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The Association of *SERPINE2* Gene with COPD in a Chinese Han Population

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Purpose: Polymorphisms of several candidate genes have been studied and associated with the development of chronic obstructive pulmonary disease (COPD). One such candidate is the *SERPINE2* (Serpin peptidase inhibitor, clade E member 2) gene. **Materials and Methods:** To assess whether the *SERPINE2* gene is associated with COPD in a Chinese Han population. Samples were collected from a Chinese Han population and analyzed for the association of single nucleotide polymor phisms (SNPs) or haplotypes of *SERPINE2* gene with COPD in a case-control study. Three SNPs including rs840088 G/A in intron 1, rs1438831 A/G in 5' upstream sequence and rs3795879 G/A in intron 3 were detected using the polymerase chain reaction (PCR)-based restriction fragment length polymorphism technique in 409 COPD subjects and 411 controls. Genotyping of the SREPINE2 polymorphisms at positions rs840088, rs1438831and rs3795879 was performed. **Results:** We found that none of the rs840088G/A, rs1438831G/A and rs3795879 G/A polymorphisms were associated with the disease. The *p*-values were 0.630, 0.208 and 0.398 respectively. **Conclusion:** Our data suggested that there was no significant association between *SERPINE2* polymorphism and COPD susceptibility in the Chinese Han population.

Key Words: Chronic obstructive pulmonary disease, *SERPINE2* gene, polymorphism, susceptibility

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by irreversible and progressive bronchial obstruction and persistent airway inflammation. It is a common, complex disease that causes severe morbidity and mortality.[1] It is the fourth leading cause of death in the world,[2] and its prevalence and mortality are expected to be increased in the coming decades.[3] Therefore, a clear understanding of the various aspects of susceptibility to this lung disease is necessary in order to develop better treatments.

So far, cigarette smoking has been proven the most important risk factor for COPD. Alpha 1-antitrypsin (AAT) deficiency is another proven genetic risk factor for COPD. However, only 15% of smokers have this disease and AAT deficiency is present in only 1-2% of COPD cases.[4] Furthermore, nonsmokers may also develop chronic airflow obstruction.[5,6] All of these data suggest that genetic constitution as well as living habits play a role in the development and severity of COPD.[7,8] Several studies have suggested that other genetic factors may also be involved in the patheogensis of COPD. Polymorphisms of several candidate genes have been studied and associated with the development of COPD.[9] One such candidate is the *SERPINE2* [Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2] gene. Genetic associations with COPD-related traits can help to define distinct subtypes of COPD.[10-12]

SERPINE2 is located on chromosome 2q33-35. Using gene-expression microarray and animal model experiments, DeMeo and colleagues identified *SERPINE2* as a novel COPD-susceptibility gene.[13] Zhu and colleagues also confirmed the association between *SERPINE2* and COPD in a large case-control collection from Bergen, Norway, which included 973 cases with COPD and 956 control subjects.[14] However, Chappell and colleagues' prospective study of 1,018 COPD cases and 911 controls coming from six European centers suggested that there was no significant association between *SERPINE2* and COPD.[15] In order to clarify these contradictory results of *SERPINE2* in COPD, we conducted a case-control association study of three known single nucleotide polymorphisms (SNPs) of the *SERPINE2* gene in the Chinese population.

MATERIALS AND METHODS

Diagnosis

COPD was diagnosed on the basis of medical history, chest radiographic findings, physical examination and spirometric data, according to a definition of COPD which was consistent with that of the American Thoracic Society (ATS) consensus statement.[16] Pulmonary function is critical to identifying whether COPD exists. We performed pulmonary function tests (Jaeger Master Screen Diffusion, Germany) to determine FVC, FEV1, FEV1% predicted, FEV1/FVC and post-bronchodilator FEV1/FVC. The inclusion criteria for COPD patients were based on the diagnosis standards of COPD according to the ATS with post-bronchodilator FEV1/FVC <70%. We also excluded patients with bronchial asthma, bronchiectasis, interstitial pulmonary disease, tuberculosis and pulmonary tumors. The control group was made up of healthy individuals who were admitted for an annual checkup. The criteria for control subjects included: age >40 years, with post-bronchodilator FEV1 >80% predicted and FEV1/FVC >70%, with no respiratory symptoms and normal manifestation on chest X-ray (CXR).

Patients

All study subjects involved in this investigation were recruited upon giving informed consent. The study design was approved by the ethical committee of Shandong University. This prospective observational study involved 409 patients with clinical diagnosis of COPD and 411 unrelated healthy controls without any respiratory system diseases. These 820 selected participants were of the Chinese Han population. All the patients and case-controls were recruited from Qilu Hospital and the Second Affiliated Hospital of Shandong University of Traditional Chinese Medicine. The average ages of the COPD patients and case-controls were 65.1 years and 63.7 years, respectively.

Specimens

Blood samples were drawn from the antecubital vein and stored at 4°C before genomic DNA was isolated.

DNA preparation

Genomic DNA was extracted from leucocytes of 2 mL peripheral blood using a standardized method.[17] The DNA concentration and purity of each sample were analyzed by ultraviolet spectrophotometry. All DNA samples were stored at -20°C until analysis.

PCR-RFLP (PCR-restriction fragment length polymorphism)

In order to detect the polymorphism of the *SERPINE2* gene in COPD, we performed a restriction fragment length polymorphism analysis. In brief, primers were designed according to the published sequence (Gene Bank: NC_000002.10). The forward and reverse primers for the amplifications of rs840088 (304bp), rs1438831 (402bp) and 3795879 (194bp) were: 5'-TACACTGGAGGTAGGTTGCC-3' and '5-CCT TTAGAGTCAGGGATTT-3'; 5'-TGGGTCATTACT TCTCTC-3' and '5-ATAGGGTCCAAATTCTTC-3'; 5'-TTGAAGTGCCTTTTGTTAC-3' and '5-TTGAACC

CTGGAGTCTAAC-3', respectively. A total of 50 ng genomic DNA was mixed in a total volume of 25 μL containing 1.0 U Taq DNA polymerase, 200 μmol/L each dNTP (mixture of dATP dTTP dCTP dGTP), 10 mmol/L Tris-HCL (PH 8.3), 1.5 mmol/L MgCL2, 0.2 μmol/L of each primer (previously described). Amplification was performed with an initial denaturation step at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 40 s each, annealing at optimum temperature for 40 s, and extension at 72°C for 60 s. A final extension was carried out at 72°C for 10 min. The optimal annealing temperatures for the three SNPs were: 54°C for rs840088, 55°C for rs143883, and 50°C for rs3795879 respectively. PCR amplification was performed in a programmable PCR thermal cycler (GeneAmp PCR System 2400, Perkin-Elmer, Wellesley, MA, USA). 10 μL of PCR product was used for electrophoresis on 2.0% agarose gels stained with ethidium bromide. The gels were visualized and photographed using an ultraviolet light transilluminator.

For genotyping the polymorphism of SNPs rs840088, rs1438831, rs3795879, the PCR products were digested with MLY1, Taq1, MSC1 (Gene corporation, Shanghai, China) for 3 h at 37°C, 3 h at 65°C, or 3 h at 37°C, respectively, according to the product's instructions. After electrophoresis in 2.0% agarose gel and staining with ethidium bromide, the genotypes were determined.

The possible outcomes were: the rs840088G/A allele was genotyped by PCR-RFLP. MLY1 digestion cleaved the 304 bp PCR products into two fragments of 177 bp and 127 bp when the G allele was present; The SNP rs1438831G/A was also analyzed by RFLP using Taq1 enzyme. Taq1 digestion cut the 402 bp PCR products into two fragments of 292 bp and 110 bp when the G allele was present; In the case of the rs3795879 G/A allele, the cleavage products of 134 bp and 60 bp were obtained when G allele was present.

DNA sequencing

Direct sequencing of a subgroup of samples with the same primers was used to further validate the authenticity of genotype analysis. Sequencing was performed using the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems), and analyzed on an ABI PRISM 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Patient and case-control age is expressed as mean±SD. We evaluated each variation for deviations from Hardy-Weinberg equilibrium (HWE) by means of χ^2 test. The genotype distributions and allelic frequencies for studied polymorphisms of the COPD patients and control-group individuals were statistically compared using the χ^2 test as well as SPSS software for Windows (version 13.0; SPSS Inc., Chicago, IL, USA). The haplotype distributions between patients and control groups were assessed by the Monte Carlo method using shesis. A *p*-value of less than 0.05 was considered to indicate statistical significance.

RESULTS

Clinical characteristics

A total of 820 subjects, including 409 COPD patients and 411 healthy controls, were studied. The demographic characteristics and relevant clinical parameters of all patients and controls are showed in Table 1. The average age of the COPD patients, consisting of 251 men and 158 women,

Table 1. Clinical Features of the Study Population*

Characteristics	Patients	Controls	*p* value†
Total number	409	411	
Gender (Male/Female)	251/158	266/145	0.84
Age (yrs)	65.14±11.82	63.70±10.51	0.07
Smoking status (smoker/nonsmoker)	219/190	223/188	0.32
FEV1, (L)	1.44±0.63	2.55±0.69	<0.001
FEV1% pred	56.69±21.45	102.02±14.33	<0.001
FEV1/FVC, %	52.80±12.07	79.84±5.75	<0.001

COPD, chronic obstructive pulmonary disease.
Four hundred and nine patients with clinical diagnosis of COPD and 411 unrelated healthy controls without any respiratory system diseases, all of Chinese Han nationality, were selected for study. COPD was diagnosed according to the guidelines of the American Thoracic Society (ATS) consensus statement. Pulmonary function tests were performed to determine FVC, FEV1, FEV1% predicted, FEV1/FVC and post-bronchodilator FEV1/FVC.
*Data are presented as No. or mean SD.
†*p* values were calculated using the Mann-Whitney U-test.

was 65.1 years old, compared with 63.7 years of the controls, consisting of 266 men and 145 women. No significant differences in sex, age or smoking history were observed between the COPD cases and controls. The parameters of FEV1, FVC, FEV1% predicted and FEV1/FVC were significantly lower in COPD patients than the controls (p<0.001).

Distributions of rs840088, rs1438831, rs3795879 genotypes

The genotype distributions of rs840088, rs1438831, rs3795879 among the controls and the COPD patients are shown in Table 2. There was no significant deviation in the genotype frequency from the Hardy-Weinberg equilibrium (p>0.05). For rs840088, the frequency of the homozygote AA genotype was 13.9% in COPD and 16.3% in controls, the frequency of homozygote AG was 51.6% in the cases and 49.6% in the controls, the frequency of homozygote GG genotype was 34.5% in the cases and 34.1% in the controls. The frequencies of genotypes rs840088 AA, AG, GG in cases were not significantly different from those of the controls (p=0.630). Besides, for rs1438831, the frequency of homozygote AA genotype was 2.9% in COPD and 4.9% in the controls. The frequency of homozygote AG was 30.6% in the controls and 34.7% in the cases; the frequency of homozygote GG genotype was 62.4% in the cases and 64.5% in the controls. For rs3795879, the frequency of homozygote AA genotype was 75.6% in COPD and 72.5% in the controls. The frequency of homozygote AG was 22.7% in COPD and 24.6% in the controls, and the frequency of homozygote GG genotype was 1.7% in the cases and 2.9% in controls. The frequencies of genotypes rs1438831 in patients were also not significantly different from those of controls (p=0.208). Moreover, we found no association with rs3795879 (p=0.398).

The allele frequencies of *SERPINE2*

The *SERPINE2* genotypes and allele frequencies for the two groups are shown in Table 3; The χ^2 test showed that the patient group did not have a significantly higher frequency than the control group of rs840088 G/A alleles, rs1438831 G/A alleles and rs3795879 G/A alleles (p=0.567, p=0.960 and p=0.217, respectively).

Haplotype analysis

Three SNPs were used to construct haplotypes. Using SHEsis software, we constructed four haplotypes (frequency >0.05). Their frequencies are listed in Table 4. There was no significant association in terms of haplotype frequencies of the four haplotypes in COPD patients and controls (p>0.05).

Verification of *SERPINE2* genotypes

In this study, the polymorphism analysis of *SERPINE2* was performed by PCR-RFLP, and the representative data are shown in Fig. 1, Fig. 2, and Fig. 3. As for rs840088, the AA

Table 2. Genotype Frequencies of *SERPINE2* between COPD Patients and Controls, and Corresponding ORs for COPD

Genotype	COPD patients n=409 (%)	Controls n=411 (%)	p value*	Odds ratio (95% CI)‡
rs840088			0.630	
G/G	141 (34.5)	140 (34.1)		1.000
A/A	57 (13.9)†	67 (16.3)		0.993 (0.702, 1.404)
G/A	211 (51.6)	204 (49.6)		0.880 (0.530, 1.461)
rs1438831			0.208	
G/G	255 (62.4)	265 (64.5)		1.000
A/A	12 (2.9)	20 (4.9)		1.499 (1.009, 2.225)
G/A	142 (34.7)	126 (30.6)		0.870 (0.331, 2.284)
rs3795879			0.398	
A/A	309 (75.6)	298 (72.5)		1.000
G/A	93 (22.7)	101 (24.6)		0.740 (0.492, 1.113)
G/G	7 (1.7)	12 (2.9)		0.709 (0.225, 2.236)

The genotype distributions of rs840088, rs1438831, rs3795879 in controls and COPD patients were calculated. Each variation for deviations from Hardy-Weinberg equilibrium (HWE) was evaluated by means of an χ^2 test.
COPD, chronic obstructive pulmonary disease.
*p values were calculated using the χ^2 test.
†Data are presented as No. (%).
‡Odds ratios were calculated using logistic regression to measure the ORs for COPD of specific genotypes.

Table 3. Allelic Frequencies of *SERPINE2* between COPD Patients and Controls, and Corresponding ORs for COPD

Allelic frequency	COPD patients n=818 (%)	Controls n=822 (%)	*p* value*
rs840088			0.567
Allele A	325 (39.7)†	338 (41.2)	
Allele G	493 (60.3)	484 (58.8)	
rs1438831			0.960
Allele A	166 (20.3)	166 (20.2)	
Allele G	652 (79.7)	656 (79.8)	
rs3795879			0.217
Allele A	711 (86.9)	697 (84.8)	
Allele G	107 (13.1)	125 (15.2)	

The *SERPINE2* genotypes and allele frequencies for the two groups were statistically compared using the χ^2 test.
COPD, chronic obstructive pulmonary disease.
*p-values were calculated using the χ^2 test.
†Data are presented as No. (%).

Table 4. The Haplotype Frequencies of Three Single Nucleotide Polymorphisms of *SERPINE2* in COPD Patients and Controls

Haplotype	rs840088	rs1438831	rs3795879	Haplotype frequency		χ^2 (global p value)*
				Control	COPD	
Hap1	A	G	G	0.557	0.572	0.996
Hap2	A	A	G	0.207	0.205	
Hap3	G	A	A	0.112	0.109	
Hap4	A	A	A	0.076	0.078	

Three SNPs were used to construct haplotypes. Using SHEsis software, four haplotypes were constructed (frequency >0.05). There is no significant association of haplotype frequencies of four haplotypes with COPD patients and controls (p>0.05).
SNPs, single nucleotide polymorphisms; COPD, chronic obstructive pulmonary disease.
*The global p-value was calculated with haplotype frequencies greater than 0.03 by the online software.

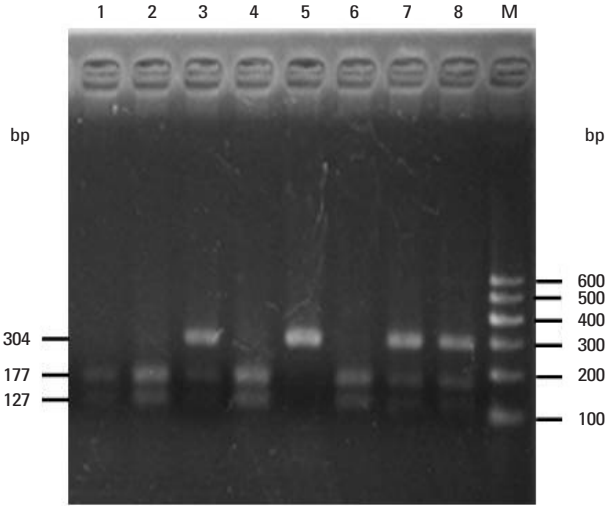

Fig. 1. The genotype analysis of SNP rs840088 by the PCR-restriction fragment length polymorphism (PCR-RFLP) method. rs840088G/A allele was genotyped by PCR-RFLP. PCR products were digested with MLY1 for 3 h at 37ºC. After electrophoresis in 2.0% agarose gel and staining with ethidium bromide, the genotypes were determined. When the GG allele was present, MLY1 would cut the 304 bp PCR products into two fragments of 177 bp and 127 bp. The AA homozygous case showed a 304 p uncleavage product, while the GA heterozygous case showed 304 bp, 177 bp and 127 bp products. Lane M: DNA Marker; Lane1, 2, 4, 6: GG genotype; Lane3, 7, 8: GA genotype; Lane5: AA genotype. 90×66 mm (300×300 DPI). SNP, single nucleotide polymorphism.

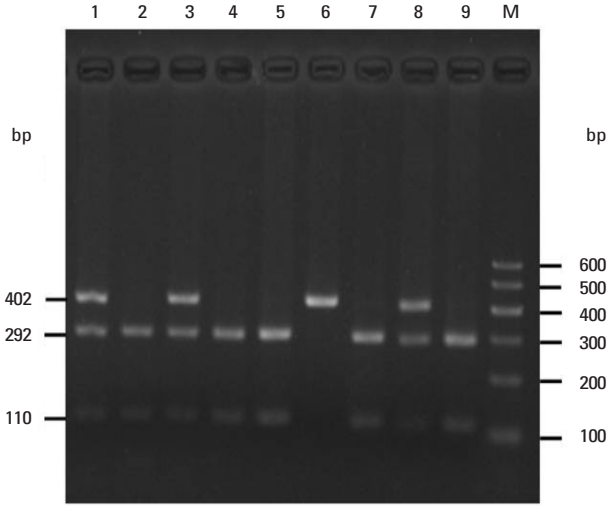

Fig. 2. The genotype analysis of SNP rs1438831 by the PCR-RFLP method. The SNP rs1438831G/A was also analyzed by PCR-RFLP using Taq1 enzyme. Taq1 digestion for 3 h at 65ºC cut the 402 bp PCR products into two fragments of 292 bp and 110 bp when the GG allele was present. The AA homozygous case showed 402 bp uncleavage product, while the GA heterozygous case showed 402 bp, 292 bp and 110 bp products. Lane M indicates the 100-bp molecular markers. Lanes 2, 3 and 6 indicate three kinds of genotypes: GG, GA, AA respectively. 81×58 mm (300×300 DPI). PCR-RFLP, PCR-restriction fragment length polymorphism; SNP, single nucleotide polymorphism.

homozygous case showed a 304 p uncleavage product, the GG homozygous case showed 177 bp and 127 bp while the GA heterozygous case showed 304 bp, 177 bp and 127 bp cleavage products (Fig. 1). As for rs1438831, the AA homozygous case showed 402 bp uncleavage product, the GG homozygous case showed 292 bp and 110 bp, while the GA heterozygous case showed 402 bp, 292 bp and 110 bp cleavage products (Fig. 2). As for rs3795879, the AA homozygous case showed 194 bp uncleavage product, the GG homozygous case showed 134 bp and 60 bp, while the GA heterozygous case showed 194 bp, 134 bp and 60 bp cleavage products (Fig. 3).

The DNA sequencing results of the three genotypes of *SERPINE2* further validated the aforementioned results of the PCR-RFLP (Figs. 4, 5 and 6).

DISCUSSION

At present, most studies of the pathogenesis of COPD have concentrated on the association analysis of candidate genes. It is believed that COPD is most likely a polygenic disorder with considerable genetic heterogeneity.[18] As a way of gaining insight into the possible role of genetic factors in the pathogenesis of COPD, we investigated the polymorphisms of *SERPINE2* gene in a Chinese population.

The *SERPINE2* gene belongs to the serpin family of proteins as well as α1-antitrypsin. It is composed of nine exons, which encode a 44-kD protein that is a thrombin, plasmin and urokinase inhibitor and plays an important role as a cellular regulatory inhibitor of serine protease, mainly involved in coagulation and fibrinolysis and extracellular matrix-associated and mitosis.[19] *SERPINE2* also prohibits neuron apoptosis and injury-mediated cell death.[20] In addition, tumor necrosis factor (TNF)-α, IL-1β, and transforming growth factor-β can induce expression of *SERPINE2*.[21] As *SERPINE2* and α1-antitrypsin are from the same family and α1-antitrypsin is associated with COPD, it is interesting to dissect the role of *SERPINE2* in the pathogenesis of COPD.

However, its role in the development of COPD is still unclear. DeMeo and associates observed that *SERPINE2* was highly expressed in embryonic mouse lungs as well as in airway epithelial cells and vascular adventitia of adult human lungs. In addition, they suggest that the overexpression of *SERPINE2* is associated with COPD.[13] Other studies have confirmed the findings of DeMeo and colleagues. Zhu

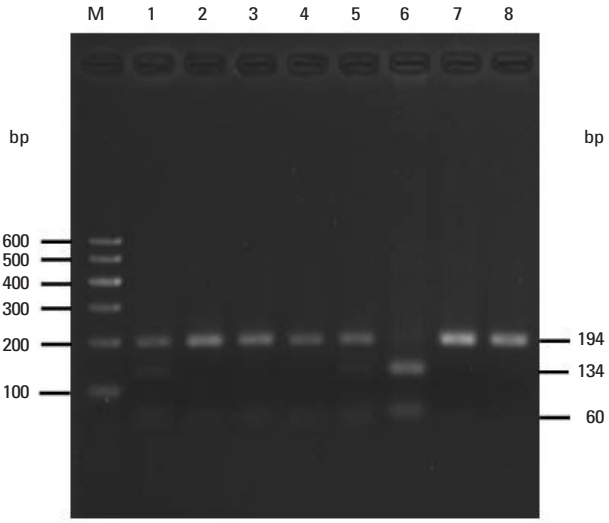

Fig. 3. The genotype analysis of rs3795879 by the PCR-RFLP method. In the case of rs3795879, MSC1 cleaved PCR products into 134 bp and 60 bp when GG allele was present. AA homozygous case showed 194 bp uncleavage product, while the GA heterozygous case showed 194 bp, 134 bp and 60 bp products. Lane M was loaded with 100-bp molecular markers. Lanes 1, 2 and 6 indicate three kinds of genotypes: GA, AA, GG respectively. 81×60 mm (300×300 DPI). PCR-RFLP, PCR-restriction fragment length polymorphism.

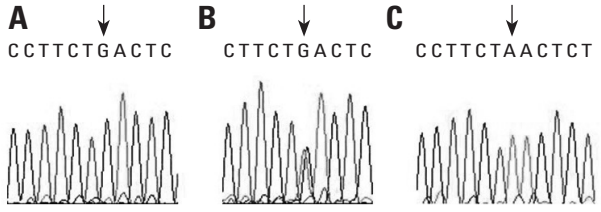

Fig. 4. The DNA sequencing of rs840088. Direct sequencing of a subgroup of samples with the same primers for PCR-RFLP was performed using the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems), and analyzed on an ABI PRISM 3100 DNA sequencer (Applied Biosystems, Foster City, USA). (A) GG genotype. (B) GA genotype. (C) AA genotype. 82×48 mm (300×300 DPI).

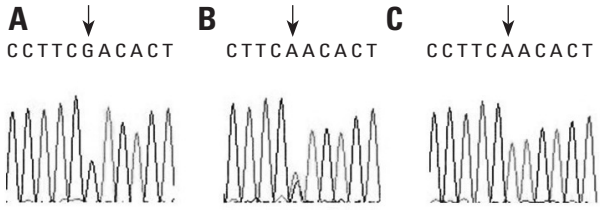

Fig. 5. The DNA sequencing of rs1438831. DNA sequencing was performed as described in Fig. 4. (A) GG genotype. (B) GA genotype. (C) AA genotype. 82×48 mm (300×300 DPI).

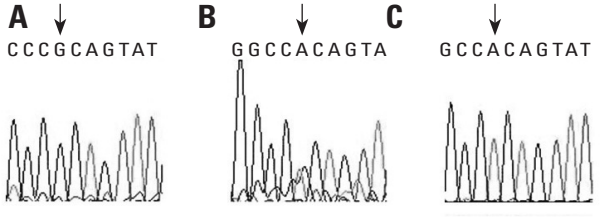

Fig. 6. The DNA sequencing of rs3795879. DNA sequencing was performed as described in Fig. 4. (A) GG genotype. (B) GA genotype. (C) AA genotype. 82×48 mm (300×300 DPI).

and colleagues genotyped 25 sSNPs from *SERPINE2* and analyzed qualitative and quantitative COPD phenotypes in two large populations. Six SNPs were found to have significant associations with COPD in the family-based association analysis ($0.0016 \leq p \leq 0.042$). Five of these SNPs were demonstrated to be replicated associations in the case-control analysis ($0.021 \leq p \leq 0.031$).[14]

The three SNPs in this study were rs840088 G/A in intron 1, rs1438831 A/G in 5' upstream sequence, and rs3795879 G/A in intron 3. Their polymorphisms in the 409 patients with COPD and the 411 unrelated controls obtained from a Chinese Han population were analyzed. These SNPs have been reported to have a role in defining susceptibility phenotypes of COPD by influencing inflammatory responses to environmental exposure.[13] The results of our study showed that there was no association between the three SNPs in *SERPINE2* and COPD in a Chinese population, consistent with those of Chappell and colleagues' study.[15] The reason for this may be complicated. First, association studies offer a potentially powerful approach to identify genetic variants that influence susceptibility to common disease, but are plagued by the impression that the results are not consistently reproducible.[22] Second, as in many complex-disease genetic association studies, and particularly in previous COPD genetic association studies, the results are inconsistent.[23] Third, different populations and different levels of environmental exposure may induce different results. Thus, it is possible that different SNPs of *SERPINE2* contribute to COPD in different ethnic populations. All of these inconsistent results can induce false-positive and/or false-negative results.[24] It is certain that more SNPs of *SERPINE2* genes will be identified with advances in SNP genotyping, which will allow us to clarify the details of the functional relationship between *SERPINE2* and COPD in the future.

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Aihua Wang and Wei Xiao conceived and designed the study. Yingqiu Yin and Pin Chen conducted the laboratory experiments, developed protocols, did the statistical analysis, and drafted the manuscript, Qinfen Yu polished the final version. Qiji Liu helped data analysis.

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