The *ACE2* G8790A Polymorphism: Involvement in Type 2 Diabetes Mellitus Combined with Cerebral Stroke

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Background: We aimed to investigate the correlations between *ACE2* polymorphisms and type 2 diabetes mellitus (T2DM) combined with cerebral stroke (CS). *Methods:* A total of 346 patients treated or hospitalized in our hospital were enrolled, including 181 cases without cerebrovascular complications (T2DM group) and 165 cases combined with CS (T2DM + CS group); 284 healthy individuals were selected as the control group. PCR-RFLP and ELISA were used to analyze *ACE2* G8790A polymorphisms and serum ACE2 levels, respectively. *Results:* Significant differences were observed in the genotype/allele frequency of *ACE2* G8790A between the T2DM + CS and control groups, and the T2DM and T2DM + CS groups, and in the genotype frequency of *ACE2* G8790A between the T2DM and the control groups. The A allele may increase the risk of T2DM combined with CS. The AA genotype may also increase the risk of T2DM combined with CS (OR = 3.733, 95%CI = 2.069–6.738; OR = 3.597, 95%CI = 1.884–6.867). Serum ACE2 levels showed statistically significant differences among the groups. Systolic pressure and diastolic pressure were protective factors of T2DM combined with CS. *Conclusion:* The *ACE2* G8790A polymorphism in T2DM patients was correlated with CS, and the A allele might be a risk factor of T2DM combined with CS. J. Clin. Lab. Anal. 31:e22033, 2017. © 2016 Wiley Periodicals, Inc.

Key words: *ACE2*; allele frequency; cerebral stroke; gene polymorphism; regression analysis; type 2 diabetes mellitus

INTRODUCTION

Type 2 diabetes mellitus (T2DM), the most common type of diabetes mellitus (DM), is a heterogeneous group of metabolic conditions characterized by increased levels of blood glucose due to impairments in insulin action and/or insulin secretion (1). It has been estimated that T2DM comprises approximately 90% of diabetes cases, with the other 10% attributed primarily to type 1 diabetes mellitus (T1DM) and gestational diabetes. Asia has emerged as the "diabetes epicenter" with more than 92 million people diagnosed with DM (2–4). In addition, the incidence and prevalence of T2DM are notably different in ethnicity, with higher rates in several ethnic groups, including Asian populations, African American, Pacific Islander, and Hispanic populations, and native American and Australian Indigenous populations (5, 6). T2DM has many associated complications, including cerebral stroke (CS), which was identified as an important source of morbidity and mortality in addition to being an enormous economic burden (7, 8). CS, the fourth leading cause of death worldwide, is due to a disruption in the blood supply to the brain, which results in approximately 15 million injuries every year worldwide (9). Existing research has indicated that the main risk factor for CS is high blood pressure, and other factors also contribute to the development of CS, including DM, smoking, obesity, and high blood cholesterol (10–12). Due to the high incidence of T2DM and CS and their linkage, identifying treatments for patients diagnosed with T2DM combined with CS is urgent.

Angiotensin-converting enzyme 2 (ACE2) is a homolog to ACE that chiefly catalyzes the conversion of Ang II into Ang-(1-7), thus leading to a balance between the two peptides, and accordingly, it has been

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Received 20 April 2016; Accepted 1 July 2016
DOI 10.1002/jcla.22033
Published online in Wiley Online Library (wileyonlinelibrary.com).

identified as a crucial modulator of the two axes of the renin–angiotensin system (13). ACE2 is a type I transmembrane glycoprotein with 805 amino acids and a single extracellular catalytic domain (14). The *ACE2* gene, discovered by Tipnis and Donoghue, located on Xp22, is localized to a region where genes are known to escape X-inactivation, which may contribute to the phenotypic differences between sexes and the tissue-specific differences in X-inactivation (15–17). Similar to ACE, ACE2 also has two domains: the amino-terminal catalytic domain and the carboxy-terminal domain (18). Moreover, ACE2 is predominantly observed in the heart, kidneys, and testes and at lower levels in various tissues, especially the colon and lung (19, 20). ACE2 has been correlated with the development of hypertension, kidney diseases, and DM, and the single nucleotide polymorphism (SNP) G8790A (rs2285666) polymorphism was related to the risk of T2DM (21–23). However, few studies have focused on the association of the *ACE2* G8790A polymorphism with T2DM combined with CS; thus, we investigated the genotype frequency of the *ACE2* polymorphism and measured the serum level of ACE2 to explore the associations between the *ACE2* G8790A polymorphism and T2DM combined with CS, providing a potential molecular marker for the diagnosis and treatment of T2DM combined with CS patients.

MATERIAL AND METHODS

Ethical Statement

This study was approved by the Ethical Committee of The Second Affiliated Hospital of Harbin Medical University. Written informed consents were obtained from all study subjects and/or their legal guardians. This study complied with the guidelines and principles of the Declaration of Helsinki (24).

Study Subjects

A total of 346 DM patients hospitalized in the Second Affiliated Hospital of Harbin Medical University between February 2013 and May 2014 were selected for our study. Using the World Health Organization (WHO) diagnostic criteria (25), 181 T2DM patients (82 males and 99 females) without cerebrovascular complications were identified, with an age of 60.9 ± 10.7 years (T2DM group). Additionally, a T2DM + CS group containing 165 patients (87 males and 78 females; age: 61.9 ± 10.3 years old) diagnosed with T2DM combined with CS was identified by the combination of WHO diagnostic criteria and head computed tomography and/or magnetic resonance imaging (26). Moreover, 284 healthy people (156 males and 128 females; age: 59.6 ± 11.4 years old) receiving physical examinations in our hospital were selected as the control group. Patients were excluded in this study based on the following criteria: (a) patients with T1DM, gestational diabetes, or other types of DM; (b) patients who could not complete the questionnaire due to unconsciousness, abnormal communication or other reasons; (c) patients with miss rates over 5% because of incomplete information; (d) foreigners (non-Chinese citizen).

Clinical Data and Biochemical Indicator Measurement

The height, weight, waistline, hipline, and blood pressure at rest were measured. A volume of 10 ml fasting ulnar vein blood was collected from all study subjects in the early morning. Of the 10 ml blood, 3 ml was added to an anticoagulant tube to detect glycosylated hemoglobin. Then, 4 ml of blood was added to a coagulation-promoting tube for centrifugation after the blood was completely coagulated (incubation for 30 min). Blood sugar, total cholesterol (TC), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), and triglycerides (TGs) were detected after the serum specimen was conventionally separated. The remaining serum specimens were stored in a −80°C freezer until further use, and ACE2 level was detected after the collection of all samples. The remaining blood samples were collected in tubes containing ethylenediaminetetraacetic acid as an anticoagulant and stored in a −80°C freezer for genomic DNA extraction of white cells.

Preparation of DNA and Genotyping

The remaining blood samples were centrifuged, and the plasma was eliminated followed by the addition of red blood cell lysate. The red blood cells were dissolved after intensive mixing and oscillating for 5 min, followed by a 30 sec centrifugation (10,000 × *g*), and then, the white cells were precipitated. After the addition of the cell lysate, all steps were conducted according to the instructions of DNA kit (Qiagen Company, Dusseldorf, Germany). An ultraviolet (UV) spectrophotometer (UV2600, Zhengzhou Nanbei Instrument Equipment Co., Ltd., Zhengzhou, Henan, China) was used to measure the DNA content and absorbance (A) values (A260 and A280; A260 vs. A280: 1.8–2.0). The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) was used to search for SNPs, and we found that the sequence number of the G8790A polymorphism of *ACE2* is AY217547. Oligo6.0 software (Oswel Research Products, Beijing,

China) was used to design primer sequences for G8790A, which were synthesized by Shanghai Yingjun Biotechnology Co., Ltd. (Shanghai, China). The primers were as follows: forward: 5-CATGTGGTCAAA AGGATATCT-3; reverse: 5-AAAGTA AGGTTGG-CAGACAT-3. The polymerase chain reaction (PCR) amplification was performed in a total volume of 25 μl in a gradient PCR system (Biometra Company, Jena, Germany) containing human genomic DNA (1 μl), 10 × PCR buffer (2.5 μl), 25 μmol/l $MgCl_2$ (1.5 μl), 10 mmol/l dNTP (0.5 μl), 10 μmol/l upstream primer (0.8 μl), 5 U/μl Taq DNA polymerase (0.2 μl), and distilled water (18.5 μl). The PCR protocol was as follows: predenaturing (95°C for 2 min), denaturing (94°C for 30 sec), annealing (50.6°C for 30 sec), and elongation (72°C for 45 sec), amplified for 34 cycles, and a final elongation for 7 min to terminate all reactions. All the PCR reagents were provided by Shanghai Jinpan Biotech Company (Shanghai, China). PCR-amplified products were directly detected by electrophoresis. It was confirmed by sequencing that the *ACE2* G8790A polymorphism was located in the third intron, fourth base, and it was at a restriction site (AGCT) for Alu I. Figure 1 shows the results of sequencing. Enzyme digestion was performed in a total volume of 20 μl containing Alu I enzyme (2 U), 10 × buffer (2 μl), and DNA products (5 μl), followed with a 16 h incubation at 37°C. After enzyme digestion, 6 × loading dye was added to terminate the reaction. PCR products were analyzed by 2% agarose gel electrophoresis. All reagents used in enzyme digestion were purchased from Wuhan Boster Bio-Engineering Ltd. Company (Wuhan, China).

Serum ACE2 Level Measurement

A human serum ACE2 enzyme-linked immunosorbent assay (ELISA) kit, produced by Shanghai Bluegene Biotech Co., Ltd. (Shanghai, China), was used and tested by a Thermo Scientific Varionskon Flash (Thermo Fisher Scientific, Waltham, MA) in our study.

The kit was taken out from refrigerator and then incubated at room temperature (20–25°C) for 30 min. The ELISA plate was opened, and 100 μl standard solutions was added into the micropores, while 100 μl phosphate-buffered saline was added in the blank group. Six standards were, respectively, added with 50 μl enzyme-labeled solutions. The ELISA plate was incubated in a 37°C incubator (steady temperature and humidity) for several hours after sealing with sealing film. After incubation, the ELISA plate was removed, the sealing film was torn, and the reaction mixture was discarded, followed by five washes of the ELISA plate by cleaning solution. Then, the ELISA plate was completely dried using a absorbent paper. In each well, 50 μl reagent A and 50 μl reagent B were sequentially added in the dark, followed by a dark reaction in a 37°C incubator. The reaction was stopped by adding 50 μl of stop buffer in each well after 15 min. Measurements were made within 10 min at 450 nm in an ELISA reader. All A values were calculated after the deduction of the A value of the blank well, and standard curves were generated with concentration as the abscissa, with the corresponding A values as the ordinate by the four parameters method.

Samples to be tested were diluted by 1% bovine serum albumin (1:19). Each well contained 100 μl diluted samples. The remaining steps were the same as the above. Concentration of ACE2 was calculated by standard curve equation and A values of every well, followed by multiplication by 20 to determine the concentration of ACE2 in the samples.

Statistical Analysis

The Hardy–Weinberg equilibrium was applied for genetic equilibrium tests. The data were analyzed with SPSS 21.0 software (SPSS Inc., Chicago, IL). Continuous data are presented as the mean ± standard deviation (SD) and were tested by Student's *t*-test. The genotype and allele frequency was calculated by the gene counting method and tested by χ^2 test. Comparisons of mean among groups were analyzed by the homogeneity test of variance and one-way analysis of variance (ANOVA). Logistic regression analysis was

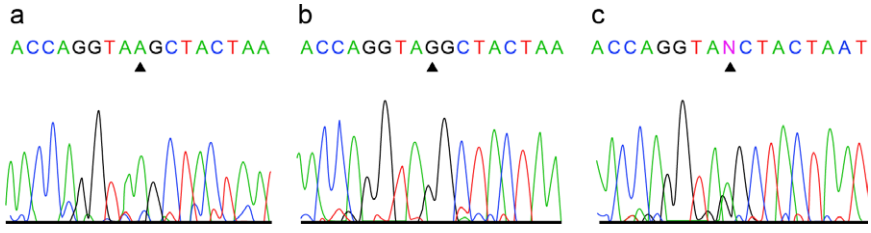

Fig. 1. Sequencing of the *ACE2* G8790A polymorphism (*ACE2*: angiotensin-converting enzyme 2; (a) the sequencing result of the *ACE2* gene containing the A allele; (b) the sequencing result of the *ACE2* gene containing the G allele; (c) the sequencing result of the *ACE2* gene containing [C/T] heterozygotes; ▲: polymorphic site).

conducted to analyze the pathogenic factors of patients. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Comparisons of Clinical Parameters Among Control, T2DM, and T2DM + CS Groups

Body mass index (BMI), systolic pressure (SBP), TC, TG, HDL, LDL, fasting blood glucose (FBG), and glycosylated hemoglobin (HbA1c) in the T2DM and T2DM + CS groups were evidently higher than those in the control group; additionally, SBP and diastolic pressure (DBP) in the T2DM + CS group were higher than those in other two groups, with statistically significant differences (all $P < 0.05$). Comparison of FBG and HbA1c between the T2DM and T2DM + CS groups indicated significant differences (both $P < 0.05$), but no differences were found for BMI, TC, TG, HDL and LDL (all $P > 0.05$; Table 1).

ACE2 G8790A Polymorphism

The fragment length *ACE2* PCR product was 369 bp. After digestion by AluI, different genotypes showed different cleavage patterns. The GA genotype had three fragments of 369, 219 and 152 bp; the AA genotype had two fragments of 219 and 152 bp; and the GG genotype had one fragment of 369 bp (Fig. 2).

TABLE 1. The Clinical Parameters in Control, T2DM and T2DM Combined with CS Group

Clinical parameters	Control group	T2DM group	T2DM combined with CS group
Number of cases	284	181	165
Age (years)	59.6 ± 11.4	60.9 ± 10.7	61.9 ± 10.3
BMI (kg/m^2)	22.5 ± 4.1	25.2 ± 6.3[a]	24.9 ± 8.1[a]
SBP(mmHg)	111 ± 16	136 ± 25[a]	147 ± 16[a,b]
DBP (mmHg)	75 ± 14	79 ± 12	88 ± 16[a,b]
TC (mmol/l)	4.57 ± 0.87	5.69 ± 1.37[a]	5.48 ± 1.29[a]
TG (mmol/l)	1.30 ± 0.38	2.15 ± 1.45[a]	2.10 ± 1.11[a]
HDL (mmol/l)	1.43 ± 0.33	1.15 ± 0.35[a]	1.12 ± 0.28[a]
LDL (mmol/l)	2.13 ± 1.04	3.15 ± 1.08[a]	3.27 ± 1.15[a]
FBG (mmol/l)	5.51 ± 0.30	9.50 ± 0.48[a]	8.94 ± 0.38[a,b]
HbA1c (%)	4.18 ± 1.32	9.44 ± 2.82[a]	8.73 ± 2.27[a,b]

T2DM, type 2 diabetes mellitus; CS, cerebral stroke; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic pressure; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; FBG, fasting blood glucose; HbA1c, glycosylated hemoglobin.
[a]Compared with control group, $P < 0.05$.
[b]Compared with T2DM group, $P < 0.05$.

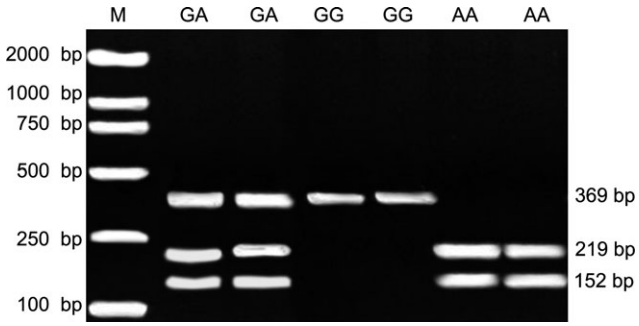

Fig. 2. Electrophoresis of *ACE2* gene G8709A polymorphism by enzyme digestion (*ACE2*: angiotensin-converting enzyme 2; M: marker).

Genotype and Allele Frequency of the *ACE2* G8790A Polymorphism Among the Control, T2DM, and T2DM + CS Groups

In the control group, the Hardy–Weinberg equilibrium was examined by a goodness of fit test, and the results showed that the distribution of the *ACE2* G8790A genotype was consistent with the Hardy–Weinberg equilibrium ($P > 0.05$), suggesting a representative sample.

Significant differences were found in the genotype and allele frequency of *ACE2* G8790A between the T2DM + CS and control groups, and the T2DM and T2DM + CS groups (all $P < 0.05$). The comparison of the genotype frequency of *ACE2* G8790A between the T2DM and control groups showed statistical differences ($P < 0.05$), while no differences were observed for allele frequency ($P > 0.05$; Table 2).

Additionally, we found significant differences in the distribution of the *ACE2* G8790A alleles between the T2DM + CS and control groups and the T2DM and T2DM + CS groups (all $P < 0.05$). The A allele may increase the risk of T2DM combined with CS (OR = 2.504, 95%CI = 1.849–3.391; OR = 2.502, 95% CI = 1.802–3.474). Additionally, the distribution frequencies of homozygous mutations (AA genotype) and the wild type (GG genotype) between the T2DM + CS and control groups, and the T2DM and T2DM + CS groups were significantly differences (all $P < 0.05$) (AA: OR = 3.733, 95%CI = 2.069–6.738; GG: OR = 3.597, 95%CI = 1.8884–6.867) (Table 3).

Correlations Between Three Genotypes and Serum ACE2 Level

The levels of serum ACE2 of the three genotypes were AA genotype > GA genotype > GG genotype, suggesting statistically significant differences (all $P < 0.01$). Thus, the A allele may increase the level of

TABLE 2. *ACE2* G8790A Genotypes in Control, T2DM and T2DM with CS Groups [*n* (%)]

Genotypes	Control group	T2DM group	T2DM combined with CS group	P^a	P^b	P^c
GG	64 (22.53)	37 (20.44)	18 (10.91)			
GA	120 (42.25)	84 (46.41)	42 (25.45)	<0.001	<0.001	<0.001
AA	100 (35.22)	60 (33.15)	105 (63.64)			
G	248 (43.66)	158 (43.65)	78 (23.64)	<0.001	0.996	<0.001
A	320 (56.34)	204 (56.35)	252 (76.36)			

ACE2, angiotensin-converting enzyme 2; T2DM, type 2 diabetes mellitus; CS, cerebral stroke.
[a]Comparison between T2DM combined with CS and Control group.
[b]Comparison between T2DM and Control group.
[c]Comparison between T2DM and T2DM combined with CS group.

TABLE 3. Correlations Between G8790A Polymorphism of *ACE2* and T2DM Combined with CS

Genotype	OR (95%CI)[a]	P^a	OR (95%CI)[b]	P^b	OR (95%CI)[c]	P^c
GG	Ref.					
GA	1.244 (0.663–2.337)	0.496	1.211 (0.741–1.980)	0.445	1.028 (0.524–2.017)	0.936
AA	3.733 (2.069–6.738)	<0.001	1.038 (0.620–1.739)	0.888	3.597 (1.884–6.867)	<0.001
G vs. A	2.504 (1.849–3.391)	<0.001	1.001 (0.767–1.305)	0.996	2.502 (1.802–3.474)	<0.001

ACE2, angiotensin-converting enzyme 2; T2DM, type 2 diabetes mellitus; CS, cerebral stroke; OR, odds ratio; CI, confidence interval; Ref, reference.
[a]Comparison between T2DM combined with CS and control group.
[b]Comparison between T2DM and control group.
[c]Comparison between T2DM and T2DM combined with CS group.

ACE2, resulting in increased CS risk. Moreover, there were no significant differences in serum ACE2 levels in the same genotype carriers among the three groups (all $P > 0.05$; Table 4).

Multivariate Stepwise Logistic Regression Analysis

In the multivariate stepwise logistic regression analysis, age, BMI, SBP, DBP, TC, TG, HDL, LDL, FBG, HbA1c, the genotype, and allele frequency of *ACE2* G8790A, and serum ACE2 level were used as the independent variables, with CS as the dependent variable. Table 5 shows that the A allele of *ACE2* G8790A was a risk factor for T2DM combined with CS ($P = 0.001$, OR = 2.479), while ACE2 level ($P = 0.006$, OR = 0.953), SBP ($P = 0.006$, OR = 0.971), and DBP ($P = 0.027$, OR = 0.961) were protective factors for T2DM combined with CS.

DISCUSSION

In this study, we showed that the *ACE2* G8790A polymorphism in T2DM patients was correlated with CS, and the A allele might be a risk factor of T2DM combined with CS. This study had four steps. In the first step of our study, patients were selected. After the selection of enrolled patients, we determined the height, weight, waistline, hipline, and blood pressure of the subjects. The results revealed that BMI, SBP, TC, TG, HDL, LDL, FBG, and HbA1c in the T2DM and T2DM + CS groups were evidently higher than

TABLE 4. Serum ACE2 Level in Control, T2DM and T2DM Combined with CS Group (U/l)

Genotypes	Control group	T2DM group	T2DM combined with CS group	*P* value
GG	$20.80 \pm 5.31^{a,b}$	$20.96 \pm 6.06^{a,b}$	$22.81 \pm 6.79^{a,b}$	0.498
GA	27.63 ± 6.44^{c}	28.68 ± 5.64^{c}	27.17 ± 4.24^{c}	0.302
AA	32.45 ± 6.72	33.10 ± 8.78	34.83 ± 6.37	0.051

ACE2, angiotensin-converting enzyme 2; T2DM, type 2 diabetes mellitus; CS, cerebral stroke.
[a]Comparison between GG and GA genotypes, $P < 0.05$.
[b]Comparison between GG and AA genotypes, $P < 0.05$.
[c]Comparison between GA and AA genotypes, $P < 0.05$.

TABLE 5. Variables Associated with T2DM Combined with CS

Variables	*B*	SE	Wald	df	Sig.	Exp (*B*)
A allele	0.908	0.273	11.056	1	0.001	2.479
SBP	0.030	0.011	7.648	1	0.006	0.971
ACE2 level	0.048	0.017	7.511	1	0.006	0.953
DBP	0.039	0.018	4.914	1	0.027	0.961

ACE2, angiotensin-converting enzyme 2; T2DM, type 2 diabetes mellitus; CS, cerebral stroke; SBP, systolic blood pressure; DBP, diastolic pressure; B, partial regression coefficient; SE, standard error; Wald, Wald χ^2; df, degree of freedom; Sig., *P* value; Exp(B), odds ratio.

that in the control group, which was consistent with previous studies (27, 28).

Subsequently, genotyping was conducted, and significant differences were found in the genotype and allele frequency of *ACE2* G8790A between the T2DM + CS and control groups and the T2DM and T2DM + CS groups. ACE2, which acts as a monocarboxypeptidase, can hydrolyze dynorphin A 1-13 and apelin-13 with high activity; the most significant biological effect of ACE2 is the degradation of Ang II to Ang 1-7, and the catalytic efficiency is 400-fold higher with Ang II than with Ang I (29). Thus, ACE2 may restrict the vasoconstrictor action of Ang II via its degradation and offset the actions of Ang II by the formation of Ang 1-7, which has antifibrotic and vasodilatory activity at the *mas* receptor of Ang 1-7 (30, 31). There are also several other cardiovascular functions of Ang 1-7, including diuresis, natriuresis, inhibition of cell growth as well as anti-atherosclerotic effects (32). In the regions of the mouse brain involved in control of cardiovascular function, ACE2 has also been identified, with *ACE2* gene deletion leading to impaired autonomic function and baroreflex sensitivity, suggesting a vital role of ACE2 in the regulation of central blood pressure (33, 34). Additionally, ACE2 might act in a counter-regulatory manner to ACE, controlling the balance between vasodilators and vasoconstrictors in the kidney and heart, and plays an essential role in modulating cardiovascular and renal function (35).

Then, the A allele may increase the risk of T2DM combined with CS, which was verified by our study. Hypertension has been reported as a common risk factor for both T2DM and CS, and DM was also identified as a risk factor for CS (36). Furthermore, *ACE2* has been proposed as a new candidate gene for hypertension (29). Two studies focusing on the associations between the G8790A polymorphism and hypertension or systolic or diastolic BP conducted by Yi et al. (35) and Niu et al. (37), respectively, also demonstrated a correlation between higher pressure and the A allele. Additionally, one recent meta-analysis with a relatively large sample size identified a significant association between the G8790A AA genotype and hypertension (38). Likewise, the last result in our study obtained by logistic regression analysis also further confirmed our conclusion that A allele might be a risk factor of T2DM combined with CS, and SBP and DBP were found to be protective factors of T2DM combined with CS.

In summary, our study showed that the *ACE2* G8790A polymorphism in T2DM patients was correlated with CS, and the A allele might be a risk factor of T2DM combined with CS. However, the limitation of the relatively small sample size should be noted. Thus, further studies with larger sample sizes should be conducted to confirm our conclusion.

ACKNOWLEDGMENTS

This project was supported by funding for Heilongjiang Provincial Department of Education Science and Technology research project (NO. 12511237). We would like to acknowledge the reviewers for their helpful comments on this paper.

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