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Differential Contribution of the CysLTR1 Gene in Patients with Aspirin Hypersensitivity

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Abstract In this study, we compared the roles of CysLT receptor type 1 (*CysLTR1*) and leukotriene C4 synthase (*LTC4S*) gene polymorphisms in two major aspirin-related allergic diseases, aspirin-intolerant asthma (AIA) and aspirin-induced chronic urticaria/angioedema (AICU). *CysLTR1*-634C>T and *LTC4S*-444A>C polymorphisms were genotyped and its functional effect on the promoter activity was compared. As in vivo functional study, changes of peripheral mRNA level of *CysLTR1* were measured by real-time PCR before and after aspirin challenge. A significant association was found for the *CysLTR1* promoter polymorphism and the AIA phenotype compared to AICU (P=0.015). In U937 cells, the variant genotype reporter construct showed significantly higher promoter activity than the common genotype (P<0.05). The *CysLTR1* mRNA levels increased significantly after aspirin challenge in AIA patients (P=0.013). In conclusion, the *CysLTR1* polymorphism may contribute to develop to the AIA phenotype and be used as a genetic marker for differentiating two major aspirin hypersensitivity phenotypes.

Keywords Aspirin-induced asthma · genetic polymorphism · leukotriene receptor

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Introduction

The distinct clinical syndromes of aspirin hypersensitivity, including aspirin-intolerant asthma (AIA) and aspirin-induced urticaria/angioedema (AICU), result from the inhibitory action of aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) on prostaglandin formation from arachidonic acids, leading to enhanced production of cysteinyl leukotrienes (CysLTs), such as LTC4, LTD4, and LTE4, which are potent inflammatory mediators [1–4]. In the Korean population, the prevalence of AIA is about 20% of adult asthmatics, based on bronchial lysine-aspirin provocation tests [5], and AICU occurs in more than 30% of chronic urticaria patients, based on oral aspirin provocation tests [6]. Interest in the pathologic role of CysLT in AIA has increased after the demonstration of therapeutic efficiency for CysLT receptor antagonists in the management of AIA patients [7, 8]. However, in contrast to AIA, the association of the cutaneous reaction with aspirin hypersensitivity has not been investigated. Up to 40% of patients with chronic urticaria exhibit increased wheal size and swelling after intake of aspirin [6, 9], and it has been suggested that increased levels of CysLTs resulting from alterations in arachidonate metabolism may exacerbate late-phase allergic reactions in AICU patients, as in AIA patients [9–11]. However, the pharmacologic effects of CysLT receptor antagonists on AICU patients have not been investigated.

CysLTs, such as LTC4, LTD4, and LTE4, are known as slow-acting anaphylactic substances and important lipid mediators of the pathogenesis of eosinophilic airway inflammation characterized by bronchoconstriction, mucus secretion, and airway hyperresponsiveness [12]. CysLT synthesis involves three major enzymes: 5-lipoxygenase, 5-lipoxygenase-activating protein, and leukotriene C4 syn-

thase (LTC4S) [13, 14]. LTC4S is the key enzyme, and the promoter polymorphism -444A>C in the *LTC4S* gene, which increases the expression of this gene, has been reported to be modestly associated with the AIA phenotype [15, 16]. Moreover, the variant allele of the *LTC4S*-444A>C polymorphism may be inherited in families with aspirin-induced urticaria [17]. The bronchoconstrictive effects of CysLTs are exerted via their G-coupled receptor, CysLT receptor type 1 (CysLTR1) [18], and these effects can be efficiently reversed by CysLTR1 antagonist [7, 8]. We have previously reported the potential contribution of a *CysLTR1* promoter polymorphism to AIA susceptibility compared to aspirin-tolerant asthma (disease control) [19].

To investigate whether polymorphisms of the *LTC4S* and *CysLTR1* genes contribute to the differentiation of aspirin hypersensitivity, we conducted a genetic case–control study of Korean subjects who were classified into AIA and aspirin-induced chronic urticaria groups. We also conducted in vivo and in vitro functional studies.

Subjects and Methods

Study Subjects

Two groups of patients (159 AIA and 116 AICU patients) were enrolled at Ajou University Hospital, Suwon, Korea. In the present study, AICU patients were defined as having more than 6 weeks of daily itchy wheals, having a history of chronic urticaria/angioedema, which was aggravated by ingestion of more than two kinds of NSAIDs, and showing positive responses in the oral aspirin challenge test. AIA patients were diagnosed by a positive lysine-aspirin bronchoprovocation test from asthmatic patients who showed the typical clinical features of asthma. The AIA patients had upper and lower respiratory symptoms without any history of drug-induced cutaneous symptoms. Patients who had both AIA and AICU were excluded from the present study. Skin prick tests were performed with 12 common aeroallergens (Bencard Co., Bredford, UK) with histamine and saline controls. A positive reaction was defined as a mean wheal diameter of more than 3 mm. Atopy was defined as one or more positive reactions in the skin prick test. All subjects gave informed consent and the protocol was approved by the institutional review board of Ajou University Hospital, Korea.

Genomic DNA Preparation and Single Nucleotide Polymorphism Genotyping

Genomic DNA was prepared from peripheral blood samples using the Puregene DNA purification kit (Gentra, Minneapolis, MN, USA) according to the manufacturer's protocol. As the three single nucleotide polymorphisms (SNPs) of *CysLTR1* (-634C>T, -475A>C, and -336A>G) were in perfect linkage disequilibrium with each other [18], we genotyped the -634C>T polymorphism only as a tagging SNP. Genotyping was performed using the SNaPSHOT ddNTP primer extension kit (Applied Biosystems, Foster City, CA, USA). The sequences of the amplifying and extension primers for *CysLTR1* -634C>T were as follows: forward, 5′-CAGGAAACAGCTATGACCTGGCATTATGTGAAACTTGA-3′; reverse, 5′-TGTAAAACGACGGCCAGTACCACCACAATCCAGAACT-3′; and extension, 5′-CTCAATATGGCTTCTGGAGAAA-3′. The sequences of the amplifying and extension primers for *LTC4S* -444A>C were as follows: forward, 5′-AGAGGAGGGTTTGGCAGG-3′; reverse, 5′-CCCAGGCTCCCGGCTAAC-3′; and extension, 5′-GTTGCCAGGAACAGCCTGGATGGGGAC-3′.

In Vitro Promoter Activity Assay

Two luciferase reporter constructs for the common and rare genotypes, which comprised a 2,466-bp fragment (from −2158 to +313), were transiently transfected into a myelomonocytic cell line (U937) by electroporation. Briefly, 2×10^5 cells were seeded in 24-well plates and transfected with 0.5 μg of the reporter constructs and 0.5 μg of pSV-β-galactosidase control vector using a microporator (Incyto, Suwon, Korea) at 1,300 V and pulse width of 30 ms. At 48 h posttransfection, the cells were lysed and assayed for firefly luciferase activity according to the manufacturer's instructions (Promega). The transfections and luciferase assays were repeated four times (twelve times in total) according to the method described previously [19].

Electrophoretic Mobility Shift Assay

U937 cells were washed with ice-cold phosphate-buffered saline before being suspended in ice-cold hypoosmotic buffer (10 mM Hepes/KOH [pH 7.9], 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 1 mM PMSF, 1 mM $NaVO_3$, 10 mM NaF, and complete protease inhibitor cocktail [Roche, Basel, Switzerland]). The cells were incubated on ice for 10 min and for an additional 15 min in the presence of 0.2% Nonidet P-40. After centrifugation at 15,000×*g* for 30 s, the pellet was resuspended in a cold saline buffer that contained 50 mM Hepes/KOH (pH 7.9), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% (v/v) glycerol, 1 mM DTT, 1 mM PMSF, and protease inhibitors. The mixture was incubated on ice for 30 min. After centrifugation at 15,000×*g* for 30 min at 4°C, the supernatant, which contained the nuclear proteins, was stored at −80°C until use.

The following double-stranded oligonucleotides were used as probes for the electrophoretic mobility shift assay (EMSA): for *CysLTR1* -634C>T, 5′-CTGCTATG TCCC[c/t] TTTCTCCAGAAG-3′; for *CysLTR1* -475A>C, 5′-GAAA[a/c]AAC AGTTTTAATCCAATGG-3′; for *CysLTR1* -336A>G, 5′-AGAGAGAGAGAGA AAC[a/g]GGAGAGATG-3′; and for NF-κB as a nonspecific competitor, 5′-GGTTACAAGG GACTTTCCGCTG-3′. The double-stranded oligonucleotides were annealed by incubation at 95°C for 5 min followed by gradual cooling to room temperature. The double-stranded DNA probes were radiolabeled at their 5′-ends with DNA polymerase I (Klenow fragment) (Promega) and [α-^{32}P]CTP. The reaction mixtures (10 μl), which contained 5 μg of nuclear extract, 10 mM HEPES (pH 7.9), 250.ng poly(dI:dC), 0.005 mM $MgCl_2$, 75 mM NaCl, 5 mM DTT, 1 mM $NaNO_3$, 10 mM NaF, 1 mM EDTA, 400 ng bovine serum albumin, and 5% glycerol, were preincubated on ice for 20 min, and then incubated for 20 min on ice in the presence of 0.2 pmol of radiolabeled probe. The reaction mixtures were then subjected to electrophoresis on a native 6% polyacrylamide gel. For competition experiments, unlabeled blunt-ended competitor oligonucleotides were added to the binding reaction mixtures before the addition of the radiolabeled oligonucleotide probes. The gels were dried and radioactivity was detected using a FLA3000 scanner (Fuji Photo Film, Tokyo, Japan).

Aspirin Challenge Test

All medications, including theophyllines, beta-2 agonists, leukotriene receptor antagonist, antihistamines, and inhaled corticosteroids, were stopped 3 days before the procedure. After the placebo challenge, the AIA patients underwent the lysine-aspirin bronchoprovocation test with increasing doses of aspirin (75–300 mg/ml Althargyl, Dong A Pharmaceutical Co., Ltd., Korea) and were observed for up to 7 days. For the AICU patients and normal healthy controls, 500 mg of aspirin (Rhonal, KunWha Pharmaceutical Co., Seoul, Korea) in tablet form was administered orally and the patients were observed for 4 h, with monitoring for urticaria and changes in lung functions every 30 min. The appearance within 4 h of urticaria without any changes in FEV_1 was considered a positive result for the AICU patients, and a change in FEV_1 within 7 h without any cutaneous symptoms was considered a positive result for the AIA patients.

Real-time PCR

Peripheral blood samples (10 ml) were collected from study subjects in two phases, at the basal status before the oral provocation test and at the appearance of the disease symptom (respiratory symptom for AIA and cutaneous symptom for AICU patients) after the provocation. Peripheral blood mononuclear cells were isolated from the blood using Ficoll-Paque Plus (Amersham Biosciences, Sweden) density gradient centrifugation. Total RNA was isolated from peripheral blood mononuclear cells using the Easy-BLUE Total RNA Extraction Kit (iNtRON Biotechnologies, Seoul, Korea). Total RNA (2 μg) was reverse-transcribed using the oligo(dT) primer and MMLV reverse transcriptase (Promega, Madison, WI, USA) in a volume of 20 μl at 42°C for 90 min. Real-time PCR was performed using the ABI Prism 7700 instrument. The PCR consisted of the cDNA product (20–200 ng), 125 nM of primer, and 12.5 μl of 2×SYBR Green PCR Master Mix (SYBR Green dye, AmpliTaq Gold, DNA polymerase, dNTP Mix, and optimized buffer components; Applied Biosystems) in a total volume of 25 μl. The following primer pairs were used: for *CysLTR1*, forward 5′-TGACCGCTGCCTTTTTAGTC-3′ and reverse, 5′-GAGAGGGTCAAAGCAACAATTG-3′; and for β-*actin*, forward, 5′-TCCTTCTGCATCCTGTCGGC-3′ and reverse, 5′-CAAGAGATGGCCACGGCTGC-3′. Amplification was performed for 40 cycles. After an initial hot start for 10 min, each cycle consisted of 10 s of denaturation, 30 s of annealing at 58°C, and 40 s of extension. Data were acquired and analyzed with the Sequence Detection System (Applied Biosystems). The amplification kinetics was recorded as sigmoid progress curves, for which fluorescence was plotted against the number of amplification cycles. The threshold cycle number (C_T) was used to define the initial amount of each template. The C_T was the first cycle for which a detectable fluorescence signal was observed. The mRNA expression levels were determined and standardized to that of β-*actin*.

Data Analysis

A significant departure of genotype frequency from the Hardy–Weinberg equilibrium (HWE) at each SNP was tested by a chi-square goodness-of-fit test. The genotype distribution of the *CysLTR1* polymorphisms was in HWE within the female subject group but not within the male subject group because *CysLTR1* is on the X chromosome. A difference in genotype frequency between the case and control groups was assessed by a chi-square test and calculation of odds ratios (ORs) with 95% confidence intervals (CIs). Logistic regression models were used for analyses of SNPs, controlling for age and sex as covariates with three alternative models (codominant, dominant, and recessive models). Differences of clinical characteristics between groups were examined by using the Student *t* test (for continuous variables) or a chi-square test (for categorical variables). Differences in the mRNA levels of target genes between paired samples were assessed with the Wilcoxon's signed-ranks test. A *P* value of less than 0.05

denoted a statistically significant difference. The statistical analyses were completed using the SPSS version 11 software (SPSS Inc., Chicago, IL, USA).

Results

The characteristics of the study subjects, including mean age, sex, prevalence of atopy, and serum total IgE, are summarized in Table I. AIA was more commonly found in middle-aged women, and the AIA patients showed a higher prevalence of rhinosinusitis (P=0.015) than the AICU patients. AICU patients showed a higher rate of atopy (P=0.005) and a tendency toward higher total serum IgE levels in comparison with the AIA patients, although this difference was not statistically significant (P>0.05).

Case–control association analysis of the allele and genotype frequencies of the two promoter SNPs, *CysLTR1* -634C>T and *LTC4S* -444A>C, are described in Table II. The genotype and allele frequencies of the *CysLTR1* -634C>T polymorphism were significantly different (P<0.05); the frequency of the CT or TT genotype was significantly higher in the AIA group than in the AICU group (OR=1.400, 95% CI=1.067–1.836), and the minor allele frequency was also significantly higher in the AIA group than in the AICU group (OR=1.218, 95% CI=1.004–1.478). No significant association of *LTC4S* -444A>C was noted for the two common phenotypes of aspirin hypersensitivity.

To evaluate whether the -634C>T polymorphism was associated with altered promoter activity in human myelomonocytic cells, we used luciferase reporters in the *CysLTR1* promoter to compare the reporter activities of plasmids that contained either common alleles (-634C, -475A, and -366A) or rare alleles (-634T, -475C, and -336G) (Fig. 1). Constructs that contained rare alleles exhibited significantly higher luciferase activities than constructs that contained common alleles (P<0.001).

Table I Clinical Characteristics of the Study Subjects

	AIA (n=159)	AICU (n=116)	P value AIA vs AICU
Age[a]	43.8±13.41	34.9±11.58	<0.001
Sex (female/total)	102/159 (64.15%)	59/116 (50.86%)	0.035
Atopy (presence/total)	84/150 (56.00%)	85/116 (73.28%)	0.005
Log [serum total IgE][a]	2.2±0.56	2.3±0.48	0.658
Rhinosinusitis (presence/total)	106/131 (80.92%)	17/29 (58.62%)	0.015

AIA = ASA-intolerant asthma, AICU = ASA-induced chronic urticaria
[a] Mean ± SEM

Table II Allele and Genotype Frequencies of the CysLTR1 and LTC4S Genes

	AIA	AICU	P value AIA vs AICU
CysLTR1 -634C>T			
CC	65 (41.67%)	62 (59.05%)	0.101
CT	49 (31.41%)	15 (14.29%)	0.656
TT	42 (26.92%)	28 (26.67%)	0.015[a]
q	0.426	0.338	0.045[b]
n	156	105	
LTC4S -444A>C			
AA	116 (72.96%)	80 (68.97%)	0.454
AC	42 (26.42%)	33 (28.45%)	0.393
CC	1 (0.63%)	3 (2.59%)	0.570
q	0.138	0.168	0.467
n	159	116	

Each P value was calculated with codominant, dominant, and recessive models. Logistic regression analysis was applied to control for age and sex as covariables.
AIA = ASA-intolerant asthma, AICU = ASA-induced chronic urticaria, n = number of patients, q = minor allele frequency.
[a] OR=1.400 (95% CI=1.067–1.836)
[b] OR=1.218 (95% CI=1.004–1.478).

To investigate whether the genetic polymorphism created a transcription factor binding site, we performed EMSA with nuclear extracts from U937 cells using double-stranded oligonucleotide probes that corresponded to -634C>T, -475A>C, and -366A>G. Of the -634C, -634T, -475A, -475C, -366A, and -366G probes, one shifted band was noted

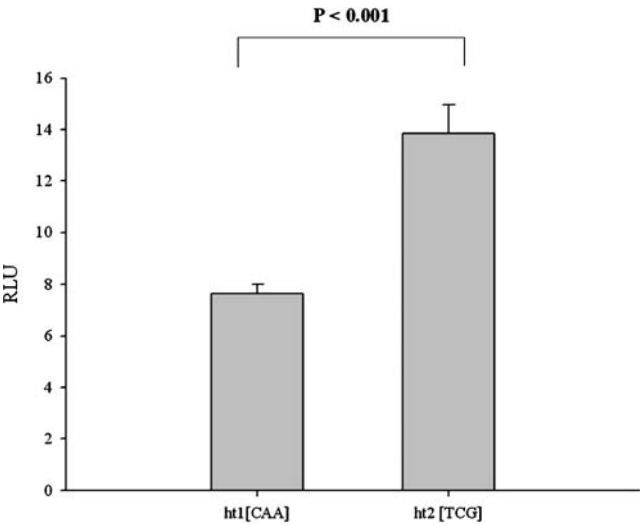

Fig. 1 Promoter activity assay of human *CysLTR1* gene according to the promoter polymorphism in U937 cells. The relative luciferase activity (*RLU*) is represented as the ratio of the activity to the luciferase activity in the cells transfected with the promoterless basic vector, pGL3B. Data are the mean values of four independent experiments (in total, triplicated for each sample, n=12), plus or minus the SD. Statistical analysis was done by Mann–Whitney U test.

for the -475A probe in U937 cells (Fig. 2b). The shifted band produced by the -475A probe disappeared in the presence of the nonlabeled -475A probe but remained visible in the presence of the nonlabeled -475C probe (Fig. 2c). A survey of a transcription factor database conducted using the Signal Scan program (http://www-bimas.cit.nih.gov/molbio/signal) suggests that the A to C conversion of the -475A>C polymorphism does not generate a transcription-factor-binding motif.

The *CysLTR1* mRNA level significantly increased after aspirin challenge, together with the development of asthmatic symptoms in AIA patients (P=0.013), whereas no significant changes were noted in AICU patients (P> 0.05) (Fig. 3). No significant changes were noted in normal healthy subjects after aspirin challenge (data not shown). Moreover, the *LTC4S* mRNA levels were not significantly changed in the AIA and AICU patients after aspirin challenge (data not shown).

Discussion

Although the two distinct clinical symptoms of aspirin hypersensitivity, AIA and AICU, are commonly exaggerated by aspirin and/or NSAIDs, many investigators have speculated that different pathogenic mechanisms and genetic backgrounds may be involved. AIA affects about 20% of adult asthmatics in the Korean population and occurs more frequently in middle-aged women [5]. The AIA patients showed a higher prevalence of rhinosinusitis than the aspirin-tolerant asthmatic patients. AICU prevalence was 35.7% for chronic urticaria patients in the Korean population, and these patients were younger and showed higher atopic rates and higher serum total IgE levels compared to aspirin-tolerant chronic urticaria patients [16]. These distinct clinical features of AIA and AICU patients were confirmed in the present study.

Although recent data suggest that abnormal arachidonate metabolism is a common finding in both AIA and aspirin-induced urticaria [9–11], there have been very few studies to suggest a genetic predisposition to aspirin-induced urticaria for patients with aspirin hypersensitivity. In the present study, we speculated that the *CysLTR1* and *LTC4S* genes might be useful for differentiating the two major phenotypes of aspirin hypersensitivity.

Based on a study of a Caucasian population, it has been suggested that the activity of *LTC4S*, which encodes a key enzyme in CysLT synthesis, is genetically regulated in the pathogenesis of AIA [15, 20]. After aspirin challenge, patients who carry the *LTC4S* -444C allele show a greater increase in urinary LTE4 than patients who carry the -444A allele [16]. However, the association of this promoter polymorphism with the phenotype of AIA patients has not been replicated in other populations, including Japanese, American, and Korean populations [21–23]. The *LTC4S* -444A>C polymorpism has recently been reported to be involved in the pathogenesis of AICU in a Caucasian population [17], which indicates that the frequency of the *LTC4S* -444C allele in AICU patients is significantly higher than in chronic urticaria patients without aspirin hypersensitivity. However, in a Korean population, the *LTC4S* -444A>C polymorphism was not associated with either

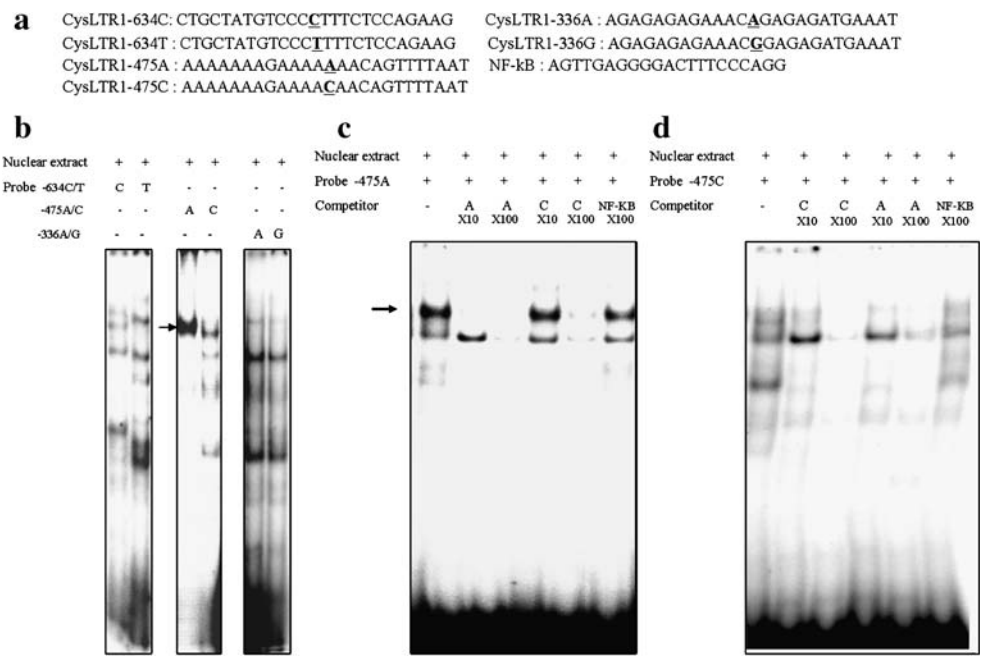

Fig. 2 Results of an EMSA for the promoter polymorphisms using nuclear extracts obtained from U937 cells. **a** Nucleotide sequences of oligonucleotides used as probes and competitors. **b** Differential binding of a nuclear protein to -475A/C alleles. **c** Competition assay of -475A allele. **d** Competition assay of -475C allele.

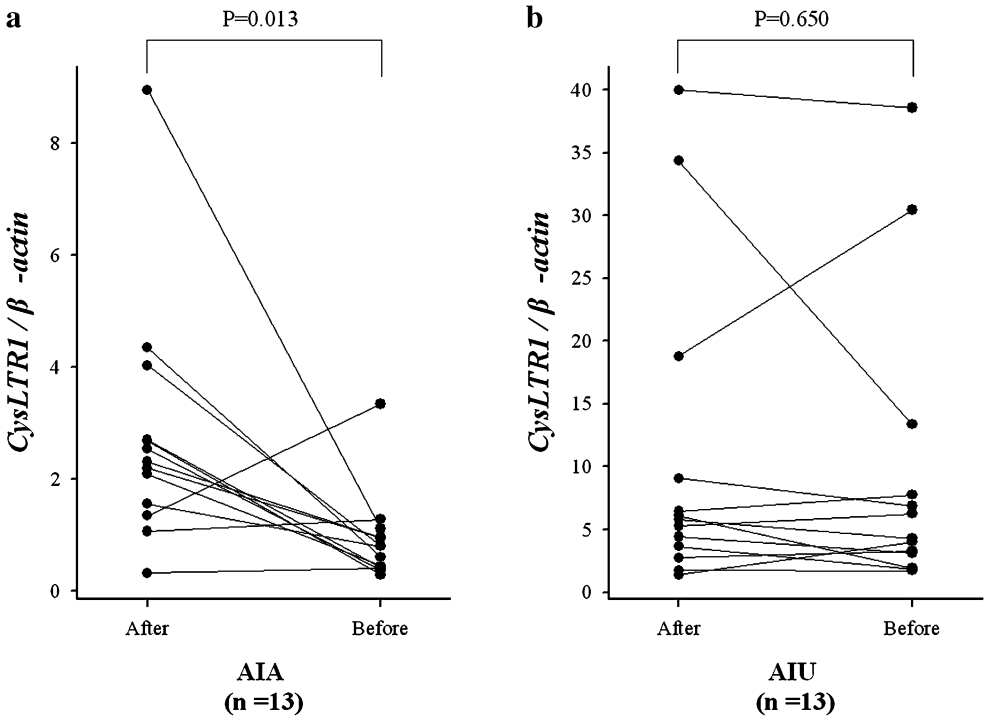

Fig. 3 mRNA expression level of *CysLTR1* gene after aspirin challenge test. **a** Comparison of *CysLTR1* expression level before and after aspirin provocation in AIA patients. **b** Comparison of *CysLTR1* expression level in AIU patients. Statistical analysis was done by the Wilcoxon's signed-ranks test.

AIA or AICU [23, 24]. In the present study, we reconfirm the lack of association of the *LTC4S* -444A>C polymorphism with AIA and AICU. Moreover, the level of *LTC4S* mRNA was not changed after aspirin provocation in either the AIA or AICU patients. These findings suggest that an *LTC4S* promoter polymorphism is not associated with the phenotype of AIA or AICU in the Korean population.

In our previous report, we evaluated the contributions of three promoter polymorphisms of the *CysLTR1* gene, -634C>T, -475A>C, and -336A>G, in increasing the transcriptional activity of *CysLTR1* and the development of AIA [19]. These effects were compared to aspirin-tolerant asthma, whereas the contribution to AIA in comparison to AICU was not evaluated. In the present study, the *CysLTR1* -634C>T polymorphism was chosen as a tagging SNP for three promoter polymorphisms, which included -634C>T, -475A>C, and -336A>G, as these three polymorphisms are perfectly linked to each other. We found a significant association of the *CysLTR1* -634C>T polymorphism with the AIA phenotype, but not with the AICU phenotype. Furthermore, the level of *CysLTR1* mRNA was significantly increased concomitant with the appearance of asthmatic symptoms provoked by aspirin challenge in AIA patients, but not in AICU patients or normal healthy controls even after aspirin provocation. The previous study [25] demonstrated elevated number of nasal inflammatory leukocytes expressing the CysLTR1 in aspirin-sensitive rhinosinusitis patients than those of aspirin-tolerant patients and downregulation of this receptor after aspirin desensitization. However, *CysLTR1* mRNA level after the desensitization was not examined in this study because the ASA desensitization has not been applied for the AIA patients in Korea.

Moreover, comparing the *CysLTR1* mRNA expression levels according to promoter polymorphism, the patients with the variant genotypes (CT or TT) tended to express higher mRNA levels than those with the common genotype, although statistical significance was not reached. In the present in vitro functional study, the *CysLTR1* promoter polymorphism enhanced the transcriptional activity in the human monocytic cell line U937. In addition, the EMSA demonstrated that a -475A-specific DNA-binding protein is involved in the transcriptional regulation of the *CysLTR1* gene, although the -475A-specific DNA-binding protein was not identified. Further studies will be needed to evaluate the changes of peripheral mRNA level of CysLTR1 after desensitization to aspirin in AIA patients according to the CysLTR1 genotype.

Taken together, our results indicate that the *CysLTR1* -634C>T polymorphism aggravates asthmatic symptoms after aspirin/NSAID exposure by increasing the expression level of *CysLTR1* in the asthmatic airways of AIA patients, whereas this SNP does not appear to be involved in AICU.

In conclusion, these findings suggest that the *CysLTR1* promoter polymorphism is critical to the determination of AIA susceptibility, and may represent the first candidate of SNP marker to differentiate the two major phenotypes of aspirin hypersensitivity, AIA and AICU.

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