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Association of *AGTR1* gene methylation and its genetic variant in Chinese farmer with hypertension

A case-control study

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Abstract

The objective was to determine the potential associations of the angiotensin II receptor type 1 (*AGTR1*) gene polymorphism, methylation, and lipid metabolism in Chinese farmers with hypertension.

A case-control study was conducted in Wuzhi county of Henan province in China in 2013 to 2014. A total of 1034 local residents (35–74 years, 386 hypertensive cases, and 648 normotensive subjects) were enrolled in this study. Triglyceride (TG), total cholesterol (TC), high-density lipoprotein, and low-density lipoprotein were measured using automatic chemistry analyzer. The *AGTR1* gene promoter methylation level was measured using quantitative methylation-specific polymerase chain reaction method. The single nucleotide polymorphism rs275653 was genotyped with TaqMan probe assay at an applied biosystems platform.

The gender, body mass index (BMI), TG, TC, and family history of hypertension in the hypertension group were significantly higher than those in control group ($P < .05$). No significant difference was observed in the distribution of AGTR1 rs275653 polymorphism in the hypertension and controls ($P > .05$). The *AGTR1* gene methylation in subjects carrying different genotypes was not significantly observed ($P > .05$). The logistic regression analysis found the *AGTR1* gene methylation level was negative correlation with hypertension in the present study (odds ratio, 0.946, 95% confidence interval, 0.896–0.999) through adjusting for age, gender, BMI, education, smoking, alcohol drinking, fruit and vegetable intake, pickles intake, and family history of hypertension.

The association of *AGTR1* gene hypomethylation and essential hypertension was observed in Chinese farmers; no significant difference was observed in the distribution of *AGTR1* rs275653 polymorphism.

Abbreviations: AGTR1 = angiotensin II receptor type 1, DBP = diastolic blood pressure, HDL = high-density lipoprotein, LDL = low-density lipoprotein, RAS = renin-angiotensin system, SBP = systolic blood pressure, TC = total cholesterol, TG = triglyceride.

Keywords: *AGTR1* gene, DNA methylation, hypertension, lipid metabolism

1. Introduction

Hypertension is a primary risk factor in cardiovascular system,[1,2] increasing prevalence rate both in developed and developing countries.[3,4] The number of people who suffer from hypertension is estimated to be about 1.5 billion all over the world by 2025.[5] Essential hypertension mainly involves interaction between genetic and environmental factors.[6] Alterations in genetic or environmental factors may contribute to the pathogenesis of essential hypertension. Several genetic variations involved in pathogenesis of hypertension have been found, including single nucleotide polymorphisms of rs4343 and rs4351 in the angiotensin I converting enzyme gene, rs7194256 in the ETS transcription factor ETS domain-containing protein (*ELK3*) gene, and rs1042039, rs1054889, and rs2073316 in the xanthine dehydrogenase gene.[7]

The renin-angiotensin system (RAS) is a vital humoral regulation system, which is related to the adjustment of blood pressure.[8] Angiotensin II is a major effector peptide in RAS, leading to vasoconstriction and water-sodium retention.[9] Angiotensin II receptor plays a critical role in mediating the RAS pathway, helping to manage the blood pressure and fluid

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volume.[10] Angiotensin II receptor type 1 (AGTR1) and type 2 (AGTR2) are subtypes of AGTR, and it is documented that the *AGTR1* gene is extremely associated with the etiology of hypertension and other cardiac outcomes.[11] The *AGTR1* gene, located on chromosomes 3q21 to 25 and spanning > 55 kb, is composed of 5 exons, with the first 4 encoding the 5′-untranslated region and the fifth being the coding region.[12] Recently, the studies on genetic polymorphisms have reported that the single nucleotide polymorphism (SNP) of the *AGTR1* gene is related to the pathogenesis of hypertension.[13] However, few studies on the relationship between epigenetic markers like DNA methylation, another important regulating factor in gene expression, and hypertension were reported,[14] and whether the epigenetic modifications of the *AGTR1* gene were associated with hypertension still remains unclear. In fact, studies on metabolism-related diseases showed that DNA methylation can affect lipid metabolism and lead to metabolic diseases, including gestational diabetes mellitus, child obesity, and hypercholesterolemia.[15,16]

Therefore, a case-control study was conducted in rural areas of China, to explore whether genetic variant and DNA methylation status are related to lipid metabolism, even hypertension.

2. Methods

2.1. Location and Population

Based on previous study, 3 administrative villages in Wuzhi county of Henan province were selected as the investigated sites using random sampling method from 2013 to 2014. Local farmers aged 35 to 74 years who lived in the county for > 5 years were considered as subjects by cluster sampling. Participants with following conditions were excluded: a) stress-induced hypertension or high altitude hypertension; b) kidney and liver transplantation or dysfunction; c) craniocerebral injuries; d) endocrine disorders; e) malignant tumor or other severe systemic diseases; f) mental illness and anxiety; g) women in gestation or lactation period, or child-bearing period without contraception; h) substance abuse. A total of the 427 hypertension cases and 717 normotensive people were selected according to diagnosis for hypertension. Then the 41 hypertension cases and 69 normotensive people were removed out as incomplete data, which is defined as the missing *AGTR1* gene methylation data. Finally, the 386 hypertension cases and 648 normotensive subjects were enrolled in our study. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional review board at Zhengzhou University in China. Informed consent was obtained from all individual participants included in the study.

2.2. Diagnosis for Hypertension

Blood pressure of each individual was measured in sitting position using electronic sphygmomanometer (Omron HEM-770AFuzzy, Kyoto, Japan) by trained operators. The systolic blood pressure (SBP) and diastolic blood pressure (DBP) of every single participant was measured 3 times, with an interval of half minute, then the average value was calculated. Before each measurement, subjects were at resting status for at least five minutes. Hypertensive patients were defined as SBP ≥ 140 mm Hg and/or DBP ≥ 90 mm Hg.

2.3. Questionnaires and Biological Samples Collection

An in-person interview was performed at the village clinics using a standardized and structured questionnaire to collect demographic characteristics, individual behavior (such as diet, smoking, and alcohol consumption), occupational, medical conditions, and medication use. A total of 10 mL of fasting blood was collected from each subject, with 5mL anticoagulation and 5 mL nonanticoagulation. After centrifugation within 2h, serum and white blood cells were separated and frozen at -80 ℃ for subsequent analyses. The triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL) in serum were measured by Hitachi-7080 automatic biochemical analyzer (Hitachi Ltd., Tokyo, Japan) according to the manufacturer's protocols. Each sample was run in duplicate, and 15% to 20% of total samples were retested randomly. The intra- and inter-assay coefficients of variation were < 10% for these assays. The dyslipidemia was defined as the presence of ≥1 abnormal serum lipid concentrations according to the Chinese guidelines on prevention and treatment of dyslipidemia in adults, or usage of antidyslipidemia medicines in the past 2 weeks.

2.4. Determination of AGTR1 Methylation Level

Genomic DNA was extracted using whole blood genomic DNA extraction kits with paramagnetic particle method (BioTeke Corporation, Beijing, China). Bisulfate modification of genomic DNA was performed by using the EZ DNA Methylation-Gold Kit (Zymo Research, CA, USA). The *AGTR1* gene methylation was performed by quantitative methylation-specific polymerase chain reaction (PCR) method (Agilent MX3000P, Santa Clara, CA, USA). The sequence in promoter region of *AGTR1* gene was searched using University of California Santa Cruz (UCSC)/Ensembl (http://genome.ucsc.edu/) and the methylated specific primers were designed by MethPrimer v1.0 (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). Two pairs of PCR primers were used to conduct methylation-specific PCR (methylated specific primers: L, 5′-AATGTTGAAGAATACGAATTTTC-GT-3′; R, 5′-CTCCCTCTCGAAATATT AACGAC-3′; unmethylated specific primers: L, 5′-AATGTTGAAGAATAT-GAATTTTTGT-3′; R, 5′-CTCCCTCTCAAAATATTAA-CAAC-3′). PCR amplification was performed in a 20.0 μL reaction mixture with the following concentrations: 7.5 μL of 2 × Power SYBR Green PCR Master Mix (Applied Biosystems, Cheshire, United Kingdom), 1.5 μL primer with a concentration of 1.25 μmol/L, and 60.0 ng of bisulfite-treated DNA template with approximate concentration of 100.0 μg/mL. PCR conditions were as follows: predegeneration at 95°C for 10 minutes, 40 cycles for degeneration at 95°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. Negative controls were set for each experiment. Each sample was run in duplicate, and 15% of total samples were retested randomly. The intra- and inter-assay coefficients of variations < 10% were considered being qualified. The rate of DNA methylation was calculated using the reference method.

2.5. Genotyping

The *AGTR1* gene was genotyped at the rs275653 site in promoter region with TaqMan probe assay using the Applied Biosystems platform (ABI 7500 Fast Real-Time PCR system, Foster City, USA). The primers and probes for SNPs were designed by Applied Biosystems Inc (Applied Biosystems, Cheshire, United Kingdom), and the allelic discrimination was detected automatically using Sequence Detection Systems 2.1 software on the 7500 Fast Real-Time PCR system. Real-time PCR reaction was carried out in a 12.0 μL volume using 0.1 μL TaqMan probe, 6.0 μL Mix, and 100 ng template DNA. Amplification was obtained by predegeneration at 95°C for 10 minutes, 40 cycles for degeneration at 95°C for 15 seconds, and annealing at 60°C for 60 seconds.

2.6. Statistical Analysis

The database was established using the Epidata 3.0 software (Epidata 3.0 for windows, Epidata Association Odense, Denmark) and all the data was doubled entered into the database by different operators. Differences in age, body mass index (BMI), TG, TC, HDL, LDL, and the *AGTR1* methylation level between 2 groups were examined by independent sample *t* tests. A Chi-square test was used to evaluate the differences in gender, education, the prevalence of alcohol drinking and smoking, fruit and vegetable intake, pickles intake, and family history of hypertension. The binary logistic regression considered hypertension as dependent variable, in which methylation level, TC, TG, HDL, LDL, and SNP of rs275653 were included as covariates. Data were presented as mean ± standard deviation. The $P < .05$ was considered statistically significant. All analyses were performed using the SPSS 21.0 (SPSS Inc., Chicago, USA).

3. Results

3.1. General Characteristics of Included Subjects

As shown in Table 1, a total of 1034 local farmers were enrolled in the study, with 386 hypertension and 648 control subjects. The gender (male/female), BMI, SBP, DBP, TG, and TC in the hypertension group were significantly higher than those in the control group ($P < .05$). The prevalence of family history of hypertension was 43.5% in hypertension and 34.1% in controls, with significant difference between the 2 groups. No significant differences on education, smoking, drinking, fruit and vegetable intake, pickles intake, HDL, LDL, and AGTR1 methylation were observed in the 2 groups ($P > .05$).

Table 1

General characteristics of included population (n = 1034).

Characteristics	Hypertension (n = 386)	Controls (n = 648)	t/χ^2	*P*
Age (year)	54.99 ± 9.12	55.19 ± 9.81	−0.303	.762
Gender			4.922	**.027**
Male	211 (54.7)	308 (47.5)		
Female	175 (45.3)	340 (52.5)		
BMI (kg/m^2)	26.43 ± 3.79	25.31 ± 3.41	4.921	**<.001**
SBP (mm Hg)	145.75 ± 14.13	120.01 ± 10.34	31.177	**<.001**
DBP (mm Hg)	91.93 ± 8.68	76.56 ± 7.05	29.463	**<.001**
Triglyceride	1.96 ± 1.48	1.75 ± 1.37	2.346	**.019**
Total cholesterol	4.69 ± 0.96	4.56 ± 0.97	2.025	**.043**
High-density lipoprotein	1.23 ± 0.29	1.24 ± 0.31	−0.568	.570
Low-density lipoprotein	2.62 ± 0.73	2.55 ± 0.73	1.304	.193
AGTR1 methylation (%)	3.07 ± 2.28	3.33 ± 2.90	−1.479	.140
Education				
≤ Primary school	147 (38.2)	293 (45.4)		
Junior high school	175 (45.5)	275 (42.6)		
≥ Senior high school	63 (16.4)	78 (12.1)		
Smoking			0.649	.420
Yes	143 (37.0)	224 (34.6)		
No	243 (63.0)	424 (65.4)		
Alcohol drinking			0.435	.509
Yes	81 (21.0)	125 (19.3)		
No	305 (79.0)	523 (80.7)		
Fruits and vegetables intake			0.475	.491
≥ 500 g/d	131 (34.0)	207 (31.9)		
< 500 g/d	254 (66.0)	441 (68.1)		
Pickles intake*			3.115	.078
≥ 2 g/d	121 (31.4)	238 (36.8)		
< 2 g/d	264 (68.6)	408 (63.2)		
Family history of hypertension			9.144	**.002**
Yes	168 (43.5)	221 (34.1)		
No	218 (56.5)	427 (65.9)		

Values in bold are significant at $P < 0.005$.

AGTR1 = angiotensin II receptor type 1, BMI = body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure.

*Pickles indicate the additional salt intake except for salt in daily diet.

3.2. Genotype Distribution of AGTR1 rs275653

Table 2 showed the AGTR1 genotype frequencies of locus rs275653 in the hypertension and control groups. All the SNP genotype frequencies in control group were consistent with the Hardy-Weinberg equilibrium ($\chi^2 = 2.314$, $P = .314$). No significant difference was observed in the distribution of *AGTR1* rs275653 polymorphism in the hypertension and controls ($P > .05$). Subjects carrying AG/GG genotypes of rs275653 did not increase the risk of essential hypertension compared with subjects carrying the AA genotype.

3.3. Association Between AGTR1 rs275653 Genotypes and DNA Methylation

The levels of TG, TC, HDL, LDL, and DNA methylation stratified by genotypes were summarized in Table 3. It turned out that no significant results were observed for the above biomarkers among different farmers carrying different genotypes of *AGTR1* rs275653 ($P > .05$, respectively).

3.4. The Effect of AGTR1 Promoter Region Methylation on the Risk of Hypertension

We further analyzed the lipid metabolism and genetic risk factors of hypertension using binary logistic regression, in which AGTR1 rs275653 genotypes, methylation level, TC, TG, HDL, and LDL were included as covariates (Table 4). It turned out that the AGTR1 promoter region methylation level (odds ratio, 0.946, 95% confidence interval, 0.896–0.999) was a protective factor of hypertension in the present study ($P = .047$).

4. Discussion

Our findings suggest a significant association between the *AGTR1* gene methylation and hypertension. However, the *AGTR1* rs275653 locus polymorphism may not be a valuable genetic marker for differential risk of essential hypertension among Chinese farmers.

The RAS is associated with the onset of various cardiovascular diseases.[17,18] The *AGTR1* gene is a key gene of RAS system and closely related to the occurrence and development of hypertension. However, few studies on the relationship between the *AGTR1* gene methylation and hypertension. In the present study,

Table 2

Genotype and allele frequencies of polymorphisms across *AGTR1* rs275653 between hypertension and control group.

AGTR1 rs275653	Hypertension, n (%)	Controls, n (%)	*P*	OR (95% CI)
Genotype				
AA	297 (78.0)	490 (77.0)	—	—
AG	81 (21.3)	141 (22.2)	.734	1.06 (0.78–1.44)
GG	3 (0.8)	5 (0.8)	.989	1.01 (0.24–4.26)
Allele				
A	675 (88.9)	1121 (88.5)	—	—
G	84 (11.1)	146 (11.5)	.754	1.05 (0.79–1.39)

AGTR1 = angiotensin II receptor type 1, CI = confidence interval, OR = odds ratio.

Table 3

Lipid metabolism and DNA methylation status in farmers carrying different genotypes.

	Hypertension group					Control group				
Markers	**AA**	**AG**	**GG**	**F**	**P**	**AA**	**AG**	**GG**	**F**	**P**
TG (mmol/L)	2.00 ± 1.54	1.76 ± 1.17	1.47 ± 1.59	1.00	.37	1.72 ± 1.32	1.83 ± 1.48	2.33 ± 2.71	0.76	.47
TC (mmol/L)	4.69 ± 0.99	4.69 ± 0.87	4.28 ± 0.79	0.27	.76	4.56 ± 0.96	4.61 ± 1.05	4.82 ± 0.89	0.31	.74
HDL (mmol/L)	1.23 ± 0.28	1.22 ± 0.30	1.41 ± 0.47	0.59	.56	1.24 ± 0.30	1.25 ± 0.32	1.32 ± 0.51	0.21	.81
LDL (mmol/L)	2.59 ± 0.74	2.41 ± 0.71	2.20 ± 0.83	1.18	.31	2.55 ± 0.72	2.56 ± 0.82	2.88 ± 0.50	0.40	.67
Methylation (%)	3.11 ± 2.23	2.90 ± 2.48	4.71 ± 2.48	1.02	.36	3.46 ± 3.07	2.92 ± 2.28	1.64 ± 1.10	2.73	.07

HDL = high-density lipoprotein, LDL = low-density lipoprotein, TC = total cholesterol, TG = triglyceride.

Table 4

Association of *AGTR1* gene polymorphism, methylation and lipid metabolism with hypertension using the logistic regression analysis.

	β	SE	Wald χ^2	*P*	OR (95% CI)
AGTR1 rs275653/AA	—	—	0.537	.765	—
AGTR1 rs275653/AG	−0.035	0.169	0.043	.836	0.965 (0.693–1.346)
AGTR1 rs275653/GG	0.542	0.783	0.479	.489	1.719 (0.371–7.976)
AGTR1 methylation	−0.056	0.028	3.952	**.047**	**0.946 (0.896–0.999)**
TG (mmol/L)	0.084	0.109	0.591	.442	1.088 (0.878–1.348)
TC (mmol/L)	0.119	0.101	1.381	.240	1.127 (0.923–1.374)
HDL (mmol/L)	−0.181	0.337	0.287	.592	0.835 (0.431–1.617)

Values in bold are significant at $P < 0.005$.

Adjusted for age, gender, body mass index, education, smoking, alcohol drinking, fruit and vegetable intake, pickles intake, and family history of hypertension.

AGTR1 = angiotensin II receptor type 1, CI = confidence interval, HDL = high-density lipoprotein, OR = odds ratio, TC = total cholesterol, TG = triglyceride.

there was significant association between the *AGTR1* gene promoter region methylation and hypertension. Meanwhile, Fan et al[19] identified a significantly lower CpG1 methylation level in the *AGTR1* gene promoter region in essential hypertension cases than in controls. Lin et al used melting temperature (T_m) values to differentiate the *AGTR1* gene methylation level, the results showed that the T_m value was lower in the hypertension than in the normotensive group.[20] Those results[21] were consistent with our findings in this study.

Essential hypertension is mainly induced by multiple genetic and environmental factors,[6] and it is not conformed to the Mendelian inheritance model. The pathogenesis of hypertension involves major and minor genes and ambient influences,[22,23] in which the gene-gene and gene-environment interaction exist.[24–26] In this study, we did not observe the genetic variant, rs275653, in *AGTR1* promoter region that was associated with hypertension. It suggested that *AGTR1* rs275653 locus polymorphism may not be a valuable genetic marker for differential risk of essential hypertension among Chinese farmers. However, the genetic variation of AGTR1 gene rs275653 was related to the promoter region methylation level in this study. Subjects in hypertension carrying AA genotype have a relative lower methylation level compared with controls. It suggested that lower methylation level of individuals with AA genotype were more susceptible to suffer from hypertension. Since the polymorphism of rs275653 may influence the susceptibility of hypertension in some sophisticated ways.

Several limitations in this study need to be illustrated. Despite the sample size in this study being relatively large, some potential confounders may have not been completely adjusted, such as the pharmacological classifications of the antihypertensive medicines. However, the descriptions of diagnosis, inherited traits, and demography were adequately characterized, with estimates for variables impacting blood pressure including dietary habits, daily exercises, cigarettes and alcohol consumption, and other parameters; therefore, our results were entirely trustworthy. It is well known that many risk factors for hypertension and their mechanisms of action and risk intensity are also different. The study of these factors is limited by the research population, sample size, methods, and other conditions. Therefore, future studies should be based on mechanism research and take into consideration the details of the combined effects of genes and environment involved in the RAS system on essential hypertension.

In conclusion, the hypomethylation of *AGTR1* gene promoter region is associated with the hypertension, and no significant difference was observed in the distribution of AGTR1 rs275653 polymorphism.

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Author contributions

Zhi-yuan Li, Qiang Ma, and Fang-fang Yu participated in the study design, performed the experiments, analyzed the data, and drafted the article. Yue Ba and Fang-fang Yu provided funding, designed and supervised the study. Qiang Ma, Xing Li, Shui-yuan Yu, and Juan Zuo recruited study participants, collected the samples, and performed DNA methylation examination. Chong-jian Wang and Wen-jie Li contributed with scientific input to the design of the experiment. All authors participated in the article editing and approved the final article.

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