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Association between SNPs in the promoter region in cathepsin S and risk of asthma in Chinese Han population

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Abstract. – OBJECTIVE: Cathepsin S (CTSS) is a lysosomal cysteine protease and is predominantly expressed in antigen-presenting cells, which plays an important role in the allergic response. In this study, we explored the association between single nucleotide polymorphisms (SNPs) in the promotor regions in CTSS and risk of asthma.

PATIENTS AND METHODS: A total of 591 cases and 621 controls were recruited for this study. Five SNPs in the CTSS were selected including rs7534124, rs16827671, rs34495036, rs3754212, and rs1136774. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was employed for genotyping.

RESULTS: Logistic regression analysis showed that rs7534124 CT and CT + CC genotypes had significantly decreased risk of asthma (CT *vs*. TT, OR: 0.576, 95% CI: 0.425-0.780, $p < 0.001$; CT + CC *vs*. TT, OR: 0.638, 95% CI: 0.479-0.849, $p < 0.001$; respectively) compared to TT genotype. Similarly, the rs1136774 AG and AG + GG genotypes (AG vs. AA, OR: 0.581, 95% CI: 0.426-0.793, $p = 0.001$; AG + GG *vs*. AA, OR: 0.647, 95% CI: 0.483-0.867, $p = 0.004$, respectively) were also associated with a decreased risk of asthma. However, there was no significant association between genotypes of the remaining SNPs and the risk of asthma ($p > 0.006$). Moreover, the alleles in all SNPs are also not associated with the risk of asthma.

CONCLUSIONS: Our study provided strong evidence that polymorphism of rs7534124 and rs1136774 in CTSS promoter may decrease the susceptibility of asthma in a Chinese Han population.

Key Words:
Cathepsin S, Single nucleotide polymorphism, Asthma, Susceptibility, Chronic obstructive pulmonary disease.

Introduction

Asthma is a chronic inflammatory disease of the airways that is characterized by reversible airflow limitation, wheezing, coughing, chest tightness, and breathlessness[1]. Asthma is influenced by genetic and environmental factors[2]. It is a serious health problem worldwide; however, the precise mechanism of asthma is still unclear[3]. Exposure to various environmental factors may cause an asthma attack in some people[4-6], while many other people remain unaffected. This implies that genetic factors may play important roles in the pathogenesis of asthma[7,8].

Allergic asthma is most frequently induced by aeroallergens such as pollen, dust mite excreta and animal dander[9]. Inhalation of allergen leads to sensitization and development of primary immune response, which contributes to the development of asthma[10]. Inhaled allergens are endocytosed by antigen-presenting cells (APCs), which are usually dendritic cells (DCs) in the airways that function in surveying the environment for pathogens. Subsequently, APCs present antigens on the cell surface via major histocompatibility complex class (MHC) II molecules, which play an important role in this procedure[11]. Several lysosomal proteases are also involved in the allergen presentation process[12]. In particular, cathepsin S (CTSS), a lysosomal cysteine protease, plays a critical role in invariant chain degradation and antigen presentation in both professional and nonprofessional MHC class II-expressed APCs[13,14].

Cumulative evidence has suggested that proinflammatory cytokines such as interleukin (IL)-13, interferon (IFN)-γ, can induce CTSS expression in several antigen-driven inflammatory lung models in mice[14-19]. For example, CTSS is induced by IL-13 in chronic obstructive pulmonary disease (COPD)[16]. This protease inhibition ameliorated airway inflammation in a murine asthma model[1] and decreased IFN-γ-induced emphysema and lung inflammation[15]. Cimerman et al[20] re-

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ported that CTSS concentrations were significantly lower in steroid-independent asthmatics compared to controls. However, there was no difference between healthy subjects and steroid-dependent asthmatics.

Studies have shown that the single nucleotide polymorphisms (SNPs) in the CTSS were associated with several diseases. For example, a G > A change at nucleotide-25 within the promoter of the CTSS might be helpful in studying associations between atherosclerosis and related phenotypes in a Caucasian and Canadian Inuit population[21]. However, the CTSS -25G/A polymorphism was not related to coronary heart disease in a Chinese population[22]. CTSS variants (rs7511673, rs11576175, rs10888390, and rs1136774) might be associated with obesity-related traits[23]. Minematsu et al[24] also found that five functional polymorphisms (including rs7534124, rs1136774, rs16827671, rs34495036 and rs3754212) in the promoter region in *CTSS* were possibly associated with pulmonary emphysema in a Japanese population, and the concentration of serum CTSS increased with the development of COPD. However, the relationship between these five SNPs in the promoter region in *CTSS* and asthma, similar to the symptoms of COPD, has not been reported. In this study, we aim to investigate SNPs in the promoter region in *CTSS* of a Chinese Han population and analyze their contribution to the risk of asthma.

Patients and Methods

Patients

This case-control study included 591 asthma patients and 621 healthy controls, and was performed with the approval of the Medical Ethics Committee of Wannan Medical College. The demographics of the patients and controls enrolled in this study are shown in Table I. All of the subjects were periodically enrolled between September 2008 and February 2012 at the Yijishan Hospital of Wannan Medical College in China. The diagnostic criteria of the Chinese Society of Allergology (2008) were adopted as follows: (1) continual episodes of wheezing and dyspnea for at least 1 year, with shortness of breath, cough, or chest tightness; (2) clinically diagnosed wheezing; (3) lung function measurement showing significant reversibility to bronchodilator [≥ 12% in 1-s forced expiratory volume (FEV_1) and peak expiratory flow (PEF) after delivering bronchodilator][25]. Healthy individuals with no history of asthma were recruited from the Healthy Testing Center in the same hospital as controls. The written informed consent was obtained from each participant before blood samples were taken.

DNA Extraction

The genomic DNA was extracted from the 200 μL EDTA-added peripheral blood samples using DNA isolation kits (Sangon Biotech, Shanghai, China) strictly according to the manufacturer's instructions.

SNP Genotyping Analysis

Five SNPs were selected including rs7534124, rs16827671, rs34495036, rs3754212, and rs1136774. Using the unique rs accession numbers, SNP details and sequence data were obtained from NCBI databases (http://www.ncbi.nlm.nih.gov). The polymorphic region was amplified by PCR with a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) in a 10 μL reaction solution. This solution included the following constituents, 0.3 μL (about 30 ng) of DNA, 1.0 μL of 10× PCR buffer with 25 mM of $MgCl_2$, 0.5 μL of a 10 mM solution of dNTPs (2.5 mM each), 0.1 μL of each primer (50 pmol/μL each), 0.06 μL (5 U/μL) of Dream *Taq*

Table I. The demographics of the patients and controls.

	Cases (n = 591)	Controls (n = 621)	*p*-value
Age, years (mean ± SD)	44.9 ± 15.3	44.7 ± 15.5	0.959[a]
Gender, n (male/female)	356/235	385/236	0.530[b]
FVC (mean ± SD)	3.10 ± 1.15	4.10 ± 0.95	0.045[a]
FEV_1/FVC (%) (mean ± SD)	64.80 ± 11.41	77.25 ± 13.01	0.031[a]
FEF 25%-75% (mean ± SD)	1.30 ± 0.43	2.81 ± 1.22	0.001[a]
PEF (mean ± SD)	3.75 ± 1.68	6.77 ± 2.25	0.002[a]

FVC: forced vital capacity; FEV_1: forced expiratory volume in 1 s; FEF: forced expiratory flow; PEF: peak expiratory flow. [a]*p*-value was calculated by *t*-test for the categorical data; [b]*p*-value was calculated by Chi-square test for the categorical data.

Table II. Primers and PCR programs for promoter of CTSS PCR-RFLP genotyping.

SNP ID		Primer sequence
rs7534124	Forward	5'-TATTCATGGAAAAAAGGATACACTC-3'
	Reverse	5'-AATGCAAATTTAAAAGATGATAGT**A**C-3'
rs16827671	Forward	5'-AGCCTGGATGACATATCAAGAC-3'
	Reverse	5'-GAGTTTGATGAACGAAGGAATG-3'
rs34495036	Forward	5'-AGCACTTTGGGAG**C**CTGAGG-3'
	Reverse	5'-GACTACAAGCATGCACCACCA-3'
rs3754212	Forward	5'-CTTTGTCCCCAAGACCATAGG-3'
	Reverse	5'-ACCTAGCAGGCAGAACAAGTTAC-3'
rs1136774	Forward-1	5'-CCCACTAATTCAAGGACTCTTACT**C**T-3'
	Reverse-1	5'-CCTAGCAGGCAGAACAAGTTAC-3'
rs1136774	Forward-2	5'-AGTACCTCATGTGACAAGTTCCA-3'
	Reverse-2	5'-CGTGATAGAACCAGCAGTTG**G**TC-3'

The reverse primer in rs7534124 contained a mismatched nucleotide A instead of T (shown underlined and bold) to incorporate a restriction endonuclease site for Tai I; the forward primer in rs34495036 contained a mismatched nucleotide C instead of G (shown underlined and bold) to incorporate a restriction endonuclease site for *Bsl* I. The forward-1 primer in rs1136774 contained a mismatched nucleotide C instead of G (shown underlined and bold) to incorporate a restriction endonuclease site for Fsp BI, while the reverse-2 primer in rs1136774 contained a mismatched nucleotide G instead of C (shown underlined and bold) to incorporate a restriction endonuclease site for Nla IV. PCR program: 98°C 4 min; 20 cycles, 94°C 45 s, 68°C 45 s, 72°C 1 min; 20 cycles, 94°C 45 s, 58°C 45 s, 72°C 1 min; 72°C 6 min.

DNA polymerase (MBI, Worcester, MA, USA), and 8 μL of nuclease-free water. Genotyping primers and PCR programs are shown in Table II. Some primers were also mutated to increase the number of restriction sites (Table II). PCR was accomplished by an initial denaturation at 95 °C for 5 min, followed by 20 cycles at 95 °C for 30 s, 68 °C for 45 s (gradient of -0.5 °C/cycle) and 72 °C for 60 s. Next it includes 20 cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 40 s, with a final elongation at 72 °C for 6 min. The PCR products were purified using SanPrep® PCR purification kit (Sangon Biotech, Shanghai, China) for further analysis.

For restriction fragment length polymorphism (RFLP) analysis, PCR products were digested with appropriate restriction endonucleases. The restriction enzymes are shown in Table III. The restriction enzyme digestion mixture was added directly to the 10 μL of purified PCR products to

Table III. Restriction enzymes and length of digested fragments.

SNP ID	Enzyme	Genotype	Length of digested fragments (bp)
rs7534124	*Tai* I	T/T	139
		C/C	46, 93
		C/T	139, 46, 93
rs16827671	*Hinl* II	C/C	233
		T/T	73, 160
		C/T	233, 73, 160
rs34495036	*Bsl* I	-/-	172
		TCCC/TCCC	39, 117
		-/TCCC	172, 39, 117
rs3754212	*BseD* I	T/T	302
		C/C	201, 101
		C/T	302, 201, 101
rs1136774	*FspB* I	A/A	46, 119
		-/G	165 or 164
		-/G/A	165 or 164, 46, 119
	Nla IV	G/G	43, 131
		-/A	173 or 174
		-/G/A	173 or 174, 43, 131

obtain a final volume of 15 μL This mixture included 0.1 μL of restriction enzyme (10 U/μL, Fermentas®, Beijing, China), 3.5 μL of nuclease-free water, and 1.5 μL of 10× buffer. The restriction enzyme digestion mixture was incubated at 55 °C overnight, according to the manufacturer's protocol, and analyzed by 4% agarose gel electrophoresis. The genotypes were assessed according to size of the digested fragments as shown in Table III.

Statistical Analysis

The frequencies of genotypes and alleles were determined by direct counts. Hardy-Weinberg equilibrium (HWE) of each SNP in controls and cases was examined by Chi-squared (χ^2) test to compare the observed and expected genotype frequencies. These differences of variants in the genotypes or alleles between the cases and controls were also evaluated using the χ^2 test. The association between SNPs and risk of asthma was estimated using logistic regression analysis. The differences with $p < 0.05$ were considered statistically significant. The odds ratio (OR) was calculated with a 95% confidence interval (CI). Because of multiple comparisons, Bonferroni correction was used to determine the significance levels of two-tailed *p*-values[26]. This was achieved by dividing the common *p*-value threshold 0.05 by the number of comparisons. Pairwise linkage disequilibrium (LD) block and haplotypes were evaluated using Haploview 4.2 software (Daly Lab, Cambridge, MA, USA)[27]. All statistical analyses were performed using SPSS software (version 16.0, SPSS Inc., Chicago, IL, USA).

Results

Characteristics of the Study Population

Among the DNA samples of 591 asthmatic patients and 621 healthy controls, the genotyping for a total of 24 samples including 15 controls and 9 cases in rs16827671 failed. For the other SNPs, the genotyping was successful in all asthma cases and controls using the DNA samples of 591 patients and 621 controls. The characteristics of the subjects such as age, sex, forced volume vital capacity (FVC), forced expiratory volume at 1-s intervals (FEV_1)/FVC%, forced expiratory flow (FEF) 25%-75%, and peak expiratory flow (PEF) in this study are summarized in Table I. The χ^2 tests showed that age and sex were not significantly different between the cases and the controls. The χ^2 tests did reveal statistically significant differences in FVC, FEV_1/FVC%, FEF 25%-75% and PEF between the cases and the controls (Table I).

Associations Between the CTSS rs7534124 and rs1136774 Genotypes and Risk of Asthma

The genotype distributions of CTSS rs7534124, rs16827671, rs34495036, rs3754212 and rs1136774 in all subjects are shown in Table IV. The observed genotype frequencies for five examined SNPs were all in HWE.

The genotype frequencies of CTSS rs7534124 were 23.4% (TT), 44.3% (CT), and 32.3% (CC) in the asthma patients and 16.3% (TT), 53.6% (CT), and 30.1% (CC) in the controls. The CT and CT+TT genotypes of CTSS rs7534124 were significantly associated with a decreased risk for asthma; CTSS rs7534124 CC homozygote genotype served as the reference group. However, the CTSS rs7534124 CC variant genotype was not associated with the risk of asthma, compared with the CTSS rs7534124 TT wild-type homozygote.

The genotype frequencies of CTSS rs1136774 were 21.8% (AA), 44.3% (AG), 33.9% (GG) in the asthma patients, and 15.3% (AA), 53.5% (AG), and 31.2% (GG) in the controls. Using the CTSS rs1136774 AA genotypes as the reference group, our analysis revealed that the AG genotype and AG+GG genotype of CTSS rs1136774 were associated with a significantly decreased risk of asthma. However, the CTSS rs1136774 GG variant genotype was not associated with the risk for asthma.

Associations Between the Alleles of Five CTSS SNPs and Risk of Asthma

There were no significant differences in allele frequencies of CTSS rs7534124, rs16827671, rs34495036, rs3754212 and rs1136774 between cases and controls, as illustrated in Table V.

Intragenic LD Structure of CTSS

To evaluate the LD block and haplotypes of the five tested SNPs, we used Haploview 4.2 software. As shown in Figure 1, a LD block was made between rs3754212 and rs7534124 without the LD magnitude ($D' = 1.0$, $r^2 = 0.382$). Moreover, the LD between rs7534124 and rs1136774 also existed ($D' = 0.98$, $r^2 = 0.926$).

Table IV. Logistic regression analysis of associations between SNPs in CTSS and risk of asthma.

SNP ID	Genotype	Cases		Controls		OR (95% CI)	*p*-value*	HWE *p*-value
		n	%	n	%			
rs7534124 C/T	TT	138	23.4	101	16.3	1.00	–	0.860
Cases, n = 591	CT	262	44.3	333	53.6	**0.576 (0.425-0.780)**	**0.000**	
Controls, n = 621	CC	191	32.3	187	30.1	0.748 (0.539-1.036)	0.081	
	CT+CC	453	76.6	520	83.7	**0.638 (0.479-0.849)**	**0.002**	
rs16827671	TT	247	42.4	254	41.9	1.00	–	0.001
Cases, n = 582	CT	276	47.4	303	50.0	0.937 (0.737-1.190)	0.592	
Controls, n = 606	CC	59	10.1	49	8.1	1.238 (0.816-1.880)	0.316	
	CC+CT	335	57.6	352	58.1	0.979 (0.777-1.232)	0.854	
rs34495036	TCCC/TCCC	200	33.8	242	39.0	1.00	–	0.001
Cases, n = 591	-/TCCC	319	54.0	306	49.3	1.261 (0.988-1.610)	0.062	
Controls, n = 621	-/-	72	12.2	73	11.7	1.193 (0.820-1.737)	0.356	
	-/- + -/TCCC	391	66.2	379	61.0	1.248 (0.987-1.578)	0.064	
rs3754212	TT	209	35.4	220	35.4	1.00	–	0.000
Cases, n = 591	CT	335	56.6	355	57.2	0.993 (0.781-1.264)	0.957	
Controls, n = 621	CC	47	8.0	46	7.4	1.076 (0.687-1.684)	0.750	
	CC+CT	382	64.6	401	64.6	1.003 (0.792-1.269)	0.982	
rs1136774	AA	129	21.8	95	15.3	1.00	–	0.996
Cases, n = 591	AG	262	44.3	332	53.5	**0.581 (0.426-0.793)**	**0.001**	
Controls, n = 621	GG	200	33.9	194	31.2	0.759 (0.546-1.057)	0.102	
	AG+GG	462	78.2	526	84.7	**0.647 (0.483-0.867)**	**0.004**	

OR: Odds Ratio. CI: Confidence Intervals. HWE: Hardy-Weinberg equilibrium. *p-value < 0.006 was considered as significant for data after Bonferroni correction. Bold number indicates statistically significant association.

Discussion

Some studies[15,16,19,28] have reported that CTSS also plays important roles in lung diseases. For example, CTSS plays important roles in IFN-γ-induced apoptosis of alveolar epithelial cells[15], IL-13-induced emphysema[16], and ozone-induced airway hyperresponsiveness/inflammation[28]. CTSS deficiency can improve alveolarization, and attenuate macrophage influx and fibroproliferative changes in the pathogenesis of hyperoxia-induced lung injury, as shown with mice models[19]. Although CTSS is a key in antigen-induced lung inflammation, it may have a weaker role in the downstream effector inflammatory phase[1].

Table V. The allele frequencies of CTSS in asthma patients and control subjects.

SNP ID	Allele	Asthma n (%)	Controls n (%)	OR (95% CI)	*p*-value
rs7534124 C/T					
Asthma, n = 591	C	644 (54.5)	707 (56.9)	0.906 (0.772-1.063)	0.227
Controls, n = 621	T	538 (45.5)	535 (43.1)	1.00	
rs16827671 C/T					
Asthma, n = 582	C	394 (33.8)	401 (33.1)	1.035 (0.873-1.227)	0.694
Controls, n = 606	T	770 (66.2)	811 (66.9)	1.00	
rs34495036 -/ TCCC					
Asthma, n= 591	–	463 (39.2)	452 (36.4)	1.125 (0.955-1.326)	0.159
Controls, n = 621	TCCC	719 (60.8)	790 (63.6)	1.00	
rs3754212 C/T					
Asthma, n = 591	C	429 (36.3)	447 (36.0)	1.013 (0.858-1.196)	0.876
Controls, n = 621	T	753 (63.7)	795 (64.0)	1.00	
rs1136774 A/G					
Asthma, n = 591	A	520 (44.0)	522 (42.0)	1.083 (0.922-1.273)	0.329
Controls, n = 621	G	662 (56.0)	720 (58.0)	1.00	

OR: Odds Ratio. CI: Confidence Intervals.

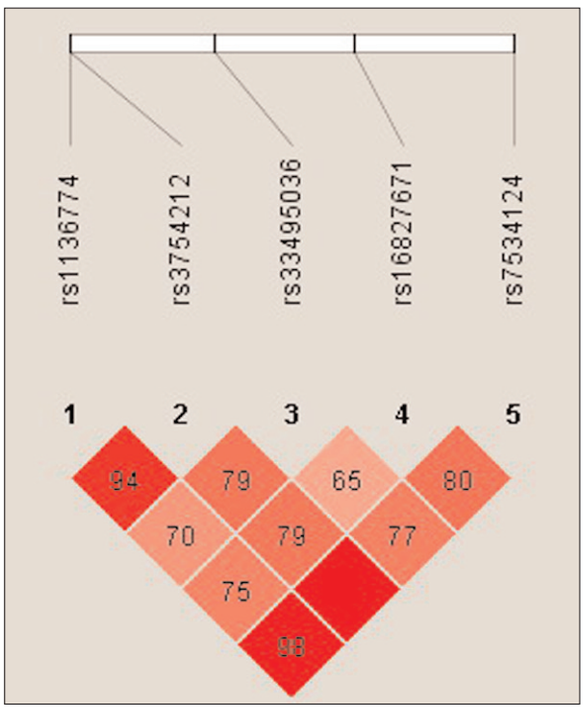

Figure 1. Pairwise linkage disequilibrium (LD) pattern of CTSS measured by D′. The location of each tested SNP along the chromosome is indicated on top. The number in each diamond indicates the magnitude of LD (D′ × 10-2) between respective pairs of SNPs. Squares without the LD magnitude represents perfect LD (D′ = 1.0).

Several studies have demonstrated that SNPs in *CTSS* are related to certain diseases such as atherosclerosis[21], obesity[23], and COPD[24]. Since CTSS has also associated to COPD[24], we hypothesized SNPs in *CTSS* may play an important role in the pathogenesis of asthma. In this case-control study, we investigated the relationship between the *CTSS* gene and asthma and evaluated the risk of asthma in a Chinese Han population. We first found that the rs7534124 CT and CT+CC genotypes were associated with a decreased risk of asthma in a Chinese Han population. Moreover, the rs1136774 AG and AG+GG genotypes also decreased asthma risk. Although the genotypes and alleles of rs3754212 had no association with susceptibility to asthma, rs3754212 and rs7534124 were in linkage disequilibrium. Minematsu *et al*[24] reported the functional association of five SNPs in *CTSS* in our study with COPD in a Japanese population. Four novel genetic SNPs in *CTSS* were found; three haplotypes derived from these SNPs were also identified as having a possible association with pulmonary emphysema. The association of other SNPs with asthma was also detected, as well as all allele frequencies of these five SNPs, but we did not find a significant association between them and asthma risk. The possible reasons may include bias in the selection of SNPs and lack of detailed information about asthma pathogenesis.

Conclusions

Our study provides strong evidence that SNPs of rs7534124 and rs1136774 affect the susceptibility of asthma in Chinese Han population. However, a larger sample size is needed to increase the reliability of the results. A future study could also combine functional evaluation with more rigorous study designs of other ethnic populations.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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