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Association analysis of *N*-acetyl transferase-2 polymorphisms with aspirin intolerance among asthmatics

Aims: Cysteinyl leukotrienes are inactivated by acetyl coenzyme A-dependent *N*-acetyltransferase (NAT). Thus, functional alterations of the *NAT* gene may contribute to the risk of aspirin-intolerant asthma. **Materials & methods:** Asthmatics ($n = 438$) were categorized into aspirin-intolerant asthma (15% or greater decrease in the forced expiratory volume in 1 s or cutaneous reactions, $n = 170$) or aspirin-tolerant asthma ($n = 268$) groups. In total, 14 polymorphisms of the *NAT2* gene were genotyped by a single-base extension method. **Results:** The distributions of all loci of the 14 SNPs were in Hardy–Weinberg equilibrium ($p > 0.05$). Among the 14 SNPs, six common SNPs (minor allele frequency $> 5\%$) in a Korean population were used for haplotype construction and further statistical analysis. The logistic regression analysis demonstrated that *NAT2* -9246G>C and haplotype 2 (TCACGG) were significantly associated with the risk of aspirin-intolerant asthma. The rare allele frequencies of the SNP and *Ht2* were significantly higher in the aspirin-intolerant asthma group than in the aspirin-tolerant asthma group ($p^{\text{corr}} = 0.03$ and $p^{\text{corr}} = 0.02$ in codominant model). **Conclusion:** In a large genetic epidemiology study of aspirin-intolerant asthma in a Korean population, genetic polymorphisms of *NAT2* were found to be related to a risk of aspirin hypersensitivity among asthmatics.

KEYWORDS: arylamine *N*-acetyltransferase • aspirin-induced asthma
• cysteinyl leukotriene • SNP

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Aspirin-intolerant asthma (AIA) refers to the development of bronchoconstriction in individuals with asthma following the ingestion of aspirin or other NSAIDs. This syndrome is characterized by the ‘aspirin triad’ syndrome of aspirin hypersensitivity, bronchial asthma and nasal polypsis, along with chronic hyperplastic eosinophilic sinusitis [1,2]. As in other individuals with asthma, the airways of patients with AIA show signs of persistent inflammation, with marked eosinophilia, epithelial disruption, cytokine production and upregulation of inflammatory molecules [3].

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 overexpression of the CysLT receptor on inflammatory cells occur within the respiratory tract [4]. The overproduction of CysLTs has been demonstrated in the airways and circulation of people with asthma who are intolerant to aspirin, via the inhibition of COX by the aspirin shunting of arachidonic acid to the 5-lipoxygenase pathway [5–7]. The enhanced activities of key synthetic enzymes, such as leukotriene C4 synthase (LTC4S) [8] and 5-lipoxygenase (ALOX5) [9], have been implicated in the elevated production of CysLTs in patients with AIA. CysLTs are important inflammatory

mediators in the development of asthma, as they mediate bronchoconstriction while increasing mucus secretion, vascular permeability and cellular infiltration [10]. The CysLTs, comprising leukotriene C4 and its metabolites, are the most biologically active mediators and are eliminated from blood circulation by the liver and kidneys. The proinflammatory CysLTs can also be inactivated by *N*-acetylation [11], and the ω -backbone of leukotrienes is subject to carboxylation and β -elimination [12]. The CysLT Ω -carboxy-*N*-acetyl-LTE4 was exclusively degraded in peroxisomes [13]. Thus, *N*-acetylation and ω -modification of the leukotriene structure are associated with a loss of leukotriene bioactivity [14].

Human acetyl coenzyme A-dependent *N*-acetyltransferase (NAT; EC 2.3.1.5) catalyzes the biotransformation of many arylamine and hydrazine compounds. NAT isozymes are encoded at two loci; one encodes NAT1, formerly known as the monomorphic form of the enzyme, while the other encodes the polymorphic NAT2, which is responsible for individual differences in the ability to acetylate certain compounds. The human *NAT1* and *NAT2* genes are located on chromosome 8p23.1-p21.3 [15]. Expression of both NAT1 and NAT2 mRNAs has been observed in the liver, GI tract tissues, urinary tract and lungs [16]. Considering

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the inactivation of CysLTs by *N*-acetylation, the genetic variants of *NAT* genes may be associated with development of aspirin hypersensitivity in asthma. However, to the best of our knowledge, no previous study has analyzed the potential associations between *NAT2* gene polymorphisms and aspirin intolerance in persons with asthma. In an effort to discover additional polymorphism(s) in these genes, the variants of which have been implicated in aspirin intolerance, we scrutinized the genetic polymorphisms of the *NAT2* gene, and evaluated this locus as a potential candidate for aspirin intolerance in people with asthma. The SNPs were analyzed in a Korean population ($n = 438$), and statistical analyses were performed to examine the genetic effects on aspirin intolerance in those with asthma.

Materials & methods

■ Subjects

The subjects were recruited from the Asthma Genome Research Center, which consists of six university hospitals (Soonchunhyang University Hospital, Chungbuk National University Hospital, Chonnam National University Hospital, Seoul National University Hospital, Chung-Ang University Yongsan Hospital and Ajou University Hospital) in Korea. All the subjects were Korean. All the patients were diagnosed by a physician and met the definition of asthma set forth in the Global Initiative for Asthma (GINA) guidelines [17]. All the patients had a history of dyspnea and wheezing during the previous 12 months, plus one of the following:

- More than 15% increase in forced expiratory volume in 1 s (FEV_1) or a more than 12% plus 200 ml increase following inhalation of a short-acting bronchodilator;
- Less than 10 mg/ml for the provocative concentration of methacholine producing a 20% decline in FEV_1 (PC_{20});
- More than 20% increase in FEV_1 following 2 weeks of treatment with inhaled steroids and long-acting bronchodilators.

A total of 24 common inhalant allergens (e.g., dust mites [*Dermatophagoides farinae* and *D. pteronyssinus*], cat fur, dog fur, cockroaches, grasses, trees and ragweed pollen; Bencard Co. Ltd, Brentford, UK) were used for a skin prick test. Atopy was defined as one or more positive reactions (>3mm in diameter) on the skin prick test. Total IgE was measured by the CAP system (Pharmacia Diagnostics, Uppsala, Sweden).

The patients with asthma had experienced no exacerbation of the disease and respiratory tract infection in the 6 weeks preceding the oral aspirin challenge. 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 previously described [18,19].

Changes in FEV_1 were followed for 5 h after the last aspirin challenge dose. Aspirin-induced bronchospasms, as reflected by the rate (%) of FEV_1 decline, were calculated as the prechallenge FEV_1 minus the post-challenge FEV_1 divided by the prechallenge FEV_1 . Oral aspirin challenge reactions were categorized as follows: 15% or greater decrease in FEV_1 or the appearance of cutaneous reactions (AIA) and 15% or less decrease in FEV_1 without naso-ocular or cutaneous reactions (aspirin-tolerant asthma). The subjects and study protocols were approved by the Institutional Review Board of Soonchunhyang University Hospital (Bucheon, South Korea). All subjects gave informed consent to participate in the study, and the protocols were approved by the Institutional Review Board of Soonchunhyang University Hospital.

■ Genotyping

A total of 14 polymorphisms of the *NAT2* gene found in the National Center for Biotechnology Information (build 36) were selected for genotyping in this study. The amplification and extension primers were designed for genotyping the polymorphic sites by a single-base extension [20]. All primer extension reactions were performed using the SNaPshot® ddNTP Primer Extension Kit (Applied Biosystems, CA, USA) and extension primers were designed for genotyping of the polymorphic sites by a single-base extension. For subsequent clean-up, 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 analyzed using GeneScan® and Genotyper® (Applied Biosystems). Each genotype was verified using the single-base extension method. The accuracy was verified by duplicate in eight samples as positive controls and eight blanks as negative controls. A total of

438 subjects were genotyped. The genotyping success rates of *NAT2* SNPs were 99.2–100%. Among the 438 patients genotyped, eight subjects had incomplete clinical parameters. Thus, genotyping data from a total of 430 subjects were analyzed for statistics and presented in the results.

■ Statistics

Six out of 14 common SNPs (minor allele frequency >5%) from a Korean population were used for haplotype construction and statistical analysis. We applied widely used measures of linkage disequilibrium (LD) to all pairs of biallelic loci: Lewontin's D' ($|D'|$) [21] and r^2 . The haplotypes of individuals were inferred using the PHASE algorithm (version 2.0) developed by Stephens *et al.* [22]. The genotype and haplotype distribution was analyzed using logistic regression models with age (continuous value), gender (male = 0, female = 1), smoking status (nonsmoker = 0, exsmoker = 1, smoker = 2), atopy (absence = 0, presence = 1) and BMI as covariates. The differences in the rates of decline in FEV₁ following the aspirin challenge among the genotypes and haplotypes were examined using a logistic regression model. The data were managed and analyzed using SAS version 9.1 (SAS Inc., NC, USA) and SPSS version 11.0 (SPSS Inc., IL, USA). For the correction of p-values, the effective number of independent markers in *NAT2* was calculated using the SNPSpD software [23,101], which is based on the spectral decomposition of matrices of pairwise LD between SNPs ($p\text{-value} \times 3.5863$).

The data are expressed as the mean $\pm$ standard deviation of the mean (SD). A p-value of less than 5% was regarded as being statistically significant.

Results

The clinical characteristics of the study subjects are summarized in TABLE 1. Significant differences were observed in mean age, age of onset, smoking status and BMI between the aspirin-tolerant asthma and AIA groups. The maximum fall in FEV₁ during the follow-up period of 5 h post-challenge with aspirin was used as the value for aspirin-induced fall in FEV₁. An aspirin-induced fall in FEV₁ of -15 to 68% was observed.

A total of 14 polymorphisms of *NAT2* were genotyped in the study subjects. The minor allele frequencies of these 14 SNPs in the Korean population are shown in TABLE 2. The distributions of all loci were in Hardy–Weinberg equilibrium ($p > 0.05$). The linkage disequilibrium coefficients ($|D'|$) and r^2 among six SNPs of the *NAT2* gene were calculated for all of the study subjects (FIGURE 1A & C). Absolute LD ($|D'| = 1$ and $r^2 = 1$) was present among -4759T>C, -4105C>G and +803A>G, and between +341T>C and +481C>T on the *NAT2* gene. The minor allele frequencies of the polymorphisms ranged from 0.005 to 0.5. Among the 14 SNPs, six common SNPs of a frequency greater than 0.05 were used for haplotype construction and statistical analysis (FIGURE 1).

The association of each polymorphism and haplotype with the risk of aspirin hypersensitivity was analyzed using multiple logistic

Table 1. Clinical profiles of the study subjects.

Clinical characteristic	ATA	AIA	p-value
Patients (n)	268	170	–
Age, years (range)	47.84 (15–79)	43.66 (17–73)	0.002
Duration, years (range)	6 (1–40)	7 (1–57)	0.38
Age of onset, years (range)	41 (1–76)	36 (1–70)	0.01
Sex (male, %)	32.46%	36.47%	0.39
Smoker/exsmoker (%)	15.67/17.54	13.53/8.82	0.02
Atopy (%)	56.34%	50.91%	0.27
FVC (% predicted)	87.83 $\pm$ 14.90	90.37 $\pm$ 14.59	0.08
FEV ₁ (% predicted)	89.75 $\pm$ 18.46	87.12 $\pm$ 17.44	0.14
log (PC ₂₀ methacholine; mg/ml)	0.46 $\pm$ 0.31	0.50 $\pm$ 0.44	0.33
BMI (kg/m ²)	24.56 $\pm$ 3.52	23.69 $\pm$ 3.29	0.01
Blood eosinophil (%)	6.22 $\pm$ 5.61	5.84 $\pm$ 5.13	0.47
log (total IgE; IU/ml)	2.17 $\pm$ 0.62	2.23 $\pm$ 0.53	0.31
Rate (%) of FEV ₁ decline after aspirin challenge	3.48 $\pm$ 4.52	24.77 $\pm$ 6.33	<0.0001

Values are mean $\pm$ standard deviation.
p-values are obtained using the t-test or the χ^2 -test between ATA and AIA.
AIA: Aspirin-intolerant asthma; ATA: Aspirin-tolerant asthma; FEV₁: Forced expiratory volume in 1 s; FVC: Forced vital capacity; PC₂₀ methacholine: Provocative concentration of methacholine producing a 20% decline in FEV₁.

Table 2. Frequency of *NAT2* gene polymorphisms in total study subjects.

Locus	Amino acid change	rs number	Region	Genotype				Frequency	Heterozygosity	Hardy–Weinberg equilibrium
				C/C	C/R	R/R	N			
-9796T>A		rs4646244	Promoter	258	153	25	436	0.233	0.357	0.713
-9246G>C		rs4271002	Promoter	272	144	21	437	0.213	0.335	0.730
-8853A>G		rs4646246	Promoter	111	215	111	437	0.500	0.500	0.738
-6592T>C		rs9987109	Intron 1	421	17	0	438	0.019	0.038	0.679
-4759T>C		rs1390358	Intron 1	420	16	0	436	0.018	0.036	0.696
-4105C>G		rs923796	Intron 1	422	15	0	437	0.017	0.034	0.715
-1638C>T		rs1112005	Intron 1	273	140	25	438	0.217	0.340	0.216
-855C>T		rs973874	Intron 1	434	4	0	438	0.005	0.009	0.924
-234C>T		rs7832071	Intron 1	424	14	0	438	0.016	0.031	0.734
+341T>C	Ile114Thr	rs1801280	Exon 2	426	12	0	438	0.014	0.027	0.771
+481C>T	Leu161Leu	rs1799929	Exon 2	426	12	0	438	0.014	0.027	0.771
+590G>A	Arg197Gln	rs1799930	Exon 2	278	134	23	435	0.207	0.328	0.201
+803A>G	Lys268Arg	rs1208	Exon 2	421	16	0	437	0.018	0.036	0.697
+1395G>A		rs4646247	3'-UTR	275	138	25	438	0.215	0.337	0.171

Loci are calculated from the translational start site. Frequency means the frequencies of rare alleles.
NAT2: *N*-acetyltransferase 2.

regression models. One SNP (-9246 G>C) and a haplotype (*ht2* [TCACGG]) were significantly associated with aspirin intolerance asthma (odds ratios of 1.61 and 1.62 with both being codominant, respectively). The rare allele frequencies of -9246G>C and *ht2* (TCACGG) were significantly higher in the AIA group than in the aspirin-tolerant asthma group using the codominant model ($p = 0.007$ and $p = 0.006$, respectively; ONLINE SUPPLEMENTARY TABLE 1; WWW.futuremedicine.com/doi/suppl/10.2217/pgs.10.65) and the statistical significances of these two polymorphisms were retained after correction (p -values after multiple correction [p^{corr}] = 0.03 and 0.02 in the codominant model, respectively).

Discussion

The molecular genetics of *NAT* in humans has revealed the presence of two loci that encode distinct but very similar enzymes [15]. In humans, two classes of substrate are metabolized by different gene products, referred to as *NAT1* and *NAT2* [15]. *NAT2* acetylates isoniazid, certain sulfonamides (e.g., sulfamethazine), dapsone [24] and arylamine carcinogens (e.g., aminofluorene) [25]. The tissue distribution of *NAT2* is that of a typical drug-metabolizing enzyme found in the liver and intestinal epithelium [26,27]. The CysLTs, comprising leukotriene C4 and its metabolites, are biologically the most active mediators, which are eliminated from blood circulation by the liver and kidneys. Exogenously administered LTE4 can be *N*-acetylated, or the ω -backbone of the leukotriene can be subjected to carboxylation and β -elimination [11,12,28,29].

The *N*-acetylation and ω -modification of the leukotriene structure are associated with a loss of leukotriene bioactivity [14]. Thus, the nucleotide variants in *NAT* genes may be associated with the development of aspirin hypersensitivity in asthma.

The limitation of the present study is the absence of direct experimental evidence demonstrating the role of *NAT2* in synthesis of *N*-acetyl-LTE₄. However, in a study of LTE₄ metabolism performed in three healthy human subjects by Sala *et al.*, in the urine collection after LTE₄ administration, the minor metabolite was identified as *N*-acetyl-LTE₄ [30]. Owing to its low concentration of *N*-acetyl-LTE₄ in urine under pathological conditions such as liver cirrhosis and hepatorenal syndrome [31]. There has been no definite experimental evidence of the presence of *N*-acetyl-LTE₄ in the urine of asthmatics. However, our genetic association study may highlight the possible role of *NAT2* in the metabolism of CysLTs, which is to be established in further research.

In the present study, we are the first to demonstrate that asthmatics with allele polymorphisms in *ht2* and -9246G>C are associated with enhanced response to aspirin provocation. These data indicate that *NAT2* polymorphisms are associated with aspirin intolerance in asthmatics. The frequency of *ht2* haplotype is 21.1% in our study. *ht2* haplotype is tightly tagged with -9246G>C. Almost all rare allele of -9246G>C compose 99.5% of *ht2* haplotype. Thus, the association of *Ht2* haplotype with aspirin hypersensitivity is derived from the nucleotide sequence variant -9246G>C.

The functional significance of -9246G>C polymorphism in *NAT2* gene regulation is still unknown. We searched the Asian data in the HapMap database [102]. -9246G>C (rs4271002) has no linkage with the SNPs on the adjacent region (-15 to +10 kb) of *NAT2* gene. This SNP was not included in CpG island methylation when searched for methylator for DNA methylation prediction [103]. We revealed putative binding sites for candidate transcription factor around the G allele of -9246 G>C SNPs such as upstream stimulatory factor (USF) 1 using a Transcription Element Search System (TESS) to predict transcription factor-binding sites in DNA sequence [104]. USF1 is a member of the USF family, along with USF2, and the USFs are key regulatory

elements of the transcriptional machinery mediating the recruitment of chromatin-remodeling enzymes and interacting with coactivators and members of the preinitiation complex [32]. Thus, the different binding affinity of USF or other transcription factors might be responsible for the induction of the *NAT2* gene expression. These data suggest that the promoter SNP may modulate the amount of *NAT2* gene products, leading to aspirin hypersensitivity. Acetylation status of *NAT2* gene seems to be associated with several kinds of diseases, such as colon cancer, rheumatoid arthritis and systemic lupus erythematosus. Rapid and slow acetylators reflect the genetically determined variation in the elimination of xenobiotics as well as in *NAT2* in the liver and other

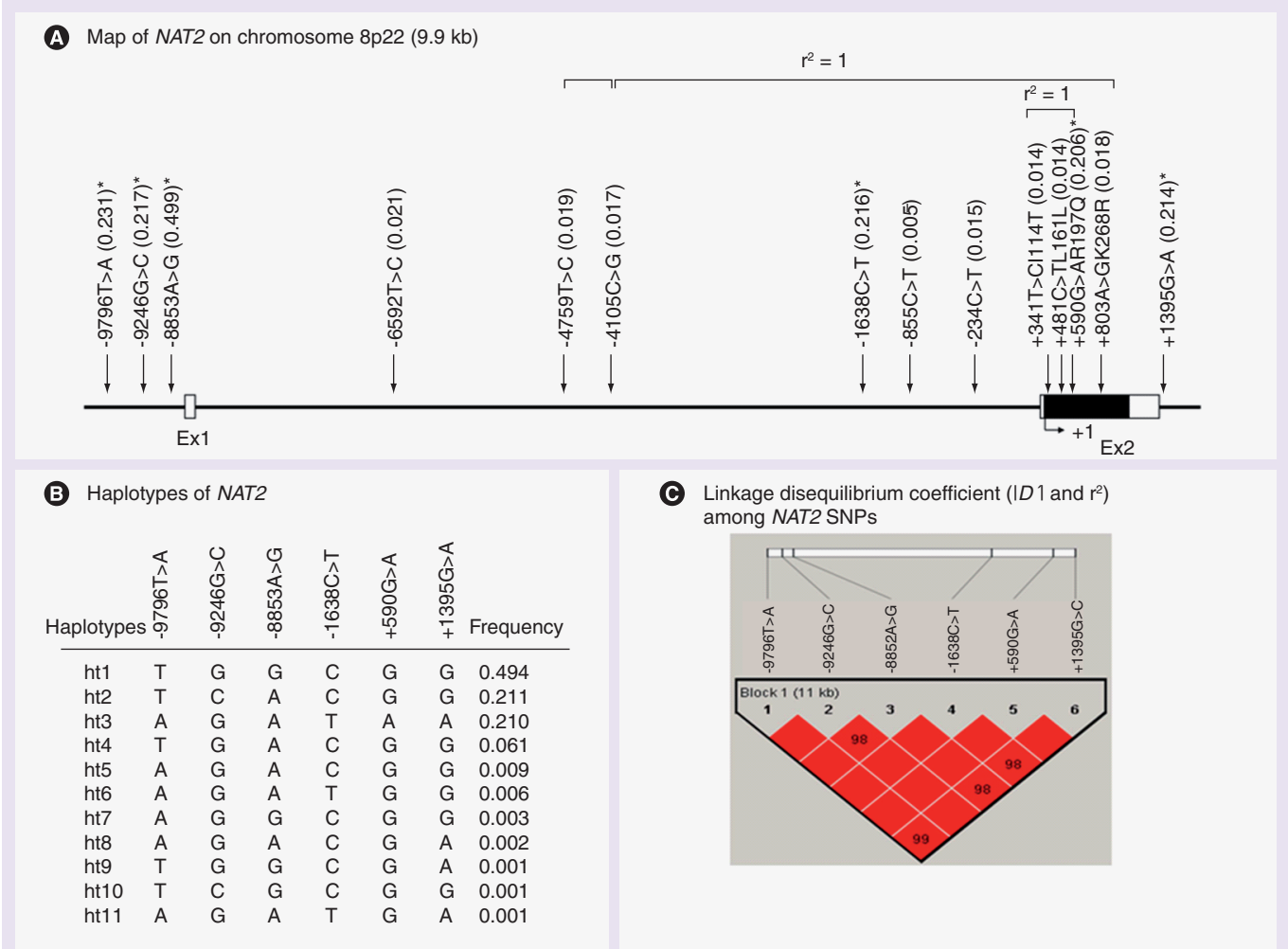


Figure 1. Gene map, haplotypes and linkage disequilibrium of SNPs in the *NAT2* gene. (A) Gene map and SNPs in the *NAT2* gene on chromosome 8p22. Coding exons are marked as black blocks and 5'- and 3'-UTRs as white blocks. The first base of the translation site was denoted as nucleotide+1. A total of 14 SNPs, as shown here, were genotyped. The linkage disequilibrium coefficients (*ID'* and *r*²) of these 14 SNPs are shown in the ONLINE SUPPLEMENTARY TABLE 2; www.futuremedicine.com/doi/suppl/10.2217/pgs.10.65. Among these SNPs, six SNPs (indicated by asterisks) of minor allele frequencies greater than 5% were selected for association analysis. The haplotypes and linkage disequilibriums of *NAT2* were constructed using these SNPs. (B) Haplotypes of *NAT2*. (C) Linkage disequilibrium coefficient (*ID'* and *r*²) among *NAT2* SNPs. *NAT2*: N-acetyltransferase 2.

tissues. [33]. It may be one of these factors that make an individual susceptible to the development of an atopic disease. It was demonstrated that a slow acetylation rate may be a factor that influences the development of allergic diseases. A *NAT2* genetic polymorphism (+590G>A) was found to be associated with the risk of developing atopic diseases among a white Polish population [34,35], along with the development of childhood asthma [36,37]. The putative slow acetylator *NAT1* genotypes posed a 2.54-fold risk of di-isocyanate-induced asthma [38]. Based on the above considerations, subjects with asthma and slow *N*-acetylation capacity may be at increased risk of aspirin-induced hypersensitivity. As we did not evaluate the effect of promoter activity of -9246G>C on the *NAT2* gene in the present study, it remains unknown whether the subjects carrying the rare allele of the SNPs have decreased NAT activity. An alternative possible mechanism is the effect of aspirin on *NAT2* gene activity. Aspirin exerts inhibitory effects on the *in vitro* NAT enzyme activity and *in vivo* acetylation of 2-aminofluorene in laboratory rats [39], human bladder tumor cells [40] and intact bacteria [41]. However, the exact genetic mechanism of this variant on aspirin hypersensitivity is unclear.

A few studies have demonstrated the genetic association of *NAT2* gene variants with the development of asthma and subphenotypes. Tamer *et al.* demonstrated that the *NAT2* slow acetylator phenotype may be a determinant in susceptibility to asthma [42]. Batra *et al.* also demonstrated that SNPs of the *NAT2* gene are closely associated with

asthma-related traits, such as high serum IgE and blood eosinophil counts [43]. Taken together with our study, the genetic polymorphisms of *NAT2* may be related to the risk of asthma, atopy and aspirin hypersensitivity in asthmatics.

Conclusion

In conclusion, we determined that human *NAT2* gene polymorphisms are associated with the development of aspirin intolerance in people with asthma. This information on genetic polymorphisms in the *NAT2* gene may be useful in the development of new methods to diagnose aspirin intolerance, and in finding new strategies for the control of aspirin intolerance.

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Financial & competing interests disclosure

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Executive summary

Cysteinyl leukotriene & aspirin hypersensitivity in asthma

- Aspirin-intolerant asthma (AIA) refers to the development of bronchoconstriction in individuals with asthma following the ingestion of aspirin or other NSAIDs.
- Cysteinyl leukotrienes are important inflammatory mediators in the development of asthma, as they mediate bronchoconstriction while increasing mucus secretion, vascular permeability and cellular infiltration. The cysteinyl leukotrienes, comprising leukotriene C4 and its metabolites, are biologically the most active mediators and are eliminated from blood circulation by the liver and kidneys. Thus, *N*-acetylation and ω -modification of the leukotriene structure are associated with a loss of leukotriene bioactivity.
- The present study is the first to investigate the genetic polymorphisms of the *NAT2* gene as a potential candidate for aspirin intolerance in people with asthma.

NAT gene polymorphism is associated with aspirin hypersensitivity in asthma

- Asthmatics ($n = 438$) were categorized into AIA ($\geq 15\%$ decrease in the forced expiratory volume in 1 s or cutaneous reaction, $n = 170$) or aspirin-tolerant asthma ($n = 268$) groups.
- Polymorphisms of the *NAT2* gene were genotyped by a single-base extension method.
- The distributions of all loci of the 14 SNPs were in Hardy–Weinberg equilibrium ($p > 0.05$). Among the 14 SNPs of the *NAT2* gene, six common SNPs (minor allele frequency $> 5\%$) in a Korean population were used for haplotype construction and further statistical analysis.
- The logistic regression analysis demonstrated that *NAT2* -9246G>C and haplotype 2 (TCACGG) were significantly associated with the risk of AIA. The rare allele frequencies of the SNP and *ht2* were significantly higher in the AIA group than in the aspirin-tolerant asthma group ($p^{\text{corr}} = 0.03$ and $p^{\text{corr}} = 0.02$ in codominant model).

Conclusion

- In a large genetic epidemiology study of AIA in a Korean population, genetic polymorphisms of *NAT2* were found to be related to a risk of aspirin hypersensitivity among asthmatics.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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