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ORIGINAL ARTICLE

A new association between polymorphisms of the *SLC6A7* gene in the chromosome 5q31–32 region and asthma

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The human chromosomal 5q31–33 region has been implicated as a susceptibility locus for several immune-mediated diseases including asthma in several populations. Recently, the extraneuronal GABAergic system has been implicated as a new link to airway obstruction in asthma. In addition, the *SLC6A7* gene, which is positioned at 5q31–32 and encodes the transporter for an excitatory neurotransmitter of L-proline, has never been studied for its association with asthma. In this study, resequencing of all exon, promoter region (2 kb), and exon–intron boundary regions in the *SLC6A7* gene found a total of 33 single nucleotide polymorphisms (SNPs) in 24 Korean asthmatic patients. After the initial SNP survey, a total of 17 common SNPs with minor allele frequency (MAF) over 10% were genotyped in 498 asthmatic patients and 303 normal controls. Logistic analyses revealed significant associations between genetic variants of the *SLC6A7* gene and asthma (*P*-value up to 6.0×10^{-4}; P^{corr} value up to 0.009). In further regression analyses, minor alleles of intronic +11431T>C, +12213C>T and +12927A>G in linkage disequilibrium block 2 and +20113T>C in 3′UTR significantly increased the bronchodilator response in asthmatics (*P*-value of recessive model up to 0.008; which are not significant after multiple correction). Therefore, our findings suggest that *SLC6A7* could be a susceptible gene for asthma.

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INTRODUCTION

According to the World Health Organization, it is estimated that over 300 million people worldwide suffer from asthma. As a chronic inflammatory airway disease, asthma is characterized by the infiltration and activation of eosinophils in the lung.[1] Asthma and its phenotypes are complex traits resulting from interactions between multiple disease susceptibility factors and the surrounding environment, which has a major influence on the onset and severity of asthma.[2] Despite our advanced knowledge of the pathological basis of asthma, its comprehensive etiology is not fully understood.

Recently, genome-wide association studies have identified susceptible genes for asthma, such as genetic variations of *metallopeptidase domain 33* in patients with asthma and bronchial hyper-responsiveness, *phosphodiesterase 4D* in childhood asthma, and variants regulating the expression of *orosomucoid 1-like 3* in childhood asthma.[3–5] In addition, the human chromosomal region 5q31–33, which contains a cluster of cytokines and other immune-related genes, has been mapped as a susceptibility locus for several inflammatory or autoimmune diseases including asthma in a number of populations.[6–9] Functional variations of genes in chromosomal region 5q31–33, such as *interleukin* (*IL*)*-13*, *IL-9*, *IL-12B* and *DCNP1* were also found to be associated with asthma.[10–13] However, the exact genetic and functional mechanisms underlying asthmatic pathology remain unclear.

The *SLC6A7* gene, which is located at the chromosomal 5q31–33 region, encodes a neurotransmitter L-proline transporter and is a member of the γ-aminobutyric acid (GABA) neurotransmitter gene

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family. As the *SLC6A7* gene was first discovered,[14,15] there have been few reports about its exact functions or molecular mechanisms, except for its expression in the brain regions related to the glutamatergic pathways.[16] In addition, there has been no evidence of association between *SLC6A7* and asthma. However, airway remodeling for the pathogenesis of asthma and its correlation with excitatory neurotransmitters have been suggested.[17,18] Moreover, new evidence has recently emerged suggesting a link between the extraneuronal signaling system (the *excitatory* rather than inhibitory GABAergic system) and mucus overproduction in asthma.[19,20]

To investigate this new link between genetic variation(s) of the *SLC6A7* gene in the chromosomal 5q31–33 region and asthma, we discovered the polymorphisms of the *SLC6A7* gene and studied its association with asthma and related phenotypes.

MATERIALS AND METHODS

Study subjects

A total of 498 asthmatic patients were recruited from the Asthma Genome Research Center, which consists of three tertiary hospitals in Korea (Soonchunhyang University, Seoul; Bucheon and Chunan Hospitals). Ethical approvals were obtained from the institutional review board of each hospital. All subjects were Korean. All patients had the clinical symptoms and the physical examination results compatible with asthma, based on the Global Initiative for Asthma guidelines.[21] All patients showed a history of dyspnea and wheezing during the previous 12 months, plus one of the following: (1) $>15\%$ increase in FEV_1 or $>12\%$ increase plus 200 ml following inhalation of a short-acting bronchodilator, (2) $<10\,mg\,ml^{-1}$ PC20 methacholine, or (3) $>20\%$ increase in FEV_1 following 2 weeks of treatment with inhaled steroids and long-acting bronchodilators. Airway reversibility was applied to rapid improvements in FEV_1, measured at 15 min after inhalation of a rapid-acting bronchodilator (400 mg salbutamol (albuterol), MDI).[22] Bronchodilator-induced change of FEV_1 was measured by the difference of post FEV_1 (l/sec) minus pre FEV_1 (l/sec) divided by pre-bronchodilator FEV_1 (l/sec) and represented as percentages ((post FEV_1 minus pre FEV_1)/pre-bronchodilator $FEFV_1\times100$). We evaluate the association between the bonchodilator-induced change and genotypes using regression analysis. Acute or chronic eosinophilic pneumonia and Churg-Strauss syndrome were excluded from the study population. A total of 303 normal subjects were recruited from the general population and spouses of the patients who answered negatively to a screening questionnaire for respiratory symptoms and had FEV_1 greater than 75% predicted, the provocation concentration causing a fall in the FEV_1 of 20% (PC20) by methacholine greater than $10\,mg\,ml^{-1}$, and normal findings on a simple chest radiogram.

Total immunoglobulin E (IgE) and specific IgE to *Dermatophagoides farinae* and *D. pteronyssinus* were measured using the UniCAP system (Pharmacia Diagnostics, Uppsala, Sweden). Twenty-four common inhalant allergens (for example, 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 having a wheal reaction by allergen extract that was equal to or greater than that by histamine ($1\,mg\,ml^{-1}$), or 3 mm in diameter. The clinical parameters are summarized in Table 1.

Resequencing and genotyping

Genomic DNA was isolated from the peripheral blood of patients using the Wizard genomic DNA purification kit (Promega, Fitchburg, WI, USA). New pairs of primers were generated for PCR amplification (Supplementary Table 1) based on the *SLC6A7* gene (NM_014228) in the chromosome 5q31–32 genomic sequence (NT_029289). All exons and their flanking regions, including the promoter region (2 kb), were resequenced from genomic DNA from the initial 24 Korean asthmatics using the ABI PRISM 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA). Sequence variants were verified by chromatograms.

After the initial SNP survey, a total of 17 common single nucleotide polymorphisms (SNPs) with minor allele frequency (MAF) over 10% were selected to increase the statistical power of the results. In the case of SNPs with complete LD ($r^2=1$), one SNP in the regulatory region rather than an intron was selected among the SNPs. In addition to the SNPs detected from resequencing, eight additional common dbSNPs were included in the total of 17 SNPs so as to fully analyze the association between *SLC6A7* and asthma. The SNPs were genotyped in 498 asthmatic patients and 303 normal controls using BeadExpress (Illumina, San Diego, CA, USA). The genotype quality score for keeping data was set at 0.25. As a result of this process, 487 asthmatics and 286 normal controls were successfully genotyped.

Statistics

Haploview v4.1 software was used to determine LD of SNPs in *SLC6A7*.[23] Lewontin's D' ($|D'|$) and the LD coefficient r^2 between all pairs of biallelic loci were examined.[24] After estimation using *PHASE* software, haplotypes were computed by logistic analyses using the Statistical Analysis System (SAS). Subjects with missing genotypes were excluded from the analysis of individual SNPs and haplotypes. Genotype distribution of SNPs among the asthmatics and the normal subjects was computed by logistic analyses controlling for age

Table 1 Clinical profiles of study subjects

Clinical profiles	*Asthma patients*	*Normal controls*
Number of subjects	498	303
Age (year, mean ± s.d. (min–max))	49.47 ± 15.84 (11.03–87.30)	47.79 ± 15.71 (5.21–83.94)
Sex (male/female)	207/291	122/181
Current smoker/ex-smoker (%)	34.9	30.1
Positive rate of atopy (%)	62.4	33.1
BMI ($kg\,m^{-2}$)	24.17 ± 3.49	23.87 ± 3.01
FEV_1 %, predicted	79.33 ± 22.18	103.09 ± 14.77
FVC %, predicted	82.88 ± 18.05	93.72 ± 12.35
Bronchodilator response (%)	12.03 ± 11.73	2.19 ± 5.16
Blood eosinophils (%)	5.63 ± 5.84	2.39 ± 1.84
PC20, methacholine ($mg\,ml^{-1}$)	4.02 ± 6.17	24.29 ± 2.47
Total IgE ($IU\,ml^{-1}$)	416.22 ± 1193.16	115.97 ± 168.58
Positive rate of specific IgE (D.f., %)	37.55	16.33
Positive rate of specific IgE (D.p., %)	46.39	25.17

Abbreviations: BMI, body mass index; FEV_1, forced expiratory volume at 1 second; FVC, forced vital capacity.
D.f., Dermatophagoides farinae; D.p., Dermatophagoides pteronyssinus.

(continuous value), sex (male=0, female=1), atopy (non-atopy=0, atopy=1), and smoking status (nonsmoker=0, ex-smoker=1, smoker=2) as covariates using SAS. Associations between variants and bronchodilator response were computed with regression analyses using SAS. Significant associations were indicated by a *P*-value <0.05. Rational selection of SNPs improves the power to detect a significant association, and it also helps determine the actual sample size needed in case–control genetic association studies.[25,26] In this study, 17 common SNPs with over 10% MAF were selected to increase statistical power for determining the association using the *Power for Genetic Association Analyses* (*PGA*) software.[27] The statistical power for single associations with a false-positive rate of 5%, disease prevalence of 1%, the control-to-case ratio of 0.61 and an assumed relative risk of 1.5 in a co-dominant model with an LD value of 1.0 was about 72% ($\geqslant$80% for optimal power) for MAF 10%, indicating that our samples provide the proper statistical power, but not enough to test for statistical significance of association. The effective numbers of independent marker loci in each gene were calculated to correct for multiple testing using the software SNPSpD (http://genepi.qimr.edu.au/general/daleN/SNPSpD/), which is based on the spectral decomposition (SpD) of matrices of pairwise LD between SNPs.

RESULTS

The clinical characteristics and comparisons of asthmatic patients and normal controls are summarized in Table 1. The group of asthmatics included 207 males (41.6%) and 291 females (58.4%) with a mean age of 49.5 ± 15.8. The positive rate of atopy, blood eosinophils, total IgE and positive rate of specific IgE in asthmatics were about two to three times higher than in normal controls, whereas the PC20 methacholine level of asthmatics was lower than that of controls. Furthermore, asthmatics showed over a fivefold increase in bronchodilator response compared with controls ($P<0.05$).

Resequencing in the initial 24 Korean asthmatic patients discovered a total of 33 genetic polymorphisms (Table 2 and Figure 1a). In addition, four novel SNPs were discovered; two in the intronic region, one synonymous SNP in an exon, and one in the 3′UTR region. However, these new SNPs were rare variants with frequency of less than 5%, and were omitted for the next study. After the initial SNP survey, a total of 17 common SNPs with over 10% MAF were selected, including nine SNPs that were identified by resequencing and eight additional tagging SNPs that were based on International HapMap Project (http://hapmap.ncbi.nlm.nih.gov/), to increase statistical power for association. In the case of SNPs with complete LD and over 10% MAF, one SNP that is positioned in a regulatory region, rather than in an intron, was selected among the SNPs. These 17 SNPs were genotyped in 498 asthmatic patients and 303 normal controls (Table 2).

Successful genotypes were obtained from 487 asthmatics and 286 normal controls. Pairwise comparisons of 17 SNPs showed five tight LD blocks, and haplotypes were inferred by *PHASE* software (Figures 1b and 2). In logistic analyses adjusted for age, sex, smoking status and atopy, all SNPs in LD block 2–5 showed significant associations with asthma (Table 3, $P<0.05$). In particular, the variations of +11431T>C (rs13153971) in intron 6 and +12927A>G (rs2240792) in intron 8 were more frequent in asthmatics than in controls showing the lowest significant association (OR=1.58; 95% CI=1.22–2.04 for +11431T>C; OR=1.57; 95% CI=1.21–2.04 for +12927A>G; both $P=6.0\times10^{-4}$). For SNPs in coding and regulatory regions, the minor allele of synonymous +13410T>C (Phe386Phe) was frequent in asthmatics ($P=0.01$), whereas +13446C>T (Asp398Asp) was infrequent ($P=0.003$). Also, two SNPs in the 3′UTR region were significantly prevalent in asthmatics ($P=0.02$–0.001). After multiple testing corrections, however, among 13 associated SNPs, only five including rs13153971, rs2240788, rs2240792, rs2240794 and rs13169575 were shown to be significantly associated (Table 3, $P^{corr}<0.05$). In haplotype associations, haplotypes of *BL2*, *BL3-ht1*, *BL3-ht2*, *BL4-ht1*, *BL4-ht2* and *BL5* were almost equivalent with +6343G>A, +13446C>T, +11431T>C, +15931G>A, +15091A>G and +20113T>C, respectively; for example, *BL3-ht1* was unique to the +13446T allele among four major *BL3* haplotypes, and was observed to be associated with asthma.

Bronchodilator response, which is an indicator of the severity of airflow limitation in asthmatics, was found to be significantly increased in asthmatics compared with controls. Therefore, the association of variants of *SLC6A7* with bronchodilator response was analyzed. In regression analyses, +11431T>C, +12213C>T and +12927A>G variants in LD block 3 and +20113T>C in block 5 were associated with the bronchodilator response (Table 4, *P*=up to 0.008 for recessive model). Each heterozygous genotype brought on no increase in the bronchodilator response, whereas the homozygous genotype of each rare allele significantly increased it, indicating that the heterozygote exerts its phenotype for bronchodilator response through its recessive form, rather than its co-dominant and dominant forms. However, the significant associations of the SNPs disappeared after multiple testing corrections.

Those with atopy have a high tendency to develop asthma, as shown by about a twofold higher prevalence of atopy in asthmatics than in normal controls in this study (Table 1). In further investigating genetic effects on bronchodilator response, additional associations were observed when atopic status in asthmatics was taken into account. These additional associations included the fact that the minor alleles of variants in LD block 2 and +13410T>C (Phe386Phe) in block 3 significantly increased bronchodilator response, whereas those of +2124C>G, +13446C>T (Asp398Asp), +15770T>G and +15931G>A variants decreased it (Supplementary Table 2). These associations were not found in asthmatics without atopy ($P>0.05$).

DISCUSSION

Although genome-wide SNP screening is now available, it is still a useful strategy to investigate candidate gene(s) for disease association. Regarding this study, the chromosome region 5q31–33 contains the cytokine gene cluster, which is the most extensively studied candidate region for asthma-related traits.[8,28,29] Inspired by the newly discovered link of excitatory neurotransmitters to asthma and airway remodeling for the pathogenesis of asthma, this study identified a new association between asthma and *SLC6A7*, which is located at chromosome 5q31–33 and encodes a neurotransmitter transporter. In addition, *protocadherin 1* (*PCDH1*), as a mediator of cell adhesion, in chromosome 5q31–33 was identified as a novel susceptibility gene for asthma.[30] Therefore, our findings that common SNPs of the *SLC6A7* gene, with tight LD, are significantly associated with asthma and increase of the bronchodilator response suggest *SLC6A7* as a susceptibility gene for asthma.

Although the interplay between nerve and bronchial airway microvasculature is complex and not yet fully understood, it has been suggested that neurogenic inflammation might have important functions in allergic diseases such as asthma.[31] Many neuropeptides have been identified to have inflammatory effects in airway diseases.[32,33] An excitatory, rather than inhibitory, GABAergic system was also observed to have a key role in asthma.[19,20] In addition, the GABA-agonist baclofen has been reported as a potential therapeutic agent for the treatment of asthma.[34] Therefore, since *SLC6A7* acts as a transporter for the excitatory neurotransmitter of L-proline and has a presynaptic regulatory role in excitatory synaptic transmission,[15,35]

Table 2 Polymorphisms of *SLC6A7* gene investigated in this study

					HWE		
SNP	*rs no.*	*Location*	*Amino acid change*	*Frequency*	*Asthmatics (n=487)*	*Controls (n=286)*	*Reference*
−1921G>A	rs3776083	Promoter		0.396	—	—	Resequencing
−1798G>A	rs3776084	Promoter		0.391	—	—	Resequencing
−1395G>A*	rs3756316	Promoter		0.426	0.674	0.802	Resequencing
−1256G>A*	rs3756317	Promoter		0.489	0.937	0.881	Resequencing
−1123C>T	rs3756318	Promoter		0.075	—	—	Resequencing
−1062A>T	rs3756319	Promoter		0.020	—	—	Resequencing
−841T>C*	rs2342277	Promoter		0.490	0.975	0.925	Resequencing
−451G>T	rs1860409	Promoter		0.063	—	—	Resequencing
−341C>G	rs3822326	Exon1 (5′utr)		0.021	—	—	Resequencing
−183A>G	rs3764886	Exon1 (5′utr)		0.458	—	—	Resequencing
+296C>A	Novel	Intron1		0.043	—	—	Resequencing
+2124C>G*	rs3776085	Intron1		0.466	0.897	0.682	dbSNP
+4260T>G	Novel	Intron1		0.021	—	—	Resequencing
+4644C>T	rs17652448	Intron2		0.091	—	—	Resequencing
+4683C>T	Novel	Intron2		0.042	—	—	Resequencing
+6343G>A*	rs2270145	Intron2		0.381	0.995	0.569	dbSNP
+6383C>T*	rs2270146	Intron2		0.381	0.998	0.569	Resequencing
+8029G>A*	rs758593	Intron4		0.392	0.985	0.683	dbSNP
+8937C>T	Novel	Exon5	Ile207Ile	0.021	—	—	Resequencing
+9109A>T	rs731376	Intron5		0.438	—	—	Resequencing
+10929G>A	rs3733680	Intron6		0.167	.	.	Resequencing
+11104A>G	rs41287116	Intron6		0.125	.	.	Resequencing
+11340A>C	rs6579777	Intron6		0.478	—	—	Resequencing
+11431T>C*	rs13153971	Intron6		0.239	0.740	0.514	dbSNP
+12179T>C	rs10875556	Intron7		0.150	.	.	Resequencing
+12213C>T*	rs2240788	Intron7		0.237	0.635	0.570	Resequencing
+12420G>A	rs57219956	Intron8		0.075	—	—	Resequencing
+12927A>G*	rs2240792	Intron8		0.235	0.689	0.431	dbSNP
+13410T>C*	rs2240793	Exon9	Phe386Phe	0.423	0.981	0.322	Resequencing
+13446C>T*	rs2240794	Exon9	Asp398Asp	0.430	0.981	0.932	Resequencing
+15091A>G*	rs4705425	Intron12		0.301	0.996	0.989	dbSNP
+15770T>G*	rs7704850	Intron13		0.458	0.840	0.922	dbSNP
+15931G>A*	rs2270147	Intron13		0.449	0.954	0.993	dbSNP
+19323G>C	rs10078509	Exon14 (3′UTR)		0.052	—	—	Resequencing
+19423G>A	rs4705114	Exon14 (3′UTR)		0.333	.	.	Resequencing
+19435T>C	rs12653451	Exon14 (3′UTR)		0.333	.	.	Resequencing
+19757A>G	rs2240795	Exon14 (3′UTR)		0.348	.	.	Resequencing
+20113T>C*	rs13169575	Exon14 (3′UTR)		0.244	0.466	0.865	Resequencing
+20243A>G*	rs13153325	Exon14 (3′UTR)		0.292	0.995	0.631	Resequencing
+20411G>A	rs13153325	Exon14 (3′UTR)		0.021	—	—	Resequencing
+20661C>A	Novel	Exon14 (3′UTR)		0.033	—	—	Resequencing

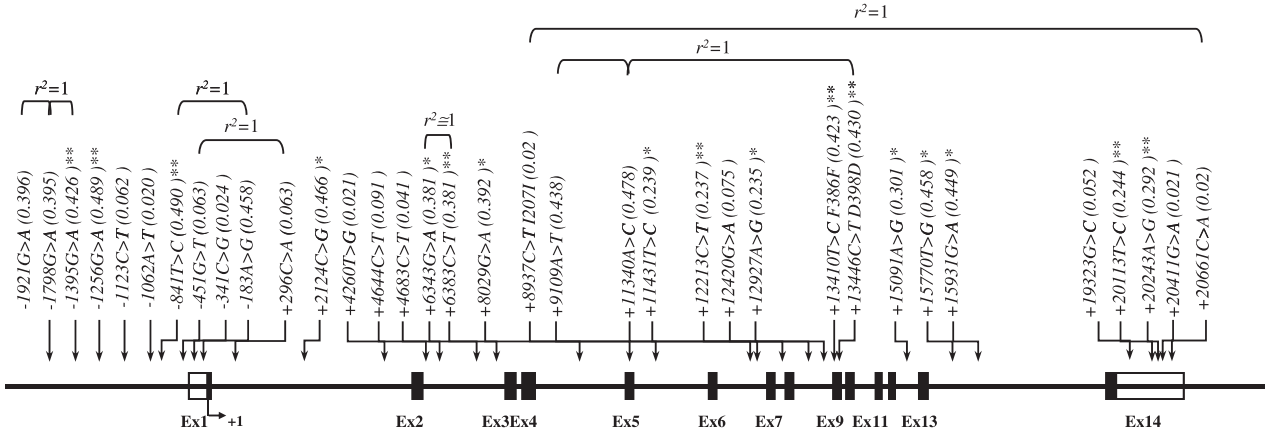
Abbreviations: HWE, Hardy–Weinberg equilibrium; UTR, untranslated region; dbSNP, public SNP of National Center for Biotechnology Information (NCBI).
Asterisks indicate SNPs that were genotyped in total case and control subjects.
The first base of the transcription start site is denoted as nucleotide +1.

it likely has an effect on allergic and inflammatory diseases including asthma. Moreover, Pin1, a proline isomerase, was identified as an essential factor for the onset of allergic asthma,[36] suggesting that L-proline-related mechanisms and variations of related genes also could contribute to asthma.

From linkage analysis of *IL-14* and other markers in chromosome 5q31.1 with total serum IgE concentration,[37] genes in the chromosome 5q31–33 region, such as *IL-4*, *IL-9*, *IL-13*, *interferon regulatory factor-1* and β_2*-adrenergic receptor*, have been reported to be associated with asthma-related phenotypes.[9,38–40] More recently, genetic polymorphisms or haplotypes of the *T-cell immunoglobulin and mucin domain* (*TIM*)*-1* gene were shown to confer susceptibility to asthma.[41,42] The *TIM-1* gene, also known as *hepatitis A virus cellular receptor 1*, is a recently identified gene in the chromosome 5q31–33 region and has been implicated in the regulation of T_H1- and T_H2-cell-mediated immunity.[43] Moreover, the imbalance of T_H1 and T_H2 immune regulation is known to affect asthma.[44] However, despite these previous studies attempting to identify a link between chromosome 5q31–33 regions and asthma, no specific gene(s) thus far have been clearly implicated in the disease's pathogenesis.

Since asthmatic patients frequently have poor recognition of their symptoms, measurements of lung function provide them with a

a Map of *SLC6A7* (solute carrier family 6 (neurotransmitter transporter, L-proline), member 7) on chromosome 5q31-q32 (21.1 kb)



b Haplotypes in *SLC6A7*

Block1						Block2					Block3							Block4					Block5			
Hap.	-1395G>A	-1256G>A	-841T>C	+2124C>G	Freq.	Hap.	+6343G>A	+6383C>T	+8029G>A	Freq.	Hap.	+11431T>C	+12213C>T	+12927A>G	+13410T>C	+13446C>T	Freq.	Hap.	+15091A>G	+15770T>G	+15931G>A	Freq.	Hap.	+20113T>C	+20243A>G	Freq.
ht1	G	G	T	G	0.464	ht1	G	C	G	0.604	ht1	T	C	A	T	T	0.426	ht1	A	G	A	0.447	ht1	T	A	0.697
ht2	A	A	C	C	0.424	ht2	A	T	A	0.379	ht2	C	T	G	C	C	0.233	ht2	G	T	G	0.303	ht2	C	G	0.230
ht3	G	A	C	C	0.062	ht3	G	C	A	0.013	ht3	T	C	A	C	C	0.182	ht3	A	T	G	0.238	ht3	T	G	0.060
ht4	G	G	T	C	0.046	ht4	G	T	G	0.002	ht4	T	C	A	T	C	0.148	ht4	A	G	G	0.007	ht4	C	A	0.012
ht5	A	A	C	G	0.002	ht5	A	C	G	0.001	ht5	C	C	A	C	C	0.004	ht5	G	G	A	0.002				
ht6	**A**	**A**	**T**	**G**	**0.001**	**ht6**	**A**	**C**	**A**	**0.001**	**ht6**	**T**	**T**	**A**	**T**	**T**	**0.004**	**ht6**	**G**	**G**	**G**	**0.002**				
ht7	G	A	T	C	0.001						ht7	C	C	G	C	C	0.002									
ht8	G	G	C	C	0.001						ht8	T	C	G	C	C	0.001									

Figure 1 Physical map and haplotypes of the *SLC6A7* gene. (**a**) Physical map of *CEP68* and its targeted SNPs. The coding exons are represented by black blocks, and 5′UTR and 3′UTR by white blocks. Numbering of SNP position +1 corresponds to the first base of the first methionine referred to NM_014228 (http://genome.ucsc.edu/). Asterisks (*) indicate the SNPs that were discovered from resequencing; **denotes additional tagging SNPs. Complete linkage disequilibrium is denoted as r^2=1. UTR, untranslated region. (**b**) Haplotypes of 17 SNPs in the *SLC6A7* gene. Associations of haplotypes with frequency >0.05 are shown in Table 3.

diagnostic confidence of asthma. Among several methods to assess airflow limitation, the response to an inhaled bronchodilator, such as the measurement within minutes after inhalation of a rapid-acting bronchodilator, is a useful indicator for the diagnosis of asthma.[45,46] Although the significance disappeared after multiple testing correction, the association of variants of *SLC6A7* with bronchodilator response showed a possibility that homozygous rare alleles of four SNPs, +11431T>C, +12213C>T, +12927A>G and +20113T>C, could increase the bronchodilator response in the recessive model. In particular, considering that the frequencies of *BL3-ht2* (unique to +11431T>C, +12213C>T and +12927A>G) and *BL5* (equivalent with +20113T>C) in asthmatics were higher than those of normal controls, the *BL3-ht2* and *BL5* haplotypes of *SLC6A7* could functionally affect asthma susceptibility.

In the case of the exon region, this study found that two synonymous SNPs, +13410T>C (Phe386Phe) and +13446C>T (Asp398Asp), were frequently and infrequently associated with asthma, respectively. Recently, the tissue-specific differences in transfer RNA expression and the tissue-specific codon usage of the human gene have been investigated because codon-mediated translational control may be an important step in regulating the expression of genes.[47,48] In fact, a synonymous polymorphism of the *multidrug resistance 1* gene was elucidated to alter drug and inhibitor interactions.[49] The amino acids Phe386Phe and Asp398Asp in the SLC6A7 protein were predicted to be positioned at transmembrane alpha-helical domain 8, and the frequencies of phenylalanine and aspartic acid were found to be different for the alpha helix region depending on synonymous codons (Supplementary Figure 1).[50] Thus, different transfer RNA expression for codon usage could have an effect on the activity of the protein and the subsequent susceptibility to asthma.

The alternative splicing database creates a database of alternative splice events on genome-wide scale.[51] Using an EMBL-EBI splice site prediction tool (http://www.ebi.ac.uk/asd-srv/wb.cgi?method=2), which computes positions and scores of branch point in intronic sequence for alternative splicing, the substitution from G to A of rs2270145, the fourth position of branch site of TTG(t/**a**)G, was predicted as a possible branch point adenosine with a score of 3.41 (Supplementary Figure 2). However, further experimental studies for its functions are needed to determine whether the predicted alternative splicing could have effects on asthma. Also, in additional analysis of LD near *SLC6A7* in Asian populations (Japanese and Chinese) from the International HapMap Project, the *SLC6A7* gene showed no LD with other genes in chromosome 5q31–33 region (Supplementary Figure 3).

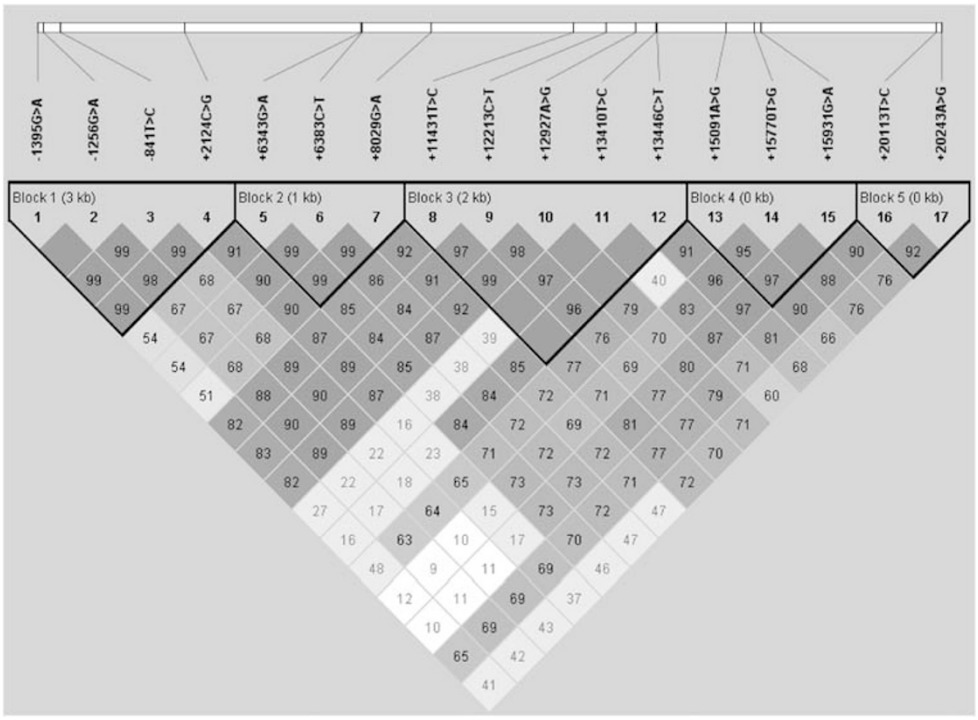


Figure 2 LD and haplotype block analysis of 17 *SLC6A7* polymorphisms in a Korean population. LD plot prepared using Haploview v4.1 software downloaded from the Broad Institute (http://www.broadinstitute.org/mpg/haploview). The numbers indicate pairwise r^2 values shown as a percentage. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Table 3 Logistic regression analysis of *SLC6A7* polymorphisms between asthmatics and controls

LD block	*SNP or haplotype*	*SNP ID.*	*Location*	*Amino acid change*	*Asthmatics (n=487)*	*Controls (n=286)*	*OR (95% CI)*	P-*value**	P^{corr}**
Block 1	−1395G>A	rs3756316	Promoter		0.431	0.418	1.12 (0.90–1.41)	0.31	—
	−1256G>A	rs3756317	Promoter		0.497	0.475	1.16 (0.94–1.45)	0.17	—
	−841T>C	rs2342277	Promoter		0.500	0.474	1.19 (0.96–1.48)	0.12	—
	+2124C>G	rs3776085	Intron1		0.454	0.486	0.83 (0.66–1.04)	0.10	—
	BL1-ht3				0.065	0.058	1.19 (0.76–1.84)	0.45	
	BL1-ht4				0.049	0.044	1.02 (0.60–1.75)	0.93	
Block 2	+6343G>A	rs2270145	Intron2		0.404	0.360	1.28 (1.02–1.61)	**0.04**	—
	+6383C>T	rs2270146	Intron2		0.403	0.360	1.28 (1.01–1.61)	**0.04**	—
	+8029G>A	rs758593	Intron4		0.418	0.362	1.36 (1.08–1.71)	**0.009**	—
Block 3	+11431T>C	rs13153971	Intron6		0.270	0.192	1.58 (1.22–2.04)	**6.0×10^{-4}**	**0.009**
	+12213C>T	rs2240788	Intron7		0.266	0.195	1.52 (1.18–1.96)	**0.001**	**0.015**
	+12927A>G	rs2240792	Intron8		0.266	0.189	1.57 (1.21–2.04)	**6.0×10^{-4}**	**0.009**
	+13410T>C	rs2240793	Exon9	F386F	0.448	0.385	1.32 (1.06–1.64)	**0.01**	—
	+13446C>T	rs2240794	Exon9	D398D	0.396	0.475	0.71 (0.57–0.89)	**0.003**	**0.045**
	BL3-ht3				0.176	0.192	0.90 (0.68–1.18)	0.43	
	BL3-ht4				0.158	0.138	1.15 (0.84–1.57)	0.38	
Block 4	+15091A>G	rs4705425	Intron12		0.323	0.276	1.32 (1.03–1.68)	**0.03**	—
	+15770T>G	rs7704850	Intron13		0.429	0.495	0.74 (0.60–0.93)	**0.009**	—
	+15931G>A	rs2270147	Intron13		0.418	0.489	0.72 (0.58–0.90)	**0.004**	—
	BL4-ht3				0.241	0.234	1.02 (0.79–1.31)	0.91	
Block 5	+20113T>C	rs13169575	3′UTR		0.270	0.206	1.52 (1.18–1.96)	**0.001**	**0.015**
	+20243A>G	rs13153325	3′UTR		0.309	0.265	1.33 (1.04–1.69)	**0.02**	—

BL1-ht1, *BL1-ht2*, *BL3-ht1*, *BL3-ht2*, *BL4-ht1* and *BL4-ht2* were almost equivalent with +2124C>G, −1395G>A, 13446C>T, 11431T>C, 15931G>A and 15091A>G, respectively.
Haplotypes of *BL2* and *BL5* were also equivalent with 6343G>A and 20113T>C, respectively.
Bold values indicate the statistical significance ($P<0.05$).
**P*-values of the co-dominant model were obtained by logistic analysis, controlled for age, sex, smoking status and atopy as co-variables.
**p^{corr} values after multiple testing correction.

Table 4 Regression analysis of *SLC6A7* polymorphisms with bronchodilator response adjusted for age, sex, smoking and atopic status

		Asthmatics			P-*value*			
LD block	*SNP or haplotype*	*C/C*	*C/R*	*R/R*	*Co-dominant*	*Dominant*	*Recessive*	P^{corr}*
Block 1	−1395G>A	150 (10.97 ± 10.41)	230 (12.54 ± 12.78)	86 (11.86 ± 11.12)	0.36	0.23	0.84	—
	−1256G>A	122 (10.50 ± 10.74)	223 (12.36 ± 12.12)	119 (12.61 ± 12.05)	0.17	0.15	0.42	—
	−841T>C	120 (10.68 ± 10.80)	223 (12.36 ± 12.12)	120 (12.49 ± 12.08)	0.25	0.20	0.54	—
	+2124C>G	138 (12.54 ± 12.11)	232 (12.06 ± 11.90)	96 (10.66 ± 10.89)	0.22	0.33	0.30	—
	BL1-ht3	414 (11.76 ± 11.73)	51 (13.75 ± 12.63)	5 (12.80 ± 9.88)	0.53	0.44	0.83	
	BL1-ht4	425 (12.09 ± 11.86)	45 (11.02 ± 11.38)	—	0.83	0.83	—	
Block 2	+6343G>A	165 (10.78 ± 11.96)	224 (12.15 ± 11.19)	75 (13.69 ± 12.95)	0.06	0.14	0.10	—
	+6383C>T	165 (10.74 ± 11.84)	226 (12.36 ± 11.56)	74 (13.16 ± 12.19)	0.09	0.12	0.23	—
	+8029G>A	159 (10.90 ± 12.11)	227 (12.19 ± 11.42)	80 (13.14 ± 12.00)	0.15	0.24	0.23	—
Block 3	+11431T>C	250 (11.76 ± 11.62)	177 (11.17 ± 11.62)	38 (16.58 ± 12.61)	0.21	0.78	**0.01**	—
	+12213C>T	256 (11.86 ± 11.62)	173 (10.91 ± 11.57)	39 (16.72 ± 12.48)	0.29	0.94	**0.008**	—
	+12927A>G	254 (11.89 ± 11.57)	173 (10.97 ± 11.67)	38 (16.58 ± 12.61)	0.32	0.96	**0.01**	—
	+13410T>C	144 (11.42 ± 11.94)	230 (11.93 ± 12.01)	94 (13.01 ± 11.26)	0.30	0.37	0.42	—
	+13446C>T	168 (12.78 ± 10.76)	223 (11.57 ± 11.83)	75 (10.99 ± 13.59)	0.21	0.21	0.49	—
	BL3-ht3	323 (12.11 ± 12.43)	132 (11.85 ± 9.90)	15 (10.47 ± 13.94)	0.76	0.88	0.59	
	BL3-ht4	328 (11.88 ± 12.42)	138 (12.05 ± 10.29)	4 (18.50 ± 8.96)	0.67	0.71	0.71	
Block 4	+15091A>G	205 (11.16 ± 11.76)	194 (11.46 ± 11.06)	48 (14.52 ± 11.33)	0.18	0.40	0.13	—
	+15770T>G	152 (12.80 ± 10.96)	223 (11.59 ± 11.67)	89 (11.11 ± 13.35)	0.20	0.23	0.38	—
	+15931G>A	157 (12.77 ± 10.94)	225 (11.60 ± 11.65)	83 (11.17 ± 13.52)	0.23	0.23	0.47	—
	BL4-ht3	277 (12.34 ± 12.56)	164 (11.37 ± 10.64)	29 (12.10 ± 10.79)	0.80	0.68	0.85	
Block 5	+20113T>C	252 (11.67 ± 10.57)	172 (11.40 ± 13.05)	41 (15.73 ± 12.63)	0.18	0.59	**0.03**	—
	+20243A>G	222 (12.11 ± 11.44)	199 (10.92 ± 11.92)	46 (15.04 ± 12.17)	0.56	0.73	0.06	—

C/C, C/R, and R/R indicate the homozygote of the common allele, and the heterozygote and homozygote of the rare allele, respectively.
Bold values indicate the statistical significance ($P<0.05$).
* P^{corr} values after multiple testing correction.

Atopy is considered to be an important risk factor in the occurrence of asthma.[52] It has been reported that the prevalence of asthmatic patients is high in the populations with atopy.[53] In addition, many studies have observed the association of genetic variations with asthma and atopy.[29,54] In a comparison between atopic and non-atopic asthmatics, this study found that additional alleles and haplotypes were significantly associated with atopic asthma, suggesting that genetic variations of the *SLC6A7* gene also could be a causative factor in the development of atopy. For this reason, additional association studies between *SLC6A7* and atopy are called for.

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