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CHRM2 but not *CHRM1* or *CHRM3* polymorphisms are associated with asthma susceptibility in Mexican patients

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Abstract

Asthma is a complex disease for which genetic predisposition has been widely documented. Considerable evidence supports the hypothesis that polymorphisms in the muscarinic–cholinergic (*CHRM*) genes could be involved in asthma pathogenesis, bronchial hyperresponsiveness, and mucus secretion. To determine whether single nucleotide polymorphisms (SNPs) or haplotypes in *CHRM1*, *CHRM2*, or *CHRM3* are associated with asthma in Mexican pediatric population. We performed a case–control study including 398 pediatric cases with asthma and 450 healthy controls. We analyzed 19 SNPs distributed among these three genes. Two of the seven SNPs located in *CHRM2*, the 3′ untranslated region rs8191992 and rs6962027, differed significantly in

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allele frequencies between patients with asthma and healthy controls [odds ratio (OR) 1.42, 95 % confidence interval (95 % CI) 1.14–1.77, $P = 0.001$, and OR 1.50, 95 % CI 1.21–1.87, $P = 0.0002$, respectively]. Statistical significance remained after multiple comparison corrections ($P = 0.003$ and $P = 0.005$, respectively). The haplotypes *AA* and *TT*, containing both major and minor alleles from rs8191992 and rs6962027, also differed between cases and controls. The haplotype *AA* occurred at a lower frequency in cases (OR 0.67, 95 % CI 0.53–0.85, $P = 0.001$) whereas the haplotype *TT* was overrepresented in cases compared to controls (28 vs 21 %, respectively; OR 1.46, 95 % CI 1.15–1.85, $P = 0.002$). No association was observed between *CHRM1* or *CHRM3* SNPs or haplotypes and asthma. *CHRM2* polymorphisms are implicated in the genetic etiology of asthma.

Keywords

Asthma; Cholinergic muscarinic genes; Association study; Susceptibility genes; Mexican patients

Introduction

Asthma is a chronic inflammatory disease of the airways, characterized by variable and recurring symptoms, including airflow obstruction, bronchial hyperresponsiveness, and airway remodeling over time [1]. *A*cetylcholine (ACh) is the principal neurotransmitter released from the parasympathetic nerve fibers in the airways, and it regulates the main processes associated with this lung disease, including airway smooth muscle contraction, mucus production, and airway inflammation and structural changes [2–5]. Acting on muscarinic receptors, ACh induces contraction of airway smooth muscle by Ca^{2+}-dependent and Ca^{2+}-independent pathways and airway inflammation through the secretion of the pro-inflammatory cytokines interleukin (IL)-8 and IL-6 [5, 6]. Thus, abnormal expression and enhanced or decreased function of ACh regulatory genes might play an important role in the pathophysiology and pathogenesis of asthma [6–9].

The muscarinic–cholinergic receptor (CHRM) family consists of five members (M1–M5), and the M1, M2, and M3 receptors are widely localized in the human lung, including the alveolar walls, bronchial epithelial cells, parasympathetic ganglia, neuromuscular junctions, and submucosal glands [6, 9, 10]. The preferred G-protein coupling and second messenger systems that they activate categorize these three receptor subtypes. The M1 and M3 receptor subtypes couple with the Gq/11 family and activate phospholipase C, and the M2 receptor subtype inhibits adenylate cyclase through Gi proteins [5, 10–12]. M1 receptor stimulation facilitates cholinergic neurotransmission and appears to contribute to the regulation of water and electrolyte secretion; M2 receptor activation limits further ACh release via a negative feedback mechanism to mediate airway smooth muscle contraction [10, 11]. Stimulation via M3 receptors leads to airway smooth muscle contraction, inhibits sympathetically induced relaxation, and mediates mucus, water, and electrolyte secretion [12]. Given the important biological role of muscarinic–cholinergic mechanisms in asthma, the M1–M3 receptor-encoding genes (*CHRM1*, *CHRM2*, and *CHRM3*, respectively) are excellent candidate genes for asthma susceptibility [3, 8, 11, 12]. However, few association studies have been performed, and no conclusive results are available [13–17]. Only one study has investigated

the involvement of the *CHRM1* SNPs in asthma, showing that the rs2075748 *A*/*G* and rs1942499 *A*/*G* SNPs confer asthma risk in the Japanese population [15]. Furthermore, the rs6962027 *A*/*T CHRM2* SNP has been reported to be associated with treatment response in childhood asthma in Poland [16]. Otherwise, *CHRM3* variants have been investigated in Japanese and Caucasians from the USA and Malta, but no associations were found [14, 17]. The aim of this study was to assess whether the *CHRM1*, *CHRM2*, and *CHRM3* polymorphisms or haplotypes are associated with asthma in Mexican pediatric patients.

Materials and methods

Study subjects

Genomic DNA was available from 398 Mexican patients with asthma, recruited from hospitals in Mexico City. All patients ranged from 5 to 17 years old, and the diagnosis of asthma was based on the definition used by the American Thoracic Society and the Global Initiative for Asthma criteria. The mean (±SD) age at asthma onset was 11.5 ± 3.3; 244 (61 %) of the cases were male and 154 were female (39 %). Most children had intermittent asthma (54 %) whereas mild and moderate disease was present in 16 and 29 %, respectively. Severe asthma represented the lowest percentage (>1 %). Skin prick test data were available in 319 cases, and 266 (83 %) of cases had a positive skin test to at least one aeroallergen (Table 1). A total of 450 healthy individuals, older than age 18 years, who did not report symptoms or a history of asthma, allergy, or other pulmonary disease, were enrolled as controls. Of these, 271 (60 %) were men and 179 (40 %). Patients and healthy controls were ethnically matched.

The study was carried out after approval from all hospital ethical committees. All procedures followed their recommendations; including obtaining informed written consent of all participants and their parents, where applicable.

Association analysis

Genomic DNA samples were extracted from peripheral blood leukocytes of all participants using a standard salt precipitation technique (Qiagen, Systems, Inc., Valencia, CA, USA).

In the present study, we reduced the problem associated with population stratification because our patients and controls were matched by ethnicity. We evaluated ten ancestry informative markers that mainly identify Amerindian and European ancestry in the Mexican population ($\delta > 0.44$) [18, 19]. Age was not considered as a confounding factor given the lack of evidence that *CHRM1*, *CHRM2*, or *CHRM3* polymorphisms may be related to a reduced life span that could result in a relatively higher representation of *CHRM* alleles in the childhood group.

We selected the SNPs based on their potential functional effect, previous association studies in asthma, its related phenotypes and other entities [14, 16, 20, 21] and the linkage disequilibrium (LD) structure around them (HapMap Data Phase III/Rel#2, Feb 09, on NCBI B36 assembly, dbSNP b126). Selection criteria of tag SNPs were: minor allele frequency ≥0.05 %, r^2 cutoff = 0.8, and CEU HapMap III data. Thus, five SNPs within the *CHRM1* (rs11605665, rs2067480, rs544978, rs542269, and rs2075748), seven located inside

the *CHRM2* (rs978437, rs1455858, rs1824024, rs324640, rs324650, rs8191992, and rs6962027) and seven of *CHRM3* (rs7527924, rs10802785, rs7520974, rs6669810, rs6701181, rs3738435, and rs4072234) were included (Table 2).

TaqMan allelic discrimination assays were employed for genotyping all SNPs using assays-on-demand products and according to the standard protocols from the manufacturer. The reactions were run in a 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Genotyping call rates of each marker were >98 %, and concordant genotypes were observed in all duplicate samples (200 cases and 150 controls). To confirm the genotypes obtained by the TaqMan assay, ten random samples of patients and ten controls were sequenced. Sequencing of PCR products was performed using the DNA Sequencing Kit with Big Dye Terminator on an automated ABI PRISM 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Analysis of Hardy–Weinberg equilibrium (HWE) was performed separately for patients and controls using the Chi square test with the FINETTI program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Haplotype frequencies and the r^2 measure of LD (r^2) were calculated using the Haploview software [22]. We applied the Bonferroni method and 100,000 permutations to adjust for multiple comparisons. Associations among the alleles, genotypes, and the disease were analyzed by comparing the allele and genotype frequency distributions between cases and controls. The odds ratios (ORs) with 95 % confidence intervals (95 % CIs) were calculated using the allele frequencies for the cases and controls. Statistical significance was estimated by the Chi square test implemented in the STATCALC program (Epi Info v. 6.02 software, Centers for Disease Control and Prevention, Atlanta, GA, USA). Statistical significance was inferred at $P < 0.05$ after multiple corrections and implemented simultaneously for all SNPs and haplotypes. Power calculations were performed with the QUANTO software (version 1.2; http://hydra.usc.edu/GxE/), assuming log-additive risk model, counting a 8 % of asthma prevalence in the Mexican children [23] and allele frequencies and ORs found in the present study. The statistical power for both SNPs displaying association, rs8191992 and rs6962027, reached >88.6 and 95.8 % respectively.

Screening for polymorphisms in the CHRM2 coding and 3′ untranslated region

After the association study and base on the chance to identify variants with MAF >1 %, we analyzed 200 chromosomes from healthy subjects to identify LD between the SNPs associated with asthma in our population with others located in the coding exon and 3′ untranslated region (UTR) of the *CHRM2* gene. We focused on exon 6, the only encoding region, because several studies have suggested that amino acid substitutions (including Val385, Thr386, Ile389, Leu390 and Tyr403) could afect the G-protein coupling specificity and activation [20, 24, 25]. The primers for exon 6 sequencing were described previously by Fenech et al. [14]. Using the Primer3 software (http://frodo.wi.mit.edu/), we designed 10 primer sets on the basis of the 3′ UTR *CHRM2* genomic sequence from the GenBank database. PCR was performed in a final volume of 50 μl, using 50 ng of genomic DNA, 2.5 pmol of each primer, 2 mM of each dNTP, 1 U of Ampli*Taq* Gold DNA Polymerase, 4 mM $MgCl_2$, and 10× PCR Gold buffer (Applied Biosystems provided polymerase, $MgCl_2$, and

buffer). Cycling conditions for PCR consisted of a first denaturation step of 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at 60 °C for 20 s, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 7 min. The runs were carried out on a GeneAmp PCR system 9700 (Applied Biosystems). PCR products were sequenced directly with a DNA Sequencing Kit with Big Dye Terminator on an automated ABI PRISM 3100 DNA sequencer (Applied Biosystems), and data analysis was performed using Lasergene software (DNASTAR Inc.) according to the genomic *CHRM2* sequence reference (ENSG00000181072).

Functional prediction analysis

We predicted the potential effect of the *CHRM2* SNPs associated with asthma in our population using bioinformatics tools, including FastSNP [26], SNP Function Prediction (http://snpinfo.niehs.nih.gov/snpfunc.htm), Human-transcriptome DataBase for Alternative Splicing (http://www.h-invitational.jp/h-dbas/), SplicePort: An Interactive Splice Site Analysis Tool (http://www.spliceport.cs.umd.edu/SplicingAnalyser2.html), and SNPs 3D (http://www.snps3d.org/).

Results

CHRM2 polymorphisms associated with asthma susceptibility

The genotype distributions of AIMs or CHRMs SNPs were in HWE either in control or asthmatic cohorts ($P > 0.05$). Distribution of the AIMs did not show statistical significant differences between cases and controls.

The association analysis between *CHRM1*, *CHRM2*, and *CHRM3* SNPs and asthma demonstrated that five *CHRM2* SNPs were statistically significantly different in their distribution among controls and asthmatic cases (Table 3). Minor alleles for each SNP were higher in cases than control groups for rs978437 *A* (30 vs 36 %, OR 1.27, 95 % CI 1.04–1.56, $P = 0.019$); rs1455858 *G* (30 vs 36 %, OR 1.29, 95 % CI 1.06–1.58, $P = 0.013$); rs1824024 *T* (30 vs 35 %, OR 1.23, 95 % CI 1.01–1.51, $P = 0.048$); rs8191992 *T* (22 vs 29 %, OR 1.42, 95 % CI 1.14–1.77, $P = 0.001$); and rs6962027 *T* (22 vs 30 %, OR 1.50, 95 % CI 1.21–1.87, $P = 0.0002$). Nonetheless, statistical significance remained only for the rs8191992 ($P = 0.003$) and rs6962027 ($P = 0.005$) SNPs after multiple comparisons (100,000 permutations) and Bonferroni correction test ($P = 0.019$ and 0.0038, respectively).

In a homozygote phase, the genotypes showed statistical significance among cases and controls for rs978437 (*GG* vs *AA*: OR 1.81, 95 % CI 1.15–2.84, $P = 0.009$), rs1455858 (*AA* vs *GG* OR 1.79, 95 % CI 1.13–2.82, $P = 0.011$), and rs1824024 (*GG* vs *TT*: OR 1.66, 95 % CI 1.05–2.64, $P = 0.029$). Both the rs8191992 (OR 1.57, 95 % CI 1.20–2.06, $P = 0.0001$) and rs6962027 (OR 1.67, 95 % CI 1.25–2.22, $P = 0.0001$) SNPs showed the strongest differences in a recessive model [*AA* vs (*AT* + *TT*)].

Haplotype structure in the *CHRM* genes

We further analyzed the haplotype structure for each gene using the genotyped SNPs. The *CHRM2* markers showed a high LD among them and were located in three haplotype blocks

($r^2 \geq 0.90$). Pairwise LD analysis showed that SNPs rs978437, rs1455858, and rs1824024 belonged to one haplotype block; rs334640 and rs324650 were in the second haplotype block; and the remaining SNPs, rs8191992 and rs6962027, composed the third haplotype block (Fig. 1). When comparisons of the haplotype distribution between case and control groups were carried out, three haplotypes showed statistical significance (*AGT* block 1: OR 1.42, 95 % CI 1.05–1.91, $P = 0.022$; *AT* block 2: OR 1.49, 95 % CI 0.73–3.04, $P = 0.038$; *AA* block 3: OR 0.67, 95 % CI 0.53–0.85, $P = 0.001$; and *TT* block 3: OR 1.46, 95 % CI 1.15–3.85, $P = 0.0016$). Nevertheless, only the haplotypes *AA* and *TT* containing both major and minor alleles of rs8191992 and rs6962027 variants remained statistically significant ($P = 0.004$ and 0.006, respectively) after correction for multiple comparisons test (100,000 permutations) (Table 4).

Otherwise, as has been seen in the analysis of the SNPs, our study failed to show an association between asthma and *CHRM1* and *CHRM3* SNPs (Table 5) and haplotypes (data not shown). *CHRM3* haplotype analysis identified a high LD block ($r^2 > 0.9$), which included rs7520974, rs6669810, rs6701181, and rs3738435 SNPs; however, the haplotype distribution did not show statistical significance. The rs10802785, rs4072234, and rs7527924 variants were in high LD with each other (data not shown). Neither LD in the *CHRM1* haplotype structure nor statistical differences in its distribution were identified.

Because in previous studies we have reported gender-dependent associations [18, 19], we performed a gender stratification analysis for all SNPs included here. We found no associations for SNPs or haplotypes located in each evaluated candidate gene.

LD analysis and SNP function prediction

Because of our results, we further evaluated the LD between the associated SNPs rs8191992 and rs6962027 with other coding or 3′ UTR *CHRM2* variants. Sequencing analysis revealed neither polymorphic variants in the coding region nor new polymorphisms in the *CHRM2* 3′ UTR sequence. We documented 12 SNPs in the 3′ UTR region (rs8191993, rs17168893, rs76518506, rs6962027, rs11984039, rs74834150, rs62487067, rs11919542, rs6967953, rs74460389, and rs17500423). As we have seen in the current study, the rs8191992 SNP showed high LD between the variant rs6962027 but also with the rs6967953 SNP (data not shown). However, based on SNP functional prediction software (http://snpinfo.niehs.nih.gov/snpfunc.htm), only the rs8191992*A* allele seems to be functional; it is predicted to affect a potential miRNA-binding site for miR-508–3p.

Discussion

The cholinergic muscarinic receptors have long been recognized to influence bronchoconstriction, mucus secretion, proinflamation, etc.; trough a control over acetylcholine release. Therefore anticholinergics are particularly useful in the treatment of asthma and chronic obstructive pulmonary disease (COPD) [9]. However decreased function of muscarinic receptors and therapy heterogeneity responses to anticholinergic agents in humans and animal models with asthma and COPD are common findings [27–29]. Studies have suggested that polymorphism in the CHRM genes could contribute with both the development of lungs diseases and the widely divergences in drug response [30, 31]. To

determine whether *CHRM1*, *CHRM2*, and *CHRM3* SNPs or haplotypes are genetic risk factors for asthma susceptibility in Mexican children, we performed a case–control study. *CHRM2* rs8191992 and rs6962027 variants showed statistical significance in genotype and allele distributions between cases and controls. Although studies suggest that the elevated degree of airway hyperresponsiveness in asthma results from both inflammatory and neurogenic factors and potentially M2 receptor dysfunction [4, 6], as far as we know, no reports have shown that SNPs in the *CHRM2* gene are implicated in asthma susceptibility. Because our patients and controls were matched by ethnicity based on the ten AIMs analysis which have already been validated to ancestry in our Mexican population [18, 19], the possibilities of association because of population stratification were reduced.

Moreover we sequenced the coding and 3′ UTR regions of the *CHRM2* gene to discard LD with other functional SNPs that could influence our data. Sequencing analysis showed no coding-region variants; thus, the *CHRM2* coding region is also highly conserved in Mexicans, as has been described in Japanese, Caucasian, and Pima populations [14, 32]. The 3′ UTR sequencing showed that the associated SNPs rs8191992 and rs6962027 were in high LD with the rs6967953 SNP. The bioinformatics tools we used predicted that only the rs8191992 has a potential functional effect, and this variant is predicted to be in a binding site for the hsa-miR-508–3p.

The role of hsa-miR-508 in the physiopathology of respiratory or allergy diseases is not clear. It is possible that SNPs in the *CHRM2* gene modify M2 receptor expression through mRNA degradation induced by the miRNA binding [33, 34]. In accordance with this hypothesis, Ricci et al. [35] demonstrated that changes in *CHRM* expression in allergic rhinitis define a role of the cholinergic system of immune cells in allergic airway disease. In addition, Szczepankiewicz et al. [16] observed that homozygotes for the risk allele had a significantly poorer response to anticholinergic drugs as compared to patients with the *AA* and *AT* genotypes of the rs6962027 polymorphism. Moreover, recently Mougey et al. [31] reported an association between homozygous for the *CHRM2* rs8191992, rs6962027 and rs6967953 minor alleles and a poor response to asthma management in subjects treated with corticosteroids and bronchodilators.

CHRM2 stimulation limits ACh release in a feedback mechanism, and ACh induces hyperresponsiveness and inflammation by recruiting inflammatory cells to sites of airway inflammation, prolonging the lifespan of lymphocytes or the release of granulocyte macrophage-colony stimulating factor, leukotriene B4, and prostaglandin E2 from bronchial epithelial cells [5, 36, 37]. Moreover, because increased ACh levels could elevate nitric oxide (NO) production by T cells, this neurotransmitter may contribute to an increase in exhaled NO in asthma and consequently exacerbate the inflammation [37, 38]. Furthermore, previous studies have shown that stimulation of postjunctional M2 receptors acts to inhibit adenylate cyclase activation via coupling to an inhibitory G-protein, Gi, and therefore decrease the degree of cyclic AMP-induced airway smooth muscle relaxation [39, 40]. Thus, CHRM2 dysfunction in airway smooth muscle could lead to excessive ACh release, resulting in increased stimulation, bronchoconstriction, mucus secretion, and a reduced ability to activate relaxant mechanisms [40]. There is an extensive literature exploring the role of *CHRM2* SNPs on mental disorder such as alcoholism, nicotine and drug addictions,

depressive disorders, bipolar disorder etc. [20, 21, 41–44], however, in asthma there are few association studies involving this gene and even more, the findings reported have been controversial. Particularly, Cannon et al. documented that the rs324650, a variant within the 5′ untranslated region of *CHRM2*, was associated with abnormally decreased M2-receptor binding in patients with bipolar disorder; supporting the hypothesis that a reduced level of M2 receptor potentially underlies brain or lung entities [43].

For the *CHRM1* and *CHRM3* variants analyzed in the present study, the results suggest that these SNPs are not genetic risk factors for asthma in Mexicans, including the rs2075748 variant in *CHRM1*, which has been associated with protection against asthma in the Japanese population [15]. The CHRM1 and CHRM3 receptors are found on airway smooth muscle and mucus glands, and they mediate vagally induced smooth muscle contraction and mucus hypersecretion [4, 45]. Because of the involvement of both receptors in lung biology, other variants should be investigated to rule out the role of the *CHRM1* and *CHRM3* polymorphisms in asthma etiology [3, 45, 46].

In summary, we identified an association between *CHRM2* 3′ UTR SNPs and asthma, suggesting that variability in the *CHRM2* gene is a contributor to genetic susceptibility for this pulmonary disease with multifactorial etiology. Further studies need to be done to determine the functional aspects of these polymorphisms.

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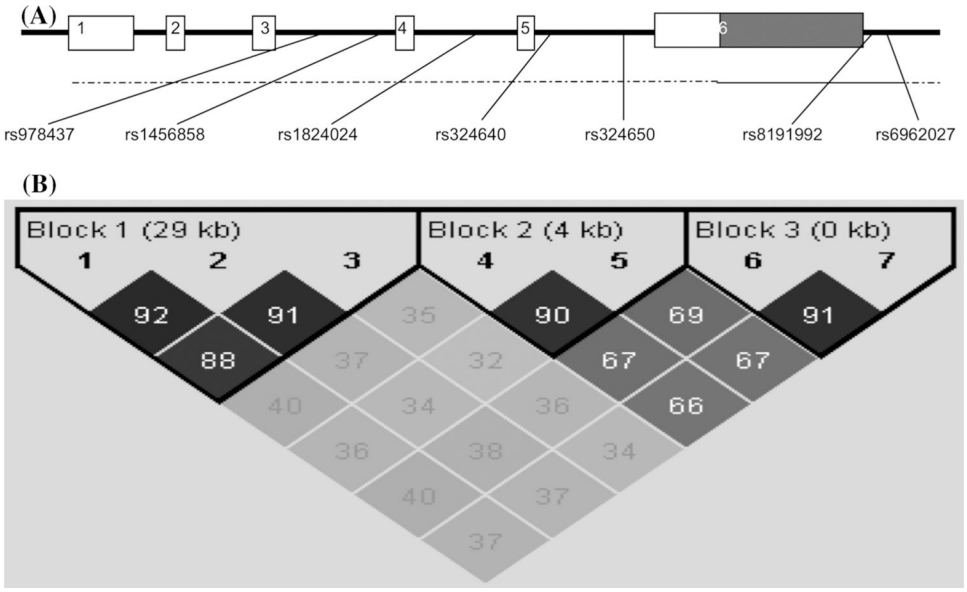

Fig. 1.

a *CHRM2* structure and SNP locations. Non-coding exons are represented by *white boxes* and coding region in *gray* (*exon 6*). **b** Pairwise LD among SNPs within the *CHRM2* gene. Figure depicts the measured r^2 between SNP pairs. Higher r^2 values are observed in three blocks

Table 1

Clinical features of patients with asthma

Clinical features	Mean (SD) or *n* (%)
Age at the study	11.5 ± 3.3
Gender	
Male	244 (61)
Female	154 (39)
Atopy*	
N = 319	266 (83)
Asthma severity[a]	
N = 324	
Intermittent	175 (54)
Mild persistent	53 (16)
Moderate persistent	94 (29)
Severe persistent	2 (<1)

SD standard deviation, *atopy* positive skin test to at least one aeroallergen

[a]Data are missing from several cases

Table 2

*CHRM g*enes and polymorphisms included in the present study

Gene	SNP	Region[a]	Location in the gene	Allele
CHRM1	rs11605665	Chr11:62433378	3′ UTR	*G/T*
	rs2067480	Chr11:62433796	Exon 2	*C/T*
	rs544978	Chr11:62441806	Intron 1	*A/C*
	rs542269	Chr11:62442113	Intron 1	*C/T*
	rs2075748	Chr11:62444845	Intron 1	*A/G*
CHRM2	rs978437	Chr7:136264718	Intron 3	*A/G*
	rs1455858	Chr7:136282243	Intron 3	*A/G*
	rs1824024	Chr7:136294234	Intron 4	*G/T*
	rs324640	Chr7:136339536	Intron 5	*A/G*
	rs324650	Chr7:136344201	Intron 5	*A/T*
	rs8191992	Chr7:136351848	3′ UTR	*A/T*
	rs6962027	Chr7:136352475	3′ UTR	*A/T*
CHRM3	rs7527924	Chr1:237859839	Intron 5	*A/G*
	rs10802785	Chr1:237875652	Intron 5	*G/C*
	rs7520974	Chr1:238133883	Intron 5	*A/G*
	rs6669810	Chr1:238135252	Intron 5	*C/G*
	rs6701181	Chr1:238136810	Intron 5	*C/T*
	rs3738435	Chr1:238137226	Intron 5	*T/C*
	rs4072234	Chr1:238139849	3′ UTR	*A/G*

[a]Positions are from NCBI dbSNP Build 137 for Human

Table 3

Association analysis of individual SNPs and genotypes in the *CHRM2* gene in cases and healthy controls

SNP		*n* (%)		OR [95 % CI]	*P*
		Healthy controls *N* = 450	Asthmatic cases *N* = 398		
rs978437	*GG*	217 (48)	171 (43)		
	GA	193 (43)	170 (43)		
	AA	40 (9)	57 (14)		
	G	627 (70)	512 (64)		
	A	273 (30)	284 (36)	1.27 [1.04–1.56]	0.019
	GG vs *AA*			1.81 [1.15–2.84]	0.009
rs1455858	*AA*	216 (48)	166 (42)		
	AG	193 (43)	177 (44)		
	GG	40 (9)	55 (14)		
	A	625 (70)	509 (64)		
	G	273 (30)	287 (36)	1.29 [1.06–1.58]	0.013
	AA vs *GG*			1.79 [1.13–2.82]	0.011
rs1824024	*GG*	216 (48)	173 (44)		
	GT	194 (43)	173 (44)		
	TT	39 (9)	52 (14)		
	G	626 (70)	517 (65)		
	T	272 (30)	279 (35)	1.23 [1.01–1.51]	0.048
	GG vs *TT*			1.66 [1.05–2.64]	0.029
rs324640	*GG*	284 (63)	228 (57)		
	GA	144 (32)	142 (36)		
	AA	22 (5)	28 (7)		
	G	712 (79)	595 (75)		
	A	188 (21)	199 (25)	1.25 [1.0–1.57]	0.051
rs324650	*AA*	274 (61)	218 (55)		
	AT	149 (33)	151 (38)		
	TT	27 (6)	29 (7)		
	A	697 (77)	589 (74)		

SNP		*n* (%)		OR [95 % CI]	*P*
		Healthy controls *N* = 450	Asthmatic cases *N* = 398		
	T	203 (23)	207 (26)	1.22 [0.98–1.53]	0.076
rs8191992	*AA*	275 (61)	199 (50)		
	AT	148 (33)	165 (42)		
	TT	27 (6)	32 (8)		
	A	698 (78)	563 (71)		
	T	202 (22)	229 (29)	1.42 [1.14–1.77]	0.001 *
	AA vs *AT*			1.55 [1.16–2.06]	0.003
	AA vs (*AT*+ *TT*)			1.57 [1.20–2.06]	0.0001
rs6962027	*AA*	278 (62)	194 (49)		
	AT	145 (32)	170 (43)		
	TT	27 (6)	34 (8)		
	A	701 (78)	558 (70)		
	T	199 (22)	238 (30)	1.50 [1.21–1.87]	0.0002 *
	AT vs *AT*			1.68 [1.26–2.24]	0.0004
	AA vs *TT*			1.80 [1.05–3.09]	0.0296
	AA vs (*AT*+ *TT*)			1.67 [1.25–2.22]	0.0001

* Significant *P* values were observed after 100,000 permutations (*P* = 0.003 and 0.005, respectively). Only statistical significant data from genotype comparison are showed

Table 4

Haplotype association analyses in *CHRM2* between case and control groups

Haplotype	Frequency		OR[a] [95 % CI]	*P*	*P***
	Healthy controls	Asthmatic cases			
Block 1 (rs978437, rs1455858, rs1824024)					
GAG	0.68	0.63	0.71 [0.56–0.9]	0.057	0.382
AGT	0.29	0.34	1.42 [1.05–1.91]	0.022	0.149
AGG	0.01	0.011	2.26 [0.88–5.85]	0.781	1.000
Block 2 (rs324640, rs324650)					
GA	0.77	0.73	1.84 [0.75–4.51]	0.069	0.432
AT	0.21	0.25	1.49 [0.73–3.04]	0.038	0.260
GT	0.015	0.013	0.87 [0.58–1.31]	0.700	1.000
Block 3 (rs8191992, rs6962027)					
AA	0.77	0.70	0.67 [0.53–0.85]	0.001	0.004
TT	0.21	0.28	1.46 [1.15–3.85]	0.002	0.006
AT	0.007	0.015	2.05 [0.70–6.24]	0.233	0.411

Only the haplotypes with frequencies >0.010 are listed

[a]ORs compared to reference of major allele haplotypes; 95 % confidence interval (95 % CI)

*P*** values after 100,000 permutations

Table 5

Association analyses of individual SNPs in the *CHRM1* and *CHRM3* genes in case–control samples

Gene	dbSNP ID	Major/minor allele	MAF		HWE *P*	*P**
			Healthy controls	Asthmatic cases		
CHRM1	rs11605665	*G*/*T*	0.138	0.124	0.455	0.444
	rs2067480	*C*/*T*	0.293	0.324	0.933	0.225
	rs544978	*A*/*C*	0.151	0.128	1.0	0.331
	rs542269	*C*/*T*	0.149	0.236	0.316	0.565
	rs2075748	*A*/*G*	0.08	0.078	0.075	0.807
CHRM3	rs7527924	*A*/*G*	0.450	0.432	0.886	0.457
	rs10802785	*G*/*C*	0.230	0.246	0.478	0.484
	rs7520974	*A*/*G*	0.430	0.423	0.077	0.797
	rs6669810	*C*/*G*	0.436	0.421	0.214	0.545
	rs6701181	*C*/*T*	0.449	0.478	0.234	0.250
	rs3738435	*T*/*C*	0.309	0.333	0.977	0.309
	rs4072234	*A*/*G*	0.427	0.416	0.985	0.660

MAF minor allele frequency, *HWE* Hardy–Weinberg Equilibrium

P values

*P** values for asthma with respect to the minor allele