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Association analysis of C6 genetic variations and aspirin hypersensitivity in Korean asthmatic patients

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ARTICLE INFO

Article history:

Received 25 November 2010

Accepted 20 May 2011

Available online 7 June 2011

Keywords:

Aspirin-intolerant asthma

C6

Haplotype

Single nucleotide polymorphism

ABSTRACT

There has been increasing evidence that genetic mechanisms contribute to the development of aspirin-intolerant asthma (AIA), a life-threatening disease. The complement component (C6) is a constituent of a biochemical cascade that has been implicated in airway epithelial damage and nasal polyposis, and therefore, may be a risk factor for AIA. To investigate the association between C6 variations and AIA in a Korean asthma cohort, 27 SNPs were selected for genotyping based on previously reported polymorphisms in the HapMap database. Genotyping was carried out using TaqMan assay, and five major haplotypes were obtained in 163 AIA cases and 429 aspirin-tolerant asthma (ATA) controls subjects. Genotype frequency distributions of C6 polymorphisms and haplotypes were analyzed using logistic and regression models. Subsequent analyses revealed a lack of association between C6 genetic variations and AIA. From the initial analyses, marginal associations of rs10512766 ($p = 0.04$ in co-dominant model) and rs4957374 ($p = 0.05$ in dominant model) with AIA did not reach the threshold of significance after multiple testing corrections; thus this study failed to find convincing evidence that variations in C6 gene influence the risk of AIA in a Korean population. However, these preliminary results may contribute to the etiology of aspirin hypersensitivity in Korean asthmatic patients.

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1. Introduction

Although aspirin is a commonly prescribed medication for a broad range of diseases, it can also induce a wide array of adverse reactions, including aspirin-intolerant asthma (AIA), an inflammation of the lungs that is characterized by a triad of chronic rhinosinusitis, presence of nasal polyposis, and exacerbated asthma akin to prolonged viral respiratory infection [1]. The estimated prevalence rate of aspirin intolerance in the general asthmatic population has been reported to be around 4.3–20% [2–4] and is predominant among women [5,6]. Previously, we have identified several variants in the solute carrier family 6 (neurotransmitter transporter, betaine/GABA) member 12 (SLC6A12), fibrous sheath interacting protein 1 (FSIP1), and calcium channel, voltage-dependent, gamma sub-

unit 6 (CACNG6) to be risk factors for AIA [7–9], suggesting that genetic mechanisms play crucial roles in disease development.

The human complement component 6 (C6) gene on 5p13 is a constituent of the complement system, a biochemical cascade that serves as a major effector of the human innate and adaptive immunity, and eliminates virally infected cells. Previously, we have observed a nominal association between C6 and AIA through genome-wide and follow-up studies [10]. During complement activation, C6 interacts with other complement proteins to form the membrane attack complex (MAC), C5b-9, a macromolecular structure that creates pores in the cell membrane, and induces cell lysis. In previous molecular studies involving animal models, C6-deficient polyvinylbenzyl glucose (PVG) rat and Peru-Coppock mouse strains were unable to form MAC [11,12], indicating that without C6, the terminal complex of MAC required for cell lysis cannot be assembled [13]. Recently, the discovery of MAC in the subepithelial basal membrane resulted in the implication of the complement system in epithelial damage and nasal polyposis [14], an important feature of AIA. One of the prevailing hypotheses on the influence of the com-

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plement proteins in AIA lies in the generation of anaphylatoxins in the airways that lead to proteolytic cascade, culminating in lytic membrane attack [15], bronchoconstriction [16], edema formation, as well as activation of mast cells [17], and eosinophils [18]. In genetically susceptible individuals, ingestion of aspirin activates the complement cascade via the classical pathway that results in the generation of anaphylatoxins, which in turn release mediators of the allergic response by a mechanism independent of Immunoglobulin E (IgE) [19,20]. A previous study has demonstrated significant associations of complement proteins, C3a and C4a with AIA as well as forced expiratory volume in the 1 second (FEV₁) during aspirin challenge [21], providing further evidence of the regulatory activity of the complement system in airway hypersensitivity. However, despite the crucial role of the complement cascade in AIA pathogenesis, the importance of the C6 gene in disease susceptibility remains speculative.

Previous candidate positional studies in different populations have provided evidences of genetic predisposition of asthma to the 5p13 locus [22,23]. Prominent asthma candidate genes within the locus include *interleukin-7 receptor (IL7R)*, leukemia inhibitory factor receptor (LIFR), and *prostaglandin E receptor 4 (PTGER4)* [22]. The mapping of C6 to chromosome 5p13 and its role in the complement system makes it a strong positional and functional candidate gene for AIA. Following the hypothesis that C6 influences aspirin hypersensitivity among asthmatic patients, we carried out a case-control analysis investigating the associations between C6 genetic variations and the risk of AIA in a Korean cohort.

2. Subjects and methods

2.1. Study subjects

Asthma patients recruited from four Korean hospitals belonging to the Asthma Genome Research Center. With AIA being a rare condition, blood samples were gathered from 2003 to 2008. Each patient, as diagnosed by trained physicians, showed clinical symptoms that met the criteria for asthma according to the Global Initiative for Asthma (GINA). Evaluation of asthma included dyspnea and wheezing during the past year plus one of the following: (1) airway reversibility measured by a positive bronchodilator response of >15% increase in FEV₁ or >12% increase in FEV₁ plus a 200-ml follow-up inhalation of a short-acting bronchodilator, (2) airway hyperreactivity to <10 mg/ml of PC₂₀ methacholine, or (3) >20% increase in FEV₁ following 2 weeks of treatment with inhaled steroids and long acting bronchodilators [24]. Twenty-four common inhalant allergens (e.g., dust mite, cat fur, dog fur, cockroach, grasses, trees, ragweed pollen; Bencard, Co, Ltd., Brentford, UK) were used in a skin-prick test. Total immunoglobulin E (IgE) was measured using the CAP system (Pharmacia Diagnostics, Uppsala, Sweden). Atopy was defined as a wheal reaction equal to or greater than histamine of 3 mm in diameter. Pulmonary function tests were performed using V_{max} Series 2130 Autobox Spirometry (Sensor Medics, Yorba Linda, CA) according to the guidelines of the American Thoracic Society (ATS) [25]. The reference values of lung functions used were according to the standards of Morris and Polgar [26]. All asthmatic patients underwent oral aspirin challenge (OAC) that was performed with increasing doses of aspirin (10–450 mg) [27] with minor modifications. Subjects reported no increase in asthma symptoms or respiratory tract infections within 6 weeks before the test. Patients with history of aspirin hypersensitivity were given 30 mg, and those having no history of aspirin hypersensitivity started with 100 mg of aspirin orally. Symptoms, external signs (urticaria and angioedema) and FEV₁ were documented every 30 minutes for a period of 2 hours. In the absence of any symptoms or signs suggestive of adverse reaction after 2 hours, 60 mg, or 100 mg of aspirin was administered and the same measurements were repeated every 1 hour, with doses of 450 mg until

the patient developed a reaction. If no reaction occurred 5 hours after the final dose, the test was deemed negative. Changes in the FEV₁ were followed for 5 hours after the final aspirin dose. Aspirin-induced bronchospasm, reflected by the rate (%) of decline in FEV₁, was calculated as the prechallenge FEV₁ minus the post-challenge FEV₁ divided by the prechallenge FEV₁. Categorization of patients was based on individual OAC reactions. Asthmatic patients exhibiting ≥20% decrease in FEV₁ or a 15–19% decrease in FEV₁ with naso-ocular or cutaneous reactions were diagnosed as AIA cases, whereas those demonstrating <15% decrease in FEV₁ without naso-ocular or cutaneous reactions were identified as ATA controls. This study was undertaken with the understanding and written consent of each subject, and the protocols were approved by the Institutional Review Board of the each hospital.

2.2. SNP selection and genotyping

Twenty-seven polymorphisms in the C6 gene were selected and screened from the International HapMap database (release #27) based on linkage disequilibrium (LD) status in the Asian population (Chinese Hans and Japanese), locations (SNPs in exons were preferred) and amino acid changes (nonsynonymous SNPs were preferred). From the 27 variants, 26 SNPs were genotyped using TaqMan [28] assay, whereas one SNP (rs3805711) was genotyped using SNaPshot method [29]. Genotyped data quality was assessed by duplicate DNA checking ($n = 10$; rate of concordance in duplicates of >99%). Assay IDs (Applied Biosystems, Foster City, CA) of each SNP is described in Supplemental Table 1.

2.3. Statistical analyses

Linkage disequilibrium (LD) between all pairs of biallelic loci were determined using Lewontin's D' ($|D'|$) and LD coefficient r^2 were examined using the Haploview algorithm [30]. To determine the association between the C6 genotype distributions in AIA cases and ATA controls, odds ratio (95% confidence interval) and corresponding p values were calculated using logistic analysis controlling for age (continuous value), gender (male = 0, female = 1), smoking status (nonsmoker = 0, ex-smoker = 1, smoker = 2), atopy (absence = 0, presence = 1), and body mass index (BMI) as covariates to eliminate or reduce any confounds that might influence the findings. Data were managed and analyzed on the Statistical Analysis System (SAS) version 9.1 (SAS Inc., Cary, NC). In addition, the differences in the fall rate of FEV₁ after aspirin challenge among C6 genotypes and haplotypes were examined using regression analysis adjusted for age, gender, smoking status and atopy as covariates.

To achieve optimal correction for multiple testing of markers representing SNPs in LD with each other, the effective number of independent marker loci (21.3107) was calculated using SNPSpD (<http://genepi.qimr.edu.au/general/daleN/SNPSpD/>), a program that is based on the spectral decomposition (SpD) of matrices of pairwise LD between markers [31].

3. Results

3.1. Clinical characteristics of study subjects

Over a 5-year period of data gathering, a total of 592 asthmatic patients were recruited for our study; 163 subjects were identified as AIA patient cases and 429 subjects were classified as ATA control subjects based on the results of the aspirin provocation test. Table 1 depicts the clinical characteristics of the study subjects. It was observed 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 clinical profiles showed a mean age of 43.1 years for AIA patients and 47.3 years for ATA controls. Results of the aspirin provocation test showed significant differences in aspirin-induced fall rate of FEV₁ between AIA patients and ATA controls (24.63 in AIA and 3.54 in ATA; $p < 0.0001$). The predicted FEV₁ percentage, smoking sta-

Table 1Clinical profiles of the study subjects ($n = 592$)

Clinical profile	Asthmatic patients (all subjects)	AIA patients	ATA patients
No. of subjects (n)	592	163	429
Age, y, mean (range)	46.15 (15.40–77.88)	43.13 (17.22–72.73) ^a	47.30 (15.40–77.88)
Gender, male/female (n)	206/386	59/104	147/282
Total smoker (current smoker; ex-smoker) (%)	27.70 (12.50; 15.20)	21.47 (12.88; 8.59) ^a	30.07 (12.35; 17.72)
Height (cm)	160.78 ± 8.63	161.72 ± 8.69	160.42 ± 8.39
Weight (kg)	62.81 ± 10.84	61.25 ± 10.38 ^a	63.40 ± 10.97
Body mass index (kg/m ²)	24.24 ± 3.39	23.39 ± 3.25 ^a	24.58 ± 3.39
% fall of FEV ₁ by aspirin provocation	9.27 ± 13.24	24.63 ± 16.11 ^b	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 ^a	91.66 ± 16.87
PC20 methacholine (mg/ml)	6.43 ± 8.67	5.02 ± 7.83 ^a	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
Positive rate of nasal polyp (%)	33.83	57.89 ^b	26.06
Positive rate of the history of aspirin hypersensitivity (%)	18.50	51.92 ^b	6.00

Values are mean ± SE. BMI, body mass index; AIA, aspirin-intolerant asthma; ATA, aspirin-tolerant asthma.

Some factors are significant at ^a $P < 0.05$; ^b $P < 0.0001$.

tus, and PC20 methacholine were significantly lower in AIA patients than those of ATA controls ($p < 0.05$). Furthermore, there were significantly higher rates of nasal polyps in AIA patients (57.89) compared with ATA controls (26.06), suggesting that aspirin-intolerance can elicit meaningful deficits in the upper airways. Histories of aspirin hypersensitivity were also found to be significantly prevalent in AIA patients (51.92) compared with ATA subjects (6.00).

3.2. Distribution of C6 variants

Among the polymorphisms, an SNP was positioned in the promoter region of the gene, 23 were localized in the noncoding introns, and three were found in the coding regions of the exons (Table 2, Fig. 1A). The minor allele frequency (MAFs) of each SNP are

Table 2

Genotype and allele distribution of C6 variants

Loci	Position	Allele	Allele change	MAF	Heterozygosity
rs2921174	Promoter	G>T		0.170	0.282
rs10512766	Intron1	C>G		0.096	0.173
rs13359770	Intron1	T>C		0.140	0.240
rs1822821	Intron1	G>C		0.351	0.456
rs1444903	Intron1	C>T		0.190	0.308
rs2305060	Exon2	C>T		0.140	0.240
rs1801033	Exon4	C>A	Ala119Glu	0.448	0.495
rs7443604	Intron4	G>T		0.448	0.495
rs6892389	Intron4	A>G		0.200	0.321
rs10473238	Intron4	C>T		0.140	0.240
rs11951782	Intron5	T>A		0.171	0.283
rs4413571	Intron5	T>A		0.278	0.401
rs6860770	Intron6	A>C		0.140	0.240
rs4245976	Intron6	C>T		0.170	0.282
rs11954878	Intron6	C>T		0.340	0.449
rs4957375	Intron9	C>T		0.276	0.399
rs6451566	Intron9	C>T		0.277	0.401
rs3805715	Intron13	A>G		0.375	0.469
rs10512764	Intron13	C>T		0.417	0.486
rs3805714	Intron13	T>A		0.042	0.081
rs2301247	Intron14	A>G		0.139	0.239
rs4957374	Intron14	C>A		0.160	0.268
rs918608	Intron15	T>C		0.381	0.472
rs1014588	Intron17	G>A		0.329	0.442
rs3805711	Intron17	C>T		0.333	0.444
rs3805710	Intron17	A>G		0.072	0.133
rs9200	Exon18	A>G		0.246	0.371
C6_h1				0.269	0.393
C6_h2				0.178	0.293
C6_h3				0.128	0.223
C6_h4				0.113	0.201
C6_h5				0.069	0.129

MAF, minor allele frequency.

shown in Table 2. Using the genotyped SNPs, five major haplotypes with frequencies >0.05 (Fig. 1B) were obtained and analyzed for comparison between AIA and ATA. The haplotypes were contained in one LD block (Fig. 1C) that was established from pairwise comparisons of the 27 genotyped SNPs.

3.3. Association of C6 variants with risk of aspirin intolerance among asthmatic patients

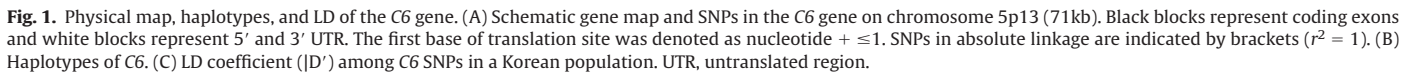
Two intronic SNPs, rs10512766C > G (OR = 0.60, 95% confidence interval [95% CI] 0.37–0.98, $p = 0.04$) and rs4957374C > A (OR = 1.47, 95% CI 0.99–2.19, $p = 0.05$) showed marginal associations with the risk of AIA via co-dominant and dominant mechanisms, respectively (Table 3). However, with 21.3107 as the effective number of independent marker loci used for corrections, the association signals detected in both C6 genetic variations disappeared after multiple testing comparisons.

Because the fall rate of FEV₁ is considered as an important diagnostic marker of AIA, we further assessed the differences in C6 variations in the fall rate of FEV₁ among AIA patients. Results from regression analysis adjusted for age, gender, smoking status, and atopy as covariates showed that the variant rs4957374, which was found to be associated with AIA susceptibility in logistic analysis, also showed nominal association signals in a dominant mode of genetic inheritance with fall rate of FEV₁ after the aspirin provocation test ($p = 0.05$). Similarly, the value did not reach the threshold of multiple comparisons.

4. Discussion

One of the serious threats of aspirin is exacerbation of acute asthma attacks that can defy the therapeutic effects of fast-acting medications. Previous studies have reported that 25% of asthma patients who required emergency mechanical ventilation are aspirin intolerant, suggesting that ingestion of aspirin may be a factor in life-threatening asthma attacks [32,33]. In the ongoing investigation for the underlying mechanisms of AIA, combinatorial effects of both environmental and genetic risk factors have been elucidated in disease pathogenesis. Etiology of airway inflammation have identified mast cell degranulation [34], subepithelial fibrosis [35], overproduction of pro-inflammatory cytokines and immunoglobulins [36], and the innate immune system [37] to be predisposing factors of asthma. Findings from this study reveal further insights into the events of immune system dysfunction in AIA.

Sequential interaction among component proteins leads to initiation and/or amplification of the complement cascade of inflammatory response [37]. This lytic effector system provides protection against microbial pathogens and mediates innate and specific



Generally, the promoter and exonic regions of the gene harbor functionally relevant SNPs that are associated with binding affinity

Table 3

Association analysis of C6 polymorphisms and haplotypes with AIA

Loci	Position	MAF		Co-dominant			Dominant			Recessive		
		AIA (n = 163)	ATA (n = 429)	OR (95% CI)	p ^a	p _{corr} ^b	OR (95% CI)	p ^a	p _{corr} ^b	OR (95% CI)	p ^a	p _{corr} ^b
rs2921174	Promoter	0.185	0.161	1.22 (0.85–1.74)	0.29	—	1.18 (0.80–1.75)	0.41	—	2.20 (0.59–8.18)	0.24	—
rs10512766	Intron1	0.061	0.105	0.60 (0.37–0.98)	0.04	NS	0.60 (0.35–1.03)	0.07	—	0.22 (0.03–1.72)	0.15	—
rs13359770	Intron1	0.129	0.146	0.89 (0.61–1.30)	0.55	—	0.91 (0.59–1.39)	0.65	—	0.64 (0.17–2.36)	0.50	—
rs1822821	Intron1	0.380	0.344	1.17 (0.90–1.51)	0.25	—	1.30 (0.89–1.90)	0.17	—	1.10 (0.66–1.86)	0.71	—
rs1444903	Intron1	0.178	0.195	0.86 (0.62–1.19)	0.36	—	0.85 (0.57–1.26)	0.41	—	0.73 (0.28–1.87)	0.51	—
rs2305060	Exon2	0.129	0.146	0.89 (0.61–1.30)	0.55	—	0.91 (0.59–1.39)	0.65	—	0.64 (0.17–2.36)	0.50	—
rs1801033	Exon4	0.445	0.449	0.99 (0.77–1.29)	0.97	—	1.09 (0.73–1.62)	0.69	—	0.88 (0.55–1.40)	0.60	—
rs7443604	Intron4	0.445	0.449	0.99 (0.77–1.29)	0.97	—	1.09 (0.73–1.62)	0.69	—	0.88 (0.55–1.40)	0.60	—
rs6892389	Intron4	0.187	0.206	0.86 (0.62–1.18)	0.34	—	0.87 (0.59–1.28)	0.47	—	0.64 (0.25–1.61)	0.34	—
rs10473238	Intron4	0.129	0.146	0.89 (0.61–1.29)	0.54	—	0.90 (0.59–1.39)	0.64	—	0.64 (0.17–2.36)	0.50	—
rs11951782	Intron5	0.190	0.161	1.27 (0.89–1.81)	0.19	—	1.24 (0.84–1.83)	0.28	—	2.17 (0.58–8.08)	0.25	—
rs4413571	Intron5	0.242	0.290	0.81 (0.60–1.08)	0.15	—	0.82 (0.57–1.20)	0.31	—	0.58 (0.28–1.20)	0.14	—
rs6860770	Intron6	0.129	0.146	0.89 (0.61–1.29)	0.54	—	0.90 (0.59–1.39)	0.64	—	0.64 (0.17–2.36)	0.50	—
rs4245976	Intron6	0.184	0.162	1.20 (0.84–1.72)	0.33	—	1.20 (0.81–1.77)	0.37	—	1.56 (0.37–6.57)	0.54	—
rs11954878	Intron6	0.368	0.332	1.16 (0.90–1.51)	0.25	—	1.26 (0.87–1.84)	0.22	—	1.16 (0.69–1.96)	0.58	—
rs4957375	Intron9	0.282	0.277	1.01 (0.76–1.34)	0.97	—	1.19 (0.83–1.73)	0.35	—	0.54 (0.25–1.16)	0.11	—
rs6451566	Intron9	0.291	0.275	1.09 (0.82–1.46)	0.54	—	1.18 (0.82–1.71)	0.38	—	0.94 (0.48–1.85)	0.86	—
rs3805715	Intron13	0.361	0.379	0.93 (0.72–1.21)	0.58	—	0.83 (0.57–1.21)	0.34	—	1.06 (0.64–1.75)	0.83	—
rs10512764	Intron13	0.411	0.418	0.97 (0.75–1.26)	0.83	—	0.92 (0.63–1.36)	0.68	—	1.03 (0.64–1.65)	0.92	—
rs3805714	Intron13	0.052	0.040	1.39 (0.75–2.60)	0.30	—	1.47 (0.77–2.80)	0.24	—	—	0.99	—
rs2301247	Intron14	0.113	0.146	0.79 (0.54–1.15)	0.22	—	0.81 (0.52–1.27)	0.36	—	0.42 (0.12–1.50)	0.18	—
rs4957374	Intron14	0.190	0.149	1.39 (0.98–1.96)	0.07	—	1.47 (0.99–2.19)	0.05	NS	1.26 (0.38–4.20)	0.71	—
rs918608	Intron15	0.365	0.385	0.93 (0.71–1.20)	0.56	—	0.83 (0.57–1.21)	0.34	—	1.04 (0.63–1.72)	0.88	—
rs1014588	Intron17	0.344	0.328	1.05 (0.81–1.37)	0.70	—	1.14 (0.79–1.65)	0.49	—	0.94 (0.54–1.63)	0.82	—
rs3805711	Intron17	0.344	0.333	1.02 (0.79–1.33)	0.87	—	1.10 (0.76–1.59)	0.63	—	0.91 (0.52–1.57)	0.73	—
rs3805710	Intron17	0.052	0.077	0.68 (0.39–1.19)	0.17	—	0.64 (0.35–1.15)	0.14	—	1.39 (0.12–16.58)	0.80	—
rs9200	Exon18	0.242	0.245	1.01 (0.75–1.35)	0.95	—	1.06 (0.73–1.54)	0.77	—	0.86 (0.42–1.76)	0.68	—
C6_h1		0.270	0.273	0.96 (0.72–1.29)	0.81	—	1.14 (0.79–1.65)	0.48	—	0.47 (0.21–1.06)	0.07	—
C6_h2		0.175	0.181	0.92 (0.66–1.28)	0.63	—	0.93 (0.62–1.39)	0.72	—	0.78 (0.30–2.01)	0.60	—
C6_h3		0.150	0.120	1.34 (0.91–1.96)	0.14	—	1.33 (0.88–2.03)	0.18	—	2.17 (0.50–9.43)	0.30	—
C6_h4		0.104	0.120	0.85 (0.56–1.29)	0.45	—	0.86 (0.54–1.36)	0.52	—	0.59 (0.12–2.89)	0.51	—
C6_h5		0.052	0.073	0.71 (0.41–1.25)	0.24	—	0.67 (0.37–1.22)	0.19	—	1.39 (0.12–16.58)	0.80	—

NS, not significant.

Co-dominant, dominant and recessive models of logistic regression analyses was used to calculate odds ratios (95% confidential interval) controlling for age, gender, smoking status, atopy and BMI as covariates.

Odds ratio (95% confidence interval [CI]) and *p* values in the co-dominant model are also reported in the supporting information of our previous paper [10].^a*p* Values at 0.05 level of significance.^b*p* Values after multiple testing corrections (*p* = 21.3107).

of transcription factors and regulation of gene expression, whereas polymorphisms located in the noncoding intron have been originally thought to have no role in protein function. However, previous reports have revealed that intronic genetic variants may also induce exon skipping, activate cryptic spliced sites, alter the balance of the alternatively spliced isoforms, or affect splicing efficiency or messenger RNA turnover [42,43]. These processes may lead to synthesis of proteins that influence disease phenotypes, and thereby may underlie the associations between intronic variants and disease susceptibility. Despite the lack of association detected in this study, the functional significance of other intronic genetic polymorphisms with aspirin hypersensitivity among asthmatic patients, therefore, may be a manifestation of the coordinated regulation of transcription and splicing in the nucleus.

To conclude, our findings do not support a major role of C6 gene variations with the risk of AIA and a related phenotype in a Korean population. To our knowledge, this study is the first to investigate the association between C6 variants and AIA. Although replication studies in larger cohorts are required to validate these findings, our results may clarify the function of C6 in aspirin-induced airway obstruction and provide useful insights for future research.

Acknowledgments

This work was supported by Korea Science and Engineering Foundation (KOSEF) funded by the Korean government (MEST) (No. 2009-0080157), a Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (No. 2009-0093822), and

a grant from the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A010249). The DNA samples were generously provided by Soonchunhyang University, Bucheon Hospital Biobank, a member of the National Biobank of Korea, supported by the Ministry of Health, Welfare and Family Affairs, Republic of Korea.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.humimm.2011.05.022.

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