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ORIGINAL ARTICLE

Polymorphisms of the PTGDR and LTC4S influence responsiveness to leukotriene receptor antagonists in Korean children with asthma

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Activation of the prostaglandin D2 receptor (PTGDR) may contribute to pulmonary vasodilation, bronchoconstriction, recruitment of eosinophils, basophils and T-lymphocytes, and enhanced synthesis of leukotriene C4. We investigated whether polymorphisms of the leukotriene C4 synthase (*LTC4S*) *−444A/C* and *PTGDR* *−441T/C* were associated with clinical phenotypes and responsiveness to leukotriene receptor antagonist (LTRA) in Korean asthmatic children. We enrolled 270 normal and 870 asthmatic children. We prescribed montelukast (5 mg per day) to 100 of asthmatic children, and analyzed the responsiveness to LTRA by exercise challenge tests. Polymorphisms were genotyped by PCR–restriction fragment length polymorphism. As the number of minor alleles of the *PTGDR* *−441T/C* and *LTC4S* *−444A/C* polymorphisms increased, the log total eosinophil counts increased in atopic asthmatic children (*P*-value=0.03). We found a significant association between responsiveness to montelukast and the *PTGDR* polymorphism (*P*-value=0.038). However, the *LTC4S* *−444A/C* and *PTGDR* *−441T/C* were not associated with the susceptibility for asthma (*LTC4S*, *AA* versus *AC+CC*, adjusted odds ratio of 0.98 (95% confidence interval, 0.73–1.31); *PTGDR*, *TT* versus *TC+CC*, adjusted odds ratio of 0.90 (95% confidence interval, 0.68–1.19)) or clinical phenotypes (*P*-value > 0.05). The effects of the *PTGDR* and *LTC4S* polymorphisms on the enhancement of eosinophil counts were additive in the Korean children with asthma. In addition, the *PTGDR* polymorphism seems to be associated with the responsiveness to LTRA. Therefore, therapies that target the *PTGDR* may be useful for modulating the responsiveness to LTRA.
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INTRODUCTION

Asthma is a complex disease that results from interactions among multiple genes and environmental factors.[1] The Th2 cytokine-driven inflammatory mechanisms have an important role in the pathophysiology of asthma.[2] Th2 cytokines, such as interleukin-4, interleukin-13 and interleukin-5, participate in the synthesis of immunoglobulin E (IgE),[3] and promote allergic eosinophilic inflammation and airway remodeling.[4]

Asthma is often triggered by mast cells that are activated by an IgE-mediated allergic challenge.[5] Activated mast cells produce a variety of chemical mediators including prostaglandin D2 (PGD2), which is the major cyclooxygenase metabolite of arachidonic acid, in response to allergen exposure.[6] PGD2 may contribute to pulmonary vasodilation, bronchoconstriction, and the recruitment of eosinophils, basophils and T-lymphocytes.[7–9] PGD2 exerts its biological actions through the PGD2 receptor (PTGDR), which is localized to chromosome 14q22.1 and has been associated with asthma.[10] The association between asthma and PTGDR function has been recently studied with a mouse model. PTGDR-knockout mice ($PTGDR^{-/-}$) show only marginal infiltration of eosinophils, reduced levels of Th2 cytokines and accumulation of lymphocytes in the lungs, and no development of bronchial hyperresponsiveness on ovalbumin challenge compared with wild-type controls.[8] These findings suggest that asthma could be inhibited when the PTGDR is absent. The existence of genetic variants in the promoter region of the *PTGDR* (*−549*, *−441* and *−197*) have been reported for several ethnic groups.[11–14] These three polymorphisms were not always related to asthma, but they may have a role in controlling PTGDR expression. Therefore, the PTGDR may serve as a therapeutic target for asthma.

Leukotriene C4 (LTC4) and PGD2 are converted from arachidonic acid by 5-lipoxygenase and cyclooxygenase, respectively.[15–17] However, the results of a recent study showed that PGD2 activated eosinophils and enhanced LTC4 synthesis *in vivo*.[18] Accumulating evidence suggests that the cysteinyl leukotrienes (cysLTs) are the primary mediators of exercise-induced bronchoconstriction (EIB), as

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demonstrated by the detection of cysLTs in the airways,[19] increased levels of urinary leukotriene E4[20] and increased levels of cysLTs in exhaled breath condensates,[21] and in induced sputum[22] from children with EIB. Therefore, we presume that the function of PGD2 may affect the response of the leukotriene receptor antagonist (LTRA), which inhibits the effect of leukotriene, although the PTGDR is not directly included in the LTRA pathway. The purpose of this study was to understand the relationship between the presence of polymorphisms in the promoter region of the genes for leukotriene C4 synthase (*LTC4S*) and *PTGDR* and the responsiveness to a LTRA.

MATERIALS AND METHODS

Subjects

We collected DNA samples from subjects who came to asthma and allergy clinics and general pediatric clinics at the Asan Medical Center (270 normal children and 870 children with asthma). The normal controls had no history of asthma, other allergic diseases or airway hyperresponsiveness (PC_{20} (concentration of methacholine that provoked a 20% fall in FEV_1) $>16\,mg\,ml^{-1}$). They had negative result of skin prick tests, normal total IgE values ($\leqslant 100\,IU\,ml^{-1}$) and normal lung function tests. We enrolled all children with asthma who were diagnosed (i) by their physician and met the criteria set forth in the American Thoracic Society guidelines; (ii) on the basis of a history of dyspnea and wheezing during the previous 12 months; or (iii) as having a $>12\%$ FEV_1 after β_2-agonist inhalation, a methacholine provocation test indicating airway hyperresponsiveness ($PC_{20} <16\,mg\,ml^{-1}$) or both. The subjects who satisfied the American Thoracic Society guidelines and had negative result of skin prick tests and normal total IgE levels ($\leqslant 100\,IU\,ml^{-1}$) defined non-atopic asthmatics.

The subjects with asthma who were included in the drug responsiveness analysis performed an exercise provocation test, from the middle of July to the beginning of November, with all measurements conducted between 1500 and 17 hours. The FEV_1 of this subjects decreased by at least 15% following the standardized exercise challenge. Subjects were excluded from the study if they had been treated in the previous 3 months with orally administered or inhaled corticosteroids, long-acting β_2-agonists or a LTRA other than a short-acting β_2-agonist, or if they had experienced an exacerbation in their asthma or a respiratory tract infection within the 4 weeks before entering the study.

This study was approved by the ethics committee of the Asan Medical Center Institutional Review Board, and written informed consent was obtained from the parents of all study participants.

Serum IgE and skin prick tests

Total serum IgE concentrations were measured by fluorescent enzyme immunoassay using the AutoCAP System (Phadia AB, Uppsala, Sweden). Skin prick tests were performed using a panel of 27 common Korean aeroallergens (Allergopharma, Reinbek, Germany). A test was considered 'positive' if the maximum wheal diameter was 3 mm.

LTRA drug responsiveness study design

In all, 100 subjects with asthma performed an exercise challenge test twice both before and after receiving their daily dose of montelukast (5 mg per day) for 8 weeks. The standardized exercise challenge consisted of 8 min of free running outdoors, as previously described.[23,24] Subjects showing a $\geqslant 10\%$ post-treatment improvement in the maximum percentage fall in FEV_1 were defined as 'responders', and those subjects exhibiting $<0\%$ improvement (worsened values) in the maximum percentage fall in FEV_1 were defined as 'non-responders'. Improvement (%) was calculated with the following formula: $100\times$ [(maximum percentage fall in FEV_1 before treatment−maximum percentage fall in FEV_1 after treatment)/maximum percentage fall in FEV_1 before treatment].

Genotyping

DNA was isolated from blood samples from each subject using a G-DEX II kit (Intron, Seoul, Korea). PCR–restriction fragment length polymorphism was used to determine the polymorphisms. The primer pairs and annealing temperatures were as follows: 5′-cgagttcttggccaccccagttcaaacaccagcacaa-3′ and 5′-ggagcaggccagtgaaga-3′ and 57 °C for the *PTGDR* −441T/C (rs803010); 5′-CCTCAGTTTCCTCGCCTATG-3′ and 5′-GGCCAAGAACTCGAAAGATG-3′ and 56 °C for the *PTGDR* −549T/C (rs8004654); and 5′-tacaacgactaaggc tggca-3′ and 5′-gctgtgtgtgaaggcgagc-3′ and 58°C for the *LTC4S* −444A/C (rs730012). The restriction enzymes were as follows: Mfe I (New England BioLabs, Beverly, MA, USA) digested the *PTGDR*−441T allele into 195 and 35 bp fragments, HpyAV (New England BioLabs) digested the *PTGDR* −549C allele into 125 and 74 bp fragment and Msp I (New England BioLabs) digested the *LTC4S* −444C allele into 388, 169 and 32 bp fragments. To confirm the accuracy of restriction fragment length polymorphism analysis, we randomly selected 20% of the subjects for DNA sequencing of each polymorphism. The observed genotype distributions of the *PTGDR* −441T/C, −549T/C and *LTC4S* −444A/C did not deviate significantly from the Hardy–Weinberg equilibrium (P=0.377, P=0.372 and P=0.346, respectively).

Statistical analyses

The clinical parameters (for example, total IgE, PC_{20}, total eosinophil count (TEC)) were converted into log_{10}-based values in order to produce a normal distribution for the statistical analyses, and then the data were analyzed with Mann–Whitney *U*-tests. Subject demographic variables (for example, sex and age) were analyzed by multiple logistic regressions in order to determine whether or not the presence of particular alleles was disproportionate among the subjects. To analyze associations between the genotypes and asthma, we adopted a dominant model for the minor allele, assuming it was the risk allele and because relatively few individuals were homozygous for the risk alleles. An unconditional logistic regression analysis, adjusted for age and sex, was used to calculate the odds ratios, 95% confidence intervals and *P*-values. The relationship between clinical phenotype and genotype was tested using linear regression. The χ^2-tests were used to examine the relationship between responder or non-responder status and the *LTC4S* and *PTGDR* polymorphisms. All statistical analyses were performed using SPSS 18.0 for Windows (SPSS, Chicago, IL, USA), and *P*-values of $\leqslant 0.05$ were considered statistically significant. And bootstrapping (1000 times) was carried out for significant association and combination between two polymorphisms.

RESULTS

Clinical characteristics

We administered montelukast daily to 100 of the subjects with asthma, for 8 weeks, and subsequently divided the subjects into two groups: responders and non-responders. There were 92 subjects included in this analysis and, of these, 69 subjects (75%) were responders ($\geqslant 10\%$ improvement) and 23 subjects (25%) were non-responders ($<0\%$ improvement). The improvement of the 92 subjects was calculated by a numerical formula, and the percentages ranged from −113.1% to 95.0%. We compared the responders and non-responders in terms of their eosinophil fraction, log IgE levels, log PC_{20} levels, baseline maximum percentage fall in FEV_1 after exercise and pulmonary function, and there were no differences between the two groups. Sex ratio seems that there might be a difference, however, it is not different statistically (P=0.075 by χ^2-test). The responders and non-responders were randomly selected among 278, exercise-induced asthmatics who performed the exercise challenge test and who showed positive, EIB. However, as usually the severe EIB (+) subjects suggested severe asthmatic patients, LTRA treatment only may be used in mild asthmatics if their parents prefer it. Therefore, the maximum % fall in FEV_1 (%) differed in the subjects included in the LTRA study and in those who were excluded (Table 1).

Relationship between LTRA responsiveness and frequency of *LTC4S* and *PTGDR* polymorphisms

The *PTGDR* −549T/C and −441T/C polymorphisms were in almost complete linkage disequilibrium (D'=0.978), so we analyzed one among two polymorphisms. We chose a dominant model for the

Table 1 Clinical characteristics of the study patients

Clinical parameter[a]	*Responders (N=69)*	*Non-responders (N=23)*	*Subjects included in LTRA study*[b] *(N=100)*	*Subjects excluded in LTRA study*[b] *(N=178)*
Sex (M/F)	42/27	19/4	68/32	110/68
Age (years)	9.75 ± 1.75	8.89 ± 2.26	9.78 ± 2.57	9.49 ± 1.91
Log TEC (per μl)	2.71 ± 0.28	2.63 ± 0.38	2.62 ± 0.36	2.68 ± 0.31
Eosinophils (%)	8.01 ± 4.11	7.71 ± 5.10	6.94 ± 4.43	7.84 ± 4.43
Log IgE ($IU\,ml^{-1}$)	2.36 ± 0.49	2.59 ± 0.52	2.40 ± 0.57	2.43 ± 0.50
Log PC_{20} ($mg\,ml^{-1}$)	0.28 ± 0.42	0.15 ± 0.44	0.25 ± 0.62	0.23 ± 0.43
Maximum % fall in FEV_1 (%)	38.53 ± 15.24	34.67 ± 13.53	30.37 ± 15.11	38.26 ± 15.45*
FEV_1 (%)	84.07 ± 13.09	81.64 ± 13.54	85.18 ± 14.19	82.97 ± 12.87
$FEF_{25-75\%}$ (%)	74.29 ± 19.93	73.13 ± 24.63	76.77 ± 24.01	73.11 ± 21.67

Abbreviations: F, female; $FEF_{25-75\%}$, forced midexpiratory flow rate; FEV_1, forced expiratory volume in 1 s; IgE, immunoglobulin E; LTRA, leukotriene receptor antagonist; M, male; PC_{20}, concentration of methacholine that provoked a 20% fall in FEV_1; TEC, total eosinophil count.
$P>0.05$ for differences between responders and non-responders and including LTRA and not including LTRA according to the Mann–Whitney *U*-test, but *P-value of maximum % fall in FEV_1 was <0.001.
[a]Mean ± s.d.
[b]Subjects included or excluded in LTRA study among all EIB (+) asthmatics.

minor allele and analyzed the distributions of the *LTC4S −444A/C* and *PTGDR −441T/C* polymorphisms because the percentage of homozygous for the minor allele of the two polymorphisms was not >7.69%. The prevalence of the *LTC4S −444A/C* polymorphism did not differ between the responders and non-responders (P=0.702), but there was a higher number of non-responders that were heterozygous or homozygous for the C allele of the *PTGDR −441T/C* polymorphism (P=0.038, Table 2).

We investigated how combinations of the *LTC4S −444A/C* and *PTGDR −441T/C* polymorphisms influenced the responsiveness of the subjects to a LTRA. The possible polymorphism combinations were determined according to the dominant model, as listed in Table 3, afterward group II, III and IV compared with group I. A significant difference in combination was not observed among each group (Table 3).

Table 2 Drug responsiveness (<0% versus ⩾10% improvement) in an exercise-induced bronchoconstriction challenge in patients with polymorphisms

		Frequency		
Polymorphisms	*Genotypes*	*Responders, N (%)*	*Non-responders, N (%)*	P-*value*
LTC4S −444A/C	*AA*	45 (65.2)	16 (69.6)	0.702
	AC+CC	24 (34.8)	7 (30.4)	
PTGDR −441T/C	*TT*	44 (63.8)	9 (39.1)	0.038
	TC+CC	25 (36.2)	14 (60.9)	

Abbreviations: LTC4S, leukotriene C4 synthase; PTGDR, prostaglandin D2 receptor.
A χ^2-analysis was carried out.

Table 3 Drug responsiveness (<0% versus ⩾10% improvement) in an exercise-induced bronchoconstriction challenge in patients with combinations of polymorphisms

		Frequency			
Group	*Genotypes (LTC4S/PTGDR)*	*Responders, N (%)*	*Non-responders, N (%)*	*aOR (95% CI)*	P-*value*
I	*AA/TT*	29 (42.03)	6 (26.09)	1.00	
II	*AA/TC+CC*	16 (23.19)	10 (43.48)	3.02 (0.93–9.85)	0.061
III	*AC+CC/TT*	15 (21.74)	3 (13.04)	0.97 (0.21–4.43)	0.965
IV	*AC+CC/TC+CC*	9 (13.04)	4 (17.39)	2.15 (0.49–9.35)	0.302

Abbreviations: aOR, adjusted odds ratio (by age and sex); CI, confidence interval; LTC4S, leukotriene C4 synthase; PTGDR, prostaglandin D2 receptor.
Logistic regression analysis was carried out.

Frequency of *LTC4S* and *PTGDR* polymorphisms in children with asthma

We analyzed the frequency of the *LTC4S −444A/C* and *PTGDR −441T/C* polymorphisms among the normal children and the children with asthma, atopic asthma or non-atopic asthma. The frequency of the minor allele of the *LTC4S −444A/C* polymorphism was 0.18 for the normal children and 0.17 for the children with asthma. The frequency of the minor allele of the *PTGDR −441T/C* polymorphism was 0.26 for the normal children and 0.25 for the children with asthma. The frequency of the minor allele for the children with asthma was identical to the frequency for the children with atopic asthma. The *LTC4S −444A/C* and *PTGDR −441T/C* polymorphisms were not significantly associated with children with asthma, atopic asthma or non-atopic asthma compared with normal children in an additive, recessive and dominant model (Table 4).

Relationship of eosinophils to the *LTC4S −444A/C* and the *PTGDR −441T/C* polymorphisms

Because leukotriene is secreted from eosinophils, a high TEC is one of the distinguishing features of asthma, and because PGD2 may contribute to the recruitment of eosinophils, we investigated the relationship between the number of eosinophils and the *LTC4S −444A/C* and *PTGDR −441T/C* polymorphisms in children with asthma or atopic asthma. Interestingly, as the number of minor alleles of these two polymorphisms increased, the log TEC increased in the children with atopic asthma (P=0.03). There was a tendency for the log TEC to increase in the children with asthma, but the effect was not statistically significant (Figure 1). We compared the difference in eosinophil counts in non-atopic asthmatics and atopic asthmatics in each combination group. According to the comparative results, log TEC was higher in atopic asthmatics than in non-atopic asthmatics (Supplementary Table 1).

DISCUSSION

In this study, we have shown a significant effect of the presence of the *PTGDR −441T/C* polymorphism on responsiveness to a LTRA during

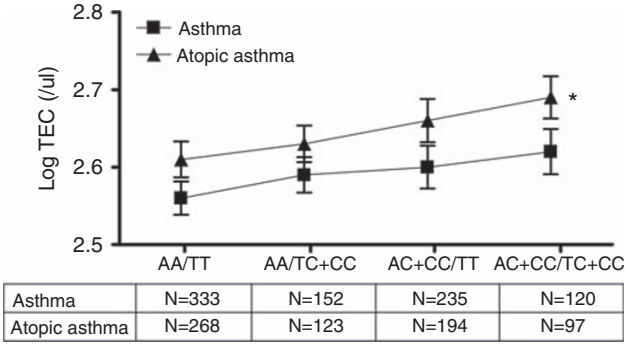
Table 4 Allele frequencies of the *LTC4S* and *PTGDR* polymorphisms in patients with asthma, atopic asthma and non-atopic asthma compared with normal

	Normal	Asthma		Atopic asthma		Non-atopic asthma	
Genotype	N *(%)*	N *(%)*	*aOR (95% CI)*	N *(%)*	*aOR (95% CI)*	N *(%)*	*aOR (95% CI)*
LTC4S −444A/C							
AA	176 (66.92)	583 (67.48)	1.00	476 (67.61)	1.00	107 (66.88)	1.00
AC	81 (30.80)	261 (30.21)	0.97 (0.72–1.31)	212 (30.11)	0.97 (0.71–1.32)	49 (30.63)	1.00 (0.65–1.53)
CC	6 (2.28)	20 (2.31)	1.01 (0.40–2.55)	16 (2.27)	0.99 (0.38–2.57)	4 (2.50)	1.10 (0.30–3.99)
AA	176 (66.92)	583 (67.48)	1.00	476 (67.61)	1.00	107 (66.88)	1.00
AC+CC	87 (33.08)	281 (32.52)	0.98 (0.73–1.31)	228 (32.39)	0.97 (0.72–1.31)	53 (33.13)	1.00 (0.66–1.52)
AA+AC	257 (97.72)	844 (97.69)	1.00	688 (97.73)	1.00	156 (97.50)	1.00
CC	6 (2.28)	20 (2.31)	1.02 (0.41–2.57)	16(2.27)	1.00 (0.39–2.58)	4 (2.50)	1.10 (0.31–3.96)
Frequency (C)	0.18	0.17		0.17		0.18	
PTGDR −441T/C							
TT	144 (55.38)	496 (57.94)	1.00	400 (57.64)	1.00	96 (59.26)	1.00
TC	96 (36.92)	288 (33.64)	0.87 (0.65–1.17)	235 (33.86)	0.88 (0.65–1.19)	53 (32.72)	0.83 (0.54–1.27)
CC	20 (7.69)	72 (8.41)	1.05 (0.62–1.78)	59 (8.50)	1.06 (0.62–1.82)	13 (8.02)	0.98 (0.47–2.06)
TT	144 (55.38)	496 (57.94)	1.00	400 (57.64)	1.00	96 (59.26)	1.00
TC+CC	116 (44.62)	360 (42.06)	0.90 (0.68–1.19)	294 (42.36)	0.91 (0.68–1.21)	66 (40.74)	0.85 (0.57–1.26)
TT+TC	240 (92.31)	784 (91.59)	1.00	635 (91.50)	1.00	149 (91.98)	1.00
CC	20 (7.69)	72 (8.41)	1.10 (0.66–1.84)	59 (8.50)	1.11 (0.65–1.88)	13 (8.02)	1.05 (0.51–2.17)
Frequency (C)	0.26	0.25		0.25		0.24	

Abbreviations: aOR, adjusted odds ratio (by age and sex); LTC4S, leukotriene C4 synthase; PTGDR, prostaglandin D2 receptor.
Logistic regression analysis was performed.

an exercise challenge model of bronchial responsiveness in children with asthma. In addition, we have found a correlation between TEC increment and the combined presence of the *PTGDR −441T/C* and *LTC4S −444A/C* polymorphisms in children with atopic asthma. However, the presence of these two polymorphisms was not associated with asthma susceptibility in Korean children with asthma.

The PTGDR is located at chromosome 14q22.1 and is the receptor for PGD2 that may contribute to the asthma phenotype, which is characterized by pulmonary vasodilation, bronchoconstriction and recruitment of eosinophils.[7–9] The effects of the PTGDR on asthma were shown with a mouse study; the asthmatic symptoms of $PTGDR^{-/-}$ mice were improved compare with wild-type mice.[8] In addition, PGD2[15] and LTC4[16,17] are both derived from arachidonic acid. However, the relationship between the PTGDR and responsiveness to a LTRA has not been investigated. We considered three single-nucleotide polymorphisms (SNPs; *−197T/C*, *−441T/C* and *−549T/C*) in the *PTGDR* gene. These three SNPs have been investigated in many previous studies. The *−441T/C* polymorphism was associated with asthma development in the white population[11] but not in the Mexican population.[12] The *−549T/C* and *−197T/C* polymorphisms are associated with asthma development in the black[11] and Caucasian populations[13], respectively. However, three SNPs were not significant in the Chinese population, as noted in our results.[14] These findings may be caused by the difference in allele frequency in a particular ethnic group. In fact, we confirmed the difference of 0.2 to 0.4 in Asians and CEPH (Utah residents with ancestry from northern and western Europe) according to the HapMap data. Although it is not a data from Asian, a minor allele frequency of *−197T/C* polymorphism is very low (about 10%) in European. And two SNPs (*−441T/C* and *−549T/C*) were in strong linkage disequilibrium. Therefore, we examined the relationship between the *PTGDR −441T/C* polymorphism and responsiveness to LTRA, and found that subjects with heterozygous or homozygous C alleles were more likely to be non-responders. In addition, we analyzed the effect of the combined presence of the *PTGDR −441T/C* and *LTC4S −444A/C* polymorphisms because LTRA improves the asthmatic symptoms of



Asthma	N=333	N=152	N=235	N=120
Atopic asthma	N=268	N=123	N=194	N=97

Figure 1 This figure shows the combined effect of the leukotriene C4 synthase (*LTC4S*) *−444A/C* and the prostaglandin D2 receptor (*PTGDR*) *−441T/C* polymorphisms on the log total eosinophil count (TEC) in children with asthma or atopic asthma. Groups with the LTC4S −444A/C and PTGDR −441T/C polymorphisms were combined. The data are presented as the mean ± s.e.m. and were analyzed by linear regression. *P=0.03 in atopic asthma.

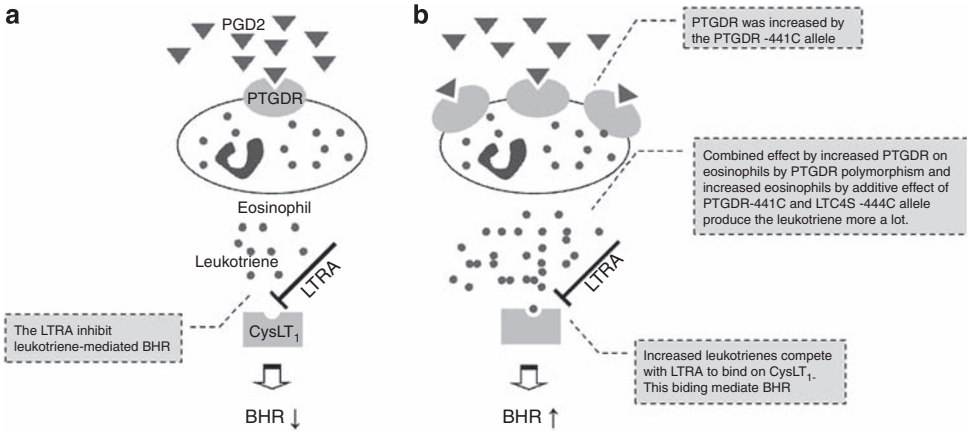


Figure 2 Followed figure is a scheme of pathway including the leukotriene C4 synthase (*LTC4S*) and prostaglandin D2 receptor (*PTGDR*) polymorphisms in terms of the responsiveness to a leukotriene receptor antagonist (LTRA). (**a**) The pathway of usual leukotriene production by prostaglandin D2 (PGD2) in eosinophils. The binding of PGD2 and PTGDR on eosinophil induce production and secretion of leukotrienes from eosinophil. Secreted leukotrienes induce bronchial hyperresponsiveness in subjects with asthma via cysLT$_1$. The LTRA (montelukast) is administered in order to inhibit leukotriene-mediated bronchial hyperresponsiveness. (**b**) The pathway of enhanced leukotrienes production by PGD2 in eosinophils, which is affected by *PTGDR* and *LTC4S* polymorphisms. Increased expression of PTGDR by the *PTGDR −441C* allele augments the function of PGD2. Also increased function of PGD2 recruits more eosinophils and induces the synthesis of leukotriene by stimulating the eosinophils. Increased eosinophils by the PGD2 via increased PTGDR by polymorphism and an additive effect of the *PTGDR −441C* and the *LTC4S −444C* allele produce the leukotriene more a lot. In the event, increased concentrations of the leukotriene may inhibit the LTRA responsiveness, which is affected by exercise-induced bronchoconstriction.

subjects by impeding the function of leukotriene. The PGD2 recruits eosinophils[8] and induces the synthesis of LTC4 by stimulating the eosinophils.[18] Also the *LTC4S −444A/C* polymorphism was associated with leukotriene production and asthma in several independent studies.[25,26] Although the LTC4S *−444A/C* polymorphism was always not associated with asthma susceptibility or severity,[26,27] this polymorphism is universally most common SNP. Therefore, we investigated a combined effect of the *PTGDR (−441T/C)* and *LTC4S (−444A/C)* polymorphisms in EIB model after treatment with LTRA. However, there were no significant differences between the responders and non-responders of this study when we analyzed the combined frequencies of the PTGDR and LTC4S polymorphisms.

PGD2 may contribute to the recruitment of eosinophils,[8] and LTC4, a potent biomarker produced by eosinophils,[28] has been shown to be a strong mediator of bronchoconstriction in EIB.[29] We previously reported that children with asthma who also experienced EIB had significantly higher TEC.[30] In addition, the results of several studies suggest that the presence and severity of EIB is significantly associated with the number of eosinophils measured from the blood and sputum of subjects with asthma.[31–34] Therefore, we hypothesized that eosinophils contribute to the mechanisms underlying EIB and the therapeutic effects of LTRA therapy, and then we investigated the effects of polymorphism combinations on LTRA drug responsiveness and on TEC in children with asthma. Interestingly, we found that the presence of the *PTGDR −441C* and *LTC4S −444C* alleles appeared to be associated with increased TEC in children with asthma, especially children with atopic asthma. In Supplementary Table 1, the reason for the higher TEC in atopic asthmatics than in non-atopic asthmatics, may be atopy and is not a combination of two polymorphisms. In fact, we confirmed that log TEC increases if log IgE increased in our asthmatics (Supplementary Figure 1). We additionally analyzed a change of eosinophil count according to combination of the two SNPs in responder (69 subjects), non-responder (23 subjects) and total 100 subjects of LTRA, respectively. However, there were not increasing trend of eosinophil counts (data not shown). Therefore, the association between responsiveness to LTRA in 100 subjects and increased number of eosinophils in atopic asthmatics may be not direct because of two different groups of subjects. However, individuals with C allele of the PTGDR have high eosinophil counts, the PTGDR was expressed on eosinophil and leukotrienes were secreted from eosinophils. We also analyzed the association between TEC and each polymorphism. Children who were heterozygous or homozygous for the minor allele of the *PTGDR −441T/C* or *LTC4S −444A/C* polymorphisms tended to have higher TEC than children who were homozygous for the common allele; however, the effect was not statistically significant (data not shown). Altogether, we thought that increment of eosinophil count in atopic asthmatics is result worth considering certainly. Increased levels of cysLTs have been detected in airways,[19] exhaled breath condensates[21] and induced sputum,[22] and bronchial hyperresponsiveness has been induced by administration of cysLTs.[35] PTGDR expression was increased by the region including *−441C* allele[14] and increased expression of the PTGDR augmented the ability of PGD2 to recruit eosinophils and stimulate them to synthesize LTC4.[18] According to our data, the combined effects of the *LTC4S −444C* and *PTGDR −441C* alleles may have an additive effect on increasing TEC. Ultimately, higher levels of LTC4 disrupt LTRA responsiveness in children with asthma (Figure 2).

A weakness of our study is that there is not a clear cutoff value that distinguishes responders from non-responders. However, to our knowledge, there are no published research findings that address this issue. The classification system used in this study enabled us to include 92 subjects, which was sufficient to analyze drug responsiveness. Second, other genes may be connected on LTRA responsiveness, for instance, cysLT$_1$, which is receptor of leukotriene and interleukin-5, which is cytokine that acts as a growth and differentiation factor for eosinophils may be associated with LTRA responsiveness. However, we only investigated two polymorphisms in two genes. In the future, more studies are needed to validate these results with more genes in

larger population. Third, EIB test is more accurate indoor than outdoor. We conducted free running at outdoor to provoke maximal stimulation of EIB and calculated temperature ($19 \pm 58^{\circ}$C) and humidity ($57 \pm 12\%$) during day of testing.[30] We performed all of these study population using the same maneuver, although there might have been limited differences in the climatic conditions compared with the standard treadmill test. The last, two SNPs in this study were associated by genotype and combination at nominal level of significance. Despite these limitations, our data may use for drug responsiveness because we compared result of medication before and after in same subjects, also the current finding of association study on LTRA responsiveness helps to possible personalized medication.

In summary, we report for the first time that the *PTGDR −441C* allele seems to be associated with LTRA responsiveness and that increases in TEC are associated with the *LTC4S −444C* and *PTGDR −441C* allele. Individual polymorphisms may have a small influence in complex diseases such as asthma. However, a combination of polymorphisms in entire pathway gives help to understand complex disease. Our results suggest that there is variability in the response to LTRA that is related to genotype, and this information may help identify more selective therapeutic strategies.

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