

ORIGINAL PAPER

Responsiveness to montelukast is associated with bronchial hyperresponsiveness and total immunoglobulin E but not polymorphisms in the leukotriene C4 synthase and cysteinyl leukotriene receptor 1 genes in Korean children with exercise-induced asthma (EIA) (responsiveness to montelukast on EIA in children)

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Clinical and Experimental Allergy

Summary

Background As previous studies have shown that cysteinyl leukotrienes are important mediators in exercise-induced bronchoconstriction (EIB), and leukotriene receptor antagonists (LTRAs) such as montelukast have been shown to improve post-exercise bronchoconstrictor responses, we herein investigated whether clinical responsiveness to montelukast was associated with polymorphisms in the genes encoding leukotriene C4 synthase (LTC4S) and cysteinyl leukotriene receptor 1 (CysLTR1) and/or clinical parameters in Korean asthmatic children with EIB.

Methods The study population consisted of 100 asthmatic children with EIB. The individuals studied were given exercise challenge tests before and after receiving montelukast (5 mg/day) for 8 weeks. Responders were defined as children showing >10% post-treatment improvement in forced expiratory volume in 1 s (FEV₁). The LTC4S A(–444)C and CysLTR1 T(+927)C polymorphisms were genotyped by PCR-restriction fragment length polymorphism analysis.

Results Of 100 enrolled children, 68 were classified as responders and 32 were classified as non-responders. No significant association was observed between montelukast responsiveness and LTC4S or CysLTR1 genotype, either alone or in combination. In contrast, montelukast-induced improvement in FEV₁ after exercise was correlated with higher pre-treatment PC₂₀ (methacholine) values ($r = 0.210$, $P = 0.036$) and lower total IgE levels ($r = -0.216$, $P = 0.031$).

Conclusions The LTC4S A(–444)C and CysLTR1 T(+927)C genotypes do not appear to be useful for predicting clinical responsiveness to montelukast, whereas bronchial hyperresponsiveness and total IgE appear to predict the degree of montelukast responsiveness in Korean asthmatic children with EIB.

Keywords Cysteinyl leukotriene, Cysteinyl leukotriene receptor 1, exercise-induced bronchoconstriction, Leukotriene receptor antagonist, LTC4S promoter, Pharmacogenetics

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Introduction

Asthma patients, particularly children and young adults with high activity levels, often develop exercise-induced bronchoconstriction (EIB) following long and/or intense

exercise [1]. Although the reported prevalence of EIB in asthmatics is high, ranging from 40% to 90% [2], its pathogenesis is poorly understood. Drying and cooling of the intrathoracic airways by inspired air is believed to act as the initial trigger for EIB [3], but the subsequent airway

responses are not yet completely understood. Unlike methacholine, which confers bronchoconstrictive activity by acting directly on smooth muscles, exercise likely bronchoconstricts via mediators released from intermediary cells (e.g. inflammatory cells, epithelial cells, or nerves) [4, 5]. The pathogenesis of EIB appears to involve the release of cysteinyl leukotrienes (CysLTs), as demonstrated by detection of released CysLTs in airways [6], increased urinary leukotriene E4 levels [7], increased CysLTs in exhaled breath condensates from children with EIB [8], and the improvement of EIB symptoms following administration of CysLT antagonists [9, 10].

The CysLT synthesis pathway involves the sequential action of enzymes, including 5-lipoxygenase (ALOX5), 5-lipoxygenase activating protein (ALOX5AP), and leukotriene C4 synthase (LTC4S) [11]. Of these, LTC4S, an integral membrane protein localized to the nuclear envelop of eosinophils and mast cells, is a key enzyme in CysLT synthesis, where it converts LTA(4) to LTC(4) [12]. The gene encoding LTC4S is located at chromosome 5q35 [13, 14]. Previous studies have reported weak associations between LTC4S promoter polymorphisms and the asthma phenotype [15], bronchial responsiveness to methacholine [16], and asthma severity regardless of aspirin sensitivity [17, 18]. In addition, our lab previously reported that the C allele of the LTC4S A(−444)C promoter polymorphism may be associated with the severity of bronchoconstriction after exercise in Korean children with asthma [19]. Furthermore, the CysLTs appear to function through at least two receptors; cysteinyl leukotriene receptor 1 (CysLTR1) and cysteinyl leukotriene receptor 2 (CysLTR2). Most of the biologic activities ascribed to CysLTs, including bronchospasm, plasma exudation, vasoconstriction, mucus secretion, and eosinophil recruitment, are mediated through interaction with the CysLTR1 subtype [20]. The CysLTR1 gene maps to the X chromosome near a region of interest in asthma (Xq13-21) [21]. CysLTR1 T(+927)C polymorphism previously was not associated with aspirin-induced asthma (AIA) in the Korean population [22]. From the previous results, LTC4S and CysLTR1 may be important for the function of leukotriene; we additionally wanted to investigate this single nucleotide polymorphisms (SNP) in children with EIB.

In recent years, several effective anti-asthma therapeutic strategies have been developed based on receptor antagonists including leukotriene receptor antagonists (LTRAs). For example, CysLTR1 is selectively antagonized by the LTRA, montelukast [23]. However, as in the case of most other asthma therapies, the efficacies of LTRA-based treatments have been limited by a large variability in clinical responsiveness. Thus, researchers have sought to develop strategies for predicting a given patient's response to various LTRAs.

In the present pharmacogenetic study, we examined the possible use of LTC4S A(−444)C and CysLTR1 T(+927)C

genotyping for predicting the efficacy of montelukast therapy in asthmatic Korean children with EIB, and we also investigated whether drug responsiveness was associated with clinical parameters.

Materials and methods

Subjects

A total of 100 asthmatic children with EIB were enrolled from the Asthma and Allergy Clinics of the Asan Medical Center (Korea). Following the American Thoracic Society (ATS) guidelines [24], asthma was confirmed on the basis of a history of dyspnoea and wheezing during the previous 12 months, a >15% reversal of forced expiratory volume in 1 s (FEV₁) either spontaneously or after β_2 -agonist inhalation, and/or a methacholine provocation test result with a PC₂₀ <16 mg/mL. The study was conducted from mid-July to early November, with all tests conducted between 15:00 and 17:00 hours. All the patients included had an FEV₁ of at least 80% of the predicted value, and a decrease in FEV₁ of at least 15% following the application of a standardized exercise challenge. Subjects were excluded from the study if they had been treated with orally administered or inhaled corticosteroids (ICS), long-acting β_2 -agonists, or LTRAs, with the exception of occasional use of short-acting β_2 -agonists. In addition, subjects were excluded from the study if their asthma had been exacerbated by a respiratory tract infection within 4 weeks before entering the study, or if the patient had become sensitized to outdoor pollen, as determined by skin prick test. These asthmatic children were enrolled prospectively following the first exercise challenge test. The study was approved by the ethics committee of the Asan Medical Center Institutional Review Board, and informed consent was obtained from all parents.

Study design

Before treatment, blood samples were collected for determination of peripheral blood eosinophil count, eosinophil %, and total serum IgE levels. Exercise challenge tests were given on day 1 of the study, between 15:00 and 17:00 hours. Thereafter, montelukast (5 mg) was administered daily at 19:00–20:00 hours for 8 weeks, and then the exercise challenge tests were repeated, again between 15:00 and 17:00 hours. Subjects showing a >10% post-treatment improvement in the maximal % fall of FEV₁ were defined as 'responders', and those exhibiting a smaller improvement in maximal % fall of FEV₁ were defined as 'non-responders.' Improvement (%) was calculated as follows: $100 \times (\text{maximal \% fall in FEV}_1 \text{ before treatment} - \text{maximal \% fall in FEV}_1 \text{ after treatment}) / \text{maximal \% fall in FEV}_1 \text{ before treatment}$.

Exercise challenge test

The standardized exercise challenge consisted of 8 min of free running outdoors while mouth breathing, as described previously [10]. This workload was performed based on a mean temperature of $19 \pm 5^\circ\text{C}$ and a relative humidity of $57 \pm 12\%$, as obtained from a local weather centre on the testing days. Short-acting β_2 -agonists and montelukast were withheld for at least 48 h before the exercise challenges.

Spirometry was performed on each subject using a standard spirometer (Microspiro HI-298, Tokyo, Japan). The pre-exercise FEV₁ was required to be at least 80% of the predicted value for the exercise challenge to be performed. Lung function was measured immediately after exercise, as well as at 5, 10, 15, 20, 25, 30, 45, and 60 min post-exercise. All spirometric measures met the ATS criteria for acceptability and reproducibility [25].

Genotyping

The LTC4S A(−444)C and CysLTR1 T(+927)C alleles were analysed with a modification of the PCR-restriction fragment length polymorphism (RFLP) protocol originally described by Unoki et al. [26]. A promoter fragment containing the LTC4S A(−444)C polymorphism was PCR amplified from patient DNA using specific primers (5′-GCT GTG TGT GAA GGC GAG C-3′ and 5′-TAC AAC TAC TAA GGC TGG CA-3′). The PCR products were digested with 10 U of *MspI* (Promega, Madison, WI, USA) at 37°C for 4 h, and separated on 3% agarose gels (FMC BioProducts, Rockland, ME, USA). The LTC4S −444C allele was identified by the presence of 169 and 32 bp digestion products. Similarly, the fragment containing the CysLTR1 T(+927)C polymorphism was PCR amplified using specific primers (5′-CTC TCC TAT ATT TCT TTT CTG G-3′ and 5′-CTA TAC TTT ACA TAT TTC TTC TCC-3′). The PCR products were digested with 5 U of *Hpy188 I* (New England BioLabs, Boston, MA, USA) at 49°C for 4 h, and separated on 2.5% agarose gels (FMC BioProducts). The CysLTR1 927C allele was identified by the presence of 97 and 53 bp digestion products. For validation of our RFLP analysis, 20 DNA samples from the study subjects were randomly selected and genotyped by DNA sequencing. In each case, the sequencing and RFLP data were consistent with one another.

Statistical analysis

Statistical analyses were performed using SPSS 11.0 software for Windows (SPSS Inc., Chicago, IL, USA). All data are presented as mean $\pm$ standard deviation. Clinical parameters and genotype frequencies were compared using the Mann–Whitney test, Kruskal–Wallis analysis, or χ^2 analysis. Correlations were evaluated by using the

Spearman's rank test. For analysis of combined effects, *P*-values were determined by logit analysis, with adjustment for age and sex. Results were considered to be significant at a *P*-value of <0.05 .

Results

Baseline characteristics

In total, 100 children were enrolled in the study (see Table 1 for clinical and demographic information). Following 8 weeks of treatment with montelukast, 68 of the subjects (68%) showed more than a 10% improvement in the maximal % fall in FEV₁ after exercise (responders), whereas 32 subjects (32%) showed $<10\%$ improvement (non-responders). When we analysed drug responsiveness in relation to eosinophil fraction, log IgE, log PC₂₀, baseline maximal % fall in FEV₁ after exercise, and pulmonary function test, our results revealed that the log PC₂₀ values were lower in non-responders (0.13 ± 0.08 mg/mL) than in responders (0.28 ± 0.05 mg/mL), and the log IgE values were higher in non-responders (2.55 ± 0.09 IU/mL) vs. responders (2.38 ± 0.06 IU/mL), but neither difference reached statistical significance. The total eosinophil counts, eosinophil fractions, FEV₁, FEF_{25–75%}, and baseline maximal % fall in FEV₁ after exercise did not differ significantly between responders and non-responders.

Correlations between clinical parameters and drug responsiveness

Among the 100 enrolled asthmatic children with EIB, the degree of improvement in maximal % fall of FEV₁ after exercise was significantly correlated with log IgE levels ($r = -0.216$, $P = 0.031$) and baseline log PC₂₀ ($r = 0.210$,

Table 1. Univariate analysis of clinical responsiveness to montelukast treatment

Clinical parameter*	Total	Responders	Non-responders
Number (M/F)	100 (68/32)	68 (41/27)	32 (27/5)
Age (years)	9.08 ± 1.78	9.25 ± 1.82	8.71 ± 1.66
TEC (/ μL)	588 ± 350	610 ± 334	542 ± 382
Eosinophil (%)	7.84 ± 4.43	8.00 ± 4.14	7.50 ± 5.05
Log IgE (IU/mL)	2.43 ± 0.50	2.38 ± 0.48	2.55 ± 0.54
Log PC ₂₀ (mg/mL)	0.23 ± 0.43	0.28 ± 0.42	0.13 ± 0.45
Maximum % fall in FEV ₁ (%)	38.3 ± 15.5	38.9 ± 15.1	37.0 ± 2.89
FEV ₁ (%)	83.0 ± 12.9	83.9 ± 13.1	81.0 ± 12.3
FEF _{25–75%} (%)	73.1 ± 21.7	74.2 ± 20.1	70.7 ± 25.3

*Mean $\pm$ standard deviation.

$P > 0.05$ for difference between responders and non-responders according to the Mann–Whitney *U*-test.

FEV₁, forced expiratory volume in 1 s; M, male; F, female; TEC, total eosinophil count.

Table 2. Correlation between clinical parameters and the degree of improvement in maximal % fall of FEV₁ after exercise

	Correlation coefficient*	P-value
TEC (/ μ L)	-0.074	0.464
Eosinophil (%)	-0.081	0.420
Log IgE (IU/mL)	-0.216	0.031
Log PC ₂₀ (mg/mL)	0.210	0.036
Maximum % fall of FEV ₁ (%)	0.064	0.524
FEV ₁ (%)	0.012	0.906
FEF _{25-75%} (%)	-0.003	0.979

*Spearman's correlation coefficient.

FEV₁, forced expiratory volume in 1 s; TEC, total eosinophil count.

Table 3. Drug responsiveness in patients with wild-type (AA) and variant (AC+CC) genotypes at the LTC4S A(-444)C polymorphism

	Genotype frequency (%)			P-value
	Number	AA	AC+CC	
Responders	68	44 (67%)	24 (71%)	0.822
Non-responders	32	22 (23%)	10 (29%)	

LTC4S, leukotriene C4 synthase.

$P=0.036$), but not the baseline maximal % fall in FEV₁ after exercise, total eosinophil count, eosinophil fraction, or FEF_{25-75%} (Table 2).

Influence of the LTC4S A(-444)C and CysLTR1 T(+927)C polymorphisms on drug responsiveness

To explore whether genetic variation in the LTC4S and/or CysLTR1 genes could influence the effect of montelukast on EIB, we genotyped each patient for two relevant polymorphisms: LTC4S A(-444)C and CysLTR1 T(+927)C. Our results revealed that homozygosity for the LTC4S AA genotype was found in 44 responders (65%) and 24 non-responders (35%), while the LTC4S variant allele (C) was carried by 22 responders (69%) and 10 non-responders (31%). No significant difference in carrier status was observed between the groups ($P > 0.05$, Table 3). Similarly, the CysLTR1 TT genotype was carried by 36 responders (62%) and 22 non-responders (38%), while the variant allele (C) was carried by 32 responders (76%) and 10 non-responders (24%), indicating no significant difference between the groups ($P > 0.05$, Table 4). Lastly, we investigated whether the LTC4S A(-444)C and CysLTR1 T(+927)C polymorphisms had any combined effect on the response of montelukast in EIB. We found no evidence of a synergistic effect between the LTC4S and CysLTR1 polymorphisms on the montelukast response in EIB (Table 5, Fig. 1). Thus, our results indicate that the LTC4S A(-444)C and CysLTR1 T(+927)C polymorphisms do not influence the effect of montelukast on EIB in asthmatic Korean children, either alone or in combination.

Table 4. Drug responsiveness in patients with wild-type (TT) and variant (CT+CC) genotypes at the CysLTR1 T(+927)C polymorphism

	Genotype frequency (%)			P-value
	Number	TT	CT+CC	
Responders	68	36 (62%)	32 (76%)	0.192
Non-responders	32	22 (38%)	10 (24%)	

CysLTR1, cysteinyl leukotriene receptor 1.

Table 5. Combined pharmacogenetic effect of the LTC4S A(-444)C and CysLTR1 T(+927)C polymorphisms on montelukast responsiveness

Group	Genotype		Responder	Non-responder	OR (95% CI)
	- 444/+927				
Total			68 100%	32 100%	
I	AA/TT		23 33.8%	15 46.90%	1.000
II	AA/CT+CC		13 19.10%	7 21.80%	0.83 (0.27-2.56)
III	AC+CC/TT		21 30.90%	7 21.80%	0.51 (0.17-1.49)
IV	AC+CC/CT+CC		11 16.20%	3 9.40%	0.42 (0.10-1.76)

CysLTR1, cysteinyl leukotriene receptor 1; LTC4S, leukotriene C4 synthase; OR, odds ratio; CI, confidence interval.

Discussion

Previous studies have shown that LTRAs provide overall beneficial effects for EIB [9, 10]. In the present study, 68% of the patients showed a significant improvement following an 8-week montelukast treatment course, while 32% did not. This is consistent with previous findings that 30-40% of the subjects failed to show an improvement in EIB following LTRA treatment [27], and underscores the importance of understanding the genetic and/or clinical bases underlying this differential responsiveness.

It is not yet known which step in the cysteinyl leukotriene production pathway is responsible for its overproduction in patients with EIB. Previous studies have shown that montelukast has a high binding affinity to CysLTR1 in receptor-binding models [23]. As human CysLTR1 is expressed primarily in airway smooth muscle cells, eosinophils, spleen, and macrophages [23] and a polymorphism in CysLTR1 has been associated with AIA, especially in males [28], we hypothesized that genetic differences in CysLTR1 might underlie the observed variations in patient response to montelukast. The +927T CysLTR1 polymorphism in male patients and female patients showed an allele frequency of 0.71 and 0.64, respectively. However, our results did not indicate a significant association between the CysLTR1 T(+927)C polymorphism and montelukast responsiveness in male or female patients, respectively (data not shown).

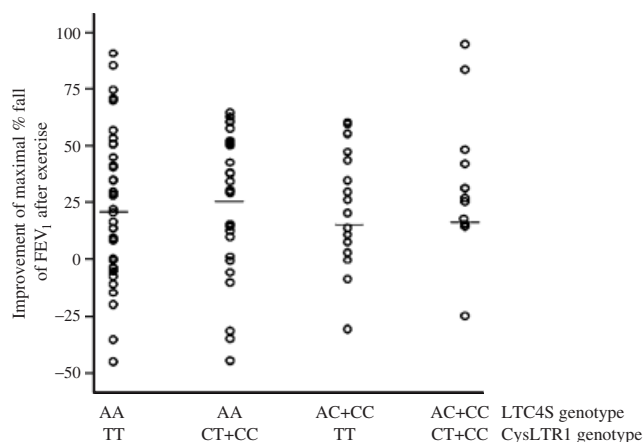


Fig. 1. Combined pharmacogenetic effect of the LTC4S A(–444)C and CysLTR1 T(+927)C polymorphisms on montelukast responsiveness. CysLTR1, cysteinyl leukotriene receptor 1; LTC4S, leukotriene C4 synthase; FEV₁, forced expiratory volume in 1 s.

We also examined the possible correlation of montelukast responsiveness with the A(–444)C LTC4S polymorphism, which has been associated with overproduction of cysteinyl leukotrienes, especially by eosinophils [29]. However, although this polymorphism has been associated with AIA [30, 31], the severity of exercise-induced asthma [19], and good pharmacological responses to zafirlukast in adults in some studies [18, 32] but not others [16, 33], our results did not support an association between this polymorphism and montelukast responsiveness in Korean children with EIB.

Because CysLTs are synthesized via a cascade of enzymes, it is likely that a variety of polymorphisms may underlie the observed variation in LTRA responsiveness among patients. Indeed, other genes encoding enzymes in the leukotriene biosynthetic pathway have been isolated, and some have been found to contain polymorphisms associated with the therapeutic response to leukotriene modifiers. In particular, polymorphisms in the genes encoding 5-lipoxygenase (5-LO) and LTC4S [34], which catalyse the conversion of arachidonic acid to leukotriene A₄ and the formation of cysteinyl LTC(4), respectively, have been shown to play important roles in the response to leukotriene modifier therapy [35, 36]. Furthermore, a number of metabolic proteins have been shown to mediate the effects of leukotriene, and several polymorphisms have been identified in the genes encoding these proteins [32, 37, 38]. Thus, future studies will be warranted to examine fully the mechanisms that underlie variability in montelukast responsiveness.

Although we were unable to identify an association between drug responsiveness and either of our candidate gene polymorphisms, our data showed that higher PC₂₀ values and lower IgE levels were associated with good montelukast responsiveness in our EIB patients. A previous study evaluating the effects of pranlukast for 4 weeks in

50 adults with moderate to severe asthma showed that responders had significantly lower baseline FEV₁ scores and a better response to salbutamol [32]. In the present study, we examined patients with mild asthma because LTRA is considered to be an alternative to ICS in mild persistent asthma and is considered to be a supplementary therapy to ICS for moderate persistent asthma [39]. While the previous study examined improvement in FEV₁ [32], we determined montelukast responsiveness based on improvement in the maximal % fall of FEV₁ after exercise challenge. We consider the change of maximal % fall in FEV₁ after exercise to be a more important parameter because we observed no significant change in overall FEV₁ following montelukast treatment (data not shown), and previous studies had shown no relationship between baseline lung function and EIB severity [40]. We also analysed whether genetic variation in the LTC4S A(–444)C and CysLTR1 T(+927)C genes could influence the effect of montelukast on the improvement of lung function (FEV₁). We could not find any significant differences in the improvement in FEV₁ post-drug and post-exercise between phenotypic and genotypic association (data not shown).

LTAs is effective in reducing bronchial hyperresponsiveness [41], allergen-induced bronchoconstriction [42], and the bronchoconstriction in aspirin-intolerant asthmatics [43]. However, there was no previous report to present clinical predictive factors such as PC₂₀, total IgE to evaluate the response of montelukast in asthmatic children with EIB.

When we performed the calculation of power for these SNPs, our studies showed poor power values (power <0.8). To obtain statistical significance, it is necessary to recruit more than 1000 study subjects because the difference in allele frequency is not prominent. Although we could not show the association between LTC4S and CysLTR1 polymorphisms and drug responsiveness, the possibilities of false negativity and additional polymorphisms related to drug responsiveness on EIB after montelukast treatment in the Korean children with asthma still remain.

In sum, here we report for the first time that the LTC4S A(–444)C and CysLTR1 T(+927)C polymorphisms do not appear to be associated with the effect of the LTRA, montelukast, on EIB in asthmatic Korean children, whereas low IgE levels and high PC₂₀ values appear to correlate with montelukast responsiveness. These novel findings may provide a useful method of predicting the clinical response to montelukast and we need further studies in larger cohorts of EIB patients with montelukast and other linked polymorphisms with the SNPs of these genes.

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