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Lack of an Association between a Newly Identified Promoter Polymorphism (-1702G > A) of the Leukotriene C4 Synthase Gene and Aspirin-Intolerant Asthma in a Korean Population

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Choi, J.-H., Kim, S.-H., Bae, J.-S., Yu, H.-L., Suh, C.-H., Nahm, D.-H. and Park, H.-S. *Lack of an Association between a Newly Identified Promoter Polymorphism (-1702G > A) of the Leukotriene C4 Synthase Gene and Aspirin-Intolerant Asthma in a Korean Population*. Tohoku J. Exp. Med., 2006, **208** (1), 49-56 —— Aspirin-intolerant asthma (AIA) is a distinct clinical syndrome that refers to the development of bronchoconstriction in asthmatic individuals following the ingestion of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs). It is widely recognized that increased cysteinyl leukotriene (cysLT) biosynthesis is associated with the development and progression of AIA. Leukotriene C4 synthase (LTC4S) is the terminal enzyme in cysLT production and is a strong candidate gene in the pathogenesis of aspirin-intolerant asthma (AIA). In this paper, we report a new single nucleotide polymorphism (SNP) of the LTC4S promoter, -1702G>A, in AIA patients and evaluate its genetic role in the association with the *LTC4S -444 A>C* polymorphism. We enrolled 110 AIA patients, 125 aspirin-tolerant asthma (ATA) patients, and 125 normal controls. SNP genotyping of the *LTC4S-1702G>A* and *-444A>C* polymorphisms was performed using SNP-IT™ assays. Haplotype analyses were performed using Haploview version 2.05, which is based on an estimation-maximization (EM) algorithm. There were no significant differences in the allele or genotype frequencies of the *LTC4S -1702G>A* and *-444A>C* polymorphisms among the three groups ($p > 0.05$), with no significant differences in the observed haplotype frequencies ($p > 0.05$). Moreover, no significant associations were found between the genotype of each SNP in AIA patients with the clinical characteristics, including a forced expiratory volume in one second (FEV_1) %, a provocation concentration of methacholine to induce more than 20% decrease of FEV_1 (PC_{20}) to methacholine, and serum total IgE levels ($p > 0.05$). These results indicate that there is no association between these two promoter polymorphisms of LTC4S and the phenotype of AIA in a Korean population. ———— aspirin-intolerant asthma; genetic polymorphism; leukotriene C4 synthase

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Aspirin-intolerant asthma (AIA), which affects 10-20% of adults with asthma, is characterized by aspirin hypersensitivity, bronchial asthma, and chronic rhinosinusitis with nasal polyposis (Samter and Beers 1967; Szczeklik and Stevenson 2003). Although the pathogenesis of AIA is not completely understood, the overproduction of cysteinyl leukotrienes (cysLTs) plays a major role in the pathogenesis of AIA, because cysLTs are important inflammatory mediators in AIA, and promote bronchoconstriction, mucus hypersecretion, vascular permeability, and cellular infiltration (Henderson 1994). CysLTs are synthesized by three major enzymes: 5-lipoxygenase (ALOX5), 5-lipoxygenase activating protein (ALOX5AP), and leukotriene C4 synthase (LTC4S) (Foegh et al. 1998). Of these, LTC4S is the key enzyme in cysLTs production, and *LTC4S* is an important candidate gene for the genetic pathogenesis of AIA. LTC4S spans 2.51 kb on chromosome 5q35, containing five exons; it is located in a region implicated in asthma by linkage analysis and is adjacent to a region (5q31-33) that includes several candidate asthma genes (Penrose et al. 1996; Bleecker 1998).

The genetic polymorphism of *LTC4S -444A>C* is strongly associated with the AIA phenotype in a Polish population, which is supported by functional data demonstrating an additional transcription factor binding site to increase the transcription rate of this gene (Sanak et al. 1997, 2000). In contrast, no significant associations were detected in American (Van Sambeek et al. 2000), Japanese (Kawagishi et al. 2002), Australian (Kedda et al. 2004), or Spanish (Isidoro-García et al. 2005) populations. We also did not find an association between *LTC4S -444A>C* and AIA in a Korean population (Choi et al. 2004). In this regard, the genetic pathogenesis of AIA might not be explained with a single nucleotide polymorphism (SNP) of *LTC4S -444A>C* despite the confirmed association in a Polish population.

Recently, Sayers et al. (2003) identified a novel promoter polymorphism, -1702G>A, in the *LTC4S* gene, which was not associated within an asthma phenotype and had no functional role for gene expression in a British population. However, no study has examined the association between this novel polymorphism and AIA, particularly in Asian populations. We hypothesized that the novel promoter polymorphism or haplotypes of the *LTC4S -1702G>A* and *-444A>C* polymorphisms might be associated with AIA in a Korean cohort. To test this hypothesis, we screened these two promoter polymorphisms in the *LTC4S* gene in an association study involving a large-scale haplotype analysis of AIA patients in a Korean population, using patients with aspirin-tolerant asthma (ATA) and normal healthy volunteers as controls.

Materials and Methods

Subjects

We enrolled 110 patients with AIA, 125 patients with ATA, and 123 normal healthy controls from the Department of Allergy and Rheumatology, Ajou University Hospital, Korea. All subjects were Korean. AIA was diagnosed from positive results on the lysine-aspirin (Lys-ASA) bronchoprovocation test, performed with increasing doses of aspirin (75-300 mg/ml; Althargyl®, Arthromedica, Switzerland) using a modification of a previously described method (Park 1995). Lys–ASA bronchoprovocation tests were performed in all of the patients with ATA to exclude aspirin hypersensitivity. Normal controls were recruited from members of the general population who answered a screening questionnaire on asthmatic symptoms negatively and had no past history of aspirin hypersensitivity, a forced expiratory volume in one second (FEV_1) greater than 80% of the predicted value, a PC_{20} (provocation concentration of methacholine to induce more than 20% decrease of FEV_1) methacholine greater than 25 mg/ml, and normal readings on a simple chest radiogram. The presence of rhinosinusitis and nasal polyps were evaluated using a paranasal sinus (PNS) x-ray and rhinoscopy. The oral steroid requirement was calculated as the cumulative prednisolone equivalent dose (mg/year). All subjects gave their informed consent and the study was approved by the institutional review board of Ajou University Hospital, Suwon, Korea. Skin prick tests were performed with 12 common aeroallergens (Bencard UK Ltd., Brentford, UK). Atopy was defined as one or more positive reactions to the skin prick test.

LTC4S genotyping

We genotyped the *-1702G>A* and *-444A>C* polymorphisms in the promoter region of the *LTC4S* gene. SNP genotyping was performed using SNP-IT™ assays with the SNPstream 25K™ System (Orchid Biosciences, Princeton, NJ, USA). Briefly, the genomic DNA region spanning the polymorphic site was amplified using one phosphothiolated primer and one regular PCR primer, consisting of 5´-AAACCCACTCAAGCCAAAA-3´(forward) and 5´-AAGGTGGCACCCGAGGAC-3 (reverse) for LTC4S-1702G>A and 5´-CATTCTGAAGCCAAAG GCACT-3 (forward) and 5´-TGCACCACCCCACTTTC TC-3 (reverse) for LTC4S -444A>C. The amplified PCR products were digested with exonuclease. The 5´-phosphothiolate protected one strand of the PCR product from exonuclease digestion, resulting in the generation of a single-stranded PCR template. The single-stranded PCR template was overlaid on a 384-well plate containing a covalently attached SNP-IT™ extension primer designed to hybridize immediately adjacent to the polymorphic site (5´-CCTGCCTGGAGTTCTGGXTGTCTCCCT-3 for LTC4S -1702G>A, X = C3 spacer phosphoramidite, and 5´-AACAGCCTGGATGGGGAC-3 for LTC4S -444A>C). The SNP-IT™ primer was extended for a single base with a DNA polymerase and a mixture of an appropriate acycloterminator, which was labeled with either FITC or biotin and was complementary to the polymorphic nucleotide. The identity of the incorporated nucleotide was determined with serial colorimetric reactions with anti-FITC-AP and streptavidin- horseradish peroxidase (HRP). The development of a yellow or blue color was analyzed using an ELISA reader and the final genotype calls were made with the program QCReview™.

Statistical analysis

The differences in the clinical characteristics of the two groups were determined using Student's *t*-test for continuous variables and the chi-square test for categorical variables. The Hardy-Weinberg equilibrium (HWE) was estimated using the chi-square test. The haplotype was analyzed using Haploview version 2.05 based on the expectation-maximization (EM) algorithm (Barrett et al. 2005), and linkage disequilibrium between loci was measured using Lewontin's |D'| (Hedrick 1987). Logistic regression models were used to analyze the allele and haplotype frequencies controlling for age and sex as co-variables with alternative models (codominant, dominant, and recessive models). A p values of 0.05 or less were regarded as significant. All statistical analyses were performed using the software SPSS, version 10.0 (Chicago, IL, USA).

Results

The clinical characteristics of the study subjects

The clinical characteristics of the study subjects are summarized in Table 1. There were significant differences in the mean age and sex distribution between AIA and the other two groups ($p < 0.05$). The prevalence of atopy in the AIA patients (50%) was significantly higher than in the normal controls (29.2%, $p < 0.05$), but was not significantly different from that in ATA

Table 1. *Clinical characteristics of the study subjects*

	AIA (n = 110)	ATA (n = 125)	NC (n = 125)	p value	
				AIA vs ATA	AIA vs NC
Age (years)*	44.87 ± 13.37	40.75 ± 10.00	36.70 ± 16.02	0.028	< 0.001
Sex (male)	38 (34.5%)	62 (49.6%)	61 (48.8%)	0.020	0.027
Atopy	55/110 (50.0%)	73/124 (58.9%)	26/89 (29.2%)	0.174	0.003
Asthma duration (years)*	6.37 ± 5.47	5.28 ± 6.24	NA	0.201	NA
FEV_1 (%)*	81.92 ± 22.98	87.48 ± 21.69	NA	0.062	NA
PC_{20} methacholine (mg/ml)*	5.10 ± 8.43	5.61 ± 9.01	NA	0.69	NA
Log serum total IgE (IU/ml)*	2.22 ± 0.53	2.22 ± 0.69	NA	0.958	NA
Rhinosinusitis (presence/total)	86/105 (81.9%)	31/34 (91.2%)	NA	0.198	NA
Nasal polyp (presence/total)	58/102 (56.9%)	1/18 (5.6%)	NA	< 0.001	NA

* This value was presented as the mean ± S.D.; NA, not applicable.

(58.9%, $p > 0.05$). There were no significant differences between the AIA and ATA patients in the prevalence of chronic rhinosinusitis. The prevalence of nasal polyps under rhinoscopic examination in AIA patients (56.9%) was significantly greater than in the ATA patients (5.6%, $p < 0.05$).

Genotyping of the promoter polymorphisms in LTC4S

The *LTC4S-1702G>A* and *-444A>C* polymorphisms were in HWE ($p > 0.05$). The allele and genotype frequencies of the two SNPs are shown in Table 2. No significant associations were found in the allele and genotype frequencies among the three groups using alternative models (codominant, dominant, and recessive models). When the clinical parameters were analyzed according to the genotypes, there were no significant differences between AIA patients with the variant alleles (AG or AA genotypes of *LTC4S*

TABLE 2. *The allele and genotype frequencies of the promoter polymorphisms of LTC4S gene*

Loci	Genotype	AIA (n = 110)	ATA (n = 125)	NC (n = 125)	p value	
					AIA vs ATA	AIA vs NC
-1072 G>A	GG	76 (69.1%)	85 (68.0%)	83 (66.4%)	0.679 (co-dominant)	0.268 (co-dominant)
	AG	32 (29.1%)	36 (28.8%)	38 (30.4%)	0.453 (dominant)	0.615 (dominant)
	AA	2 (1.8%)	4 (3.2%)	4 (3.2%)	0.836 (recessive)	0.282 (recessive)
	q	0.164	0.176	0.184	0.683	0.282
-444 A>C	AA	80 (73.4%)	84 (67.2%)	94 (75.8%)	0.241 (co-dominant)	0.799 (co-dominant)
	AC	29 (26.6%)	39 (31.2%)	26 (21.0%)	0.999 (dominant)	0.999 (dominant)
	CC	0	2 (1.6%)	4 (3.2%)	0.344 (recessive)	0.475 (recessive)
	q	0.133	0.172	0.137	0.269	0.800

Each p value was calculated using codominant, dominant, and recessive models.

Logistic regression analysis was applied to control for age and sex as covariables. q, minor allele frequency.

TABLE 3. *Comparisons of the clinical characteristics according to the genotype of the LTC4S polymorphisms within AIA patients*

	-1072 G>A		p value	*-444 A>C*		p value
	GG	AG or AA		AA	AC or CC	
Sex (M)	24/76 (31.6%)	14/34 (41.2%)	0.328	26/80 (32.5%)	12/29 (41.4%)	0.390
Atopy (presence/total)	38/76 (50.0%)	17/34 (50.0%)	1.000	39/80 (48.8%)	16/29 (55.2%)	0.553
Asthma duration (years)*	6.12 ± 4.86	6.94 ± 6.66	0.485	6.51 ± 5.89	5.91 ± 4.24	0.629
FEV_1 (%)*	79.88 ± 23.70	86.49 ± 20.90	0.164	82.26 ± 21.40	80.97 ± 27.61	0.798
PC_{20} methacholine (mg/ml)*	4.85 ± 7.80	5.75 ± 10.02	0.666	5.83 ± 9.24	2.84 ± 4.67	0.061
Log mean serum total IgE (IU/ml)*	2.23 ± 0.51	2.20 ± 0.59	0.804	2.19 ± 0.52	2.33 ± 0.56	0.226
Rhinosinusitis (presence/total)	61/73 (83.6%)	25/32 (78.1%)	0.505	66/76 (86.8%)	20/28 (71.4%)	0.065
Nasal polyps (presence/total)	37/69 (53.6%)	21/33 (63.6%)	0.339	43/74 (58.1%)	15/27 (55.6%)	0.818

TABLE 4. *Haplotype frequencies of the LTC4S gene*

Haplotype	AIA (n = 218)	ATA (n = 250)	NC (n = 248)	p value*	
				AIA vs ATA	AIA vs NC
ht1 [GA]	162 (74.3%)	173 (69.2%)	178 (71.8%)	0.238	0.461
ht2 [**A**A]	27 (12.4%)	36 (14.4%)	40 (16.1%)	0.508	0.119
ht3 [G**C**]	20 (9.2%)	33 (13.2%)	24 (9.7%)	0.685	0.774
ht4 [**AC**]	9 (4.1%)	8 (3.2%)	6 (2.4%)	0.642	0.452

*Logistic regression analysis was used to control for age and sex as covariables.
Variant alleles are given in **bold**. N, number of chromosomes; ht, haplotype.

TABLE 5. *Genotype distributions of the haplotypes of LTC4S gene*

Haplotype	Genotype	AIA (n = 110)	ATA (n = 125)	NC (n = 125)	p value*	
					AIA vs ATA	AIA vs NC
Ht1	Ht1 / Ht1	55 (50.5%)	52 (41.6%)	58 (46.8%)		
	Ht1 / -	52 (47.7%)	67 (53.6%)	58 (46.8%)	NS	NS
	- / -	2 (1.8%)	6 (4.8%)	8 (6.5%)		
Ht2	Ht2 / Ht2	2 (1.8%)	4 (3.2%)	4 (3.2%)		
	Ht2 / -	23 (21.1%)	28 (22.4%)	32 (25.8%)	NS	NS
	- / -	84 (77.1%)	93 (74.4%)	88 (71.0%)		
Ht3	Ht3 / Ht3	0	2 (1.6%)	4 (3.2%)		
	Ht3 / -	20 (18.3%)	31 (24.8%)	20 (16.1%)	NS	NS
	- / -	89 (81.7%)	92 (73.6%)	100 (80.6%)		
Ht4	Ht4 / Ht4	0	0	0		
	Ht4 /-	9 (8.3%)	8 (6.4%)	6 (4.8%)	NS	NS
	- / -	100 (91.7%)	117 (93.6%)	118 (95.2%)		

* Each p value was calculated with co-dominant, dominant and recessive models. Logistic regression analysis was applied to control for age, sex as covariables. NS, non significant.

-1702G>A) and those with the common allele (GG) (Table 3). In addition, no significant differences were noted between the AIA patients with the AC or CC genotypes of *LTC4S -444A>C* and those with the AA genotype. Similarly, when all the asthma patients were classified according to the genotypes irrespective of aspirin tolerance, we found no significant differences in the clinical characteristics of the two groups (data not shown).

The haplotype frequencies are listed in Table 4. The two promoter polymorphisms of *LTC4S* were in complete linkage disequilibrium ($|D'| = 1$, $r^2 \neq 1$). There were no significant differences in the haplotype frequencies or genotype distributions among the three groups using the alternative models (Table 5).

DISCUSSION

We analyzed the genetic association between the two promoter polymorphisms of *LTC4S*, *-1702G>A* and *-444A>C*, and the phenotype of AIA patients. This was the first association analysis of *LTC4S -1702G>A* in AIA patients to date. Our previous analysis of the association of leukot-

riene-related gene polymorphisms (*ALOX5, ALOX5AP, COX2, LTC4S*, and *CYSLTR1*) with AIA in a Korean population found no significant associations between each SNP of those genes, including *LTC4S -444A>C*, and the phenotype of AIA, but the findings did suggest a significant association with a haplotype of *ALOX5* (Choi et al. 2004). Therefore, haplotype analysis rather than a single SNP can provide important information concerning the contribution of a gene to a specific disease. However, we found no significant associations between the phenotype of AIA and these two polymorphisms or their haplotypes in this study, and no significant differences were noted with other clinical parameters. It has been reported that *LTC4S* was expressed five times more strongly in bronchial biopsies from AIA patients compared to ATA and normal controls (Cowburn et al. 1998). Sanak et al. (1997, 2000) showed that AIA was associated with the genetic polymorphism of *LTC4S -444A>C*, which has an additional responsive element to histone H4 transcription factor-2, and increases the transcription rate of the gene in vitro and in vivo in a Polish population. Furthermore, the variant *C* allele was not associated with mild asthmatics having AIA who did not require oral steroids to control their asthma symptoms, suggesting that it is a marker of the severe, steroid-dependent phenotype of AIA. These findings suggest that these two promoter polymorphisms of LTC4S might not be associated with the phenotype of AIA nor clinical parameters in the Korean population.

It has been reported that the C_{-444} allele in the *LTC4S* gene was not significantly associated with AIA and had no functional impact on gene expression in the United States (Van Sambeek et al. 2000). In a study of a Japanese population, although the frequency of the variant *C* allele was significantly higher in AIA (q = 0.19) than in ATA patients (q = 0.11, $p = 0.42$), there was no significant relationship between the *LTC4S -444A>C* polymorphism and *LTC4S* activity (Kawagishi et al. 2002). Recently, Kedda et al. (2004) showed that the polymorphic *LTC4S -444A>C* was weakly associated with asthma *per se* in an Australian population, but found no association between the C_{-444} allele and chronic asthma severity or aspirin tolerance. Also, there were no significant differences detected in functional studies. This study involved a large population of subjects with well-stratified asthma severity, so it can be considered the most major and thorough association analysis of the *LTC4S -444A>C* promoter polymorphism conducted to date. A Spanish study also failed to detect any association between the C_{-444} allele and asthma phenotype, degree of severity, or AIA (Isidoro-García et al. 2005). Combined with our findings, although polymorphisms in the *LTC4S* gene might be related to the pathogenesis of asthma, the *-444A>C* promoter polymorphism is not associated with AIA or disease severity, except in a Polish population.

Recently, Sayers et al. (2003) identified a novel promoter polymorphism of the *LTC4S* gene (*-1702G>A*) in addition to the previously identified polymorphism (*-444A>C*). However, they did not find any significant role for these two polymorphisms in asthma susceptibility in a Caucasian population, although a preliminary study showed an association between the polymorphic C_{-444} allele and an increased capacity for cysLT synthesis in eosinophil and severe asthma phenotypes (Sampson et al. 2000). In order to investigate the role of this new promoter polymorphism, *LTC4S -1702G>A*, in AIA development, we performed an association analysis of the *LTC4S -1702G>A* polymorphism and a haplotype analysis with *LTC4S -444A>C* in AIA patients. However, we could not find any significant associations of this new polymorphism or their haplotypes with AIA patients. Although we did not conduct a functional study of this polymorphism, our findings combined with previous observations (Choi et al. 2004) suggest that the two promoter polymorphisms in the *LTC4S* gene, as well as their haplotypes, do not contribute to the pathogenesis of AIA development in a Korean population.

There have been several studies to suggest leukotriene related gene polymorphism with the phenotype of AIA. In et al. (1997) first identified the promoter polymorphism of the *ALOX5* gene consisting of a variable number of tandem repeats

of GC-rich motifs associated with the binding of Sp1 transcription factors subjects with the wild-type genotype (five repeats) had a significantly higher capacity to produce Cys-LTs compared to those with a mutant genotype (3, 4, or 6 repeats). Furthermore, a significant association between the *ALOX5* promoter polymorphism and the severity of airway hyperresponsiveness was detected in a Korean population (Kim et al. 2005); When we screened ten SNPs for key enzymes involved in arachidonate metabolism, 5-lipoxygenase (ALOX5, -1708G>A, 21C>T, 270G>A, 1728G>A), ALOX5-activating protein (ALOX5AP, 218A>G), and cyclooxygenase 2 (COX-2, -162C>G, 10T>G, 228G>A), in a Korean population, the lack of an association was noted between the *ALOX5AP*, *COX-2*, and *CYSLTR1* gene polymorphisms, and the AIA phenotype (Choi et al. 2004). However, the possible involvement of ALOX5-ht1 [G-C-G-A] of the *ALOX5* gene (*-1708G>A, 21C>T, 270G>A, 1728G>A*) in AIA development was suggested because the frequency of the [G-C-G-A] haplotype of *ALOX5* in the AIA group was significantly higher than its frequency in the ATA group. Further studies will be essential to find the key leukotriene related genes to be involved in the pathogenesis of AIA.

In conclusion, *LTC4S* has been identified as a likely candidate gene for the AIA phenotype; however, the polymorphic C_{-444} allele has been found to be associated with AIA only in a Polish population and not in American, Japanese, Korean, Australian, or Spanish populations. The new promoter polymorphism, *-1702G>A*, and its haplotypes were also not associated with the AIA phenotype in a Korean population. To evaluate the genetic pathogenesis of AIA, further study is needed to find novel polymorphisms of the candidate genes that have a functional impact.

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