

ORIGINAL ARTICLE

Transient receptor potential melastatin 8 gene polymorphism is associated with cold-induced airway hyperresponsiveness in bronchial asthma

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ABSTRACT

Background and objective: Cold-induced airway hyperresponsiveness (CAH) is common in bronchial asthma (BA) patients and represents a problem for those living in cold climate. Transient receptor potential melastatin 8 (TRPM8) channel is the main cold temperature sensor in humans that could mediate cold response in asthmatics with CAH. No associations between TRPM8 gene polymorphisms and CAH have been reported.

Methods: The present study involved 123 BA patients. CAH was assessed by 3-min isocapnic (5% CO₂) cold air (−20°C) hyperventilation challenge. The c.750G > C (rs11562975), c.1256G > A (rs7593557), c.3048C > T (rs11563208) and c.3174C > G (rs11563071) polymorphisms of TRPM8 gene were genotyped by allele-specific polymerase chain reaction (PCR) and PCR with subsequent restriction fragment length polymorphism analysis.

Results: GC genotype and C allele carriers of the c.750G > C (rs11562975) polymorphism were more frequently observed to exhibit CAH. The estimated odds ratio for the GC genotype was 3.73 95%CI (1.48; 9.37), $P = 0.005$. Furthermore, GC heterozygotes had a prominent decrease in forced expiratory volume in 1 s after the challenge as compared to GG homozygotes (−12% (−16; −8.1) vs −6.45% (−11; −2.1), $P < 0.001$). GC carriers also had a marked reduction in other spirometric parameters.

Conclusions: The GC variant of the TRPM8: c.750G > C (rs11562975) polymorphism is associated with CAH in patients with BA, which suggests a potential role of TRPM8 in CAH development.

Key words: asthma, cold temperature, polymorphism, spirometry, transient receptor potential channel.

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Received 31 July 2014; invited to revise 20 October and 27 March 2015; accepted 6 May 2015 (Associate Editor: Shu Hashimoto).

Article first published online: 13 August 2015

SUMMARY AT A GLANCE

The transient receptor potential melastatin 8 polymorphism, TRPM8:c.750G > C (rs11562975), was found to be associated with CAH in BA. Patients with heterozygous genotype were more predisposed to bronchoconstriction in response to cold air hyperventilation. This observation may be useful in defining cold airway hyperresponsiveness as a phenotype of bronchial asthma.

Abbreviations: BA, bronchial asthma; CAH, cold-induced airway hyperresponsiveness; ICAH, isocapnic cold air hyperventilation; FEV₁, forced expiratory volume in 1 s; FEF, forced expiratory flow; FEF50%, maximum expiratory flow at 50% of FVC; FEF75%, maximum expiratory flow at 75% of FVC; FVC, forced vital capacity; PEF, peak expiratory flow; SNP, single nucleotide polymorphism.

INTRODUCTION

Increased airway responsiveness to cold air is an important factor in bronchial asthma (BA). It has been shown that cold-induced airway hyperresponsiveness (CAH) is common in patients with BA and represents a serious problem for those living in cold climate. CAH is a cause of poor BA control, excessive deterioration of lung function and reduced quality of life.^{1–3} The effect of therapy in BA with concomitant CAH has been found to be decreased.^{4,5} Defining a CAH BA phenotype by describing its key phenotypic aspects and genotypic properties would make diagnostic processes more precise and may allow physicians to target therapeutic approaches.

Molecular basis of cold perception is mainly represented by specific ion channels named transient receptor potential melastatin 8 (TRPM8) channels.

Temperature threshold for TRPM8 activation commonly lies near 28°C and is affected by various factors like pH, Ca²⁺, lipids and others including ambient temperature.^{6–10} TRPM8 is expressed in various tissues and organs of the human body, including the respiratory system, where its expression has been reported in upper airways, airway epithelium and nerve endings.^{11–13} In the bronchial epithelium activation of a short isoform of TRPM8 produces inflammatory response accompanied by expression and secretion of a number of cytokines including interleukin (IL)-1 α , -1 β , -4, -6, -8, -13 and TNF- α .¹⁴ According to Li M. *et al.* cold temperature can cause mucin MUC5AC hypersecretion via TRPM8 activation and subsequent Ca²⁺/PLC δ 1/MARCKS signalling cascade.¹⁵ TRPM8 expressed in the nerve endings presumably may be responsible for reflex bronchoconstriction.

TRPM8 could be considered as a candidate gene for CAH. The aim of the present study was to investigate the effects of TRPM8 single nucleotide polymorphisms (SNPs) on lower airway reaction to cold air hyperventilation in patients with BA.

METHODS

Subjects

A total of 123 participants with an established diagnosis of mild or moderate persistent BA were recruited. The diagnosis was made according to Global Initiative for Asthma criteria.¹⁶ All asthmatics had a score 19 or less in the Asthma Control Test questionnaire.¹⁷ All subjects were Caucasians with mean age 35.3 $\pm$ 1.01 (39% male, 61% female) living in the Amur region of the Far East, Russia. No current smokers were studied; 11% reported a history of smoking. All participants underwent physical examination, blood sampling for genetic study, spirometry testing and 3-min isocapnic (5% CO₂) cold air (–20°C) hyperventilation challenge (ICAH). The study was approved by the local Ethics Committee of the Far Eastern Scientific Centre of Physiology and Pathology of Respiration and all participants signed the informed consent.

DNA extraction and genotyping

Genomic DNA was extracted from frozen peripheral blood samples using standard method with phenol/

chloroform. The four TRPM8 gene SNPs were genotyped: c.750G > C (rs11562975), c.1256G > A (rs7593557), c.3048C > T (rs11563208) and c.3174C > G (rs11563071). SNPs selection was based on the previously obtained data suggesting their functional significance in cold sensitivity, blood lipids level and anthropometric parameters.^{18,19} Genotyping of the c.750G > C (rs11562975), c.1256G > A (rs7593557) and c.3174C > G (rs11563071) SNPs was performed by allele specific polymerase chain reaction (PCR). The c.3048C > T (rs11563208) SNP was detected by restriction fragment length polymorphisms analysis with TaqI restriction endonuclease. Amplification product was incubated with 5 U TaqI during 17 h at 65°C. C-homozygotes were detected by 141 b.p. product, heterozygotes—by 141 b.p. and 165 b.p. products—and T-homozygotes—by 165 b.p. long product only (Supplementary Fig. S1). Amplification was performed for 30 cycles under the following conditions: denaturation—at 95°C for 1 min., annealing—at the temperature listed in Table 1 for 1 min., extension—at 72°C for 1 min. Primers design was performed in Vector NTI 11.0 software (Invitrogen, Carlsbad, CA, USA, 2010); sequences are shown in Table 1. The final PCR products were separated by polyacrylamide gel electrophoresis in 1x TBE buffer, stained using 4% with ethidium bromide and photographed in UV-illumination.

Lung function testing

Lung function was assessed by spirometry before and after ICAH challenge. Ambient air at room temperature was mixed with CO₂ to attain 5% concentration for prevention of hypocapnia-induced bronchoconstriction. Mixed air was cooled to –20°C, partially dried using cold air generator and delivered to the participant. Appropriate ventilation was defined as 60% from maximal minute ventilation. Respiratory rate and breathing depth were continuously controlled and nasal breathing was prevented by a nose clip. Immediately after 3 min of hyperventilation patients underwent spirometry testing. The ICAH challenge was considered positive with a 10% or greater fall in the forced expiratory volume in 1 s (FEV₁).

Table 1 Primer sequences and annealing temperature

SNP	Primers	Annealing t°C
c.750G > C	G-allele Fwd.	60
	C-allele Fwd.	
	Reverse	
c.1256G > A	G-allele Fwd.	69
	A-allele Fwd.	
	Reverse	
c.3048C > T	Forward	63
	Reverse	
c.3174C > G	C-allele Fwd.	60
	G-allele Fwd.	
	Reverse	

Table 2 Comparative characteristics of baseline lung function in the studied patients

Parameter	Group I (n = 47)	Group II (n = 76)	Significance (P)
Age (years)	34.6 ± 1.54	35.7 ± 1.35	0.58
Gender			
Male, n (%)	18 (38)	30 (39)	0.89
Female, n (%)	29 (62)	46 (61)	
Asthma duration (years)	3.4 ± 0.16	3.3 ± 0.14	0.59
FVC%	104.3 ± 2.92	107.8 ± 1.65	0.26
FEV ₁ %	86.8 ± 2.60	95.4 ± 1.80	0.006
FEV ₁ /FVC	83.1 ± 1.49	89.2 ± 1.20	0.002
Response to salbutamol, FEV ₁ %	17.2 ± 2.34	9.6 ± 1.10	<0.001

Group I: asthma patients with cold-induced airway hyperresponsiveness, Group II: asthma patients without cold-induced airway hyperresponsiveness.

FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity.

Statistical analysis

Statistical analysis was performed in Statistica 8.0 software (StatSoft Inc., Tulsa, OK, USA, 2008). *P*-value <0.05 was considered statistically significant. In case of multiple comparisons Bonferroni adjustment was employed. Kolmogorov–Smirnov and Shapiro–Wilk tests were used to determine whether the data are normally distributed. Data are presented as mean ± standard error of mean or median (lower quartile; upper quartile) in case of normally and abnormally distributed variables, correspondingly. Hardy–Weinberg equilibrium between observed and expected genotype frequencies was evaluated by Pearson’s chi-square test. Associations between qualitative variables were studied by Pearson’s chi-square test or Fisher’s exact test. Quantitative variables were assessed with *t*-test for normal or Mann–Whitney U test for abnormal distribution.

RESULTS

Group I included subjects with CAH (38.2%), group II with normal response to cold air (61.8%). FEV₁ decreased after ICAH by –15% (–25; –11) in group I and –4.4% (–6.75; –1.25) in group II. Analysis of lung function showed lower airway patency and higher response to salbutamol in patients with CAH (Table 2). A weak correlation between the change in FEV₁ and baseline FEV₁ ($r = 0.32$, $P < 0.01$), FEV₁/FVC (forced vital capacity) ratio ($r = 0.34$, $P < 0.01$), peak expiratory flow (PEF) ($r = 0.32$, $P < 0.01$), maximum expiratory flow at 50% of FVC (FEF50) ($r = 0.37$, $P < 0.01$) and 75% of FVC (FEF75) ($r = 0.32$, $P < 0.01$) was observed. No direct association between CAH and BA severity was found ($\chi^2 = 2.4$, $P = 0.12$).

Genotype frequencies were in Hardy–Weinberg equilibrium for each of the studied SNP. The distribution of genotypes and alleles stratified by main groups

are shown in Table 3. Homozygous genotypes for minor alleles were only rarely detected, AA homozygotes for the c.1256G > A (rs7593557) SNP were not found. Subjects with homozygous rare allele genotypes for each SNP were excluded from the further analysis of genotype–phenotype association.

None of the studied SNPs were linked with the disease severity. The c.750G > C (rs11562975) SNP was found to significantly influence the development of cold air-induced bronchoconstriction. GC genotype of the c.750G > C (rs11562975) SNP was identified as a risk factor for CAH in BA as it was represented with a higher frequency in group I in comparison to group II (34% vs 12%; OR 3.7 95% CI (1.48; 9.4), $P = 0.005$). Similarly, evident but less significant association with CAH was detected for C allele of the c.750G > C (rs11562975) SNP. Carriers of C allele were more frequently observed among subjects in group I than in group II (17% vs 8.7%, $P = 0.04$). In addition, GC heterozygotes had more pronounced fall in FEV₁ and other spirometric parameters in response to the bronchoprovocation with ICAH (Δ FEV₁ –12% (–16; –8.1) vs –6.5% (–11; –2.1) for GC and GG genotype carriers, respectively, $P < 0.001$) (Fig. 1).

There were no statistically significant associations observed for the other studied SNPs (c.1256G > A (rs7593557), c.3048C > T (rs11563208) and c.3174C > G (rs11563071)) with CAH, baseline spirometric parameters or their changes after the ICAH challenge test.

Haplotype analysis revealed eight main haplotypes with frequency more than 0.01%. Detailed information on structure and frequencies of distinct haplotypes is shown in Supplementary Table S1. Subjects with H3 haplotype tended to be more prevalent in the group with CAH (12.9%) than in controls (5.7%); however, it was not statistically significant ($\chi^2 = 3.07$, $P = 0.08$). The other haplotypes did not show any substantial difference between the groups. The c.1256G > A (rs7593557) SNP was found to be in almost complete linkage disequilibrium with the c.3174C > G (rs11563071) variation; the c.750G > C (rs11562975) was moderately linked to the c.1256G > A (rs7593557) and c.3048C > T (rs11563208) (Table 4).

DISCUSSION

CAH is a common condition in BA. We demonstrated that almost 40% of the studied BA patients had abnormal airway response to ICAH challenge with markedly decreased tolerance to cold air and significant fall in FEV₁.

Patients with BA and CAH have generally greater baseline airflow limitation than those without CAH, which suggests an influence of the disease severity with underlying activity of inflammation and airway remodelling. However, CAH itself can promote inflammatory responses and be the primary cause of the differences in lung function. No direct association between CAH and asthma severity has been shown in this study. We previously published evidence for genetic predisposition to airway hyperresponsiveness

Table 3 Genotypic and allelic frequencies for the *TPRM8* SNPs in the studied groups

SNP	Group I			Group II			Odds ratio (95% CI), <i>P</i>
c.750G > C	GG	GC	CC	GG	GC	CC	3.73 (1.48; 9.37), <i>P</i> = 0.005
	31 (66)	16 (34)	0	65 (86)	9 (12)	2 (2)	
	G	C		G	C		2.19 (1.003; 4.80), <i>P</i> = 0.049
c.1256G > A	GG	GA	AA	GG	GA	AA	1.27 (0.4; 3.98), <i>P</i> = 0.67
	42 (89)	5 (11)	0	66 (87)	10 (13)	0	
	G	A		G	A		1.25 (0.41; 3.79), <i>P</i> = 0.69
c.3048C > T	CC	CT	TT	CC	CT	TT	1.73 (0.79; 3.8), <i>P</i> = 0.17
	26 (55)	15 (32)	6 (13)	36 (47)	36 (47)	4 (6)	
	C	T		C	T		1.01 (0.57; 1.78), <i>P</i> = 0.97
c.3174C > G	CC	CT	TT	CC	CT	TT	1.87 (0.62; 5.59), <i>P</i> = 0.26
	40 (85)	5 (11)	2 (4)	60 (79)	14 (18)	2 (3)	
	C	T		C	T		1.26 (0.54; 2.95), <i>P</i> = 0.58
	85 (90)	9 (10)		134 (88)	18 (12)		

Group I: asthma patients with cold-induced airway hyperresponsiveness, Group II: asthma patients without cold-induced airway hyperresponsiveness. Rare allele homozygotes were excluded from the analysis of genotypes distribution but preserved in the allelic association tests.

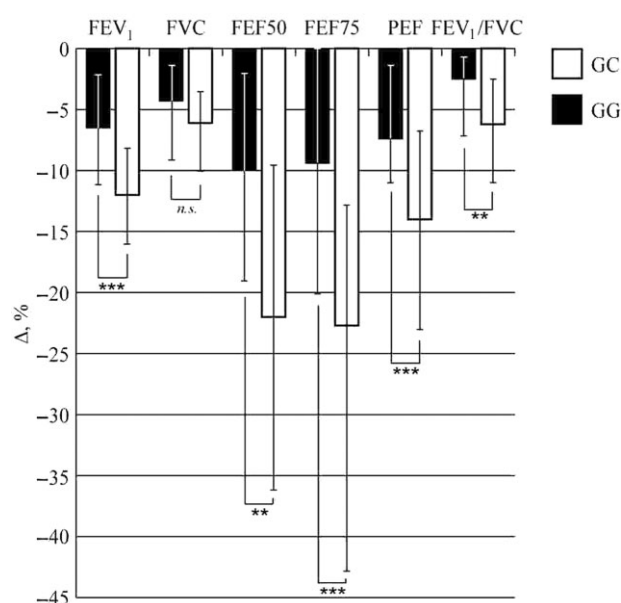


Figure 1 Relative decrease of lung function parameters after cold air hyperventilation in GG and GC genotype carriers (SNP c.750G > C (rs11562975)). Lung function was measured by spirometry: FEV₁, forced expiratory volume; FVC, forced vital capacity; FEF, forced expiratory flow; PEF, peak expiratory flow. Data are presented as median with lower and upper quartiles. n.s., *P* > 0.05; ***P* < 0.01; ****P* < 0.001 by Mann-Whitney U test.

in BA patients and described association with cold-induced airway response for the Arg16Gly SNP of the *ADRB2* gene.²⁰ Its role in CAH development may, however, be non-specific, as it also contributes to airway hyperresponsiveness to metacholine and histamine.^{21,22}

Table 4 Linkage disequilibrium of the studied *TRPM8* polymorphisms

	c.1256G > A	c.3048C > T	c.3174C > G
c.750G > C	<i>D'</i> = 0.43 <i>P</i> = 0.5	<i>D'</i> = 0.28 <i>P</i> = 0.3	<i>D'</i> = 0.07 <i>P</i> = 0.8
c.1256G > A		<i>D'</i> = 0.17 <i>P</i> = 0.3	<i>D'</i> = 0.98 <i>P</i> = 0.16
c.3048C > T			<i>D'</i> = 0.03 <i>P</i> = 0.8

D' represents a normalized deviation of the observed frequency of a haplotype from the expected as a measure of linkage disequilibrium. *P*-value shows a significance of linkage disequilibrium.

In the present study we focused on the role of *TRPM8* gene in CAH as it encodes a specific cold-sensitive receptor and participates in the regulation of temperature homeostasis. The frequencies of the genotypes and alleles of the *TRPM8* SNPs almost coincided with those determined in earlier studies. Homozygous genotypes for minor alleles of the c.750G > C (rs11562975) and c.3174C > G SNPs (rs11563071) were rarely observed. The limited sample size made it impossible to assess the role of homozygous genotypes for rare alleles.

One of the four studied SNPs was found to be significantly associated with the respiratory response to cold air. The c.750G > C (rs11562975) substitution in exon 7 resulted in prominent alteration of airway responsiveness to cold air in asthmatic patients. The GC genotype and C allele were more frequently observed in subjects with CAH than in subjects with normal cold airway responsiveness. The odds for GC carriers to have CAH were 3.7-fold higher than for GG

homozygous individuals. Heterozygotes were also characterized by excessive fall in FEV₁ which reflects the increasing degree of bronchial obstruction in response to ICAH. Some findings may be the result of linkage disequilibrium between the studied SNPs. Analysis of haplotypes allowed us to establish the eight most frequent variants and describe the one supposed to cause cold-induced airway response in asthmatics.

TRPM8 channels are potent regulators of intracellular calcium level and contribute to thermoregulation by providing molecular basis for thermoreception. Only a few studies explore the functional role of *TRPM8* gene polymorphism and none of them considered the possible role of *TRPM8* SNP in BA. Some studies investigated the relation of *TRPM8* polymorphisms to migraine. The rs7577262 SNP was shown to be associated with migraine overall, but not with any of its distinct characteristic.²³ Another study indicated that the L250L (rs11562975) and S419N (rs7593557) SNPs were correlated with lipid metabolism. The V1058V (rs11563071) and I1016I (rs11563208) SNPs were associated with the levels of total, low-density lipoprotein and high-density lipoprotein cholesterol and triglyceride content correspondingly.¹⁹ The most relevant investigation carried by Kozyreva T.V. *et al.* suggested that a difference in cold and menthol sensitivity *in vivo* depended on the rs11562975 SNP genotype.¹⁸ The authors describe that the GC genotype predisposed subjects to enhanced cold but decreased menthol sensitivity that indirectly match the findings of the present study. The temperature measured in trachea and bronchial tree of asthmatics was increased in comparison to healthy subjects.¹ Thus, bronchoconstriction reflex must be provoked from TRPM8 receptors expressed in the upper respiratory tract, especially the larynx, where the temperature may be relatively low during the hyperventilation and sufficient for the ion channel activation. The mechanism underlying the effect of the *TRPM8* SNP is not clear, although there may be several ways by which synonymous SNP can influence cold sensitivity by affecting *TRPM8* gene expression. Alterations in the gene expression resulting in higher neuronal receptor density would explain more pronounced afferent signalling and respectively strong airway response. We did not study *TRPM8* expression in the respiratory system of BA patients. It may be elevated as it has been found in the airways of chronic obstructive pulmonary disease patients, where TRPM8 mediates MUC5AC hypersecretion.¹⁵

This study has some limitations. We were unable to assess the role of homozygous genotypes for rare alleles due to the limited size of the population sample. The study must be replicated in a larger sample to attain the acceptable minor allele frequencies and rates of corresponding genotypes. Another task suggested for further research is to reveal the mechanisms involved in the development of TRPM8-mediated cold hypersensitivity and hyperresponsiveness, particularly the influence of genetic variations on TRPM8 channels expression in the upper airways.

In conclusion, the findings suggest that *TRPM8* is associated with the development of CAH in BA. Variation of the gene sequence, namely, the c.750G > C (rs11562975) SNP, can enhance the response to low temperature predisposing GC carriers to bronchial obstruction in cold air. The association between CAH and *TRPM8* as a specific molecular marker could be used to define CAH as a distinct phenotype of BA. This may lead to a search for a novel therapeutic targets approach to block the ion channels in affected individuals.

Acknowledgements

The authors thank Dr. Anna Prikhodko for assistance in conduction and interpretation of pulmonary function testing and providing valuable remarks on the study protocol. This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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Supplementary Information

Additional Supplementary Information can be accessed via the online version of this article at the publisher's web-site:

Figure S1 Representative genotyping results for the c.3048C > T (rs11563208) SNP. The 165 b.p. and 141 b.p. fragments of amplicon digestion with TaqI are shown.

Table S1 Haplotype frequencies of the TRPM8 polymorphisms in the studied population

SUPPLEMENTARY INFORMATION

The transient receptor potential melastatin 8 gene polymorphism is associated with cold-induced airway hyperresponsiveness in bronchial asthma

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Figure S1- Representative genotyping results for the c.3048C>T (rs11563208) SNP. The 165 b.p. and 141 b.p. fragments of amplicon digestion with TaqI are shown.

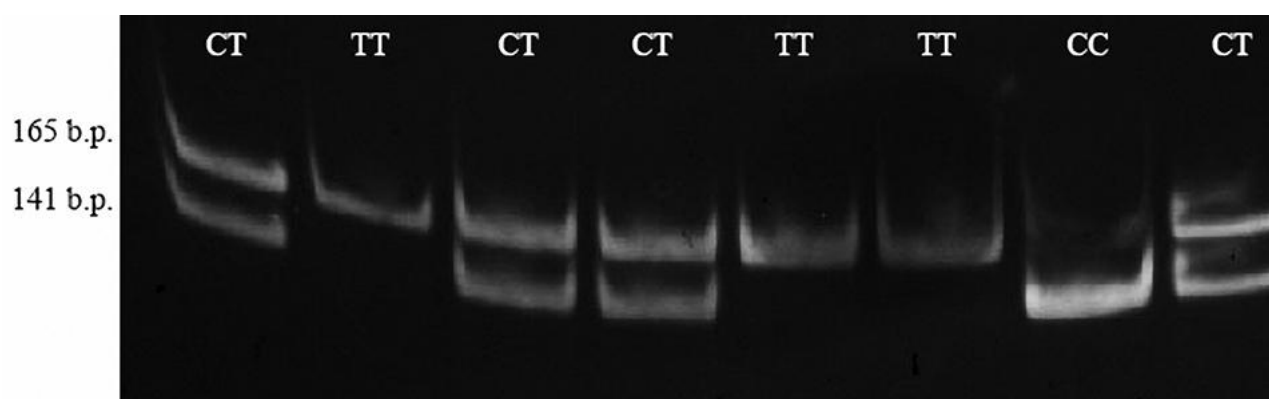


Table S1-Haplotype frequencies of the *TRPM8* polymorphisms in the studied population

Haplotype	Alleles				Frequency (%)			p-value
	c.750G>C	c.1256G>A	c.3048C>T	c.3174C>G	Group I	Group II	Total	
H1	G	G	C	C	49.6	52.3	50.9	0.7
H2	G	G	T	C	19.6	23.3	21.6	0.52
H3	C	G	C	C	12.9	5.7	9	0.08
H4	G	G	C	G	4	9.9	6.9	0.17
H5	G	A	C	C	3.7	3	3.7	0.9
H6	G	G	T	G	4.5	0	3.1	0.1
H7	G	A	T	C	1.6	2.6	2	1
H8	C	G	T	C	3	1	1.8	0.6

Group I: asthma patients with cold-induced airway hyperresponsiveness, Group II: asthma patients without cold-induced airway hyperresponsiveness.; p-value reflects comparison of haplotype frequencies between Group I and II by Pearson's chi-squared test with Yates' correction employed when necessary.