

A possible association of *EMID2* polymorphisms with aspirin hypersensitivity in asthma

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Abstract Aspirin-intolerant asthma (AIA) is an asthma phenotype characterized by the development of bronchoconstriction following ingestion of aspirin. Despite the well-defined pathological trigger, the underlying mechanisms of AIA are still unclear. With the biophysical characteristics of the human *EMI domain-containing protein 2* (*EMID2*) gene in relation to the extracellular matrix deposition and epithelial-mesenchymal transition as pivotal characteristics of airway remodeling in asthma, we hypothesized that genetic poly-

morphisms of *EMID2* might affect the development of AIA. In this study, the allelic associations of 49 single-nucleotide polymorphisms (SNPs) of the human *EMID2* gene were evaluated from 163 AIA patients and 429 aspirin-tolerant asthma (ATA) subjects as controls in a Korean population. Logistic analysis showed that five SNPs ($P=0.01$ – 0.04 , but $P^{\text{corr}} > 0.05$) and *EMID2*_BL2_h2 haplotype (unique to the minor alleles of *rs4727494* and *rs13233066*; $P=0.02$; $P^{\text{corr}}=0.02$) were significantly associated with AIA. More interest-

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ingly, regression analysis of the decline of forced expiratory volume in one second (FEV_1) by aspirin provocation revealed that 10 SNPs ($P=0.003$ – 0.04) and four relevant haplotypes ($P=0.002$ – 0.02) were significantly associated with the fall rate of FEV_1 by aspirin provocation, indicating that genetic polymorphisms of *EMID2* could cause meaningful deficits in the upper and lower airways among AIA patients. These findings provide evidence that *EMID2* may be a susceptible genetic factor for aspirin hypersensitivity among asthmatics in Korean population.

Keywords Aspirin-intolerant asthma · *EMID2* · Polymorphism · Haplotype

Abbreviations

AIA	Aspirin-intolerant asthma
EMID2	EMI domain-containing protein 2
NSAID	Non-steroidal inflammatory drugs
ECM	Extracellular matrix
GINA	Global Initiative for Asthma
ACE	Angiotensin converting enzyme
SDS	Sequence detection system

Introduction

Aspirin-intolerant asthma (AIA) is a clinically distinct phenotype induced by non-steroidal inflammatory drugs (NSAID) such as aspirin and is characterized by asthma attacks, aspirin sensitivity and inflammation of the upper airways (rhinitis and nasal polyps) (Bel 2004). It has been reported by the European Network on Aspirin-Induced Asthma that 5.8% of AIA patients had a family history of aspirin sensitivity (Szczeklik et al. 2000), and a number of studies have provided estimates of AIA prevalence to be around 20% among asthma cases (Hedman et al. 1999; McDonald et al. 1972). Despite the well-defined pathological trigger and previous reports on the genetic background of AIA, the molecular pathogenesis of the disease is still unclear and requires further investigation.

Airway limitation is the dominant physiological event that leads to the reversible clinical symptoms in asthma such as airway hyperresponsiveness and airflow obstruction (Djukanovic et al. 1990). However, it had been contended that airflow limitation may not be entirely reversible (Backman et al. 1997; Loren et al. 1978). Persistent inflammation in airway tissues results to structural alterations in the airway epithelium, subepithelium, airway smooth muscle and vasculature, leading to permanent changes in asthmatic airway commonly known as airway remodeling (Holgate and Polosa 2006). Subepithelial fibrosis has been highlighted as an integral component of the remodeling response.

Accumulation of extracellular matrix (ECM) such as fibronectin and types I, III, and V collagens in the true basement membrane, specifically the lamina reticularis (Ward et al. 2002), results in thickening of subepithelial space, a response that has been associated with asthma exacerbation and frequency and duration of symptoms (Chetta et al. 1997; Nomura et al. 2002). In addition, previous studies have shown that subepithelial thickness was correlated with decline in FEV_1 (Minshall et al. 1997; Tagaya and Tamaoki 2007). These findings suggest that the fibrotic response plays an important role in asthma exacerbation, and more importantly, it may contribute to the irreversibility of the disease due to airway remodeling.

The *emilin/multimerin domain-containing protein 2* (*EMID2*) on chromosome 7q22.1 encodes collagen $\alpha 1$ (XXVI) chain, and is predominantly expressed in mesenchymal cells. Due to its expression in undifferentiated mesenchymal cells (Haïtchi et al. 2005; Yang et al. 2008) that results in the epithelial-mesenchymal transition, a characteristic of the remodeling response in asthma (Hackett et al. 2009), *ADAM33* has been identified as an AIA susceptibility gene in Japanese and Caucasian populations (Sakagami et al. 2007; Van Eerdewegh et al. 2002). Emilin is a component of elastic fibers located at the elastin-microfibrils interface (Bressan et al. 1993; Doliana et al. 1999) whereas multimerin is a protein that has the ability to self associate and form large, branching matrix fibers (Jeimy et al. 2008). As crucial components of the ECM, pathological studies have observed an abnormal deposition of elastic fibers in large and small asthmatic airways (Chakir et al. 2003; Mauad et al. 2004; Mauad et al. 1999). In addition, *EMID2* is a glycosylated protein that is secreted and deposited in the ECM, serving a variety of structural and functional roles in membrane and secreted proteins (Leimeister et al. 2002). These implications on ECM deposition and the epithelial-mesenchymal transition in airway remodeling of asthma irreversibility led to our hypothesis that *EMID2* polymorphisms could influence an individual's susceptibility to aspirin-induced hypersensitivity in asthma.

Materials and methods

Study subjects

A total of 592 study subjects composed of 163 AIA patients and 429 ATA controls were recruited from the Asthma Genome Research Center, a part of Soonchunhyang, Yonsei, Hallym, Chung-Ang, Chunnam, Chungbuk, and Seoul National University hospitals in Korea. These subjects were diagnosed with asthma according to the criteria of the Global Initiative for Asthma (GINA)

Global strategy for asthma management and prevention study. Evaluation of the subjects included airway reversibility measured by an inhalant bronchodilator-induced improvement of more than 15% of forced expiratory volume in 1 s FEV₁ and/or hyper-reactivity of less than 10 mg/mL of metacholine (Crapo et al. 2000). The diagnosis of AIA was based on aspirin-induced bronchospasms presented by rate (%) of FEV₁ decline and the patient's history of aspirin hypersensitivity. Subjects with 20% or greater decreases in FEV₁ or 15% to 19% decreases in FEV₁ with naso-ocular or cutaneous reactions were categorised as AIA patients, whereas ATA controls consisted of asthmatics exhibiting 15% or more reduction of FEV₁ without extrabronchial nasal or cutaneous reactions. Finally, the study was undertaken with the understanding and written consent of each subject, and ethical approval was obtained from the institutional review board of the each hospital.

SNP selection and genotyping

Candidate polymorphic single-nucleotide polymorphisms (SNPs) of the *EMID2* gene with minor allele frequency (MAF) of over 0.05 based on Asian population were selected from the National Center for Biotechnology Information (build 36) and International HapMap (<http://hapmap.ncbi.nlm.nih.gov/>). Genomic DNA was extracted from peripheral blood lymphocytes using Gentra Puregene kit (Minneapolis, MN, USA) according to the manufacturer's protocol. Genotyping was performed using TaqMan assay in the ABI-PRISM 7900HT sequence detection

system (Applied Biosystems, CA, USA) and was assessed by duplicate DNAs ($n=10$) for data quality. Genotyping data were obtained using the ABI-PRISM SDS software version 2.3. A total of 49 SNPs of the *EMID2* gene were successfully genotyped.

Statistical analyses

To determine the association between the genotype distributions of AIA patients and ATA controls, logistic analysis was carried out controlling for age (continuous value), gender (male=0 and female=1), smoking status (non-smoker=0, ex-smoker=1, and smoker=2), atopy (absence=0 and presence=1) and body mass index (BMI) as covariates to eliminate or reduce any confounds that might influence the findings. The analysis was performed using SAS version 9.1 (SAS Inc., Cary, NC). Lewontin's D' ($|D'|$) and the LD coefficient r^2 were examined to measure linkage disequilibrium between all pairs of biallelic loci (Hedrick 1987). Using the 49 SNPs, haplotypes were inferred using PHASE algorithm ver. 2.0 (Stephens et al. 2001), and 14 haplotypes with frequency over 5% were analyzed for association using SAS. In addition, the differences in the fall rate in FEV₁ following aspirin challenge among the genotypes and haplotypes were examined by regression analyses. The effective number of independent marker loci detected from the *EMID2* polymorphisms were calculated for multiple testing corrections using the SNPSpD (<http://genepi.qimr.edu.au/general/daleN/SNPSpD/>) software, which is based on the spectral decomposition of matrices of pair-wise linkage disequilibrium (LD) between SNPs (Nyholt 2004).

Table 1 Clinical profiles of the study subjects

Clinical profile	Asthmatics (all subjects)	AIA	ATA
Number of subjects (n)	592	163	429
Age (year, mean (range))	46.15 (15.40–77.88)	43.13 (17.22–72.73)*	47.30 (15.40–77.88)
Sex (n , male/female)	206/386	59/104	147/282
Current smoker/Ex-smoker (%)	27.70	21.47*	30.07
Height (cm)	160.78±8.63	161.72±8.69	160.42±8.39
Weight (kg)	62.81±10.84	61.25±10.38*	63.40±10.97
Body mass index (kg/m ²)	24.24±3.39	23.39±3.25*	24.58±3.39
% fall of FEV ₁ by aspirin provocation	9.27±13.24	24.63±16.11**	3.54±4.85
Blood eosinophil (%)	6.01±5.73	5.96±5.21	6.03±5.92
FEV ₁ (% predicted)	90.54±16.97	90.35±14.04*	91.66±16.87
PC20 methacholine (mg/ml)	6.43±8.67	5.02±7.83*	6.91±8.90
Total IgE (IU/ml)	357.65±604.09	348.60±596.44	361.00±607.56
Positive rate of skin test (%)	56.42	52.76	57.81

Each clinical profile of AIA was compared with the respective ATA controls. Age indicates a first medical examination

AIA aspirin-intolerant asthma, ATA aspirin-tolerant asthma

* $P<0.05$; ** $P<0.0001$ compared with ATA control

Results

Characteristics of the study subjects

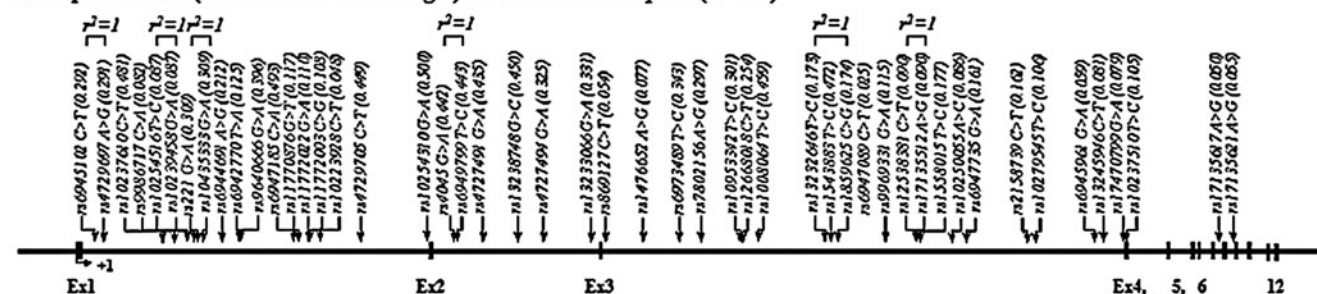
The clinical characteristics of the study subjects showed that AIA patients (23.39 ± 3.25 kg/m²) had lower BMI compared with ATA controls (24.58 ± 3.39 kg/m²). In addition, the age onset for cases of AIA was 43.1 and 47.3 for the control group, indicating that significant deficits in lung function growth could occur earlier in aspirin-intolerant asthmatics. Most importantly, our findings from the aspirin provocation test in AIA patients showed significant increase of aspirin-induced fall rate in FEV₁ compared with that of ATA controls (24.63 for AIA and 3.54 for ATA; $P < 0.0001$, Table 1). The predicted FEV₁%, smoking status and PC20 methacholine in AIA

patients were significantly lower than those of ATA controls ($P < 0.05$).

Genotyping of variants in the *EMID2* gene

A total of 49 SNPs of *EMID2* were genotyped in 163 AIA cases and 429 ATA controls in a Korean population with an average call rate of 99.9%, but only 32 genotypes failed to be detected. Among the polymorphisms, 18 SNPs are positioned in intron 1; 6 SNPs in intron 2; 23 SNPs in intron 3; each one in introns 8 and 9 (Fig. 1a and Electronic supplementary Table 1). The MAF of each polymorphism are shown in Electronic supplementary Table 1. Pair-wise comparisons of the 49 SNPs that were used to construct four LD blocks and 14 major haplotypes with frequencies over 0.05 are summarized in Figs. 1b and 2.

A. Map of *EMID2* (EMI domain containing 2) on chromosome 7q22.1 (194Kb)



B. LDs among *EMID2* polymorphisms

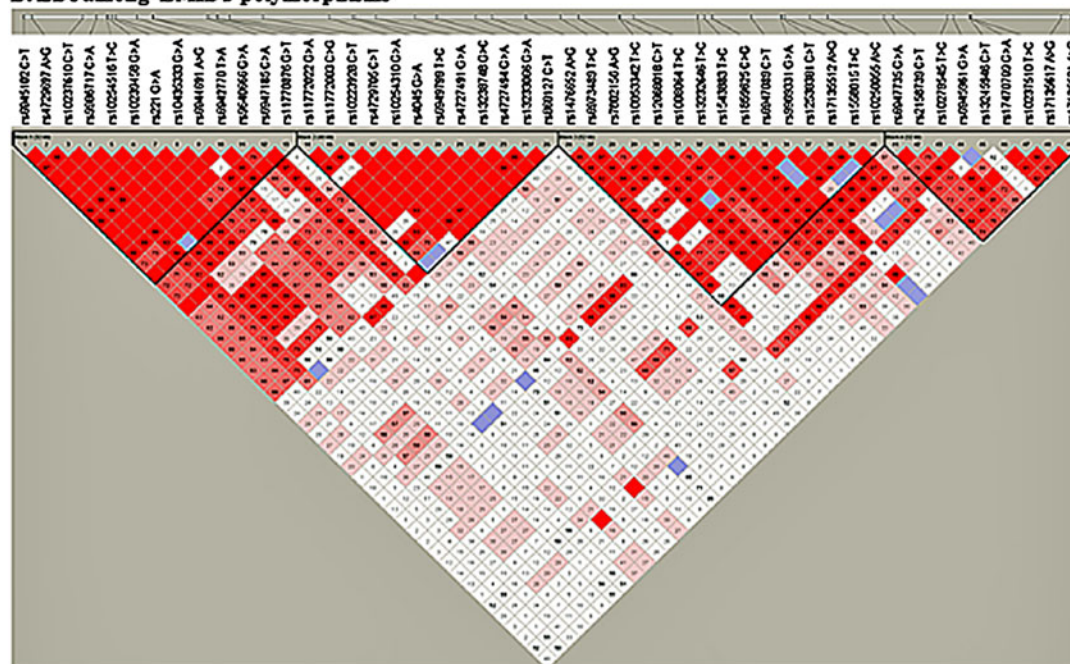


Fig. 1 Physical map and LD of the *EMID2* gene. **a** Schematic gene map and SNPs in the *EMID2* gene on chromosome 7q22.1 (194 kb). Black blocks represent coding exons and white blocks represent 5' and

3'UTR. The first base of translation site was denoted as nucleotide +1. UTR untranslated region. **b** LD coefficient (D') among *EMID2* SNPs

Fig. 2 Haplotypes of *EMID2*. Haplotypes of 49 SNPs in the *SLC6A7* gene depending on the four haplotype blocks. Associations of haplotypes with frequency >0.05 are shown in Tables 2 and 3. Significant *EMID2* variants and *EMID2*_BL2_ht2 are indicated as bold and highlighted

Results from logistic association analysis of the variants in *EMID2* between AIA and ATA from a Korean population with age, sex, smoking status, atopy and BMI adjusted as covariates showed that *rs6949799*, *rs4727494*, and *rs13233066* in intron 2 and *rs10279545* and *rs17470799* in intron 3 initially exhibited association signals with AIA ($P=0.01-0.04$ depending on models, Table 2), together with other SNPs showing nominal significances (Electronic supplementary Table 2). However, the signals disappeared after performing multiple testing corrections on the data. In further association analysis of haplotypes, *EMID2* BL2 *ht2*

Since the fall rate of FEV₁ by aspirin provocation is an important diagnostic marker for AIA, further association between *EMID2* polymorphisms and the fall rate of FEV₁ by aspirin provocation was analyzed using regression analysis. Ten SNPs (*rs6945102*, *rs4729697*, *rs221*, *rs10435333*, *rs9640666*, *rs4045*, *rs6949799*, *rs4727491*, *rs4727494*, and *rs13233066*) showed significant differences with fall rate of FEV₁ by aspirin provocation in AIA patients compared with those of ATA controls ($P=0.002-0.04$, Table 3 and Electronic supplementary Table 4).

Table 2 Associations of the significant variants among 49 SNPs in *EMID2* with AIA

SNP/Haplotype	MAF		Co-dominant			Dominant			Recessive		
	AIA (n=163)	ATA (n=429)	OR (95% CI)	P*	P ^{corr**}	OR (95% CI)	P*	P ^{corr**}	OR (95% CI)	P*	P ^{corr**}
<i>rs6949799</i> T>C	0.491	0.425	1.32 (1.02–1.72)	0.04	–	1.49 (0.98–2.26)	0.06	–	1.41 (0.90–2.21)	0.13	–
<i>rs4727494</i> G>A	0.288	0.337	0.78 (0.59–1.04)	0.09	–	0.62 (0.43–0.91)	0.01	–	1.14 (0.63–2.06)	0.68	–
<i>rs13233066</i> G>A	0.291	0.343	0.78 (0.59–1.03)	0.08	–	0.62 (0.42–0.90)	0.01	–	1.09 (0.61–1.95)	0.76	–
<i>rs10279545</i> T>C	0.077	0.118	0.61 (0.38–0.98)	0.04	–	0.60 (0.36–0.99)	0.05	–	0.35 (0.04–3.00)	0.34	–
<i>rs17470799</i> G>A	0.043	0.089	0.47 (0.26–0.85)	0.01	–	0.47 (0.25–0.87)	0.02	–	–	0.98	–
<i>EMID2</i> _BL2_h2	0.288	0.331	0.80 (0.60–1.07)	0.14	–	0.64 (0.44–0.93)	0.02	0.02	1.21 (0.66–2.20)	0.53	–

AIA aspirin-intolerant asthma, ATA aspirin-tolerant asthma, MAF minor allele frequency, OR odds ratio, CI confidence interval

* At 0.05 level of significance, P values for logistic analyses controlling age, smoking status, atopy and BMI as covariates.

**P values after multiple testing corrections (effective number of independent marker loci = 43.7927)

Furthermore, haplotypes *EMID2*_BL1_h2 (unique to the minor alleles of *rs6945102*, *rs4729697*, *rs221*, and *rs10435333*, in haplotypes with frequency >0.05), *EMID2*_BL2_h1 (unique to the minor alleles of *rs4045*, *rs6949799*, and *rs4727491*), *EMID2*_BL2_h2 and *EMID2*_BL2_h4 (unique to the minor alleles of *rs11772022* and *rs11772003*) showed significant associations with the fall rate of FEV₁ by aspirin provocation in AIA patients compared with those of ATA controls depending on the models used ($P=0.002$ – 0.02 , Table 3 and Electronic supplementary Table 5). These findings imply that genetic polymorphisms of the *EMID2* gene could be a causative factor for the reversibility of lung function abnormalities among AIA patients.

Discussion

AIA is a condition wherein asthma is exacerbated by aspirin intake. Central to the phenotypes of asthma is the presence of airway inflammation. Although the disease was thought to be reversible, current studies on asthma has shifted to a new paradigm that recognizes the long-term irreversibility of its symptoms as a response to structural changes of the contracted airway (Backman et al. 1997; Loren et al. 1978). One of the mechanisms underlying inflammation of the asthmatic airway is subepithelial fibrosis, a response brought about by abnormal deposition of ECM such as collagen and fibronectin in the basement membrane, resulting to thickening of the subepithelial space (Chetta et al. 1997; Nomura et al. 2002). Considering that airway limitation, such as airway hyperresponsiveness and airflow obstruction, is a factor in AIA, as well as the abundance of elastic fibers as a component of the ECM in the large and small airways of asthma patients, the human *EMID2* gene, which is secreted and deposited in the ECM, is presumably relevant in the pathophysiology of AIA.

With the fall rate of aspirin being a crucial diagnostic marker for AIA, we performed analysis on the association of *EMID2* variants and fall in FEV₁. Interestingly, results from regression analysis revealed that polymorphisms and haplotypes of the *EMID2* gene might have a significant effect on the fall rate of FEV₁ by aspirin provocation. Considering the predominant expression of *EMID2* in mesenchymal cells and the genetic association of the *ADAM33* gene that is selectively expressed in mesenchymal cells and is associated with bronchial hyperresponsiveness (Haitchi et al. 2005; Leimeister et al. 2002; Van Eerdewegh et al. 2002), abnormality derived from *EMID2* variants could also affect the airway hyperresponsiveness in AIA patients.

Although the key findings of this study suggest the involvement of the genetic variations in *EMID2* with

Table 3 Association analysis between variants of the *EMID2* gene and fall of FEV₁ by aspirin provocation

SNP/Haplotype	Allele	Fall rate of FEV ₁			<i>P</i> **	<i>P</i> ***	<i>P</i> ****
		C/C	C/R	R/R			
rs6945102	C>T	299 (10.40±14.10)	239 (8.72±12.77)	54 (5.05±8.43)	0.01	0.04	0.03
rs4729697	A>G	299 (10.40±14.10)	234 (8.76±12.86)	54 (5.05±8.43)	0.01	0.04	0.03
rs221	G>A	285 (10.56±14.31)	246 (8.93±12.58)	60 (4.00±8.33)	0.002	0.02	0.004
rs10435333	G>A	285 (10.56±14.31)	246 (8.99±12.60)	60 (4.00±8.33)	0.002	0.02	0.004
rs9640666	G>A	219 (10.31±13.47)	274 (9.52±13.48)	99 (6.06±11.44)	0.02	0.16	0.01
rs4045	G>A	182 (8.35±14.25)	292 (8.82±11.62)	117 (11.66±15.06)	0.05	0.33	0.02
rs6949799	T>C	181 (8.08±13.80)	293 (8.81±11.60)	117 (11.66±15.06)	0.03	0.22	0.02
rs4727491	G>A	187 (8.23±13.77)	289 (8.73±11.57)	115 (11.72±15.15)	0.04	0.30	0.01
rs4727494	G>A	272 (10.94±14.83)	258 (7.62±11.24)	62 (8.46±12.62)	0.02	0.003	0.71
rs13233066	G>A	271 (10.93±14.86)	254 (7.54±10.76)	67 (8.78±13.95)	0.02	0.004	0.83
<i>EMID2</i> _BL1_h12		335 (10.46±14.14)	218 (8.26±12.18)	39 (4.10±8.21)	0.003	0.01	0.02
<i>EMID2</i> _BL2_h11		189 (8.48±14.17)	288 (8.74±11.58)	115 (11.72±15.15)	0.06	0.41	0.02
<i>EMID2</i> _BL2_h12		275 (10.84±14.78)	257 (7.68±11.25)	60 (8.56±12.81)	0.02	0.005	0.77
<i>EMID2</i> _BL2_h14		525 (9.02±12.77)	64 (9.99±14.05)	3 (31.07±44.35)	0.11	0.30	0.002

Fall rate values are mean±SD

C/C, C/R and R/R indicate the homozygote of common allele and the heterozygote and homozygote of rare allele, respectively

*At 0.05 level of significance, *P* values of regression analysis representing co-dominant, dominant and recessive models controlling sex, smoking status and atopy as covariates.; ***P* values of the co-dominant model; ****P* values of the dominant model; *****P* values of the recessive model

hypersensitivity in asthma, the precise molecular mechanisms that account for this event remains unclear. However, we speculate that the location of these variants, which are found in the intronic region of the *EMID2* gene, may influence its effect on the pathogenesis of AIA. Although often overlooked, but the coordinated regulation of transcription and splicing in the nucleus in vivo serves as a critical pathway to diseases (Pagani and Baralle 2004). A previous study has revealed that variants in the intronic region is involved in this complex activity, particularly by inducing exon skipping, activating cryptic spliced sites or altering the balance of the alternatively spliced isoforms that are produced, thus, causing disease phenotypes (Pagani and Baralle 2004). Therefore, the functional significance of the *EMID2* variants with hypersensitivity among asthmatics detected in this study may be a manifestation of the splicing process.

In this study, we conducted an association analyses between the variants in *EMID2* and AIA involving subjects from Korean ethnicity. Although a relationship between SNPs of *EMID2* and AIA was initially detected in case/control comparison, the signals disappeared after multiple testing corrections ($P^{\text{corr}} > 0.05$). On the other hand, in the gene-based haplotype analysis, the haplotype *EMID2*_BL2_h12 showed a statistically significant evidence of association with AIA in dominant model ($P^{\text{corr}} = 0.02$). Dominance occurs when a single functional allele in the heterozygote produces a non-functional gene product

that resembles the phenotype of homozygous non-functional allele: this phenomenon is called haploinsufficiency. Recently, human non-syndromic cleft lip with or without cleft palate (NSCLP) development has been reported to be associated with intronic SNPs of the *small ubiquitin-like modifier 1* (*SUMO1*) gene; interestingly, *SUMO1* haploinsufficiency results in cleft lip and palate (Song et al. 2008). Therefore, it can be assumed that heterozygous genotypes of *rs4727494* and *rs13233066* in *EMID2*_BL2_h12 might generate mean values similar to the recessive phenotype in AIA patients. However, the underlying mechanisms of how these haplotypes affect the patients risk for AIA need to be elucidated. In this regard, it is suggested that the human *EMID2* gene may either be a causative factor in the irreversibility of asthma due to the remodeling response or may only act as a genetic marker of an underlying process for airway structural alterations in a Korean population.

Bronchial biopsy evidence has revealed that asthma inception associated with activation of the epithelial-mesenchymal transformation corresponds with the remodeling response in severe childhood asthmatic airways (Jenkins et al. 2003; Warner et al. 1998), an event that has recently emerged in the pathophysiology of airway hyperresponsiveness. The *ADAM33* gene, which is expressed in undifferentiated mesenchymal cells (Haitchi et al. 2005; Yang et al. 2008), has been implicated in bronchial hyperresponsiveness and AIA in ethnically diverse populations (Sakagami et al. 2007; Van Eerdewegh

et al. 2002). A previous result for the presence of *ADAM33* splice variants in undifferentiated mesenchymal cells suggest that epithelial-mesenchymal transformation protein may contribute to the development of bronchial, vascular, and neuronal structures of the airways and in differentiation of mesenchymal cells to myofibroblasts, such as fibroblasts in these structures (Haïtchi et al. 2005). Furthermore, it has been reported that accumulation and activation of fibroblasts beneath the reticular basement membrane is associated with remodeling of the asthmatic airway (Knight et al. 2004), a critical component of AIA. Taken together, these evidences indicate that the epithelial-mesenchymal transition might have an effect on the remodeling response in asthma-related phenotypes, such as AIA.

To our knowledge, results from this study are the first to demonstrate associations between *EMID2* variants and AIA. Our findings provide further evidence that variations in a structural protein can influence the development of several human diseases including those ordinarily thought to be associated with immune system dysfunction. Although replication of the associations reported in an independent study cohort is required to validate these findings, our study may provide a new direction in the formulation of medication for aspirin hypersensitivity in asthmatics.

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Conflicts of interest The authors declare no conflict of interests.

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