

Original article

Polymorphism of tandem repeat in promoter of 5-lipoxygenase in ASA-intolerant asthma: a positive association with airway hyperresponsiveness

Background: 5-Lipoxygenase (ALOX5) and 5-lipoxygenase-activating protein (ALOX5AP) are known as key enzymes in cysteinyl-leukotriene (cys-LT) production, critical mediators in aspirin acetylsalicylic acid (ASA)-intolerant asthma (AIA). To date, studies of the promoter region of ALOX5 gene has revealed the potential influence of a variable number of tandem repeats of a Sp1- and Egr1-binding motif, on the transcription rate.

Methods: To understand the pathological process that arises from cys-LT overproduction in AIA, we genotyped ALOX5 Sp1 and ALOX5AP poly(A) repeat promoter polymorphism by fluorescent-based capillary electrophoresis in the Korean population.

Results: No significant differences in allele and genotype frequencies of the ALOX5 and ALOX5AP promoter polymorphisms were observed between the three groups. However, there was a strong association of the ALOX5 Sp1 repeat polymorphism with airway hyperresponsiveness (AHR; PC₂₀ methacholine); AIA patients carrying a mutant allele ($n > 5$ or $n < 5$ repeats) showed increased AHR compared to AIA patients with wild-type genotype ($P = 0.003$).

Conclusion: Although the alleles of the ALOX5 and ALOX5AP promoter cannot be considered as a prominent risk factor in the development of AIA, the genetic variant of tandem repeat (GGGCGG; Sp1-binding motif) in ALOX5 promoter is associated with the severity of airway hyperresponsiveness in AIA patients.

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Key words: 5-lipoxygenase; 5-lipoxygenase-activating protein; aspirin-intolerant asthma; bronchial hyperresponsiveness; tandem repeat polymorphism.

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Aspirin acetylsalicylic acid (ASA)-intolerant asthma (AIA) is a distinct clinical syndrome (1, 2) that refers to the development of bronchoconstriction in asthmatic individuals following the ingestion of ASA and other nonsteroidal anti-inflammatory drugs (NSAIDs). The AIA affects about 5–10% of adults with asthma. It is widely recognized that increased cysteinyl-leukotriene (cys-LT) biosynthesis is associated with the development and progression of AIA, for example, patients with AIA produce higher amounts of cys-LTs in urine compared with ATA patients (3, 4). Although the pathogenesis of AIA is not completely understood, cys-LTs are recognized to be important proinflammatory mediators in the pathogenesis of AIA and drugs that inhibit the synthesis of cys-LTs have clinical efficacy in asthma (5, 6).

The soluble enzyme, 5-lipoxygenase (ALOX5), and the membrane protein, 5-lipoxygenase-activating protein (ALOX5AP), are essential for cys-LT production. The ALOX5, which catalyses the first two steps in the formation of cys-LTs, is abundantly present in alveolar macrophages (7, 8) and mRNA levels are increased

following cell activation (9). Activation of ALOX5 generates the highly reactive intermediate LTA₄, which is rapidly converted to the potent inflammatory compounds LTB₄, LTC₄ and LTD₄ (10). The ALOX5AP is an 18-kDa protein considered to be an integral part of the ALOX5 pathway of arachidonic acid metabolism (11, 12). The ALOX5AP transfers arachidonic acid to ALOX5, thereby enabling ALOX5 to efficiently produce LTA₄ (13). It has been reported that mRNA expression of both ALOX5 and ALOX5AP are increased in patients with asthma (14), and clinical studies in patients with asthma showed a therapeutic benefit with ALOX5 inhibitors (15, 16).

Recently, it has been reported that tandem repeat polymorphisms in the promoters of ALOX5 and ALOX5AP are associated with asthma phenotypes. The activity of the promoter of the ALOX5 gene is modulated by the number of Sp1 transcription factor-binding motifs present and this varies between individuals with three, four and six repeats (mutant type) and five repeats (wild type) being reported (17). This tandem repeat

polymorphism has also been reported to modify individual's response to ALOX5 inhibitors (18). In that study, it was shown that asthmatic patients carrying two mutant alleles (three, four or six repeats) of the Sp1 tandem repeat showed diminished response to treatment with ABT761, a selective inhibitor of ALOX5. A novel tandem repeat polymorphism in ALOX5AP gene promoter was identified by Koshino et al. (19) in study of Japanese subjects. They reported that the allele consisting of a mononucleotide A repeat of 21 bp in the ALOX5AP promoter was present in a higher frequency in asthmatics (73.2%), when compared with normal subjects (54.9%), but they did not investigate the effects of the polymorphism on promoter function.

To understand the pathological process that arises from cys-LT excess in AIA, we have investigated whether the polymorphisms of the ALOX5 and ALOX5AP genes are associated with AIA phenotypes in a Korean population.

Methods

Study subjects

Three groups of the study patients (107 patients with AIA, 114 normal healthy controls and 109 patients with ATA) were enrolled from the Department of Allergy and Rheumatology, Ajou University Hospital in Korea; all subjects were of Korean ethnicity. Diagnosis of asthma was confirmed using the Global Initiative for Asthma (GINA) guidelines (2002, revised) and AIA was diagnosed by positive response to a lysine (L)-ASA bronchoprovocation tests, performed according to a modified method as previously described (20). The patients showing negative responses on L-ASA bronchoprovocation tests with no history of adverse reactions to ASA/NSAIDs were classified as ATA. All subjects gave informed consent and the protocol was approved by the local ethics committee. Skin prick tests were performed with 12 common aeroallergens (Bencard, UK). Atopy was defined as one or more positive reactions on skin prick test results. All the clinical history was reviewed in detail by the investigators. Methacholine bronchial challenge tests were performed according to the previously described method (20).

Molecular methods

Peripheral blood samples (10 ml) were collected from the participant of this study. Genomic DNA was used in G-DEXTM for Blood Genomic DNA Extraction Kit (iNtRON Biotechnology, Sungnam, South Korea) according to the manufacturer's instructions. The tandem repeat polymorphisms in the promoter of ALOX5 and ALOX5AP were genotyped using an ABI3100 DNA sequencer in conjunction with GENESCAN and GENOTYPER software (Applied Biosystems, Foster City, CA, USA). Amplicons encompassing the polymorphic region were generated by duplex-polymerase chain reaction (PCR): 10 ng genomic DNA template, 0.05 U/ μ l *Taq* DNA polymerase (Solgent, Daejeon, South Korea), standard buffer, 2 mM MgCl₂, 200 nM dNTPs, 0.2 μ M of each fluorescent-labelled forward primer (6-FAM for ALOX5AP, HEX for ALOX5) and reverse primer in a reaction volume of 20 μ l. Oligonucleotides used in this study are follows – ALOX5 forward primer: HEX-cgtgaa-gagtgggagagaa, ALOX5 reverse primer: gctgaggtagatgtctgcagt, ALOX5AP forward primer: FAM-gggaagtttccatgaca, ALOX5AP

reverse primer: accattctggaccacgtgat. Thermal cycling parameters were 94°C for 5 min, then 40 cycles at 94°C for 30 s, at 50°C for 40 s, at 72°C for 45 s and a final 72°C extension period for 7 min. Depending on the alleles present in the ALOX5 promoter, PCR fragments of 268, 274 and 280 bp were obtained corresponding to four, five and six repeats of the Sp1-binding motif (GGGCGG). For ALOX5AP poly(A) repeats, PCR fragments of 358 and 362 bp were obtained corresponding to 19 and 23 repeats.

Statistical analysis

Statistical analyses were completed using SPSS version 10 (SPSS Inc., Chicago, IL, USA) 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 chi-square test of association. Differences in the mean value of the phenotypic characteristics within AIA patients were compared using ANOVA and *t*-tests. For the association, study between genotype and the disease-related phenotypes, we accepted a *P*-value using Levene's test (21) for equality of variances in order to exclude an artificial false significance.

Results

In an effort to investigate whether two genetic polymorphisms in the ALOX5 and ALOX5AP promoters contribute to the pathogenesis of AIA, the tandem repeat of GGGCGG (Sp1-binding motif) in ALOX5 promoter at positions –176 to –147 from the translational start site and a mononucleotide A repeat in ALOX5AP promoter at positions –169 to –146 relative to the translational initiation codon (ATG) were genotyped in three subject groups [AIA, nonasthmatic control (NC), ATA] in a Korean population.

The clinical characteristics of the study subjects in this study are summarized in Table 1. There were no significant differences in mean age and sex between AIA group and the other control groups. Allele and genotype frequencies of the polymorphisms are described in Tables 2 and 3. We designated an individual genotype for most commonly found allele (with five tandem repeats for ALOX5 and mononucleotide A repeats of 23 bp for

Table 1. Clinical characteristics of the study subjects

Characteristics	AIA (<i>n</i> = 107)	NC (<i>n</i> = 114)	ATA (<i>n</i> = 109)
Age (year)	44.0 $\pm$ 13.3	44.5 $\pm$ 17.8	44.5 $\pm$ 14.9
Sex (M)	38 (35.5%)	53 (46.5%)	52 (47.7%)
Atopy	52 (51.0%)	NA	60 (55.0%)
Asthma duration (year)	6.7 $\pm$ 5.9	NA	5.4 $\pm$ 6.4
FEV ₁ (%)	83.3 $\pm$ 22.5	NA	85.7 $\pm$ 22.9
PC ₂₀ -methacholine (mg/ml)	4.7 $\pm$ 7.3	NA	5.6 $\pm$ 9.2
Serum total IgE (IU/ml)	333.2 $\pm$ 428.1	NA	401.6 $\pm$ 554.8

n, number of patients; NA, not applicable; FEV₁, forced expiratory volume after 1 s; IgE, immunoglobulin E; AIA, aspirin-intolerant asthma; NC, nonasthmatic control; ATA, aspirin-tolerant asthma.

Table 2. Allele frequencies of the ALOX5 and ALOX5AP in different population

Gene	Allele type	Oriental population					Caucasian population		
		Korean*			Japanese†		American‡	British§	
		AIA (<i>n</i> = 214)	NC (<i>n</i> = 228)	ATA (<i>n</i> = 218)	Asthma (<i>n</i> = 142)	NC (<i>n</i> = 142)	Asthma (<i>n</i> = 442)	Asthma (<i>n</i> = 368)	NC (<i>n</i> = 368)
ALOX5	3	0	0	0	NA	NA	17 (3.8)	3 (0.8)	0
	4	55 (25.7)	64 (28.15)	60 (27.5)	NA	NA	76 (17.2)	52 (14.1)	51 (13.9)
	5	130 (60.7)	135 (59.2)	120 (55.0)	NA	NA	341 (77.2)	310 (84.2)	308 (83.7)
	6	29 (13.6)	29 (12.7)	38 (17.4)	NA	NA	8 (1.8)	3 (0.8)	9 (2.4)
ALOX5AP	A18	0	0	0	23 (16.2)	42 (29.6)	NA	Asthma (<i>n</i> = 366) 0	NC (<i>n</i> = 358) 0
	A19	34 (15.95)	48 (21.1)	33 (15.1)	0	0	NA	40 (10.9)	47 (3.1)
	A21	0	0	0	119 (83.8)	100 (70.4)	NA	0	0
	A23	180 (84.1)	180 (78.9)	185 (84.9)	0	0	NA	326 (89.1)	311 (86.9)

n, number of patients (or individual); NA, not applicable; asthma, asthmatic sibling 1; NC, nonasthmatic control; ALOX5, 5-lipoxygenase; ALOX5AP, 5-lipoxygenase-activating protein; AIA, aspirin-intolerant asthma.

*Our study.

†Koshino et al. (19)

‡Drazen et al. (18)

§Sayers et al. (22)

Values in parentheses expressed as percentage.

Table 3. Genotype distribution of the ALOX5 and ALOX5AP in different population

Gene	Genotype	Oriental population					Caucasian population		
		Korean*			Japanese†		American‡	British§	
		AIA (<i>n</i> = 107)	NC (<i>n</i> = 114)	ATA (<i>n</i> = 109)	Asthma (<i>n</i> = 71)	NC (<i>n</i> = 71)	Asthma (<i>n</i> = 221)	Asthma (<i>n</i> = 184)	NC (<i>n</i> = 184)
ALOX5	3/3	0	0	0	NA	NA	3 (1.4)	0	0
	3/4	0	0	0	NA	NA	0	1 (0.5)	0
	3/5	0	0	0	NA	NA	11 (5.0)	2 (1.1)	0
	4/4	9 (8.4)	11 (9.6)	8 (7.3)	NA	NA	7 (3.2)	5 (2.7)	3 (1.6)
	4/5	33 (30.8)	35 (30.7)	34 (31.2)	NA	NA	60 (27.2)	41 (22.3)	44 (23.9)
	4/6	4 (3.7)	7 (6.1)	10 (9.2)	NA	NA	2 (0.9)	0	1 (0.5)
	5/5	39 (36.4)	43 (37.7)	33 (30.3)	NA	NA	133 (60.2)	132 (71.7)	128 (69.6)
	5/6	19 (17.8)	14 (12.3)	20 (18.3)	NA	NA	4 (1.6)	3 (1.6)	8 (4.4)
	6/6	3 (2.8)	4 (3.5)	4 (3.7)	NA	NA	1 (0.5)	0	0
ALOX5AP	A18/18	0	0	0	4 (5.6)	10 (14.1)	NA	Asthma (<i>n</i> = 183) 0	NC (<i>n</i> = 179) 0
	A18/21	0	0	0	15 (20.8)	22 (31.0)	NA	0	0
	A19/19	4 (3.7)	6 (5.3)	3 (2.8)	0	0	NA	2 (1.1)	3 (1.7)
	A19/23	26 (24.3)	35 (30.7)	27 (24.8)	0	0	NA	36 (19.7)	41 (22.9)
	A21/21	0	0	0	52 (73.2)	39 (54.9)	NA	0	0
	A23/23	77 (72.0)	73 (64.0)	79 (72.5)	0	0	NA	145 (79.2)	135 (75.4)

n, number of patients (or individual); NA, not applicable; asthma, asthmatic sibling 1; NC, nonasthmatic controls; ALOX5, 5-lipoxygenase; ALOX5AP, 5-lipoxygenase-activating protein; AIA, aspirin-intolerant asthma.

*Our study.

†Koshino et al. (19).

‡Drazen et al. (18)

§Sayers et al. (22)

Values in parentheses expressed as percentage.

ALOX5AP) as a wild-type homozygote, an individual carrying at least one mutant type allele (four or six tandem repeats for ALOX5 and mononucleotide A repeats of 19 bp for ALOX5AP) as a heterozygote and an individual with no wild-type allele as a mutant-type homozygote. These designations were based on those of

Drazen et al. (18). There were no significant differences in allele frequencies between AIA patients and normal control group, and between AIA patients and ATA patients (Table 2). No significant differences in genotype frequencies with any of three alternative analysing models (co-dominant, dominant and recessive models) were

Table 4. Mean ($\pm$ SD) of the clinical characteristic in the homozygous wild type and homozygous or heterozygous mutant type of the ALOX5 and ALOX5AP promoter in AIA

Gene	Genotype*	Asthma duration (year)	FEV ₁ (%)	PC ₂₀ methacholine (mg/ml)	Serum total IgE (IU/ml)
ALOX5	Homozygous wild type ([GGGCGG]5/[GGGCGG]5)	6.8 $\pm$ 6.7	81.1 $\pm$ 22.5	7.7 $\pm$ 9.4	347.2 $\pm$ 447.5
	Other genotypes	6.7 $\pm$ 5.5	84.6 $\pm$ 22.6	3.1 $\pm$ 5.4	325.1 $\pm$ 419.6
	<i>P</i> -value	NS	NS	0.003	NS
ALOX5AP	Homozygous wild type (A23/A23)	6.3 $\pm$ 5.7	82.8 $\pm$ 22.1	5.0 $\pm$ 7.7	365.0 $\pm$ 451.7
	Other genotypes	7.8 $\pm$ 6.6	84.9 $\pm$ 23.8	3.7 $\pm$ 6.2	252.2 $\pm$ 355.0
	<i>P</i> -value	NS	NS	NS	NS

ALOX5, 5-lipoxygenase; ALOX5AP, 5-lipoxygenase-activating protein; AIA, aspirin-intolerant asthma; FEV₁, forced expiratory volume after 1 s; IgE, immunoglobulin E.

*Other genotypes did not have any wild-type allele.

found between AIA patients and the normal control group or between AIA patients and ATA patients (Table 3). Genotype distributions of both polymorphisms were in HWE ($P > 0.05$). The observed allele and genotype frequencies of each polymorphism of the ALOX5 and ALOX5AP genes in the Korean population were compared with reported frequencies in other populations (Tables 2 and 3). We identified that the frequency of the allele having five repeats for ALOX5 was about 60% in the Korean population, which was slightly lower than that observed in the UK Caucasian population (approximately 80%). We did not find the allele carrying three repeats in the Korean population and the frequency of the allele with six repeats (approximately 13%) is higher than that in the UK Caucasian population (approximately 1%). In the genotype distribution of ALOX5AP, genotype with 23 poly(A) repeat was found more frequently than genotype with 19 poly(A) repeats by according with the genotype distribution in the Caucasian study, which was quite different from the Japanese study. Genotypes homozygous for A21 or A18 repeats were predominant in the Japanese study.

We examined whether two genetic polymorphisms of ALOX5 and ALOX5AP were associated with intermediate traits [asthma duration, baseline forced expiratory volume after 1 s (FEV₁), PC₂₀ methacholine and total IgE] in AIA patients (Table 4). To investigate the direct effect of the homozygous wild genotype on the AIA phenotypes, we compared homozygote wild type (five of five, Sp1 repeats) with all other genotypes (Table 4). There were no significant associations between ALOX5 or ALOX5AP genotype and asthma duration, baseline FEV₁ (%) and serum total IgE level among the AIA patient group, but there was strongly significant association between ALOX5 genotype and PC₂₀ methacholine; AIA patients carrying at least one mutant allele ($n > 5$ or $n < 5$) of ALOX5 had a lower mean PC₂₀ (3.1 mg/ml) than AIA patients homozygous for the wild-type ($n = 5$ repeats) genotype of ALOX5 (7.7 mg/ml, $P = 0.003$).

Discussion

In this study, we have investigated the role of two genetic polymorphisms of ALOX5 and ALOX5AP in the

pathogenesis of AIA in the Korean population. Although In et al. (17) previously reported the allele frequency of the tandem repeat polymorphism of ALOX5 gene in AIA patients, their sample size was very small ($n = 6$). Therefore, this is the first study to investigate the tandem repeat polymorphism of the ALOX5 and ALOX5AP gene promoter in AIA patients on a larger scale ($n = 107$). We did not identify any statistical significance in allele or genotype distributions between AIA patients and ATA or normal control groups in the Korean population. In keeping with this, a previous study of the UK Caucasian population (22), also found no significant associations with asthma susceptibility for either the ALOX5 or ALOX5AP polymorphisms. However, it has been established that the ALOX5 polymorphism does regulate the promoter activity, the consequences of which are likely to be increased cys-LT production in asthma. Furthermore, it has been reported that the polymorphism in ALOX5 has a pharmacogenetic effect on treatment response in asthma (18, 23). In this study, genotype with five tandem repeats was more frequent than other genotypes with four or six repeats in the Korean population. This result was consistent with the genotype distribution in the Caucasian study, but the frequency of the allele having five repeats for ALOX5 in the Korean population was slightly lower than that observed in the UK Caucasian population. Furthermore, the allele carrying three repeats was not found in the Korean population and the frequency of the allele with six repeats was higher than that in the UK Caucasian population. Therefore, there was an ethnic difference in the genotype distribution of ALOX5 between Caucasian and Korean population.

In the genotype distribution of ALOX5AP, the genotype with 23 poly(A) repeat was found more frequently than genotype with 19 poly(A) repeats, in accordance with the genotype distribution in the Caucasian study, but the genotype distribution of the ALOX5AP polymorphism in Japanese study is quite different. Koshino et al. (19) documented that genotype with homozygous type A21 and A18 repeats were predominant genotypes and the incidence of 21 poly(A) repeats was significantly higher in the asthmatics than in the normal controls. These findings suggested that there might be ethnic differences in the genotype distribution of ALOX5AP.

There was no significant difference in genotype frequency in the ALOX5 promoter between AIA patients and normal control group, and between AIA patients and ATA patients. However, we identified a very significant association between ALOX5 genotype frequency and PC₂₀ methacholine in both AIA and ATA. This result suggests that individuals with mutant genotype ($n > 5$ or $n < 5$) of short tandem repeat (Sp1-binding motif) in ALOX5 promoter exhibit more severe airway hyperresponsiveness.

Airway hyperresponsiveness to methacholine is composed of heterogeneous parts of airway inflammation including structural (smooth muscle, nerve, vessel) and immunological components derived from chemical mediators, inflammatory cells and cytokine networks. Therefore, it is not easy to explain the direct relationship between one kind of chemical mediator, such as cysteinyl leukotrienes, and degree of airway hyperresponsiveness to methacholine in the asthmatic airways of ASA-intolerant patients. Although it is true that in murine models reduction of cys-LT expression through knockout of the ALOX5 gene (24) or function using antileukotriene

therapeutics (25) results in reduced airway hyperresponsiveness to methacholine, If the observations in murine models hold true for human physiology, we may have expected that patients with the mutant type of ALOX5 promoter would have a lower degree of airway hyperresponsiveness to methacholine due to a reduction in ALOX5 expression and a corresponding reduction in cys-LT production. However, in fact our observations suggest the opposite effect with patients carrying the mutant genotype ($n > 5$ or $n < 5$ short tandem repeat Sp1-binding motifs) have increased airway hyperresponsiveness. Further studies will be needed to investigate how ALOX5 alone or in interaction with other inflammatory genes and environmental influences may influence airway hyperresponsiveness in patients with AIA.

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