

Leukotriene-related Gene Polymorphisms in Patients with Aspirin-intolerant Urticaria and Aspirin-intolerant Asthma: Differing Contributions of ALOX5 Polymorphism in Korean Population

The pathogenesis of aspirin (acetylsalicylic acid, ASA)-intolerant urticaria (AIU) is still poorly understood but it has recently been suggested that it is associated with the overproduction of leukotriene (LT). This is supported by evidence that cyclooxygenase 2 inhibitor is given safely to patients with AIU. The present study was designed to investigate the role of genetic polymorphism of LT related genes in the pathogenesis of AIU via a case-control study. We screened single nucleotide polymorphisms (SNPs) in genes encoding enzymes involved in leukotriene synthesis in the Korean population with AIU (n=101), ASA-intolerant asthma (AIA, n=95) and normal healthy controls (n=123). Genotype was determined by primer extension reactions using the SNaPshot ddNTP primer extension kit. Among 8 SNPs of four LT related genes, the polymorphism of ALOX5 at positions of -1708 G>A showed significant difference in genotype frequency between AIU and AIA ($p=0.01$). Furthermore, there were significant differences observed in the frequencies of two ALOX5 haplotypes between the AIU group and AIA group ($p<0.05$). However, there were no differences in allele, genotype, or haplotype frequencies of ALOX5 between the AIU group and the normal control group. These results suggested that ALOX5 has a differing contribution in two major clinical pathogenesis related to ASA-sensitivity.

Key Words : Aspirin, Hypersensitivity; Urticaria; Polymorphism, Genetic; Leukotrienes

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INTRODUCTION

Aspirin (acetylsalicylic acid, ASA) sensitivity results in distinct clinical syndromes. Patients sensitive to ASA usually develop either a respiratory syndrome (ASA-intolerant asthma, AIA) or a cutaneous syndrome (ASA-intolerant urticaria/angioedema, AIU) (1, 2). ASA is also an occasional cause of anaphylaxis (3).

The pathogenesis of ASA sensitivity most likely involves inhibition of cyclooxygenase by ASA or other nonsteroidal anti-inflammatory drugs (NSAIDs) resulting in an enhanced production of leukotrienes (LTs), potent inflammatory mediators synthesized from arachidonic acid through a sequential pathway. It is widely recognized that LT biosynthesis is associated with the development and progression of AIA. Urinary LT E₄ excretion is higher in patients sensitive to ASA than in patients insensitive to ASA (4, 5). Drugs that inhibit the synthesis of LT have clinical efficacy in asthma (6, 7).

In contrast to AIA, the study of the cutaneous reaction

resulting from ASA sensitivity has not yet led to understanding of the mechanism underlying this reaction. Many patients with chronic urticaria exhibit increased wheal and swelling after intake of ASA (2, 8). The proportion of patients with chronic urticaria who develop exacerbation after ASA administration ranges from 20% to 30% (1), whereas ASA administration causes urticaria and/or angioedema in a percentage ranging from 1% to 6% of normal subjects (9). The mechanisms of such hypersensitivity to ASA are still poorly understood. It has been postulated that ASA sensitivity may be caused by a mechanism with increased production of cysteinyl-LT (10) as the pathogenesis of AIA. This suggestion is strengthened by several reports that antihistamine, histamine receptor 1 antagonist, may not be sufficient as a monotherapy in urticaria sensitive to ASA and LT receptor antagonist (LTRA) may be effective in chronic urticaria (11-14). It has been reported that cyclooxygenase-2 (COX2) inhibitor may be given safely to patients with AIU (15, 16). In patients with chronic urticaria, baseline urinary LT E₄ excretion is higher in patients sensitive to ASA than in patients insensitive to ASA (1). These

studies support the hypothesis that overproduction of LT may be important in chronic urticaria.

Although there is no clear evidence of the involvement of LT in AIU and/or angioedema, it has been proposed that LTs may participate in the pathogenesis of urticaria and angioedema in a similar mechanism to that of AIA. We (17) and others (18, 19) have previously shown that polymorphism in LT related genes is associated with AIA. Therefore, there is a rationale to investigate the genetic polymorphisms of candidate genes related with LT production in AIU and/or angioedema.

In this study, we investigated the genetic polymorphism in 8 candidate single nucleotide polymorphisms (SNPs) of four LT related genes, *ALOX5* (5-lipoxygenase), *ALOX5AP* (5-lipoxygenase activating protein), *PTGS2* (cyclooxygenase 2) and *LTC4S* (*LTC4* synthase), in patients with AIU compared to AIA and a normal healthy control group recruited from a Korean population

MATERIALS AND METHODS

Study subjects

One hundred one patients with urticaria sensitive to both ASA and NSAIDs (46 male subjects; mean age: 34.2 yr; 31 patients had underlying chronic urticaria with more than 6 weeks duration), 95 patients with ASA-intolerant asthma (35 male subjects, mean age: 42.3 yr), and 123 normal healthy controls (NC) enrolled from the Department of Allergy and Rheumatology, Ajou University Hospital, Suwon, Korea were enrolled in the study. In this study, ASA-intolerant urticaria group was defined as patients having a certain history of urticaria/angioedema development after the ingestion of more than two kinds of NSAIDs and positive responders on oral ASA challenge test (classified as cross reacting group by Sanchez-Borges et al. (20)). Also NSAIDs sensitivity could be confirmed because the patients visited our Allergy Clinic or emergency room presenting current urticaria/angioedema after taking NSAIDs. In order to exclude a single ASA-intolerant urticaria, we performed skin prick test with 10 mg/mL of lysine-ASA (L-ASA) and none of them had positive skin prick test. ASA-intolerant asthma was diagnosed by a positive result to L-ASA bronchoprovocation testing and they had no history of drug allergies presenting as skin manifestations. Patients having both AIA and AIU were excluded in this study. 123 normal controls, who had non-atopy, no personal and family history of allergic diseases, and no past history of ASA and other drug hypersensitivity, were recruited from the general population. Seventy (77.8%) patients among the ASA-intolerant urticaria group and 35 (43.8%) in ASA-intolerant asthma patients were atopic. All subjects provided informed consent and the protocol used were approved by the ethics committee of Ajou University Hospital, Suwon, Korea. Skin prick

tests were performed with 12 common aeroallergens (Bencard Co., U.K.) including *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat, dog, cockroach, tree pollen mixture, grass pollen mixture, mugwort, ragweed, Hop Japanese, *Aspergillus*, *Alternaria*, with histamine and saline controls.

Oral ASA challenge and L-ASA bronchoprovocation tests

All medications, including theophyllines, beta-2 agonists, LT receptor antagonist, anti-histamine and steroids were stopped 72 hr before the procedure. After the placebo challenge, 500 mg of ASA in a tablet was orally administered, and patients were observed for 4 hr with monitoring of urticaria and changes of lung functions every 30 min. Appearance of urticaria within 4 hr without any changes of FEV₁ was considered as a positive result. L-ASA bronchoprovocation test was performed according to the modified method as previously described (17, 21). The test solutions were delivered by a DeVilbiss 646 nebulizer (DeVilbiss Co., Somerset, Penn., U.S.A.) and connected to a compressed air source (5 L/min). L-ASA powder (Althargyl[®], Arthromedica, Swiss) that contained 900 mg L-ASA with 100 mg aminoacetic acid was diluted in normal saline to produce a stock solution of 300 mg/mL. A placebo solution of normal saline was used as a control. Subjects inhaled the aerosol via a mouthpiece during normal tidal breathing. The L-ASA solution was inhaled every 30 min. The FEV₁ and maximum mid expiratory flow (MMEF) are measured at 10 and 30 min after each dose. The provocation was stopped when the FEV₁ has decreased 20% or more from the baseline. The FEV₁ and MMEF after the last dose were measured at 10, 30, 60 min, and then hourly for 7 hr.

Table 1. Clinical characteristics of the study subjects

	AIU (n=101)	AIA (n=95)	NC (n=123)
Age (yr)*	34.2±12.3	42.3±14.1	35.2±14.9
Sex (M)	46 (45.5%)	35 (36.8%)	63 (51.2%)
Atopy†	70 (77.8%)	35 (43.8%)	19 (16.7%)
Total IgE	333.4±366.7	346.6±460.0	70.0±71.1
History of other drug allergy	9 (8.9%)	NA	0
History of allergic diseases	57 (51.5%)	NA	0
Provocation test	28 (27.7%)	76 (80.0%)	NA
Urticaria	42 (41.5%)	0	NA
Angioedema	18 (17.8%)	0	NA
Urti/angio.	41 (40.5%)	0	NA
Anaphylaxis	4 (4.0%)	0	NA
Chronic urticaria	31 (30.7%)	0	NA

AIU, ASA-intolerant urticaria; AIA, ASA-intolerant asthma; NC, normal controls; NA, not applicable.

**p*<0.01 (AIU vs. AIA). †*p*<0.01 (AIU vs. AIA and AIU vs. NC).

Genomic DNA preparation and SNP genotyping

Peripheral blood samples (10 mL) were collected from study subjects. Genomic DNA was extracted using a G-DEX™ for Blood Genomic DNA Extraction Kit (iNtRON Biotechnology, Korea) according to the manufacturer's instructions. For each of the 8 SNPs in the four genes, *ALOX5*, *ALOX5AP*, *PTGS2* and *LTC4S*, analyzed, pairs of forward and reverse primers were designed to amplify the genomic region surrounding the SNP of interest (Table 1). The polymerase chain reaction (PCR) was performed with 20 ng genomic DNA, 50 mM KCl, 10 mM Tris, 2 mM MgCl₂, 1.25 pmol of each primer, 200 μM dNTPs, and 0.15 U *Taq* DNA polymerase (Perkin Elmer, Emeryville, CA, U.S.A.) in standard buffer provided by the manufacturer. After initial denaturation for 5 min at 95°C, a touch-down PCR (22) was undertaken with 10 cycles consisting of 1 min denaturation at 94°C, 1 min annealing at 54°C and 2 min elongation at 72°C followed by 35 cycles of 1 min at 94°C, 1 min at 45°C and 2 min at

72°C. A final elongation step at 72°C for 10 min terminated the program. Primer extension reactions were performed with the SNaPSHOT ddNTP primer extension kit (Applied Biosystems) as recommend by the manufacturer using extension probes as previously described (17).

Data analysis

Statistical analyses were completed using SPSS version 10 (SPSS Inc., Chicago, IL, U.S.A.) and a *p*-value <0.05 was considered to be significant. Allele frequencies were calculated for each polymorphic site by the allele counting method, and significant departures of genotype frequency from the Hardy-Weinberg equilibrium (HWE) at each polymorphic site were tested by chi-square analysis. Differences in genotype frequency between patients and controls were tested by Fisher's exact test. Differences in the mean value of the phenotypic characteristics within patient groups were compared using ANOVA and t-test. Haplotypes of *ALOX5* were ana-

Table 2. Allele and genotype frequencies of the SNPs in the candidate genes

Gene	Locus	Genotype	AIU (n=101)	AIA (n=95)	NC (n=123)	<i>p</i> value*	
						AIU vs. AIA	AIU vs. NC
<i>ALOX5</i>	-1708G>A	GG	57 (56.4%)	48 (64.9%)	76 (67.9%)	0.04	NS
		AG	33 (32.7%)	25 (33.8%)	30 (26.8%)	0.01	NS
		AA	11 (10.9%)	1 (1.4%)	6 (5.4%)	NS	NS
		q.	0.272	0.182	0.188	0.06	0.04
	270G>A	GG	92 (91.1%)	72 (90.0%)	98 (85.2%)	NS	NS
		AG	9 (8.9%)	8 (10.0%)	16 (13.9%)	NS	NS
		AA	0	0	1 (0.9%)	NS	NS
		q.	0.045	0.05	0.078	NS	NS
	1728A>G	AA	80 (81.6%)	71 (92.21%)	98 (86.0%)	NS	NS
		AG	17 (17.3%)	6 (7.8%)	16 (14.0%)	NS	NS
		GG	1 (1.0%)	0	0	0.05	NS
		q.	0.097	0.039	0.07	0.04	NS
<i>ALOX5AP</i>	218A>G	AA	99 (98.0%)	90 (98.9%)	119 (98.3%)	NS	NS
		AG	2 (2.0%)	1 (1.1%)	2 (1.7%)	NS	NS
		GG	0	0	0	NS	NS
		q.	0.01	0.005	0.008	NS	NS
<i>PTGS2</i>	-162C>G	CC	95 (96.9%)	86 (98.9%)	114 (96.6%)	NS	NS
		CG	3 (3.1%)	1 (1.1%)	4 (3.4%)	NS	NS
		GG	0	0	0	NS	NS
		q.	0.015	0.006	0.017	NS	NS
	10T>G	TT	96 (97.0%)	89 (98.9%)	120 (98.4%)	NS	NS
		GT	3 (3.0%)	1 (1.1%)	2 (1.6%)	NS	NS
		GG	0	0	0	NS	NS
		q.	0.015	0.006	0.008	NS	NS
R228H* (G>A)	GG	97 (97.0%)	84 (98.8%)	114 (96.6%)	NS	NS	
	AG	3 (3.0%)	1 (1.2%)	4 (3.4%)	NS	NS	
	AA	0	0	0	NS	NS	
	q.	0.015	0.006	0.017	NS	NS	
<i>LTC4S</i>	-444A>C	AA	72 (72.0%)	64 (71.9%)	85 (72.6%)	NS	NS
		AC	24 (24.0%)	23 (25.8%)	25 (21.4%)	NS	NS
		CC	4 (4.0%)	2 (2.2%)	7 (6.0%)	NS	NS
		q.	0.16	0.156	0.167	NS	NS

AIU, ASA-intolerant urticaria; AIA, ASA-intolerant asthma; NC, normal controls; n, number of patients; q, minor allele frequency. R, arginine; H, histidine; NS, not significant. *Each *p* value was calculated with co-dominant, dominant and recessive models.

lyzed by using the Haploview version 2.05 (23).

RESULTS

The characteristics of the participants were shown in Table 1. The three groups significantly differed with respect to age ($p < 0.01$ for AIU vs. AIA) and prevalence of atopy ($p < 0.01$ for AIU vs. NC and AIU vs. AIA) between AIU and other control groups. Seventy (77.8%) patients with AIU and 35 (43.8%) AIA patients were atopic. Patients with AIU were composed of 31 (30.7%) chronic urticaria exacerbated by ASA, 18 (17.8%) combined with angioedema and 4 (4.0%) combined with anaphylaxis. Patients with both AIA and AIU were excluded in this study.

We investigated 8 SNPs of *ALOX5*, *ALOX5AP*, *PTGS2* and *LTC4S* in AIU compared to other control groups, AIA and NC. Genotype distributions of all loci were in Hardy-Weinberg equilibrium ($p > 0.05$). Allele and genotype frequencies of each SNP in four genes are shown in Table 2. Of

Table 3. Haplotype frequencies of *ALOX5* gene. The haplotype of *ALOX5* is in order of -1708G>A, 270 G>A, 1728 A>G

Haplotype*	AIU (n=196)	AIA (n=142)	NC (n=210)	p value	
				AIU vs. AIA	AIU vs. NC
Ht1 (G-G-A)	0.60	0.73	0.67	0.01	0.12
Ht2 (A-G-A)	0.28	0.18	0.20	0.05	0.06
Ht3 (G-G-G)	0.08	0.03	0.05	0.06	0.22
Ht4 (G-A-A)	0.03	0.06	0.06	0.28	0.16
Ht5 (G-A-G)	0.02	0.00	0.02	0.27	0.72

AIU, ASA-intolerant urticaria; AIA, ASA-intolerant asthma; NC, normal controls; Ht, haplotype; N, number of chromosomes.

*Minor alleles are given in bold.

all SNPs tested, the polymorphism of *ALOX5* at positions of -1708 G>A showed significant difference in genotype frequency between AIU and AIA; the frequency of minor genotype of *ALOX5*-1708G>A was significantly higher in AIU group compared to AIA group ($p = 0.01$ in dominant model, OR=8.9, 95% CI=1.13-10.73). The p value remained significant after correction for multiple comparisons ($P_c = 0.045$). For all other SNPs tested, there were no significant differences in allele and genotype frequencies among the three groups.

Using Haploview program, haplotypes were constructed for 3 SNPs and the frequency of each haplotype in the patient groups was assessed. The frequency of five haplotypes of *ALOX5* showing $\geq 1\%$ frequency in the population was shown in Table 3. There were significant differences observed in the frequency of the *ALOX5* haplotypes between the AIU group and AIA group; the frequency of *ALOX5* ht1 [G-G-A] carrying major alleles of 3 SNPs in the AIU group was significantly lower than that in the AIA group ($p = 0.01$, OR=0.541, 95% CI=0.339-0.865), but the frequencies of haplotypes carrying minor allele of *ALOX5*-1708G>A, ht2 [A-G-A] was significantly higher in AIU group compared to AIA group ($p = 0.05$, OR=1.697, 95% CI=1.00-2.88). Analysis of the genotype distributions of the *ALOX5* haplotypes (Table 4) also revealed that frequencies of genotype containing ht1 [G-G-A] and ht2 [A-G-A] were significantly different between AIA and AIU ($p = 0.01$ in dominant model for ht1, $p = 0.02$ in recessive model for ht2). However, there were no differences in allele, genotype, or haplotype frequencies of *ALOX5* polymorphisms between the AIU group and the normal control. Haplotypic analysis of *PTGS2* polymorphism was not undertaken due to the extremely low frequencies of the variant alleles.

Table 4. Genotype distributions of *ALOX5* haplotypes

Haplotype	Genotype	AIU (n=98)	AIA (n=71)	NC (n=105)	p value*	
					AIU vs. AIA	AIU vs. NC
Ht1	Ht1/Ht1	36 (36.7%)	36 (50.7%)	46 (43.8%)	0.02	NS
	Ht1/-	45 (45.9%)	32 (45.1%)	49 (46.7%)	0.01	NS
	-/-	17 (17.3%)	3 (4.2%)	10 (9.5%)	NS	NS
Ht2	Ht2/Ht2	11 (11.2%)	1 (1.4%)	6 (5.7%)	0.04	NS
	Ht2/-	32 (32.7%)	24 (33.8%)	29 (27.6%)	NS	NS
	-/-	55 (56.1%)	46 (64.8%)	80 (65.0%)	0.01	NS
Ht3	Ht3/Ht3	0	0	0	NS	NS
	Ht3/-	6 (6.1%)	0	6 (5.7%)	NS	NS
	-/-	92 (93.9%)	71 (100%)	99 (94.3%)	NS	NS
Ht4	Ht4/Ht4	0	0	0	NS	NS
	Ht4/-	6 (6.1%)	8 (11.3%)	13 (12.4%)	NS	NS
	-/-	92 (93.9%)	63 (88.7%)	92 (87.6%)	NS	NS
Ht5	Ht5/Ht5	0	0	0	NS	NS
	Ht5/-	3 (3.1%)	0	5 (4.8%)	NS	NS
	-/-	95 (96.9%)	71 (100%)	100 (95.2%)	NS	NS

[†]AIU, ASA-intolerant urticaria; AIA, ASA-intolerant asthma; NC, normal controls; Ht, haplotype.

*Each p value was calculated with co-dominant, dominant and recessive models.

DISCUSSION

In this study, we have investigated genetic association between polymorphisms of LT related genes (*ALOX5*, *ALOX5-AP*, *PTGS2* and *LTC4S*) and AIU in a Korean population.

Among 8 SNPs of four LT related genes, the polymorphism of *ALOX5* at positions of -1708 G>A showed highly significant differences in genotype frequency between AIU and AIA ($p=0.01$). Furthermore, there were significant differences observed in the frequency of the *ALOX5* haplotypes (ht1 and ht2) between the AIU group and AIA group. However there were no differences in allele, genotype, or haplotype frequencies in *ALOX5* -1708 G>A between the AIU group and the normal control. In association study of 8 SNPs in LT related genes, *ALOX5*, *LTC4S*, *PTGS2* and *ALOX5AP* with phenotypes (sex, age, atopy, tlgE), we could not find any significant difference.

In our previous study, we found that an *ALOX5*-ht1 [G-C-G-A] was associated with AIA but not ASA-tolerant asthma; the frequency of the *ALOX5*-ht1 [G-C-G-A] carrying major alleles of these 3 SNPs in the AIA group was significantly higher than its frequency in the control group. In addition, consistent with the present study, significant differences in frequency of the *ALOX5* polymorphism at positions of -1708G>A was also observed between the AIU group and the AIA group. The results of the present study however, suggested that unlike AIA, susceptibility to AIU is associated with minor genotype of *ALOX5* -1708G>A. There was a significant difference in atopy frequency between the AIA and AIU groups. However this may be unlikely to influence the results as previous studies have not identified association of LT related polymorphism with atopy (24, 25). It may be possible that *ALOX5* polymorphism may contribute to AIU susceptibility with odds ratio (OR) of 8.9 and 95% confidence interval (95% CI) of 1.13-70.73 relative to AIA. These results suggested that *ALOX5*, which catalyzes the first step in the formation of LT, has a differing contribution in two major clinical pathogenesis related to ASA-sensitivity.

This study has several potential limitations. It is possible to observe a discordant result, oral tests were positive but the bronchial tests were negative, according to the provocation route (26). Our limited power to detect modest effects may at least partially explain our modest evidence of association to *ALOX5* SNP. While the minimum p -values we observed were modest, this is consistent with our expectations regarding common, complex conditions such as AIA and AIU.

In conclusion, we have shown that *ALOX5* has a differing contribution in two major clinical pathogenesis related to ASA-sensitivity. Our studies suggested that *ALOX5* may be a potentially important gene in AIU susceptibility in the Korean population, and that promoter polymorphism of the *ALOX5* gene might contribute to AIU susceptibility relative to AIA. Further studies will be needed to confirm functional effect of the *ALOX5* -1708 G>A on the pathogenic mecha-

nism of AIU.

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