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ORIGINAL PAPER **Asthma and Rhinitis**

Association of angiotensin I-converting enzyme gene polymorphisms with aspirin intolerance in asthmatics

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Summary

Background Aspirin-intolerant asthma (AIA) refers to the development of bronchoconstriction in asthmatic individuals following the ingestion of aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs). Angiotensin I-converting enzyme (ACE), a membrane-bound peptidase present in the lung, plays a pivotal role in the metabolism of the endogenous peptides involved in the pathogenesis of asthma.

Methods We screened a Korean asthma cohort (581 asthmatics including 81 aspirin-intolerant asthmatics and 231 aspirin-tolerant asthmatics, and 181 normal controls) for four single nucleotide polymorphisms (SNPs; −262 A>T and −115 T>C in the 5′-flanking region and +5467 T>C [Pro450Pro] and+11860 A>G [Thr776Thr] in the coding region) and one ins/del (+21288 CT) in the *ACE* gene.

Results None of the SNPs or haplotypes showed any association with the development of asthma, but they were significantly associated with the risk of AIA. Logistic regression indicated that the frequency of the rare alleles of −262 A>T and −115 T>C was higher in subjects with AIA than in subjects with aspirin-tolerant asthma (ATA) ($P = 0.003$–0.01, $P^{corr} = 0.015$–0.05). Subjects homozygous for the rare alleles of −262 A>T and −115 T>C showed a greater decline in forced expiratory volume in 1 s (FEV_1) after aspirin provocation than those homozygous for the common alleles ($P < 0.05$). A luciferase reporter assay indicated that *ACE* promoters containing the rare −262 A>T allele possessed lower activity than did those containing the common allele ($P = 0.009$). In addition, *ACE* promoters bearing the rare −115 T>C allele had no luciferase activity. DNA–protein binding assays revealed a band containing the *ACE* promoter region (including −262 A) and a protein complex.

Conclusion The −262 A>T polymorphism in the promoter of the *ACE* gene is associated with AIA, and the rare allele of −262 A>T may confer aspirin hypersensitivity via the down-regulation of *ACE* expression.

Keywords AIA, angiotensin-converting enzyme, aspirin hypersensitivity, asthma, ATA, polymorphism

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Introduction

Aspirin-intolerant asthma (AIA) refers to the development of bronchoconstriction in asthmatic individuals following the ingestion of aspirin or other non-steroidal

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anti-inflammatory drugs (NSAIDs). This syndrome is characterized by the so-called aspirin triad: aspirin hypersensitivity, bronchial asthma and chronic rhinosinusitis with nasal polyposis [1]. AIA affects approximately 5–10% of adult asthmatics, with a higher incidence in women [2]. Although the pathogenesis of AIA has not been completely elucidated, a two-compartment model has been proposed in which both the augmentation of cysteinyl leukotriene (CysLT) production and the overexpression of the CysLT receptor on inflammatory cells occur within the respiratory tract [3, 4]. The CysLT receptor is selectively antagonized by several currently available leukotriene modifiers, including montelukast, pranlukast, and zafirlukast [5, 6]; however, clinical studies have demonstrated that the response to these medications is incomplete [7, 8]. This suggests the presence of an alternative pathway leading to AIA.

Angiotensin 1-converting enzyme (ACE; on chromosome 17q23, MIM number 106180) is a membrane-bound peptidase, that is present in several types of epithelial and endothelial cells [9]. ACE inactivates a wide range of peptides, including kinins and substance P, which are produced in the lungs of asthmatics [9–11]. ACE is expressed in relatively large amounts in the lungs, where it plays a pivotal role in the metabolism of peptides that may be involved in the pathogenesis of asthma. The inhibition of ACE has been linked to the suppression of kininase II activity, resulting in the accumulation of kinins, substance P, and prostaglandins (PG) in the airways and consequent stimulation of vagal afferents [12]. This in turn leads to bronchial hyper-reactivity [13] and airway inflammation [14], especially eosinophilic inflammation, in asthmatics [15]. In asthmatics, low levels of ACE expression in the epithelium are associated with increased numbers of eosinophils in the epithelium and lamina propria [15]. When considered with the increased infiltration of eosinophils into the airway in AIA compared with aspirin-tolerant asthma (ATA) [2], the low level of ACE in the airway may be responsible for eosinophilic infiltration into the airway in AIA. In asthmatics, cyclooxygenase blockade with inhaled lysine acetylsalicylate produces significant protection against bradykinin- and neurokinin A-provoked bronchoconstriction [16–18], implying a role for endogenous prostanoids in this response. In a variety of animal studies, bronchoconstriction provoked by bradykinin is mediated largely through the formation of thromboxane A2 (Tx-A2) [19]. Furthermore, bradykinin may also augment the release of newly generated spasmogenic prostanoids, such as PGs and Tx-A2 [20, 21]. This process is largely prevented by cyclooxygenase blockade [22]. Further support for the involvement of prostanoids in bradykinin-induced responses is demonstrated in human airways [23]. Considering the prostaglandin and thromoxane pathway as the basic mechanisms of AIA [2], the altered metabolism of endogenous prostaglandin and thromboxane may accentuate the airway response to bradykinin or neurokinin A in AIA. Thus, genetic variants of the *ACE* gene may have different effects on the development of airway inflammation and airway spasm in AIA and ATA.

Seventy-eight sequence variations in *ACE* have been identified in African and/or Caucasian populations [24]. ACE is well known for its role in cardiovascular disease, and the possible involvement of these polymorphisms in asthma has been suggested. A positive association of the risk of asthma with an ins/del in intron 16 has been shown in Chinese [25] and French subjects [26]. The possible involvement of ACE in the pathogenesis of asthma and the suggested functional differences caused by the polymorphisms indicate that polymorphisms in the *ACE* gene may participate in the control of ACE activity and consequently could be implicated as a factor in patients with asthma. However, to the best of our knowledge, there has been no previous analysis of the potential associations between polymorphisms in *ACE* and aspirin intolerance in asthmatics. Thus, we screened a Korean population for four single nucleotide polymorphisms (SNPs) and one ins/del in *ACE* and performed statistical analyses to examine the relationship between these polymorphic loci and asthma and/or aspirin intolerance. In addition, the functional effects of the SNPs were validated *in vitro*.

Materials and methods

Subjects

The subjects were recruited from the Asthma Genome Research Center, comprising Soonchunhyang, Chunnam, Chungbuk, Seoul national and Ajuo University Hospitals, Korea. All the subjects were Korean. The 581 asthmatics had compatible clinical symptoms and physical characteristics [27]. Each patient showed airway reversibility, as documented by inhalant bronchodilator-induced improvement of more than 15% of forced expiratory volume in 1 s (FEV_1) and/or airway hyper-reactivity of less than 10 mg/mL of methacholine. Among them, aspirin challenge was performed in 312 subjects who had a history of aspirin hypersensitivity, or presence of urticaria, nasal poly, or sinusitis on water's view.

The oral provocation test was performed with increasing doses of aspirin (10–450 mg Astrix; Mayne Pharma Ltd, Melbourne, Australia) using methods slightly modified from those described previously [28, 29]. Provocations always started between 08:00 and 09:00 hours. Antihistamine and short-acting 2-agonists were withdrawn for 24 h. Changes in FEV_1 were followed for 5 h after the last aspirin challenge dose. Aspirin-induced bronchospasm as reflected by rate (%) of FEV_1 decline was calculated as the pre-challenge FEV_1 minus the post-challenge FEV_1 divided by the pre-challenge FEV_1. Subjects were labelled as positive responders if they

showed a rate of FEV_1 decline of more than 20% without extrabronchial symptoms or appearance of skin manifestations ($n = 81$). Subjects exhibiting a rate of FEV_1 decline below 15% without extrabronchial nasal or skin symptoms were regarded as negative responders (ATA) ($n = 231$).

Normal controls ($n = 181$) were recruited from spouses of the patients and the general population who answered negatively to a screening questionnaire regarding respiratory symptoms, had no past history of aspirin (ASA) hypersensitivity, FEV_1% predicted > 80%, PC_{20} methacholine > 25 mg/mL, and normal findings on simple chest radiograms. Skin prick tests were performed with 24 common aeroallergens (Bencard Co. Ltd, Brentford, UK) [30]. Atopy was defined as one or more positive reactions (> 3 mm in diameter) on the skin prick test. Total IgE was measured using the UniCAP system (Pharmacia Diagnostics, Uppsala, Sweden). Subjects with hypertension and diabetes or those taking ACE inhibitors were excluded from the study. All subjects gave informed consent to participate in the study, and the protocols were approved by the local ethics committees of each hospital.

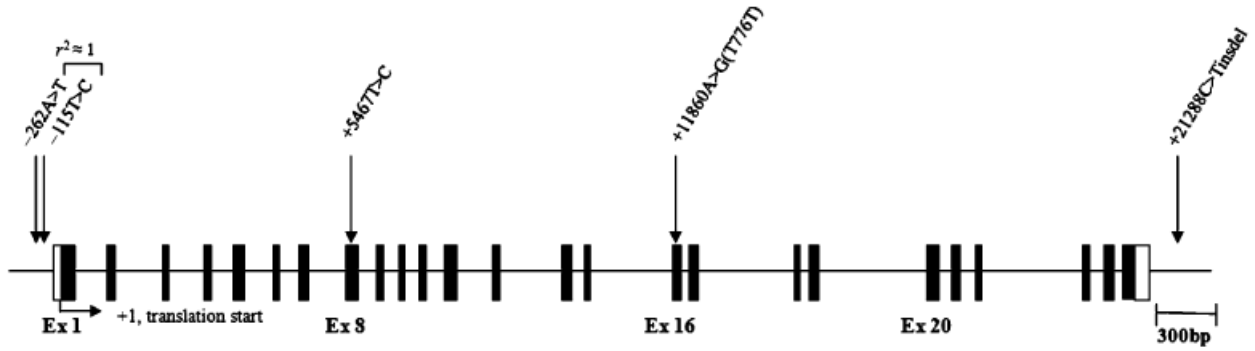
Genotyping

For genotyping in Korean subjects, five polymorphisms, including −262 A>T and −115 T>C, +5467 T>C [Pro450Pro], +11860 A>G [Thr776Thr], and one ins/del (+21288 CT) among those reported previously in Caucasians [31], were selected (Fig. 1a). Amplification and extension primers were designed for genotyping of the polymorphic sites by single-base extension (Table 1) [32]. All primer extension reactions were performed using the SNaPshot ddNTP Primer Extension Kit (Applied Biosystems, Foster City, CA, USA). For subsequent cleanup, the reaction mixture was incubated at 37 °C for 1 h with 1 U of shrimp alkaline phosphatase, followed by 15 min at 72 °C for enzyme inactivation. The extension products and Genescan 120 Liz size standard solution were then mixed with Hi-Di formamide (Applied Biosystems) according to the manufacturer's recommendations and incubated at 95 °C for 5 min, followed by 5 min on ice. The mixture was then electrophoresed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The results were analysed using GeneScan and Genotyper (Applied Biosystems).

Luciferase assay of the promoter gene according to the polymorphisms

The promoter region of the *ACE* gene was amplified by PCR using specific primers (Table 2) and genomic DNA from an allele-matched B cell line as a template (889 bp fragment). The reaction mixture was then diluted and used as a template for nested PCR using nested primers (Table 2) to produce a 291 bp fragment containing *Xho*I and *Nhe*I

(a) Map of ACE gene on chromosome 17q23



(b) Haplotypes of ACE gene

Haplotype	−262 A>T	−115 T>C	+5467 T>C	+11860 A>G	+21288 insdel	Freq.
ht1	A	T	T	A	Ins	0.450
ht2	T	C	C	G	Del	0.366
ht3	A	T	T	A	Ins	0.087
Others	–	–	–	–	–	0.097

(c) LDs among polymorphisms in ACE gene

		|D'|				
	SNPs	−262 A>T	−115 T>C	+5467 T>C	+11860 A>G	+21288 ins/del
	−262 A>T	–	0.993	0.861	0.847	0.730
	−115 T>C	1	–	0.855	0.841	0.723
r^2	+5467 T>C	0.937	0.930	–	0.844	0.739
	+11860 A>G	0.936	0.936	0.943	–	0.865
	+21288 insdel	0.893	0.886	0.890	0.988	–

Fig. 1. (a) Gene map and single nucleotide polymorphisms (SNPs) in the *ACE* gene on chromosome 17q23. Coding exons are marked by black blocks and 5′ and 3′-UTR by white blocks. The first base of translation site was denoted as nucleotide+1; (b) haplotypes of ACE; (c) linkage disequilibrium coefficient ($|D'|$ and r^2) among ACE SNPs. ACE, angiotensin I-converting enzyme.

Table 1. Sequences of amplifying and extension primers for genotyping of *ACE* polymorphisms by the single-base extension method

Locus		Primer sequence
ACE −262 A>T	Forward	5′-tgcggacagctcctgggg-3′
	Reverse	5′-ccttctcctgcgccgcgg-3′
	Extension	5′-tgatgattgccgggtccccatcttc-3′
ACE −115 T>C	Forward	5′-tgcggacagctcctgggg-3′
	Reverse	5′-ccttctcctgcgccgcgg-3′
	Extension	5′-atgcttcctcctccgctcca-3′
ACE+5467 T>C	Forward	5′-ccatgagatgggccatatacagtact-3′
	Reverse	5′-ccataccgtgtcattggtga-3′
	Extension	5′-caatgatgatctgcagtacaag gatctgcc-3′
ACE+11860 A>G	Forward	5′-ttccagctctgaaattctctgag-3′
	Reverse	5′-attgagccgggcagcctggt-3′
	Extension	5′-ataatcaatgatgatctgacgaatgt gatggccac-3′
ACE+21288 ins/del	Forward	5′-gagtaccttggagggcctgct-3′
	Reverse	5′-gctacactccagcgtctgagg-3′
	Extension	5′-tcatgataatcaatgatgattgtac cagctccatgactct-3′

ACE, angiotensin I-converting enzyme.

Table 2. PCR primer sequences used in this study

Approaches	Primer sequence	Orientation	Location*
Promoter construct			
1st PCR	TGTGT CCTCG GGGGG AA	Forward	−710/−694
	ACCTG TTCGG CGCTG GA	Reverse	+163/+179
Nested PCR			
Contain −262 A>T and −115 T>C	TCTCG AGCTC GGGT G TTCCG GCAA *Xho*I	Forward, nested	−299/−283
	AGCTA GCTTC TGCCC CTTCT CCTGC GCC *Nhe*I	Reverse, nested	−42/−23
Contain only −115 T>C	TCTCG AGATC CCGCC ACCCC CG *Xho*I	Forward, nested	−208/−194
	AGCTA GCTCT GCCC C TTCTC CTGC G CC *Nhe*I	Reverse, nested	−42/−23

*The locations of primers are numbered from the transcription start site. The length of ACE 5′-UTR is 22 bp.
Underlined nucleotide sequences are added to the 5′-end of the oligonucleotide primer for cloning.
ACE, angiotensin I-converting enzyme.

restriction sites. The amplified product was then cloned into pGEM-T easy (Promega, Madison, WI, USA) and digested with *Xho*I and *Nhe*I (New England Biolabs, Beverly, MA, USA). The restricted fragment was then subcloned into the *Xho*I and *Nhe*I sites of the promoterless luciferase reporter pGL3-basic (Promoter Neuroscience, Madison, WI, USA). The 293T cells were grown in Dulbecco's modified Eagle's medium (JBI Life Technologies, Inc., Dalseo-gu, Daegu, Korea) with 2 mM L-glutamine, 25 mM HEPES, 10% heat-inactivated fetal bovine serum (FBS; Invitrogen Life Technologies, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 ng/mL streptomycin (Life Technologies-BRL, Gaithersburg, MD, USA) at 37 °C in humidified air containing 5% CO_2. One day before transfection, 5×10^5 cells/well were seeded in a six-well plate in 2 mL of medium without antibiotics to obtain 90–95% confluence at the time of transfection. The cultured cells were transiently transfected with pGL3-*ACE* promoter using LF2000 (Invitrogen Life Technologies, recommended micrograms of DNA : microlitres of lipofectamine = 1 : 2) in serum-free OptMEM (GIBCO BRL, Eggstein, Germany); 1 μg of pSV-β-galactosidase (Promega) was co-transfected as an internal control. After 48 h, the cells were washed twice with phosphate-buffered saline (PBS) and solubilized by scraping with 400 μL of reporter lysis buffer (Promega). The lysates were then centrifuged, and the supernatant was stored at −70 °C. The level of β-galactosidase activity in the supernatant was assessed by *ortho*-nitrophenyl-D-galactopyranoside (ONPG) hydrolysis using the β-Galactosidase Enzyme Assay System Kit (Promega); the total amount of protein was measured using the BCA Protein Assay Kit (Pierce Biotechnology Inc., Rockford, IL, USA). The amount of luciferase activity was measured using the Luciferase Assay System (Promega) and a luminometer. The luciferase activity in the lysates of transfected cells with pGL3-*ACE* promoter and empty pGL3-basic vector was normalized to β-galactosidase activity. The promoter activity was expressed as the ratio of luciferase activity in pGL3-*ACE* promoter transfectants to that of empty pGL3-basic vector transfectants.

Nuclear extract preparation and electrophoretic mobility shift assays

Nuclear extracts were prepared using a modified version of the method described by Schreiber et al. [33]. The 293T cells were harvested after washing twice with Hank's Balanced Salt Solution (HyClone Lab Inc., SouthLogan, Utah, USA), washed with buffer A (10 mM HEPES, 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/mL leupeptin, and 10 μg/mL aprotinin), and collected by centrifugation. The cell pellet was then resuspended in buffer B (buffer A containing 0.2% Nonidet P-40) and incubated for 10 min. The nuclei were then pelleted by centrifugation and resuspended in buffer C (buffer A containing 0.25 M sucrose). The nuclei were then re-pelleted, resuspended in buffer D (50 mM HEPES, 400 mM KCl, 10% glycerol, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/mL leupeptin, and 10 μg/mL

aprotinin), and incubated with shaking for 30 min. All procedures were performed on ice. Finally, the mixture was centrifuged, and the supernatant was stored at −70 °C. DNA–protein binding assays were then performed using the Gel Shift Assay System (Promega) as per the manufacturer's instructions. Aliquots (10 μg) of the nuclear extracts were incubated with binding buffer (Promega) in a total reaction volume of 10 μL for 10 min, with or without the unlabelled oligonucleotide probe. The reaction mixture was then incubated for 20 min with the ^{32}P-labelled oligonucleotide probe. The products were analysed by electrophoresis in 6% polyacrylamide gel with 0.5× TBE buffer. The gels were then dried and analysed by autoradiography. The sequences of the probes were the follows; forward, 5′-catct tc[a/t]aa agaga ggagg c-3′; reverse, 5′-gcctc ctctc ttt[t/a]g aagat g-3′. To determine the specificity of the DNA–protein complexes, 1.75 pmol of unlabelled probe was incubated with the nuclear extracts for 10 min before addition of the labelled probe.

Statistics

We applied widely used measures of linkage disequilibrium (LD) to all pairs of biallelic loci: Lewontin's D' ($|D'|$) [34] and r^2. The genotype distribution was analysed using logistic regression models with age (continuous value), gender (male = 0, female = 1), smoking status (non-smoker = 0, ex-smoker = 1, smoker = 2), and atopy (absence = 0, presence = 1) as covariates. These four covariates were the confounding factors for the development of asthma and showed a significant difference between asthmatics and healthy controls ($P<0.05$; Table 3). Haplotype associations were estimated using HaploScore (http://www.biostat.wustl.edu/genetics/genetic ssoft/), which computes score statistics to test for associations between a given haplotype and a wide variety of traits, including binary, ordinal, quantitative, and Poisson. The differences in the rate of decline in FEV_1 following aspirin challenge among the genotypes and haplotypes were examined using a linear regression model. The data were managed and analysed using SAS version 8.1 (SAS Inc., Cary, NC, USA) and SPSS version 10.0 (SPSS Inc., Chicago, IL, USA). A P-value of $\leqslant 0.05$ was regarded as statistically significant. Bonferroni's corrections for multiple comparisons were performed according to the number of polymorphisms analysed and are expressed as the corrected P-value (P^{corr}). The level of luciferase reporter activity is expressed as the mean ± standard error of the mean (SE). Differences between independent groups and samples were detected using the non-parametric Kruskal–Wallis H-test for continuous data. If significant differences were found, the Mann–Whitney U-test was used to detect differences between pairs of samples. Statistical powers were calculated using Statistical Power Calculator (http://www.dssresearch.com/toolkit/spcalc/power_p2.asp). A two-tail test of allele frequency in case and control as well as 5% of alpha error level were used.

Results

Characteristics of the study subjects

There were significant differences in the mean age, gender distribution, smoking status, and prevalence of atopy between the controls and asthmatics (Table 3). The FEV_1% predicted and PC_{20} methacholine values of the asthmatics were significantly lower than those of the normal controls, whereas the total IgE level was significantly higher in the former than in the latter. A total of 312 asthmatics

Table 3. Clinical profiles of the study subjects

			ASA-challenged asthmatics	
	Normal controls	Asthmatics	ATA	AIA
Number of subjects	181	581	231	81
Age [years, mean (range)]	33.9 (7–81)	38.4 (11–80)*	46.2 (18–76)	43.7 (18–72)
Sex (male/female)	94/87	264/317*	67/164	34/47
Current smoker (%)	24.31	12.39*	12.12	8.64
FVC (% predicted)	93.21±0.50	87.84±0.77*	87.86±1.16	88.95±1.95
FEV_1 (% predicted)	88.73±0.39	80.74±0.89*	85.41±1.28	79.33±2.42†
PC20 methacholine (mg/mL)	20.19±0.25	2.45±0.07*	6.27±0.68	3.03±1.11†
Total IgE (IU/mL)	191.46±28.58	406.68±19.31*	366.00±38.95	366.99±61.27
Positive skin test (%)	41.44	58.69*	35.06	40.74
% decline of FEV_1 by aspirin provocation‡	ND		2.12±0.38 (−15–14.4)	20.23±1.77 (−9.7–68)†

Values are mean±SE. P-values are obtained using the t-test or the χ^2-test between asthmatics and normal controls and between AIA and ATA.

*$P<0.05$ for difference between asthmatics and normal control.

†$P<0.05$ for difference between ATA and AIA subjects.

‡Provocation was performed in 312 patients with asthma. Data are mean±SE (range).

AIA, aspirin-intolerant asthma; ASA, aspirin; ATA, aspirin-tolerant asthma; FEV_1, forced expiratory volume in 1s.

underwent the aspirin provocation test, and an aspirin-induced decline in FEV_1 of −15% to 68% was observed. The FEV_1% predicted and PC_{20} methacholine values for the asthmatics were significantly lower among those with AIA than among those with ATA (Table 3).

Frequency of each angiotensin I-converting enzyme gene polymorphism among the study subjects

For screening for ACE polymorphisms, the five previously reported polymorphisms [31], including two SNPs (−262 A>T and −115 T>C) in the 5′-flanking region, two SNPs (+5467 T>C [Pro450Pro] and+11860 A>G [Thr776Thr]) in the coding region, and one ins/del (+21288 CT) in the 3′-region, were selected and genotyped in a Korean asthma cohort (Fig. 1). Two SNPs in the 5′-flanking region were selected for the assay of promoter activity, although these two SNPs were in almost absolute LDs ($|D'| = 1$ and $r^2 > 0.99$; Fig. 1c).

The distribution of each locus was in Hardy–Weinberg equilibrium ($P > 0.05$; Table 4). The minor allele frequencies of the polymorphisms were 0.396 (−262 A>T), 0.400 (−115 T>C), 0.404 (+5467 T>C [Pro450Pro]), 0.390 (+11860 A>G [Thr776Thr]), and 0.418 (+21288 CT ins/del; Table 4). Based on these results, three haplotypes with a frequency > 0.05 were constructed (Fig. 1).

Association of polymorphisms with the risk of asthma and aspirin intolerance in asthmatics

The association of each polymorphism and haplotype with the risk of asthma and the risk of aspirin hypersensitivity was analysed using multiple logistic regression models. No significant association with the risk of asthma was detected using three alternative models (co-dominant, dominant, and recessive; Table 5); however, two of the SNPs (−262 A>T and −115 T>C) were significantly associated with aspirin intolerance [odds ratios (ORs) of 2.08 and 1.91, respectively] in the co-dominant model (Table 6). The rare allele frequencies of the two SNPs were significantly higher in the AIA group than in the ATA group. The rare allele frequency of −262 A>T was approximately two times higher in the AIA group than in the ATA group using the co-dominant model ($P = 0.003$, $P^{corr} = 0.015$). A similar association was observed for *ACE* −115 T>C. In terms of the haplotype, the frequency of the *ht2* haplotype was significantly different between the AIA and ATA groups ($P = 0.01$, $P^{corr} = 0.03$). Because the aspirin-induced rate of decline in FEV_1 is the most important parameter for the diagnosis of aspirin intolerance in asthmatics, we determined the association between each of the two SNPs (−262 A>T and −115 T>C) and the rate of decline in FEV_1 following aspirin challenge. Asthmatics carrying the rare allele for −262 A>T or −115 T>C

Table 4. Frequency of *ACE* gene polymorphisms in total study subjects ($n = 762$)

Locus	rs no.	Region	HWE (*P*-value)	Heterozygosity	Frequency
ACE −262 A>T	rs4291	Promoter	0.365	0.478	0.396
ACE −115 T>C	rs4292	Promoter	0.653	0.481	0.400
ACE+5467 T>C	rs4309	Exon 8	0.994	0.481	0.404
ACE+11860 A>G	rs4343	Exon 16	0.787	0.476	0.390
ACE+21288 ins/del	rs1799763	3′-UTR downstream	0.601	0.480	0.418

ACE, angiotensin I-converting enzyme.

Table 5. Comparisons of genotype and haplotype distributions between normal controls and asthmatics

			Allele frequency			
Locus	Location	Amino acid change	BA ($n = 581$)	NC ($n = 181$)	*P**	Statistical power (%)†
ACE −262 A>T	Promoter	–	0.399	0.389	0.808	8.0
ACE −115 T>C	Promoter	–	0.408	0.389	0.937	11.6
ACE+5467 T>C	Exon 8	Pro>Pro	0.411	0.404	0.841	7.9
ACE+11860 A>G	Exon 16	Thr>Thr	0.380	0.376	0.933	6.1
ACE+21288 ins/del	3′-UTR downstream	–	0.425	0.407	0.644	11.1
ht1 (ATTAIns)		–	0.460	0.422	0.231	22.6
ht2 (TCCGDel)		–	0.369	0.358	0.917	8.4
ht3 (ATTAIns)		–	0.080	0.106	0.092	30.1

*P-value for logistic analyses of co-dominant models controlling age, sex, smoking and atopy as covariates. Haplotype association analyses were performed using software HaploScore.

†Statistical powers were calculated using Statistical Power Calculator (http://www.dssresearch.com/toolkit/spcalc/power_p2.asp). The two-tailed test of allele frequency in case and control as well as 5% of alpha error level were used.

ACE, angiotensin I-converting enzyme; BA, bronchial asthma; NC, normal controls.

Table 6. Comparisons of genotype and haplotype distributions between aspirin-intolerant asthmatics and aspirin-tolerant asthmatics

		C/C		C/R		R/R		Total	Co-dominant			Dominant			Recessive			Statistical power (%)†
		N	%	*N*	%	*N*	%	*N*	OR (95% CI)	*P**	P^{corr}	OR (95% CI)	*P**	P^{corr}	OR (95% CI)	*P**	P^{corr}	
−262 A>T	ATA	74	38.54	92	47.92	26	13.54	192	2.08 (1.28–3.38)	**0.003**	**0.015**	3.14 (1.42–6.97)	**0.005**	**0.025**	2.05 (0.92–4.56)	0.078	0.39	60.1
	AIA	14	20.59	39	57.35	15	22.06	68										
−115 T>C	ATA	68	36.56	91	48.92	27	14.52	186	1.91 (1.17–3.10)	**0.010**	**0.050**	2.80 (1.258–6.24)	**0.012**	0.060	1.85 (0.83–4.11)	0.128	0.64	51.1
	AIA	14	20.59	39	57.35	15	22.06	68										
+5467 T>C	ATA	70	35.00	103	51.50	27	13.50	200	1.86 (1.13–3.05)	**0.014**	0.070	2.67 (1.201–5.93)	**0.016**	0.080	1.75 (0.78–3.91)	0.173	0.865	42.7
	AIA	14	20.00	43	61.43	13	18.57	70										
+11860 A>G	ATA	68	39.08	85	48.85	21	12.07	174	1.83 (1.10–3.03)	**0.019**	0.095	1.95 (0.926–4.12)	0.079	0.395	2.50 (1.05–5.95)	**0.038**	0.19	57.3
	AIA	13	22.03	33	55.93	13	22.03	59										
+21288 ins/del	ATA	63	31.98	100	50.76	34	17.26	197	1.67 (1.04–2.68)	**0.032**	0.160	2.03 (0.932–4.44)	0.075	0.375	1.87 (0.89–3.93)	0.096	0.48	35.2
	AIA	15	22.39	35	52.24	17	25.37	67										
ht1 (ATTAIns)	ATA	59	28.10	110	52.38	41	19.52	210	1.64 (1.02–2.63)	**0.042**	0.126	1.45 (0.708–2.98)	0.308	0.924	2.63 (1.09–6.32)	**0.03**	0.09	38.3
	AIA	11	15.71	41	58.57	18	25.71	70										
ht2 (TCCGDel)	ATA	23	10.95	98	46.67	89	42.38	210	0.54 (0.34–0.86)	**0.010**	**0.030**	0.41 (0.208–0.84)	**0.015**	**0.045**	0.51 (0.22–1.18)	0.116	0.348	48.5
	AIA	12	17.14	39	55.71	19	27.14	70										
ht3 (ATTAIns)	ATA	1	0.48	14	6.67	195	92.86	210	4.64 (0.59–36.84)	0.146	0.438	4.72 (0.58–37.9)	0.144	0.432	23.12 (0)	0.816	1	20.4
	AIA		0.00	2	2.86	68	97.14	70										

**P*-values for logistic analyses controlling age, sex, smoking, and atopy. Haplotypes and their frequencies were inferred using the algorithm developed by Stephens et al. Missing genotype data were omitted for exact haplotyping.

†Statistical powers were calculated using Statistical Power Calculator (http://www.dssresearch.com/toolkit/spcalc/power_p2.asp). Two-tail test of allele frequency in case and control as well as 5% of alpha error level were used.

Bold values signifies $P<0.05$.

AIA, aspirin-intolerant asthma; ATA, aspirin-tolerant asthma; CI, confidence interval; OR, odds ratio.

showed a greater decline in FEV_1 in response to aspirin than did those carrying the common alleles (6.47±1.26% vs. 10.03±1.99%, $P=0.021$, and 6.85±1.32% vs. 9.99±1.95%, $P=0.037$, respectively; Fig. 2). The statistical power of each polymorphism was also calculated and the values ranged from 20.4% to 60.1% (Table 6).

Comparison of angiotensin I-converting enzyme gene promoter activity between subjects with the −262 A>T and −115 T>C alleles

Because the −262 A>T and −115 T>C SNPs are located in the *ACE* promoter region, we investigated the effect of each on *ACE* mRNA expression. We compared the level of activity in promoters bearing each of the −115 T and −115 C alleles. We also compared the promoter activity of −262 A/−115 T with that of promoters bearing −262 T/−115 C based on the common haplotypes using a luciferase reporter assay. The relative level of luciferase activity for the −262 A/−115 T *ACE* promoter was significantly higher than that for the −262 T/−115 C promoter ($P=0.009$; Fig. 3). In addition, both *ACE* promoter–luciferase constructs showed a higher level of activity than the promoterless luciferase control vector ($P<0.001$). However, the level of activity for the −115 T and −115 C promoters was insignificant when compared with the promoterless luciferase reporter pGL3-basic.

The −262 A>T angiotensin I-converting enzyme gene promoter region contains a nuclear factor-binding site

To assess whether the −262 A>T locus serves as a transcription factor-binding site, gel shift assays were performed using nuclear extracts of 293T cells.

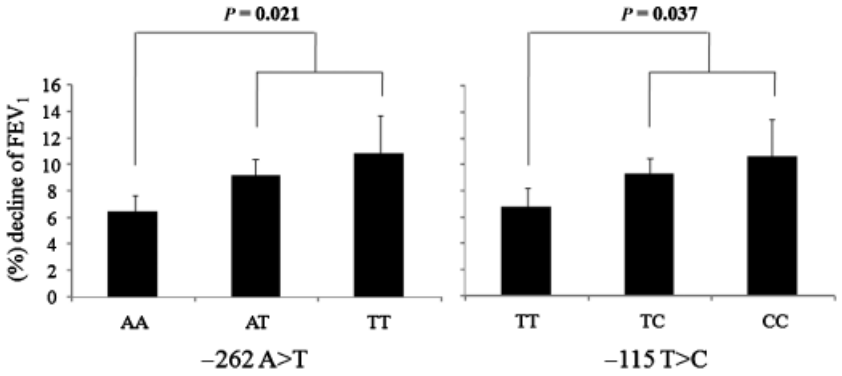


Fig. 2. Association of single nucleotide polymorphisms on the *ACE* gene with (%) a decline of forced expiratory volume in 1 s by aspirin provocation.

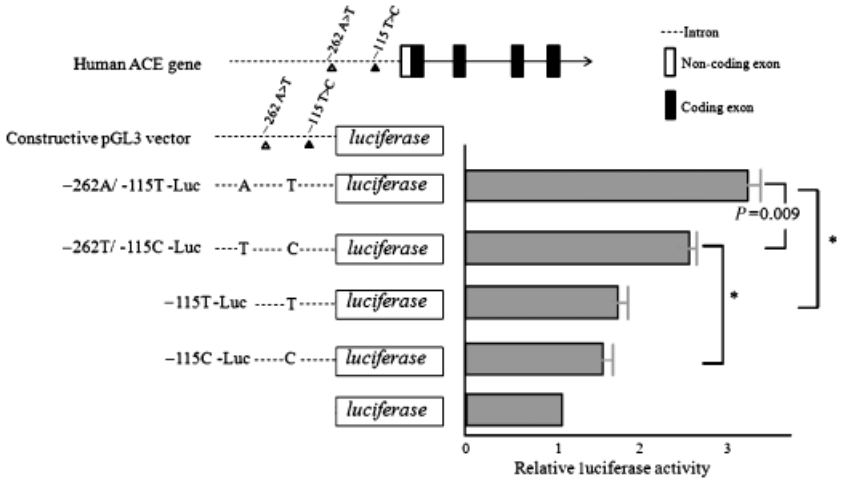


Fig. 3. Comparison of promoter activities between −262 genotypes using luciferase reporter assay. The luciferase activity assay was examined six times. Each type of promoter was inserted into the pGL3-basic luciferase reporter system, and each construct was transfected into 293T cells using lipofection as described in 'Materials and methods'. As internal control, pSV-β-galactosidase control vector was co-transfected with the reporter constructs. The data were presented by the ratio of normalized luciferase activities of each construct to that of empty pGL3-basic vector. The data were presented as mean±standard error of six independent experiments. *$P<0.001$. ACE, angiotensin I-converting enzyme.

DNA–protein complexes were detected using probes for *ACE* −262 A or T (Fig. 4). The intensity of the complex was greater using the probe for −262 A than for −262 T. The complexes disappeared due to competition when an unlabelled probe of either type was included (Fig. 4). This indicates that the region of the *ACE* promoter near −262 A may be a transcription factor-binding site.

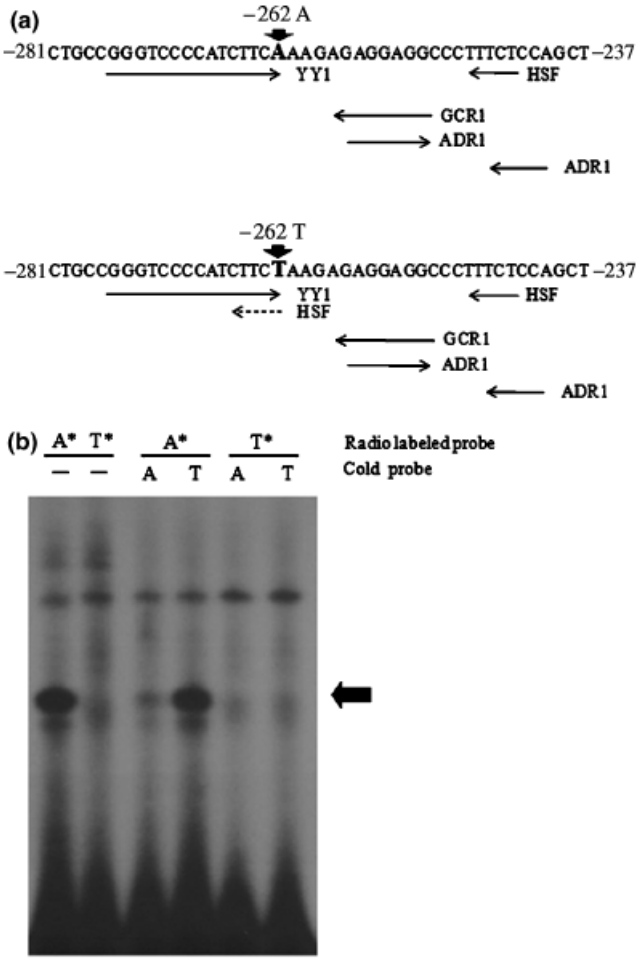


Fig. 4. Gel shift assay for −262 A>T single nucleotide polymorphism on the promoter of the *ACE* gene using nuclear extract from 293T cell line. (a) DNA sequence of the ACE promoter from −281 to −235 of the translation start site and the putative binding sites around −262 A>T. YY1 and HSF are searched as the transcription factor binding around −262 A>T loci. (b) One million cells per millilitre of 293T cell lines were cultured. The nuclear extracts of 293T cell line were incubated with the ^{32}P-labelled probe, of which sequences included the −262 A>T. To identify specific binding complexes, nuclear extract from 293T cell line was pre-incubated with unlabelled probe before addition of the labelled probe for competition. The filled arrow indicates the specific DNA-binding protein. A* and T* indicate ^{32}P-labelled probes. The arrow shows that the oligonucleotide probe having −262 A reacts with the binding proteins for transcription. The binding of proteins with the ^{32}P-labelled probes was effectively inhibited by the unlabelled probe having −262 A. ACE, angiotensin I-converting enzyme.

Discussion

In the present study, there were no significant associations between polymorphisms in the *ACE* gene and asthma; however, a positive association between the polymorphisms and aspirin hypersensitivity was detected in asthmatics in the case–control association study and functional validation of the polymorphism. Several studies have attempted to find associations between *ACE* polymorphisms and the risk of asthma. Lee et al. [35] found no significant difference between Korean controls and asthmatics in terms of a 287 bp ins/del in intron 16. Studies of the same polymorphism in Japanese [36, 37], Caucasian [38], and Turkish [39] populations also found no association with the development of asthma. In contrast, this polymorphism was recently reported to be associated with asthma and rhinitis in Taiwanese children [40]. However, to the best of our knowledge, no study has addressed the association of polymorphisms other than the 287 bp ins/del with the development of asthma and aspirin hypersensitivity in asthmatics. Because polymorphisms in *ACE* are associated with hypertension [41], subjects with hypertension and diabetes were excluded from our study. Our case–control study indicated that two SNPs (−262 A>T and −115 T>C) in the *ACE* promoter region are significantly associated with aspirin intolerance (OR of 1.85–3.14). The frequencies of the rare alleles of the SNPs were higher in aspirin-intolerant asthmatics than in aspirin-tolerant asthmatics. This suggests that asthmatics who have the rare alleles of the two SNPs are susceptible to developing aspirin hypersensitivity.

To validate the functional effect of the two SNPs (−262 A>T and −115 T>C), we used a luciferase reporter assay. The −262 A/−115 T promoter possessed a higher level of activity than did the −262 T/−115 C promoter. We attempted to determine which allele was responsible for the activity of the promoter using promoters bearing either −115 T or −115 C; however, no luciferase activity was detected in either case. Thus, we conclude that −262 A>T is responsible for the activity of the *ACE* promoter. The significant association of *ACE* −115 T>C with AIA may be due to the complete LD with *ACE* −262 A>T. Although 293T cells, whose origin is kidney epithelial cells, could not reflect the biological roles of ACE in the airway or in the lung, our data indicate that the rare allele of −262 A>T causes decreased promoter activity. When considered with the association data, the decreased activity of the −262 A>T promoter results in the decreased expression of *ACE* gene products, which induces the accumulation of inflammatory mediators in the lung, predisposing asthmatics to aspirin hypersensitivity.

In normal individuals, *ACE* is expressed in the surface epithelial cells, endothelial cells, and submucosal glands, including the mucus-secreting seromucous cells. In contrast, ACE is absent from or present in a reduced amount

in the surface epithelium in asthmatics not treated with corticosteroids compared with those treated with corticosteroids and healthy controls. To define the exact role of the polymorphisms in *ACE*, association analyses with ACE levels in the airway are needed; however, sampling of the airway secretions in subjects with symptomatic asthma is not an easy task. For this reason, we were unable to measure the ACE levels in the airway secretions of subjects with AIA.

ACE conceivably has both beneficial and detrimental effects on airway inflammation and remodelling in asthma. Increased *ACE* activity could have beneficial effects by improving the metabolism of several pro-inflammatory mediators in the airway. *ACE* may also regulate the effects of kinins and tachykinins that are released from intraepithelial C-fibre nerves [42]. Therefore, *ACE* is probably involved in regulating the pro-inflammatory effects of the bioactive peptides that are released from the surface epithelium or intraepithelial nerves in response to various stimuli such as irritants. ACE in the surface epithelium may also regulate the cough reflex caused by some peptides, including kinins [12, 16]. *ACE* expression is reduced in the surface epithelium of asthmatic subjects who are not treated intranasally with corticosteroids, whereas treatment with corticosteroids restores *ACE* expression in the epithelium to the level observed in normal subjects [15]. Evidence suggests that substance P and neurokinin A may be involved in aspirin intolerance in asthmatics. Substance P and neurokinin A are able to relax the airway via neurokinin receptor coupled to cyclooxygenase (COX)-2 activation and the subsequent release of PGE2 [43]. The endothelium-dependent contractions (EDCs) that are induced by substance P are attenuated by ASA, an inhibitor of cyclooxygenase [44]. In addition, primary cultured murine mast cells that are stimulated with substance P produce several major mediators of aspirin hypersensitivity, including LTC4 and PGD2, but they do not release the granule-associated enzyme β-hexosaminidase [45]. Thus, decreased ACE activity may enhance airway inflammation and hyperreactivity via endogenous peptides, especially in asthmatics with aspirin hypersensitivity.

Gel shift assays indicated the presence of a transcription factor-binding site in the *ACE* promoter at −262 A>T. The detection of DNA–protein complexes was better using the −262 A probe than that using the −262 T probe. Thus, the transcription factor may be an inducer. Because there are putative binding sites for several candidate factors, including HSF and YY1, based on a transcription factor search using the −262 A>T allele (http://www.cbrc.jp/research/db/TFSEARCH.html), we performed a supershift assay using antibodies against HSF and YY1, but no shift was observed (data not shown).

In summary, we screened for the presence of four SNPs in the *ACE* promoter region and one ins/del in the *ACE* gene to examine the association of each with the development of aspirin intolerance in asthmatics. Two of the SNPs, i.e. −262 A>T and −115 T>C, were suggestive of aspirin hypersensitivity in asthma. In addition, the association of −262 A>T was more pronounced when paired with functional data produced by luciferase assays and EMSA. This information may be useful in the development of new strategies for the diagnosis and control of aspirin intolerance.

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