

Effect of Genetic Polymorphism of *ALOX15* on Aspirin-Exacerbated Respiratory Disease

Young-Sin Song Eun-Mi Yang Seung-Hyun Kim Hyun Jung Jin Hae-Sim Park

Department of Allergy and Clinical Immunology, Ajou University School of Medicine, Suwon, Korea

Key Words

Aspirin-exacerbated respiratory disease · Arachidonate 15-lipoxygenase · Polymorphism · Eosinophil

Abstract

Background: Aspirin-exacerbated respiratory disease (AERD) is a clinical syndrome associated with chronic inflammation in the airways coincident with chronic rhinitis, sinusitis, recurrent polyposis and asthma. Eosinophils are the key inflammatory cells in the development of AERD. AERD has been attributed to abnormalities of the arachidonic acid metabolism, but the pathogenesis of AERD is not fully understood. Our aim was to investigate the genetic contribution of the arachidonate 15-lipoxygenase gene (*ALOX15*) to the development of AERD. **Methods:** We enrolled 171 patients with AERD, 229 patients with aspirin-tolerant asthma, and 195 normal healthy controls in a Korean population. Three polymorphisms (–427G/A, –272C/A, –217G/C) in the promoter region of *ALOX15* were genotyped. The functional variability of the promoter polymorphisms were analyzed by luciferase reporter activity assay. **Result:** No significant difference in the genotype frequency of the *ALOX15* genetic polymorphism was found. Peripheral total eosinophil count was significantly higher in the patients carrying the GG genotype of the –427G/A polymorphism ($p = 0.016$). Similarly,

the patients carrying haplotype 1 (ht1) (GCG) of –427G/A, –272C/A and –217G/C showed a significantly higher total eosinophil count compared to the other haplotypes ($p = 0.008$) in the AERD group. The promoter activity of the ht1 (GCG) construct was significantly higher compared to that of the ht3 (AGG) construct in A549 and U937 cells (both $p < 0.001$). **Conclusion:** These results suggest that the promoter polymorphisms of the *ALOX15* gene affect *ALOX15* activity leading to increased eosinophil infiltration in AERD patients.

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Introduction

Aspirin-exacerbated respiratory disease (AERD) is a clinical syndrome characterized by persistent rhinitis, nasal polyposis and chronic hyperplastic eosinophilic sinusitis after exposure to aspirin [1–3]. AERD has been attributed to abnormalities of the arachidonic acid metabolism and leads to upregulation of lipid inflammatory mediators such as leukotrienes, cytokine production and eosinophil infiltration. However, the pathogenic mecha-

Y.-S.S. and E.-M.Y. contributed equally to this work as first authors.

nisms of chronic eosinophilic inflammation found in the upper and lower airway mucosa of AERD are not completely understood [4, 5]. There have been a few genetic studies on CRTH2 and IL-13 in association with eosinophil activation in AERD patients [6, 7].

Chronic rhinosinusitis and nasal polyps are associated with remarkable infiltration of activated eosinophils [8] with increased Th2 lymphocytes, which may be a distinguishing feature of AERD compared to aspirin-tolerant asthma (ATA) [9]. These findings have also been noted in bronchial biopsy samples of AERD patients compared to ATA and normal control (NC) groups [10].

Arachidonate 15-lipoxygenase (ALOX15) is a member of the lipoxygenase family and catalyzes the conversion of AA to 15-hydroperoxy-eicosatetraenoic acid [11, 12]. ALOX15 has been considered as a proinflammatory factor, and few studies have demonstrated that activated ALOX15 was found in airway mucosa of asthmatic patients [13, 14]. Increased expression and activity of ALOX15 lead to increased release of chemokines from airway epithelial cells, leading to the recruitment of various inflammatory cells [15]. ALOX15 metabolites display important bioactivities concerning signal transduction involved in physiological or pathophysiological immune responses [16, 17]. ALOX15 is expressed predominantly in eosinophils, activated monocytes, epithelial cells and mast cells [18]. Most of the eosinophils expressing ALOX15 are inflammatory cells derived from asthmatic airway mucosa [13]. In addition, a study using ALOX12/15-deficient mice demonstrated attenuated airway inflammation with a decreased number of eosinophils in bronchoalveolar lavage fluid [11].

Considering the role of ALOX15 in eosinophil infiltration and activation, we hypothesized that ALOX15 activity is crucial to the development of AERD. The present study addressed this hypothesis. We examined the functional effect of ALOX15 gene polymorphism on the phenotype of AERD in a Korean population.

Subjects and Methods

Subjects

To investigate the genetic association of ALOX15 with AERD, three different study groups were enrolled, including 171 AERD patients, 229 ATA patients and 195 NCs. All subjects were recruited from the Ajou University Hospital, Suwon, Korea. The AERD patients showed a positive response to the lysine-acetylsalicylic acid bronchoprovocation test, which was performed as described previously [19]. Informed consent was obtained from all subjects, and the study was approved by the Ajou University Hospital Institutional Review Board.

Genomic DNA Preparation and Single Nucleotide Polymorphism Genotyping

Genomic DNA was prepared from peripheral blood samples using the Puregene DNA purification kit (Gentra, Minneapolis, Minn., USA) according to the manufacturer's protocol. Three promoter polymorphisms of ALOX15, -427G/A (rs34104097), -272C/A (rs7220870) and -217G/C (rs2664592), were targeted and genotyped using the SNaPshot Primer Extension (Applied Biosystems, Foster City, Calif., USA). The following primer pairs were used: for -427G/A of ALOX15, forward 5'-CAAAGCTGGTCTGAATGAA-3', reverse 5'-AGTCCAAAAGGCGGTGT-3', and extension 5'-TTGTGTGTGCGCGCGCTGTCCTTGY; for -272C/A of ALOX15, forward 5'-CTAAATAACCCAGCCTGAGA-3', reverse 5'-CCTGAATGTACCTTCCTCAA-3', and extension 5'-GGGAGAAAAAGAAAAGGATTTGGAC-3'; and for -217G/C of ALOX15, forward 5'-CAAAGCTGGTCTGAATGAA-3', reverse 5'-AGTCCAAAAGGCGGTGT-3', and extension 5'-TCAAGATAGTGGTTTCCACTCCCT-3'.

Promoter Activity Assay

For the functional study of ALOX15 polymorphisms, a promoter activity assay was conducted using three plasmid DNA constructs containing single nucleotide polymorphisms (SNPs) of ALOX15: ht1 (GCG), ht2 (GAC) and ht3 (GCG). A549 human lung epithelial cells and U937 human monocytes were transiently transfected by Lipofectamin reagent (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's protocol. Briefly, 1×10^5 A549 cells and 3×10^5 U937 cells were seeded in wells of 12-well plates and transfected with 1 μ g of the ALOX15 reporter construct and 5 ng of PRL SV 40 control vector using Lipofectamin (ratio 1: 5). Twenty-four to 48 h after transfection, the cells were lysed and the luciferase activity assay determined (Promega, Madison, Wisc., USA).

Statistics

Differences in the genotype frequency between cases and controls were assessed by the χ^2 test and the calculation of odds ratios, with 95% confidence intervals. Logistic regression analysis was used to analyze the SNPs and haplotypes controlling for age and gender as covariates with three alternative models. Haplotypes of the ALOX15 gene were analyzed using Haploview version 4.0 based on the expectation maximization algorithm [20]. All statistical analyses were completed using SPSS version 12.0.1 (SPSS Inc., Chicago, Ill., USA), and a p value ≤ 0.05 was considered significant.

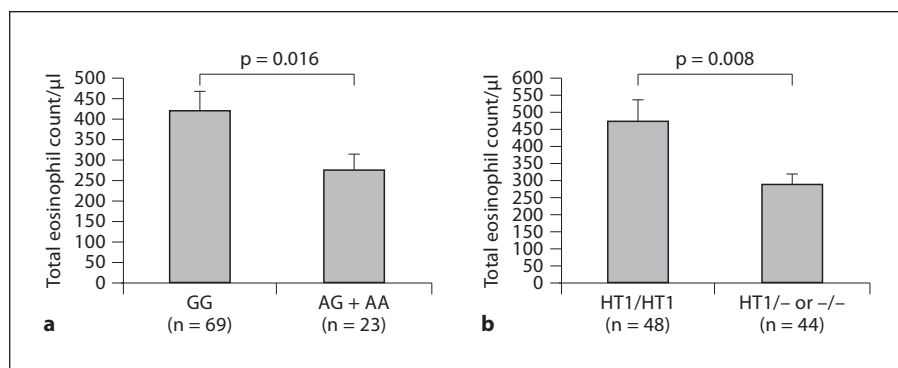
Results

Clinical Characteristics of the Study Subjects

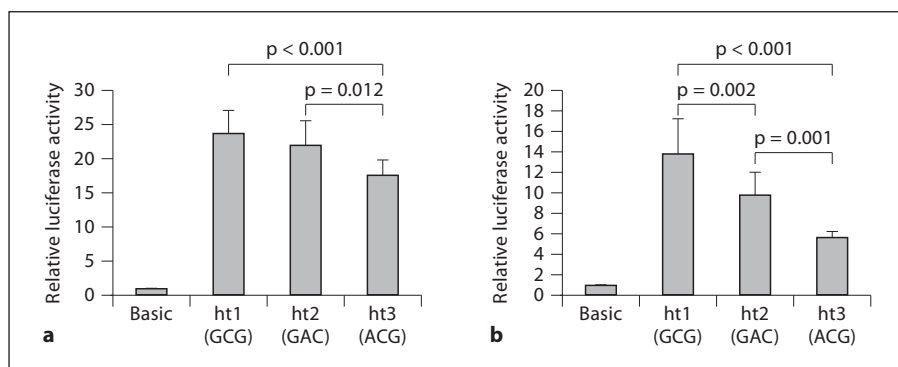
The AERD patients exhibited a significantly higher prevalence of rhinosinusitis and nasal polyps compared to ATA patients ($p = 0.05$ and $p < 0.001$, respectively) (table 1). The AERD and ATA groups showed a significantly higher atopy and total IgE than the NC group (both $p < 0.001$).

Fig. 1. Comparison of peripheral TEC according to the *ALOX15* gene polymorphism in AERD patients. **a** TEC according to the -427G/A of *ALOX15*. **b** TEC according to the haplotype. The values represent the mean $\pm$ SE. $p \leq 0.05$ was considered to be significant by t test.

Fig. 2. Effect of *ALOX15* promoter polymorphisms (-427G/A, -272G/T and -217G/C) on promoter activity. **a** Promoter activities in A549 human carcinoma epithelial cells. **b** Promoter activities in U937 human monocyte cells. The reporter gene constructs contained the *ALOX15* polymorphisms ht1 (GCG), ht2 (GAC) and ht3 (ACG). The relative luciferase activity is represented as the ratio of the activity to the luciferase activity in the cells transfected with the promoterless basic vector, pGL3 basic. Data represent the mean $\pm$ SD of three independent experiments (triplicate for each sample; $n = 9$). Statistical differences were evaluated with ANOVA and Bonferroni test.



1



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Table 1. Clinical characteristics of the *ALOX15* study subjects

	AERD (n = 171)	ATA (n = 229)	NC (n = 195)	p value		
				AERD vs. ATA	AERD vs. NC	ATA vs. NC
Males	61/171 (35.67)	102/229 (44.54)	91/195 (46.67)	0.081	0.034	0.696
Age, years	43.75 $\pm$ 13.03 [171]	42.45 $\pm$ 15.09 [229]	34.25 $\pm$ 14.16 [191]	0.357	<0.001	<0.001
Atopy	85/155 (54.84)	109/165 (66.06)	21/174 (12.07)	0.051	<0.001	<0.001
Rhinosinusitis	102/119 (85.71)	125/165 (75.76)	NA	0.050	NA	NA
Nasal polyps	49/114 (42.98)	9/164 (5.49)	NA	<0.001	NA	NA
Duration of asthma, years	6.31 $\pm$ 6 [116]	5.39 $\pm$ 6.2 [162]	NA	0.220	NA	NA
Log total IgE, IU/ml	5.11 $\pm$ 1.26 [109]	5.17 $\pm$ 1.5 [157]	3.67 $\pm$ 1.34 [48]	0.762	<0.001	<0.001
TEC/ μ l	385.32 $\pm$ 348.09 [92]	NA	NA	NA	NA	NA

Data are number of subjects/total, with percentages in parentheses, or mean $\pm$ SD, with number of subjects evaluated given in brackets. NA = Not applicable. Values in italics indicate significant p values ($p \leq 0.05$).

Genotype and Haplotype Frequencies of *ALOX15* with AERD Phenotypes

There were no significant differences among the three groups in the genotype and haplotype frequencies of the *ALOX15* gene. However, peripheral total eosinophil count (TEC) was significantly associated with -427G/A and ht1

(GCG). The patients having the GG genotype of *ALOX15* -427G/A showed significantly higher TEC levels compared to the others (AG or AA; $p = 0.0016$). The AERD patients carrying the homozygous haplotype ht1 (GCG) had significantly higher TEC than those with the other haplotypes (ht1/ht1 vs. ht1/-; $p = 0.008$; fig. 1). These re-

sults suggest that the -427G genotype and haplotype ht1 (GCG) could be the risk factors enhancing the recruitment of eosinophils into the airways of AERD patients.

Functional Effect of ALOX15 Gene Polymorphism

We performed a luciferase reporter assay to examine the functional variability of *ALOX15* according to the SNP. The ht1 (GCG) showed the highest luciferase activity when compared to the others ($p < 0.001$), both in A549 human lung epithelial cells and in U937 human monocyte cells (fig. 2). This result indicated the possibility of -427G of the *ALOX15* gene being a risk factor, which may enhance the expression of *ALOX15* and consequently increase the infiltration of eosinophils.

Discussion

Although a lack of association of the *ALOX15* gene polymorphism at -272C/A was previously reported in a Korean population [21], two additional promoter polymorphisms (-427G/A and -217G/C) were newly investigated in this study with a larger cohort of study subjects. This study demonstrates a significant association of the *ALOX15* gene polymorphisms with the phenotype of AERD. The -427G allele of *ALOX15* displayed the highest TEC level in AERD although there were no significant differences in the genotype frequency of the SNP among the study groups. The haplotype ht1 (GCG) containing the -427G allele was also related to the highest TEC level. However, these findings were not found in the ATA group. ht1 (GCG) showed the highest promoter activity. These findings suggest that the SNP -427G of the *ALOX15* gene could increase the expression of *ALOX15* in AERD patients.

Eosinophil is the key inflammatory cell of asthma, and intense infiltration of eosinophils has been observed in upper and lower airway mucosa of AERD patients [22]. A significantly increased number of eosinophils and a higher eosinophil cationic protein level were noted in

bronchoalveolar lavage fluid of AERD patients compared to controls after stimulation with lysine-acetylsalicylic acid [23, 24]. Eosinophils contain an abundant amount of *ALOX15*. Moreover, it is suggested that the *ALOX15* pathways play an important role in the pathogenesis of severe asthma. Increased *ALOX15* expression and activity is associated with the degree of airway eosinophil accumulation, as well as with an increased metabolite of *ALOX15*, 15(s)-hydroxyeicosatetraenoic acid [15-(s)-HETE], in severe asthmatics [25]. Similarly, Kowalski et al. [26] reported that peripheral blood leukocytes from patients with AERD exposed to aspirin produce more 15(s)-HETE than do patients with ATA. *ALOX15* products in asthmatics are increased, which may be derived from bronchial epithelium and infiltrated eosinophils [11]. Recently, eoxins have been reported as proinflammatory arachidonic acid metabolites produced via the 15-lipoxygenase-1 pathway in human eosinophils [27].

In our preliminary experiments, we observed a higher tendency of 15(s)-HETE and eoxin C_4 levels, the intermediate metabolites of *ALOX15*, in the AERD group compared with the NC group (data not shown). Further studies will be needed to compare the level of these metabolites according to the *ALOX15* gene polymorphisms.

Increased expression of *ALOX15* according to the promoter polymorphism may induce eosinophil infiltration into the upper and lower airway mucosa of AERD patients, in which infiltrating eosinophils could provide cytokines and growth factors leading to promote their own recruitment, proliferation and activation of eosinophils [28, 29].

Taken together, it was suggested that the *ALOX15* -427G/A polymorphism may be a risk factor of AERD by increasing *ALOX15* expression, leading to increased eosinophil infiltration in AERD patients.

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References

- 1 Lee RU, Stevenson DD: Aspirin-exacerbated respiratory disease: evaluation and management. *Allergy Asthma Immunol Res* 2011;3: 3-10.
- 2 Chang J, Simon RA, Stevenson DD: Aspirin-exacerbated respiratory disease: is it in our DNA? *Ann Allergy Asthma Immunol* 2011; 106:263-264.
- 3 Berges-Gimeno MP, Simon RA, Stevenson DD: The natural history and clinical characteristics of aspirin-exacerbated respiratory disease. *Ann Allergy Asthma Immunol* 2002;89:474-478.
- 4 Hamad AM, Sutcliffe AM, Knox AJ: Aspirin-induced asthma: clinical aspects, pathogenesis and management. *Drugs* 2004;64:2417-2432.

- 5 Nasser SM, Pfister R, Christie PE, Sousa AR, Barker J, Schmitz-Schumann M, Lee TH: Inflammatory cell populations in bronchial biopsies from aspirin-sensitive asthmatic subjects. *Am J Respir Crit Care Med* 1996;153:90–96.
- 6 Palikhe NS, Kim SH, Cho BY, Choi GS, Kim JH, Ye YM, Park HS: IL-13 gene polymorphisms are associated with rhinosinusitis and eosinophilic inflammation in aspirin intolerant asthma. *Allergy Asthma Immunol Res* 2010;2:134–140.
- 7 Palikhe NS, Kim SH, Cho BY, Ye YM, Choi GS, Park HS: Genetic variability in CRTH2 polymorphism increases eotaxin-2 levels in patients with aspirin exacerbated respiratory disease. *Allergy* 2010;65:338–346.
- 8 Hamilos DL, Leung DY, Wood R, Bean DK, Song YL, Schotman E, Hamid Q: Eosinophil infiltration in nonallergic chronic hyperplastic sinusitis with nasal polyposis (CHS/NP) is associated with endothelial VCAM-1 upregulation and expression of TNF- α . *Am J Respir Cell Mol Biol* 1996;15:443–450.
- 9 Mascia K, Borish L, Patrie J, Hunt J, Phillips CD, Steinke JW: Chronic hyperplastic eosinophilic sinusitis as a predictor of aspirin-exacerbated respiratory disease. *Ann Allergy Asthma Immunol* 2005;94:652–657.
- 10 Cowburn AS, Sladek K, Soja J, Adamek L, Nizankowska E, Szczeklik A, Lam BK, Penrose JF, Austen FK, Holgate ST, Sampson AP: Overexpression of leukotriene C₄ synthase in bronchial biopsies from patients with aspirin-intolerant asthma. *J Clin Invest* 1998;101:834–846.
- 11 Claesson HE: On the biosynthesis and biological role of eoxins and 15-lipoxygenase-1 in airway inflammation and Hodgkin lymphoma. *Prostaglandins Other Lipid Mediat* 2009;89:120–125.
- 12 Chanez P, Bonnans C, Chavis C, Vachier I: 15-lipoxygenase: a Janus enzyme? *Am J Respir Cell Mol Biol* 2002;27:655–658.
- 13 Bradding P, Redington AE, Djukanovic R, Conrad DJ, Holgate ST: 15-lipoxygenase immunoreactivity in normal and in asthmatic airways. *Am J Respir Crit Care Med* 1995;151:1201–1204.
- 14 Profita M, Sala A, Riccobono L, Paterno A, Mirabella A, Bonanno A, Guerrera D, Pace E, Bonsignore G, Bousquet J, Vignola AM: 15-lipoxygenase expression and 15(s)-hydroxyeicoisatetraenoic acid release and reincorporation in induced sputum of asthmatic subjects. *J Allergy Clin Immunol* 2000;105:711–716.
- 15 Liu C, Xu D, Liu L, Schain F, Brunnstrom A, Bjorkholm M, Claesson HE, Sjoberg J: 15-lipoxygenase-1 induces expression and release of chemokines in cultured human lung epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2009;297:L196–L203.
- 16 Gulliksson M, Brunnstrom A, Johannesson M, Backman L, Nilsson G, Harvima I, Dahlen B, Kumlin M, Claesson HE: Expression of 15-lipoxygenase type-1 in human mast cells. *Biochim Biophys Acta* 2007;1771:1156–1165.
- 17 Vanderhoeck JY: Role of the 15-lipoxygenase in the immune system. *Ann NY Acad Sci* 1988;524:240–251.
- 18 Claesson HE, Griffiths WJ, Brunnstrom A, Schain F, Andersson E, Feltenmark S, Johnson HA, Porwit A, Sjoberg J, Bjorkholm M: Hodgkin Reed-Sternberg cells express 15-lipoxygenase-1 and are putative producers of eoxins in vivo: novel insight into the inflammatory features of classical hodgkin lymphoma. *FEBS J* 2008;275:4222–4234.
- 19 Park HS: Early and late onset asthmatic responses following lysine-aspirin inhalation in aspirin-sensitive asthmatic patients. *Clin Exp Allergy* 1995;25:38–40.
- 20 Barrett JC, Fry B, Maller J, Daly MJ: Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005;21:263–265.
- 21 Lee HY, Kim SH, Ye YM, Choi GS, Park HS: Lack of association of ALOX12 and ALOX15 polymorphisms with aspirin-exacerbated respiratory disease in Korean patients. *Ann Allergy Asthma Immunol* 2009;103:84–86.
- 22 Moneret-Vautrin DA, Hsieh V, Wayoff M, Guyot JL, Mouton C, Maria Y: Nonallergic rhinitis with eosinophilia syndrome a precursor of the triad: nasal polyposis, intrinsic asthma, and intolerance to aspirin. *Ann Allergy* 1990;64:513–518.
- 23 Kowalski ML, Grzegorzczak J, Wojciechowska B, Poniatowska M: Intranasal challenge with aspirin induces cell influx and activation of eosinophils and mast cells in nasal secretions of ASA-sensitive patients. *Clin Exp Allergy* 1996;26:807–814.
- 24 Sladek K, Dworski R, Soja J, Sheller JR, Nizankowska E, Oates JA, Szczeklik A: Eicosanoids in bronchoalveolar lavage fluid of aspirin-intolerant patients with asthma after aspirin challenge. *Am J Respir Crit Care Med* 1994;149:940–946.
- 25 Chu HW, Balzar S, Westcott JY, Trudeau JB, Sun Y, Conrad DJ, Wenzel SE: Expression and activation of 15-lipoxygenase pathway in severe asthma: relationship to eosinophilic phenotype and collagen deposition. *Clin Exp Allergy* 2002;32:1558–1565.
- 26 Kowalski ML, Ptasinska A, Jedrzejczak M, Bienkiewicz B, Cieslak M, Grzegorzczak J, Pawliczak R, Dubuske L: Aspirin-triggered 15-HETE generation in peripheral blood leukocytes is a specific and sensitive Aspirin-Sensitive Patients Identification Test (ASPISTest). *Allergy* 2005;60:1139–1145.
- 27 Feltenmark S, Gautam N, Brunnstrom A, Griffiths W, Backman L, Edenius C, Lindbom L, Bjorkholm M, Claesson HE: Eoxins are proinflammatory arachidonic acid metabolites produced via the 15-lipoxygenase-1 pathway in human eosinophils and mast cells. *Proc Natl Acad Sci USA* 2008;105:680–685.
- 28 Borrich L: Sinusitis and asthma: entering the realm of evidence-based medicine. *J Allergy Clin Immunol* 2002;109:606–608.
- 29 Stevenson DD, Zuraw BL: Pathogenesis of aspirin-exacerbated respiratory disease. *Clin Rev Allergy Immunol* 2003;24:169–188.