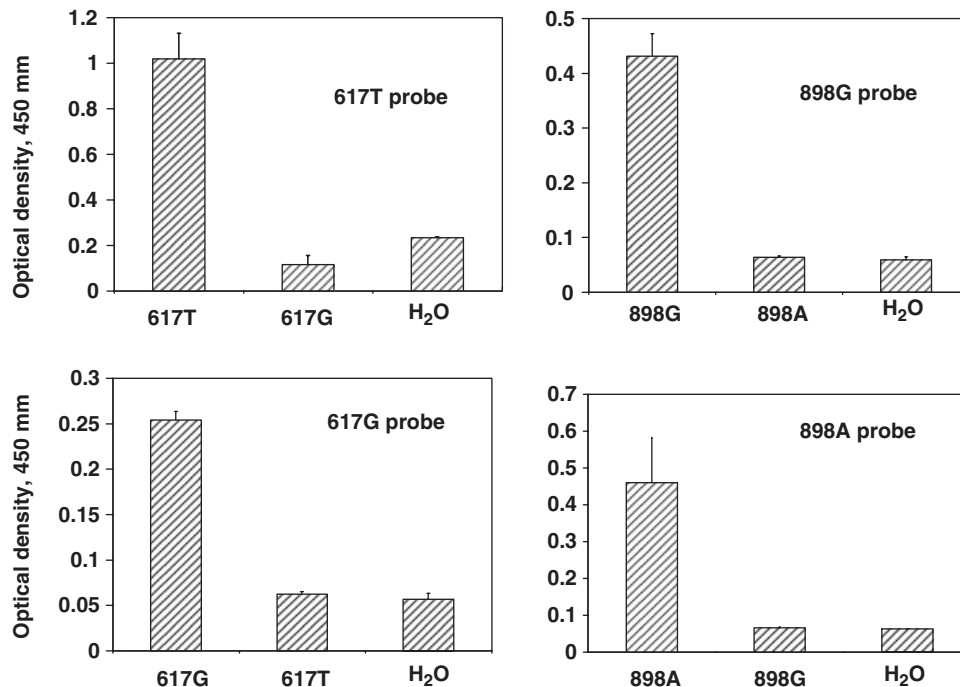


Fig. 1. Oligonucleotide ligation assay (OLA) analysis of wild-type alleles (617T and 898G) and artificial mutant alleles (617G and 898A). In each panel, the first bar refers to the analysis of DNA sample containing a specific allele using the corresponding probe; the second bar shows analysis of the DNA not containing the specific allele as an internal negative control; the third bar shows the analysis of distilled water as a negative control. The optical density was read at 450 nm and each value was the mean of three experiments. Each experiment was performed in triplicate.

plasmid DNA containing the 617G or 898A alleles showed a greater than fivefold signal compared with the negative controls (Fig. 1, lower panels). With positive controls available for all the investigated alleles, we repeated the OLA analysis on 90 non-asthmatic individuals and this replicated our previous findings.

## Discussion

This study detected and confirmed the previously identified 927T/C polymorphism in the coding region of *CYSLTR1*. We examined the association of the *CYSLTR1* 927T/C polymorphism with a number of atopy and asthma phenotypes in a large, well-defined Caucasian family cohort. While no significant associations were observed between this polymorphism and any of the asthma-related phenotypes examined, a significant association with the atopy severity score was observed in females. The frequency of two polymorphisms reported in lymphoblastoid cell lines (617T/G and 898G/A) was shown to be negligible in our UK Caucasian population.

We found a positive association of the 927T/C polymorphism of *CYSLTR1* with the atopy severity score in asthmatic families. The T allele was preferentially transmitted to offspring with more severe atopy as defined by the atopy severity score, compared with the C allele, which was undertransmitted ( $P=0.0148$ ). In contrast, no asso-

ciation was found between *CYSLTR1* 927T/C genotype and total serum IgE.

A connection between CysLT<sub>1</sub> and atopy defined as elevated IgE is not immediately apparent, but the receptor is expressed on mast cells and eosinophils, both being cell types implicated in allergic inflammation [8, 21, 22] and on activated T-cells [23]. The 927T/C polymorphism, although a synonymous change, may affect the efficiency of *CYSLTR1* mRNA processing and/or stability, or alternatively be in linkage disequilibrium with (an)other polymorphism(s) in functionally important genomic elements of the *CYSLTR1* gene. Duan et al. [24] reported that the synonymous human dopamine receptor (DRD2) mutation 957T has empirically measurable biological consequences compared with the wild-type 957C, which include decreased mRNA stability, decreased mRNA translation and a weakened response to dopamine-induced up-regulation of DRD2. It is plausible that the *CYSLTR1* 927T/C polymorphism could be analogous to the mutation in DRD2. To this end, folding of *CYSLTR1* 927T and 927C mRNA was checked using MFOLD 3.2.1 software (<http://bioweb.pasteur.fr/seqanal/interfaces/mfold.html>), but this did not reveal possible differences in mRNA secondary structure. Further studies are required to investigate the functional impact of other polymorphisms closely linked with this SNP and of other novel SNPs in the *CYSLTR1* promoter.

The fact that the association of 927T/C with atopy severity was most apparent when transmission only to female siblings was considered ( $P = 0.0087$ ) is intriguing, but it is hard to explain using current knowledge. Lynch *et al.* [3] mapped the *CYSLTR1* gene to Xq13–Xq21, a region lacking known markers of asthma. Ohd *et al.* [25] found a significantly higher frequency of CysLT<sub>1</sub> receptor staining in tissues from male patients affected by colorectal adenocarcinoma, suggesting the presence of allele-dependent dysregulation or sex-specific transcriptional subordination. The sex-specific phenomenon may be due to the location of *CYSLTR1* on the X chromosome, suggesting that X-inactivation may play a role. In accordance with recently published results in a Korean population [26], we did not find an association of the 927T/C polymorphism with asthma.

The two amino acid-changing polymorphisms 617T/G and 898G/A reported by others [13] were not found in 60 Caucasian asthmatic families (386 subjects, 60 unrelated asthmatics) or 90 unrelated non-asthmatic Caucasian individuals. Positive controls were used to confirm the validity of our OLA assay. Our results indicate that the amino acid-changing polymorphisms of *CYSLTR1* are very rare in the Caucasian population, and are unlikely to contribute either to the observed variability in clinical response to CysLT<sub>1</sub> receptor antagonists, or to asthma susceptibility or severity in a significant proportion of the population. Further investigations in other ethnic groups may be justified.

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