Cysteinyl leukotriene receptor 1 gene variation and risk of asthma

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ABSTRACT: Although it has been recognised that genetics plays an important role in the development of asthma, important causal loci remain to be identified. The aim of the present study was to examine the association of known and novel candidate genes with asthma.

Two independent samples, including 170 asthmatic cases and 347 controls in the initial sample, and 202 asthmatic cases and 332 controls in the confirmation sample, were recruited from the same region of China. Functional single nucleotide polymorphisms (SNPs; n=129) from 105 genes were genotyped using MassARRAY technology, and 119 SNPs were used for the subsequent analysis.

In the initial sample, three SNPs, rs320995 in the cysteinyl leukotriene receptor 1 gene, rs1047266 in the tumour necrosis factor receptor superfamily, member 10b, gene and rs40401 in the interleukin-3 gene, were associated with risk of asthma. Notably, under the recessive genetic model, subjects without the thymidine allele in SNP rs320995 had a 3.1 times higher risk of asthma, which remained significant after accounting for multiple testing. This association was replicated in the confirmation sample and validated by meta-analysis. Further, sex-specific analysis was performed, but no sex difference was found.

The present study provided coherent evidence that cysteinyl leukotriene receptor 1 gene variation is associated with risk of asthma.

KEYWORDS: Asthma susceptibility, cysteinyl leukotriene receptor 1 gene, single nucleotide polymorphism

Asthma, a chronic inflammatory disorder of airways, has been recognised as a serious public health problem worldwide. As estimated by the Global Initiative for Asthma in 2006, ~300 million individuals of all ages and different ethnicities suffer from asthma, and the number of annual worldwide deaths due to asthma were estimated at ~250,000. Since the burden of this disease has continued increasing worldwide, better understanding of the aetiology and underlying biological mechanisms of asthma are important for the effective prevention and treatment of asthma.

It has been well established that the development of asthma is influenced by genetic and environmental factors and their interactions. Currently, >100 asthma susceptibility genes have been reported in at least one population [1–3]. Approximately 33 genes, which have been replicated by more than five studies, have been suggested to be underlying candidate genes of asthma [3]. However, important gaps remain in

the dissection of genetic predispositions of asthma. For example, even if a genotype association is replicated, it is often associated with different phenotypes, different polymorphisms in the same gene, different alleles of the same variant or different populations [1]. Since asthma is a complex disease, genes with different biological functions and involved in various regulatory pathways may simultaneously affect the development of asthma. However, most reported genetic studies of asthma have examined only one or a few candidate genes in a single study. Only limited studies have systematically examined a large number of candidate genes in relation to asthma or asthma-related phenotypes [4–11].

The aim of the present study was to systematically examine the association of known or suspected novel candidate genes with asthma and asthma-related phenotypes in two sets of homogeneous samples recruited from the same region of China: one for an initial genetic

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STATEMENT OF INTEREST

None declared.

association test, and the other for confirmation. To the present authors' knowledge, the present study is the largest asthma candidate gene study in a Chinese population.

MATERIALS AND METHODS

Subject recruitment and phenotype definition

In the present study, 170 unrelated asthmatic cases and 347 unrelated controls (the initial sample) were selected from 3,022 families. A detailed description of the study site, subject recruitment and protocols of data collection has been provided previously [12]. Briefly, the study subjects were enrolled from the eight counties of the city of Anqing (China). The following data were available for each participant: 1) information on respiratory symptoms, disease history and smoking, collected using a questionnaire; 2) forced expiratory volume in one second (FEV₁) and forced vital capacity (FVC), measured using a standardised spirometric test; 3) airway methacholine challenge test results from five sequentially increasing combinations of numbers of breaths and methacholine concentration (1 breath of 1 mg·mL⁻¹, 1 breath of 5 mg·mL⁻¹, 4 breaths of 5 mg·mL⁻¹, 1 breath of 25 mg·mL⁻¹ and 4 breaths of 25 mg·mL⁻¹), with the test being terminated at the dose that produced a $\geq 20\%$ fall in FEV₁ from baseline (PD₂₀), or at the final dose if PD₂₀ was not observed; and 4) blood eosinophil (Eos) count, performed by use of a Coulter counter. The present study protocol was approved by the Human Subjects Committee (the institutional review board (IRB)) of Harvard School of Public Health (Boston, MA, USA), the institutional review board of the University of Illinois at Chicago (Chicago, IL, USA) and the Ethics Committee of Anhui Medical University (Hefei, China). Written informed consent was explained to, read and signed by each participant.

In the initial step of the present study, asthma cases were defined on the basis of: 1) self-reported physician-diagnosed asthma; 2) an observed PD₂₀ on or before the third dose during the five-dose test; and 3) being in the top quartile of Eos distribution; The controls were defined on the basis of: 1) no history of self-reported physician-diagnosed asthma; 2) no observed PD₂₀ during the five-dose test; and 3) being in the bottom quartile of Eos distribution. In total, 170 asthma cases and 347 controls were enrolled for genotyping.

An additional 202 unrelated asthmatic cases and 332 unrelated matched controls (the confirmation sample) were selected from the same study population in order to independently confirm the significant signal detected in the initial screening. The selection criteria for asthmatic cases and controls in the confirmation sample were almost identical to those employed for the initial sample, except that the criterion based on Eos distribution was not applied during the enrolment of the confirmation sample. It is of note that of all the cases and controls in the two data sets were unrelated.

Criteria for candidate gene and single nucleotide polymorphism selection

Asthma candidate genes were first identified from GeneCards [13]. Specifically, candidate genes were identified using the following keywords: "asthma", "bronchial responsiveness", "lung function", "airway smooth muscle", "airway allergy or inflammation", "eosinophil and allergy or inflammation", "mast cell and allergy or inflammation", "IgE", and "atopy".

A total of 989 potential asthma candidate genes were identified from the GeneCards database. Next, candidate single nucleotide polymorphisms (SNPs) were selected from among these 989 candidate genes based on the following criteria: 1) allele frequency in Asian populations (obtained from the HapMap project [14] and Affymetrix [15]); 2) BLOck Substitution Matrix with 80% identity (BLOSUM80) score [16], which represents the severity of amino acid change caused by a nonsynonymous SNP, a lower score meaning more severity in amino acid change; and 3) previously reported synonymous SNPs that showed association with asthma. All SNPs with a minor allele frequency (MAF) of $< 5\%$ and all nonsynonymous SNPs with a BLOSUM80 score of ≥ 0 were removed. If complete linkage disequilibrium (LD; $r^2=1$) existed among two or more nonsynonymous SNPs on the same gene, only the SNP with the lowest BLOSUM80 score was included. As a result, a total of 141 SNPs from 113 genes were selected and 129 SNPs from 105 genes were successfully genotyped. Detailed information regarding each genotyped SNP is provided in table E1 of the supplementary material.

Genotyping

A venous blood sample was obtained from each study participant, and genomic DNA was extracted from blood lymphocytes using a standard salting out procedure. Genotyping of 129 SNPs was performed using Sequenom (San Diego, CA, USA) MassArray technology in the Broad Institute Center for Genotyping and Analysis (Cambridge, MA, USA). In the confirmatory stage, the SNP with the identifier rs320995 was genotyped using the TaqMan allelic discrimination method, which has been described previously [17]. A random 5% of the samples were independently repeated in order to confirm genotyping results. The concordance of these duplicated samples was $> 99.5\%$. Additionally, 50 samples that had been genotyped using Sequenom MassArray technology were randomly selected. SNP rs320995 was re-genotyped using the TaqMan allelic discrimination method in these 50 samples. The data showed that the concordant rate of these two genotyping methods was 100%.

Data analysis

The primary outcome was the risk of developing asthma, and the secondary outcomes were continuous variables related to lung function, including FEV₁, FVC and FEV₁/FVC. For SNPs on the autosomal chromosomes, the Hardy-Weinberg equilibrium (HWE) in the control group was examined using a Chi-squared test and those not in HWE were removed from further analysis. Under three different genetic models (additive, recessive and dominant), multiple logistic and linear regression models were used to estimate the effect of each SNP on asthma and lung function, separately, with adjustment for age, age squared, height, height squared, weight, current smoking status and study site. In order to account for multiple testing, a permutation of 10,000 times for 119 SNPs and four phenotypes under three genetic models were performed to obtain empirical p-values. The permutations were performed using R [18].

The HWE test for the SNP on the X chromosome was performed according to the method suggested by ZHENG *et al.* [19]. Since most loci on the X chromosome are subject to X chromosome inactivation, males were treated as homozygous

females in the initial analysis, as applied previously in a genome-wide association study [20], and then the association was tested under the three genetic models. Sex-specific analysis was also performed.

Next, a meta-analysis was undertaken to evaluate the overall effect of SNP rs320995 in the cysteinyl leukotriene (Cys-LT) receptor 1 (CysLT₁) gene (*CYSLTR1*). Published reports containing the terms “*CYSLTR1* asthma”, “cysteinyl leukotriene receptor 1 asthma” and “cysteinyl-leukotriene type 1 receptor asthma” were identified by PubMed searches (1966 to April 2008) by two independent investigators (X. Hong and H-J. Tsai). From the initial search, six articles were found [21–26]. Three articles were excluded due to not having case-control study design and there being no information on sample size [23, 25, 26]. Heterogeneity was assessed using a Chi-squared test, and the meta-analysis was performed using a fixed-effect model if no significant heterogeneity existed, or using a random-effect model if huge heterogeneity existed. The pooled odds ratio (OR) and 95% confidence interval (CI) were calculated as a measure of the genetic effect of rs320995 on asthma in the whole population as well as in sex-specific subgroups. It should be noted that, in sex-specific meta-analyses, the report of Choi *et al.* [22] was excluded because it did not report sex-specific results. All of the meta-analyses were performed using R.

RESULTS

Demographic and genotyping characteristics

The phenotypic characteristics of the 170 asthmatic cases and 347 controls in the initial sample, as well as the 332 asthmatic cases and 202 controls in the confirmation sample, are summarised in table 1. In the initial sample, asthmatic cases were relatively younger, shorter, had a lower body mass index (BMI) and a higher percentage of males compared to controls. In the confirmation sample, asthmatic cases and controls were comparable in terms of age, sex, weight, height and BMI.

A total of 129 SNPs from 105 genes with different biological functions were genotyped in the initial sample (see table E1 of the supplementary material). Among them, 16 genes have previously been reported to be associated with asthma or asthma-related phenotypes in at least one published study. The mean genotyping call rate for the 129 SNPs was ~97.5%. Six SNPs with a MAF of <0.05 were excluded in the subsequent analyses. The mean MAF for the remaining 123 SNPs was ~0.23. Using a p-value of <0.01 as the cut-off for significance, four SNPs (rs3732486, rs11584340, rs2071499 and rs2274907) were not in HWE and removed from the subsequent analysis. Finally, a total of 119 SNPs from 98 genes, with a genotyping call rate of 97.9%, were used to examine the association with asthma and lung function.

Initial association analysis

In the initial sample, evidence suggestive of an association with asthma was found for three of the SNPs with a *p*-observed of <0.01, including rs320995 in *CYSLTR1* (OR 3.1; 95% CI 1.8–5.4; *p*-observed=0.00004), rs1047266 in the tumour necrosis factor (TNF) receptor superfamily, member 10b, gene (*TNFRSF10B*; OR 1.9; 95% CI 1.2–3.1; *p*-observed=0.007) and rs40401 in the interleukin (IL)-3 gene (*IL3*; OR 0.5; 95% CI 0.3–0.8; *p*-observed=0.008; table 2). Only the association between SNP

rs320995 and asthma remained significant after accounting for multiple testing by permutation (*p*-global=0.047). Interestingly, this association remained marginally significant if Bonferroni correction was applied instead of permutation.

The association between each SNP and continuous variables related to lung function are shown in table E2 of the supplementary material. SNP rs320995 tended to be associated with FEV₁/FVC (*p*-observed=0.0004), but not with FEV₁ or FVC. Evidence suggestive of association with FEV₁ was also detected for four SNPs with a *p*-observed of <0.01. However, none of these associations were significant after accounting for multiple testing.

Further subgroup analyses were performed for the relationship between rs320995 and asthma in subjects aged <25 yrs and ≥25 yrs, and similar associations detected in the younger (OR 5.2; 95% CI 1.7–16.5; *p*-observed=0.005) and older group (OR 3.0; 95% CI 1.4–6.3; *p*-observed=0.004). There was no significant interaction between age and rs320995 by log-likelihood-ratio test.

Confirmatory and pooled analyses for single nucleotide polymorphism rs320995

The association between SNP rs320995 and asthma was successfully validated in another independent sample of 202

TABLE 1 Population characteristics of the two sample sets

	Initial sample		Confirmation sample	
	Case	Control	Case	Control
Subjects n	170	347	202	332
Age yrs	22.6±14.5**	33.0±13.1	28.0±15.2	29.0±16.0
Weight kg	40.7±14.9**	51.0±9.3	44.7±15.2	45.4±12.8
Height cm	145.1±17.4**	157.5±9.6	149.5±17.6	151.9±14.3
BMI kg·m⁻²	18.5±3.3**	20.4±2.6	19.2±3.4	19.2±3.0
Asthma-related phenotype				
FEV ₁ L	2.1±0.8**	3.1±0.7	2.3±0.8**	3.0±0.9
FVC L	3.1±1.2**	3.7±0.9	3.3±1.2*	3.5±1.1
FEV ₁ /FVC	0.7±0.1**	0.8±0.1	0.7±0.1**	0.9±0.1
Log₁₀Eos cells·mm⁻³	2.7±0.2**	1.4±0.1	2.0±0.5**	1.9±0.3
Male	87 (51.2)*	145 (41.8)	119 (58.9)	196 (59.0)
Children*	90 (52.9)**	52 (15.0)	69 (34.2)	123 (37.1)
Current smoker†	17 (21.3)	76 (25.8)	54 (26.7)	85 (25.6)
Study sites				
Huanning county	42 (12.1)	9 (5.3)	49 (14.8)	37 (18.3)
Qianshan county	18 (5.2)	24 (14.1)	53 (16.0)	18 (8.9)
Susong county	16 (4.6)	20 (11.8)	11 (3.3)	12 (5.9)
Tongchen county	9 (2.6)	12 (7.1)	5 (1.5)	10 (4.9)
Taihu county	31 (8.9)	23 (13.5)	9 (2.7)	24 (11.9)
Wangjiang county	174 (50.1)	34 (20.0)	82 (24.7)	50 (24.8)
Yuxi county	8 (2.3)	7 (4.1)	48 (14.5)	10 (5.0)
Zhongyang county	49 (14.1)**	41 (24.2)	75 (22.6) **	41 (20.3)

Data are presented as mean±sd for continuous variables and n (%) for categorical variables. BMI: body mass index; FEV₁: forced expiratory volume in one second; FVC: forced vital capacity. #: aged ≤18 yrs; †: determined only in adults. *: *p*<0.05; **: *p*<0.01 versus controls (unpaired t-test for continuous variable and Chi-squared test for categorical variables).

TABLE 2 Association of single nucleotide polymorphisms (SNPs) with the risk of developing asthma in the initial screening[#]

Gene	SNP ID	Polymorphism	MAF [†]	Genotypic distribution ⁺		Genotypic analysis		
				Case	Control	Model [§]	OR (95% CI) [‡]	pobserved ^{##}
CYSLTR1	rs320995	Phe309Phe	40.0	89/26/55	204/79/60	Recessive	3.1 (1.8–5.4)	0.00004 ^{††}
TNFRSF10B	rs1047266	Ala67Val	26.9	69/77/21	186/128/28	Dominant	1.9 (1.2–3.1)	0.007
IL3	rs40401	Ser27Pro	49.7	46/94/30	93/163/91	Recessive	0.5 (0.3–0.8)	0.008
RNASE3	rs2073342	Thr124Arg	27.5	75/81/14	182/136/27	Dominant	1.8 (1.1–2.9)	0.011
CSF2	rs25882	Ile117Thr	38.7	64/88/17	136/148/58	Recessive	0.4 (0.2–0.9)	0.020
SERPINB2	rs6104	Ser413Cys	44.0	38/94/37	100/183/59	Additive	1.5 (1.0–2.1)	0.026
TPO	rs1126799	Val847Ala	37.1	70/61/29	130/163/43	Dominant	0.6(0.4–1.0)	0.042
MMP8	rs3765620	Ile32Thr	39.1	75/71/21	129/161/54	Recessive	0.5(0.2–1.0)	0.040

ID: identifier; MAF: minor allele frequency; OR: odds ratio; CI: confidence interval; *CYSLTR1*: cysteinyl leukotriene receptor 1 gene; *TNFRSF10B*: tumour necrosis factor receptor superfamily, member 10b gene; *IL3*: interleukin-3 gene; *RNASE3*: ribonuclease, RNase A family, 3 gene; *CSF2*: colony-stimulating factor 2 (granulocyte macrophage) gene; *SERPINB2*: serpin peptidase inhibitor, clade B, member 2 gene; *TPO*: thyroid peroxidase gene; *MMP8*: matrix metalloprotease-8 gene; Phe309Phe: substitution of phenylalanine 309 with phenylalanine; Ala: alanine; Val: valine; Ser: serine; Pro: proline; Thr: threonine; Arg: arginine; Ile: isoleucine; Cys: cysteine. [#]: pobserved<0.05; [†]: in control sample; ⁺: shown as homozygotes for major allele/heterozygotes/homozygotes for minor allele; [§]: most proximate model (of recessive, dominant and additive); [‡]: adjusted for age, age squared, height, height squared, weight, current smoking, sex and study site; ^{##}: p-value not accounting for multiple testing; ^{††}: pglobal=0.047 after accounting for multiple testing by permutation.

asthmatic cases and 332 controls (OR 1.6; 95% CI 1.0–2.4; p=0.032), as well as in the pooled sample (OR 2.0; 95% CI 1.4–2.7; p=0.00002; table 3).

Next, sex-stratified analyses were performed separately in each independent sample and in the pooled sample. The present results suggested that males without a T allele in SNP rs320995 had a significantly higher risk of asthma in the initial sample (OR 3.2; 95% CI 1.5–7.1; p=0.004), confirmation sample (OR 1.7; 95% CI 1.0–2.9; p=0.045) and pooled sample (OR 2.1; 95% CI 1.4–3.1; p=0.0003; table 3). Likewise, under the genetic recessive model, females without a T allele in SNP rs320995 showed a significantly higher risk of asthma in the initial sample (OR 3.3; 95% CI 1.5–7.3; p=0.003) and pooled sample

(OR 2.0; 95% CI 1.1–3.4; p=0.014). The effect of SNP rs320995 on risk of asthma was comparable in males and females.

Finally, the association of SNP rs320995 with lung function in the confirmation and pooled samples was examined. It was found that, in males, the relationship between FEV₁/FVC and SNP rs320995 in the confirmation sample and pooled sample showed a similar trend to that detected in the initial sample (see table E3 of the supplementary material).

Meta-analysis of single nucleotide polymorphism rs320995

Meta-analysis was performed to estimate the genetic effect of rs320995 on asthma using the present data and data available from previous published reports, for which mild heterogeneity

TABLE 3 Association of rs320995 with risk of developing asthma in a Chinese population

Allele	Initial sample			Confirmation sample			Pooled sample		
	Case	Control	OR [#]	Case	Control	OR [#]	Case	Control	OR [#]
Subjects n	170	343		202	332		372	675	
Total									
T	67.6	82.5	1.0	69.8	77.4	1.0	69.5	80.9	1.0
No T [†]	32.4	17.5	3.1 (1.8–5.4) ⁺	30.2	22.6	1.6 (1.0–2.4) [*]	30.5	19.1	2.0 (1.4–2.7) ⁺
Male									
T	56.3	74.7	1.0	60.5	70.4	1.0	58.7	72.2	1.0
No T [†]	43.7	25.4	3.2 (1.4–7.1) ^{**}	39.5	29.6	1.7 (1.0–2.9) [*]	41.3	27.8	2.1 (1.4–3.1) ^{***}
Female									
T	79.5	88.1	1.0	83.1	87.5	1.0	81.3	87.8	1.0
No T [†]	20.5	11.9	3.3 (1.5–7.3) ^{**}	16.9	12.5	1.4 (0.6–3.3)	18.7	12.2	2.0 (1.1–3.4) [*]

Data are presented as percentages unless otherwise indicated. OR: odds ratio; T: thymidine. [#]: adjusted for age, age squared, height, height squared, weight, current smoking, sex (only for the analysis in the total population) and study site; [†]: males with a cytosine (C) allele or females of CC genotype. ^{*}: p<0.05; ^{**}: p<0.01; ^{***}: p<0.001; ⁺: p<0.0001.

FIGURE 1. Pooled analysis of effect of single nucleotide polymorphism rs320995 on asthma. Data are presented as odds ratio (OR; ■ (size reflects weighting)), using a fixed effect model (..... line of no effect), and 95% confidence interval (horizontal bars). The centre of the diamond indicates the combined mean effect of the studies included in meta-analysis and its extremities the 95% confidence interval.

was observed ($p=0.03$). A significant association was found between rs320995 and asthma using a fixed-effect model (OR 1.3; 95% CI 1.1–1.5; fig. 1) and marginally significant association using a random-effect model (OR 1.2; 95% CI 0.8–1.9). On sex-specific analysis, heterogeneity was not observed and a significant association was detected between rs320995 and asthma in both males (OR 2.0; 95% CI 1.4–2.8) and females (OR 1.4; 95% CI 1.0–2.1) using a fixed-effect model.

DISCUSSION

Taken together, the present study has contributed to the genetics of asthma in the following aspects. First, the coding SNPs of 98 asthma candidate genes were systematically examined in relation to asthma and lung function. To the present authors' knowledge, this is the largest-scale asthma candidate gene study in a homogeneous Chinese population.

Secondly, a significant association between SNP rs320995 in *CYSLTR1* and the risk of asthma was validated in two independent Chinese samples. Although the associations between *CYSLTR1* variations and asthma or asthma-related phenotypes have previously been investigated in Caucasian [21, 23, 24] and Asian populations [22, 25–28], the results were inconsistent. In detail, ARRIBA-MENDEZ *et al.* [21] reported that the *CYSLTR1* thymidine to cytosine substitution at nucleotide 927 (rs320995) variation could predispose male children to asthma and atopic dermatitis in a Caucasian population [21]. This positive association was confirmed by SANZ *et al.* [24] in an adult Caucasian population and by KIM and co-workers [27, 28] in an adult Korean population, but KIM and co-workers [27, 28] presented a positive relationship between the promoter variations and the risk of aspirin-intolerant asthma. Inconsistent with these findings, two studies in a Japanese population found no association of the promoter variations or rs320995 in *CYSLTR1* with asthma [25, 26]. The study in a Caucasian population found that rs320995 was associated with female atopic severity but not asthma [23]. These inconsistent

results may be partly caused by limited statistical power in some previous reports of relatively small sample size ($n<400$). Another possible explanation for the inconsistency is the genetic heterogeneity of asthma and ethnicity/geometric difference, which suggests that the association between *CYSLTR1* variations and asthma should be replicated in different populations. The results of the current meta-analysis using the present data and data from three previous reports [21, 22, 24] further support the genetic effect of SNP rs320995 on asthma.

The association between *CYSLTR1* variation and risk of asthma is biologically plausible. *CYSLTR1* encodes the G-protein-coupled receptor CysLT₁. Cys-LTs are biologically active lipid mediators, which can promote human airway smooth muscle contraction, proliferation and migration [29]. Cys-LTs have also been reported to be involved in the generation of type-2 T-helper cell cytokines and expression of some mediators involved in the process of airway inflammation and remodeling, such as TNF- α , endothelin 1 [30], reactive oxygen intermediate [31], histamine receptor [32] and IL-11 [33]. These biological actions of Cys-LTs occur *via* binding to Cys-LT receptors, CysLT₁ and CysLT₂ [34]. Activation of CysLT₁ may result in proliferation and contraction of smooth muscle, oedema and Eos migration to the lungs, and thus play an important role in the development of asthma. In the present study, evidence was found suggestive that SNP rs320995 may affect asthma and also FEV₁/FVC, but not FEV₁ or FVC. Given the multiple functions of *CYSLTR1*, the specific pathways by which SNP rs320995 affects the risk of asthma should be further investigated.

Although SNP rs320995 does not cause any change in amino acid sequence, it remains likely that the nucleotide substitution at SNP rs320995 might affect the efficiency of *CYSLTR1* mRNA processing and/or stability. Alternatively, SNP rs320995 may be in strong or complete LD with some important functional SNPs in *CYSLTR1*, which are asthma causal. Therefore, the available SNPs in *CYSLTR1* were explored in a Chinese population from the HapMap project and it was found that SNP rs320995 was in strong LD with SNP rs3201029 in the promoter region ($r^2=0.87$). In addition, published data indicated that SNP rs320995 was in complete LD with SNPs rs3201029, rs2637204 and rs2806489 in the promoter regions [25]. These polymorphisms have been reported to significantly change promoter activity and increase the risk in the development of aspirin-intolerant asthma [25, 27, 28]. As such, SNP rs320995 may be a surrogate marker for a causal SNP in the promoter region of *CYSLTR1*. In the future, it might be more informative to map the causal SNP(s) in *CYSLTR1* by genotyping the complete SNP set covering the whole gene.

In addition to SNP rs320995, the present findings also suggested that SNP rs40401 in *IL3* should be of particular interest for further investigation. Specifically, the present study validated the protective effect of the proline 27 allele in SNP rs40401 in *IL3* on asthma, which has been reported previously in a Korean population [35]. Similar associations were detected between this SNP and lung function, including FVC and FEV₁/FVC. Although the results did not remain significant after correcting for multiple testing, this is probably due to a modest genetic effect and/or the relatively small sample size.

The present study has several strengths. First, asthmatic cases were defined carefully based on physician diagnosis plus airway responsiveness to methacholine challenge. This combined definition is more reliable than physician diagnosis alone since: 1) clinical definition of asthma relies on the clinical experience of physicians and thus lacks standardisation; and 2) airway responsiveness to methacholine is a reproducible and robust method associated with the diagnosis of asthma [36]. Secondly, all of the participating subjects were recruited from a rural area and were homogeneous with respect to ethnicity, lifestyle and occupation, as well as social and cultural norms, which would minimise potential confounding effects and decrease the degree of false-positive results. Thirdly, the association between SNP rs320995 in *CYSLTR1* and asthma was identified and confirmed in two independent samples. Since false-positive association has been a critical concern in candidate-gene association studies, the approach herein would ensure that the observed association was less likely to be a false-positive result.

However, there are several limitations that should be considered. In the initial sample, the controls were older than the cases. This was intentional and done to ensure that controls would not develop asthma. In order to minimise the potential confounding effects caused by age, height and weight, these three variables were included as covariates in all of the analyses, and the observed association was further confirmed in an independent set of cases and controls with matched characteristics in terms of age, height and weight. The present authors acknowledge that such study design may be problematic in terms of detection of age-related associations. However, in the present study, similar associations between rs320995 and asthma were detected in both age groups and no significant interaction was found between age and rs320995, which suggests that age may have a minimal effect on the association between rs320995 and asthma. Another limitation is that the cases and controls in the initial sample were selected based on an additional criterion, which was not applied in the confirmation sample, *i.e.* the cases/controls in the initial sample had an extremely high/low Eos level. Since no association could be observed between SNP rs320995 and Eos level in the confirmation sample, and the association between SNP rs320995 and asthma remained significant with additional adjustment of logarithmically transformed Eos density in the pooled sample, it was possible to be confident that such sample selection would not lead to a bias in the present findings. Finally, total immunoglobulin E data were not available for the study, which limited ability to explore the association of rs320995 with atopy.

In conclusion, the present study provides strong evidence that single nucleotide polymorphism rs320995 in the cysteinyl leukotriene receptor 1 gene is associated with asthma in a Chinese population, and that this effect is comparable in males and females. Further investigation into the biological functions of the cysteinyl leukotriene receptor 1 gene in relation to asthma and its related phenotypes may provide new clues for asthma therapeutic and preventive strategies.

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