Preliminary analysis of the association between methylation of the *ACE2* promoter and essential hypertension

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Abstract. The aim of the present study was to investigate whether methylation of the angiotensin I converting enzyme 2 (*ACE2)* promoter increases the risk of essential hypertension (EH). A total of 96 patients with EH were recruited and 96 sex- and age-matched healthy controls. Methylation of 5 CpG dinucleotides in the *ACE2* promoter was quantified using bisulfite pyrosequencing. Logistic regression and multiple linear regression were used to adjust for confounding factors and the generalized multifactor dimensionality reduction (GMDR) method was applied to investigate high-order interactions. Methylation of CpG4 (adjusted P=0.020) and CpG5 (adjusted P=0.036) was significantly higher in patients with EH, with frequency 97.56±5.65% and 12.75±4.15% in EH individuals and 95.73±9.11% and 11.47±3.67% in healthy controls. GMDR detected significant interaction among the 5 CpG sites (odds ratio=7.33, adjusted P=0.01). Furthermore, receiver operating characteristic curves identified that CpG5 methylation was a significant predictor of EH. Notably, CpG2 methylation was significantly higher in males than in females (adjusted P=0.018). Conversely, CpG5 methylation was significantly lower in males (adjusted P=0.032). These results indicated that aberrant methylation of the *ACE2* promoter may be associated with EH risk. In addition, sex may significantly influence *ACE2* methylation.

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Introduction

Essential hypertension (EH) is a disorder characterized by high blood pressure of unknown cause and is a major risk factor for cardiovascular and cerebrovascular disease and a serious public health problem worldwide. The prevalence was at 26.7% in 2010 in China (1), this is predicted to increase to 29.2% globally by 2025 (2). EH may be closely associated with dysregulation of the renin-angiotensin system (RAS). However, the underlying molecular mechanisms that lead to the dysregulation remain to be elucidated; however, genetic alterations, environmental factors, gene-gene, and gene-environment interactions may be considered key factors (3,4).

The RAS is a master regulator of blood pressure. Angiotensin II is an important vasoconstrictor in this system, whereas angiotensin converting enzyme 2 (*ACE2*), the discovery of which was considered to be a breakthrough in 2000 (5,6), promotes vasodilation by degrading angiotensin II, and generating the vasodilators Ang 1-7 (7). Accordingly, increasing the expression of *ACE2*, which is located on chromosome Xp22, protects against increased blood pressure, whereas inhibition or deletion promotes EH (8). Previous genetic studies have identified polymorphisms in *ACE2* as risk factors for EH in multiple populations, such as the Han-Chinese and Caucasian population (9,10).

DNA methylation, a common mechanism of reversible epigenetic regulation, usually occurs at cytosine residues in cytosine-phosphate-guanine (CpG) dinucleotides in mammalian cells (11). Environmental factors can affect DNA methylation levels in the genome and thus alter gene expression. Promoter hypermethylation silences genes, whereas hypomethylation promotes active transcription (12). Therefore, controlling methylation of relevant genes may provide novel opportunities to treat or prevent EH. Previous studies have determined that aberrant methylation of components of the RAS, including angiotensinogen, *ACE,* and angiotensin II receptor type 1 (*AGTR1*) was associated with the onset and development of EH (13-16). However, the association between EH and methylation of the *ACE2* promoter remains to be elucidated. Therefore, the present study aimed to investigate whether aberrant methylation of the *ACE2* promoter

contributed to EH and the association with age, sex and other clinical indicators, as has been determined for other genes, including adducing 1 (17) and glucokinase (18).

Materials and methods

Sample collection. A total of 192 individuals, 96 patients with EH and 96 healthy controls, were recruited at The Seventh Hospital of Ningbo (Ningbo, China). Participants were from Han Chinese families who had been residing in Ningbo for a minimum of three generations and had no history of diabetes mellitus, secondary hypertension, myocardial infarction, stroke, renal failure, drug abuse or other serious diseases. Patients were categorized as hypertensive according to the 'diagnostic gold standard' (19) and had at least three consecutive measurements of systolic blood pressure (SBP) >140 mm Hg and/or diastolic blood pressure (DBP) >90 mm Hg (19). In addition, the hypertensive patients were newly diagnosed patients and had not received therapy for hypertension. Healthy controls had SBP and DBP <120 mm Hg and <80 mm Hg respectively, had no family history of hypertension in first degree relatives and had not received therapy for hypertension. A calibrated mercury sphygmomanometer with an adult-sized cuff was used to quantify the blood pressure according to standard protocols of the American Heart Association (20). Blood pressure was measured in the supine position twice ≥10 min apart by different trained technicians. Following a 12 h overnight fast, 5 ml blood samples were collected from the antecubital vein using vacutainer tubes containing EDTA and stored at -80˚C for DNA extraction. The protocol of the present study was approved by the Ethics Committee of Ningbo Seventh Hospital of Ningbo (Ningbo, China) and written informed consent was obtained from all patients.

Biochemical analyses. Plasma levels of total cholesterol, triglyceride, alanine transaminase (ALT), uric acid, high-density lipoprotein (HDL), low-density lipoprotein (LDL), homocysteine (Hcy), and glucose were quantified enzymatically using an AU2700 automatic analyzer (Olympus Corporation, Tokyo, Japan). A Lab-Aid 820 nucleic acid extraction analyzer (Zeesan Biotech Co. Ltd., Xiamen, China) was used to extract genomic DNA from peripheral blood samples. DNA concentration was quantified using a NanoDrop 2000 ultramicro nucleic acid ultraviolet tester (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Pyrosequencing, a sequencing-by-synthesis technique, was used to determine the methylation levels. The target sequences were first treated with sodium bisulfite using an EpiTech Bisulfite kit (Qiagen GmbH, Hilden, Germany) to preferentially convert unmethylated cytosine residues to thymine and then amplified by polymerase chain reaction, finally 'sequenced by synthesis' using Pyromark Gold Q96 (Qiagen GmbH) as previously described (17,18,21). In addition, CpG island (CGI) was identified using MethPrimer (www.urogene.org/methprimer/) (22). CpG sites of interest and PCR primers were selected according to the general rules and advice of primer design as previously described (23) and the scores were automatically calculated by the PyroMark Assay Design, version 2.0.1.15 (Qiagen GmbH). Targets were amplified using a Mastercycler Nexus Gradient (Eppendorf, Hamburg, Germany) in reactions containing 8 μl DNase/RNase-free water, 12 μl ZymoTaq Premix (Zymo Research Corporation, Irvine, CA, USA), 2 μl bisulfite-converted DNA, and 1.5 μl each of forward (F) and reverse (R) primer. Reactions were initially denatured at 95˚C for 10 min, amplified over 45 cycles at 95˚C for 30 sec, 52.8˚C for 40 sec, and 72˚C for 50 sec, and extended at 72˚C for 7 min. Targets were amplified with F 5'-GGGTAG ATTAAGAGGTTAGAAG-3' and R 5'-Biotin-ATTCACCCC ATTCTCCTA-3', and sequenced with primer 5'-TTATTA AAAATATAAAAATATTAG-3'.

Statistical analyses. Data were analyzed using PASW Statistics, version 18.0 (IBM SPSS, Armonk, NY, USA). Continuous variables, including DNA methylation, age, body mass index (BMI), total cholesterol, triglycerides, glucose, ALT, uric acid, HDL, LDL and Hcy were compared by Student's t-test or rank-sum test Pearson χ^2 or Fisher's exact test were used to analyze the association between EH and categorical variables such as sex, smoking and alcohol consumption. Pearson correlation analysis was used to investigate interactions among the five CpG sites in the *ACE2* promoter. Logistic regression and multiple linear regression were applied to adjust for confounding factors. Receiver operating characteristic (ROC) curves were constructed to determine the sensitivity of *ACE2* methylation as a predictor of EH. $P<0.05$ was considered to indicate a statistically significant difference.

Generalized multifactor dimensionality reduction (GMDR) (http://www.ssg.uab.edu/gmdr/) was used to investigate potential high-order interactions between *ACE2* promoter methylation and risk of EH. In this approach, high-dimensional data is reduced to a one-dimensional variable with two levels (high risk or low risk) (24). The method may detect interactions in small sample sizes, adjust for quantitative and discrete covariates and may be used dichotomous and continuous phenotypes. Additionally, this approach does not require a genetic model and is a non-parametric alternative to linear or logistic regression for the detection and characterization of interactions between genetic and environmental attributes (24). In the present study, the data set was randomly split into 10 subsets, of which 9 were used for training and one for testing. *N* factors were selected from the training set and combined in n-dimensional space. A number of parameters were provided to estimate training balanced accuracy, testing balanced accuracy, sign test P-value, and cross-validation consistency for each candidate interaction model. From the candidate models, the one with a sign test P-value of <0.05 and the highest cross-validation consistency, training, and testing balanced accuracy was identified to be the most suitable model (24).

Results

Patient characteristics and analysis of promoter methylation. A total of 96 patients with EH were recruited, along with 96 sex- and age-matched (±3 years) healthy controls. The characteristics of the study population are summarized in Table I.

A CpG island (CGI) was identified in the *ACE2* promoter using MethPrimer (22). Subsequently, a fragment containing

Table I. Characteristics of the study population (n=192).

Characteristic	Healthy (n=96)	EH (n=96)	t/χ^2	P-value
Age (years)	56.32±8.23	56.72±8.71	-4.49	$2.02x10^{-5}$[b]
Sex (M/F)	38/58	38/58	0	1.000
Smoking (Y/N)	17/79	27/69	4.05	0.041[b]
Drinking (Y/N)	31/65	40/56	2.31	0.175
BMI (kg/m^2)	22.20±2.40	23.62±3.28	-3.48	0.001[b]
Total cholesterol (mmol/l)	5.21±0.88	5.38±0.61	-1.55	0.125
Triglycerides (mmol/l)	1.21±0.68	1.43±0.72	-2.25	0.027[b]
Glucose (mmol/l)	4.91±0.79	4.90±0.31	0.14	0.888
ALT (IU/l)	26.43±16.18	28.44±11.95	-0.96	0.340
HDL (mmol/l)	8.01±6.35	2.07±5.61	6.53	$3.32x10^{-9}$[b]
LDL (mmol/l)	3.22±0.86	3.31±0.69	-0.81	0.421
Uric acid (mmol/l)	300.81±73.38	325.75±83.07	-2.68	0.009[b]
Hcy (μmol/l)	9.38±2.04	12.33±4.28	-2.64	0.018[b]
CpG1 methylation (%)	69.97±2.40	69.07±5.09	1.42	0.147[a]
CpG2 methylation (%)	35.20±2.54	35.30±1.90	-0.37	0.870[a]
CpG3 methylation (%)	23.13±3.75	23.18±4.25	0.09	0.055[a]
CpG4 methylation (%)	95.73±9.11	97.56±5.65	-1.61	0.020[a,b]
CpG5 methylation (%)	11.47±3.67	12.75±4.15	-2.45	0.036[a,b]

Data are presented as the mean ± standard deviation. [a]P-values were adjusted by conditional logistic regression for age, sex, smoking, drinking, body mass index, triglycerides, HDL, uric acid and Hcy. [b]P<0.05 vs. control group. Y, yes; N, no; BMI, body mass index; ALT, alanine transaminase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Hcy, homocysteine.

five CpG dinucleotides in this island (ChrX:15621573-15622147) was selected (Fig. 1). The correlation among the five CpG sites is presented Fig. 1 (r<0.5).

Promoter methylation and essential hypertension. Methylation of *ACE2* in CpG4 (adjusted P=0.020) and CpG5 (adjusted P=0.036) was significantly higher in cases of EH, with frequency 97.56±5.65% and 12.75±4.15% in patients with EH and 95.73±9.11% and 11.47±3.67% in healthy controls, respectively. However, EH was not significantly associated with methylation of the remaining three CpG sites following adjusting for age, sex, smoking, alcohol use, BMI, triglycerides, HDL, uric acid, and Hcy (Table I; Fig. 2). In addition, CpG5 methylation was determined to be a significant predictor of EH based on ROC curves (Fig. 3), with area under the curve was 0.645 for all patients ($P=4.98x10^{-4}$), 0.690 for males (P=0.004), and 0.646 for females (P=0.007).

GMDR was then used to investigate high-order interactions among the five CpG sites. The best models at various orders are summarized in Table II. The five-factor model had the best training balanced accuracy (0.72), testing balanced accuracy (0.65), and cross-validation consistency (10/10). The adjusted P-value was 0.01 following the sign test and the training odds ratio (OR) was 7.33 with 95% confidence interval (2.03, 26.49).

Association of clinical variables with promoter methylation. Methylation of CpG2 was significantly higher (adjusted P=0.018) in healthy males compared with healthy females, with frequency 36.21±2.21% and 34.71±1.40%, respectively. In contrast, CpG5 methylation was significantly lower (adjusted P=0.032) in males (10.97±4.28%) compared with females (13.91±3.66%) following adjusting for confounding factors (Table III; Fig. 4). As presented in Table I, significant differences between hypertensive and healthy subjects were also detected in age ($P=2.02x10^{-5}$), smoking (P=0.041), BMI (P=0.001), triglyceride (P=0.027), HDL ($P=3.32x10^{-9}$), uric acid (P=0.009) and Hcy (P=0.018). Therefore, a multiple linear regression was used to test whether these clinical variables were associated with *ACE2* methylation in healthy controls. However, no significant difference was identified (data not shown).

Discussion

Previous studies have demonstrated that *ACE2* polymorphisms are associated with risk of EH (9,10). Therefore, it is possible that aberrant methylation of the *ACE2* promoter may also contribute to this risk. The results of the present study indicated that CpG4 and CpG5 in the *ACE2* promoter were hypermethylated in patients with EH and a significant interaction among the five CpG sites was observed. Furthermore, the present study determined that methylation of CpG2 and CpG5 was significantly different between males and females. The observations of the present study elucidated the underlying mechanism of the pathogenesis of EH.

ACE2 counterbalances the effect of RAS by degrading the vasoconstrictor angiotensin II, and generating the vasodilators Ang 1-7 (7). Since its discovery in 2000 (5,6), *ACE2* has been identified as a candidate gene that may be responsible for the development of EH and to the best of our knowledge,

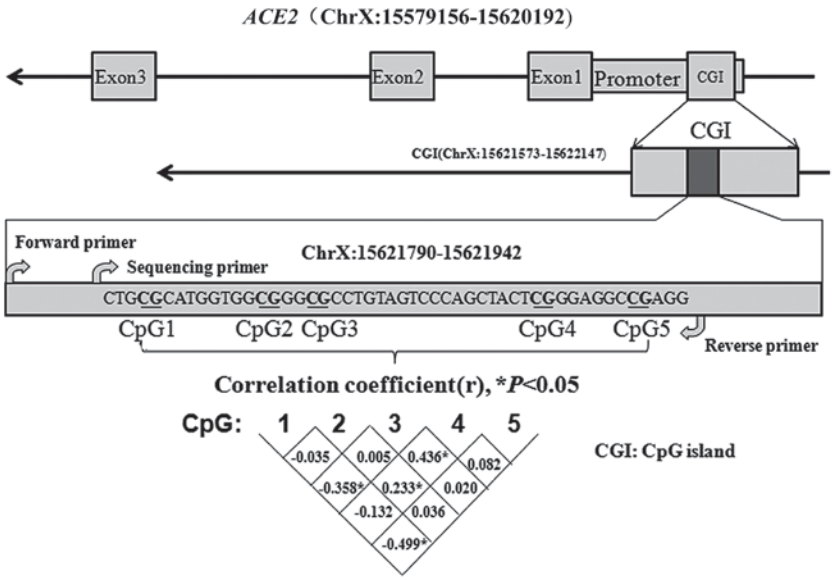

Figure 1. A total of 5 CpG sites were analyzed in *ACE2*. *ACE2*, angiotensin I converting enzyme 2; CGI, CpG island.

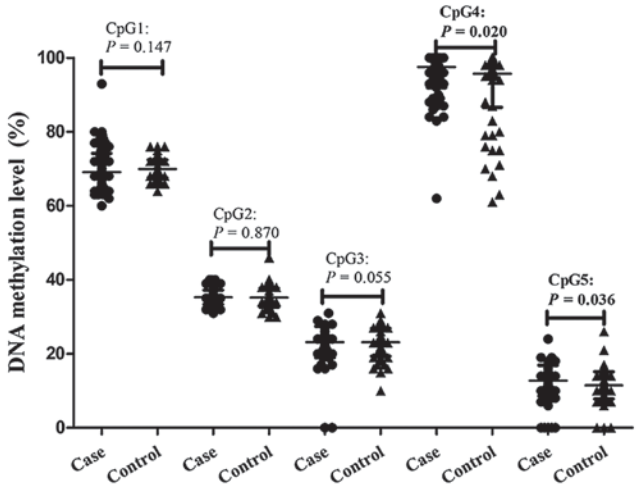

Figure 2. Angiotensin I converting enzyme 2 CpG methylation in patients with essential hypertension (n=96) and healthy controls (n=96). P-values were adjusted by conditional logistic regression for age, sex, smoking, drinking, body mass index, triglycerides, high-density lipoprotein, uric acid and homocysteine.

the present study was the first to examine the association between EH and the methylation status of the *ACE2* promoter. Promoter hypermethylation inactivates transcription, whereas hypomethylation promotes active transcription (12). A previous study determined that promoter hypomethylation upregulated *AGTR1* expression, a key gene in RAS that was closely associated with EH (25). Therefore, hypermethylation of CpG4 and CpG5 in the *ACE2* promoter may reduce expression, promoting EH pathogenesis. However, as no expression analysis was performed in the current study, the observations are only correlative and not causal. Ongoing expression analysis is required to confirm the present findings.

As EH is a multifactorial disease, gene-gene and gene-environment interactions contribute to its onset and progression. However, due to the 'curse of dimensionality,' traditional statistical methods are unsuitable to detect these potential interactions. Non-parametric methods that do not require genetic models have been previously used to identify high-order interactions efficiently. One such method is GMDR, which accommodates qualitative and quantitative phenotypes, adjusts for discrete and continuous covariates and enhances prediction accuracy (24). Using this method, the present study detected a significant five-order interaction among the five CpG sites in the *ACE2* promoter, an interaction that may contribute to the risk of EH. It is of note that there may be a 7.33-fold increased risk of developing EH in individuals with hypermethylation of all five CpG sites (OR=7.33). Nevertheless, this interaction is purely theoretical at present, based on statistical analyses, and it is only descriptive of variations in the population (24). The physiological relevance of such an interaction, if any, remains to be elucidated and should be investigated in future experiments.

It is of note, that as the *ACE2* gene is located on the X chromosome and the prevalence and progression of EH, and the methylation of hypertension-associated genes have been determined to display sex differences (17,26). In order to maintain equal gene expression between males and females, one female X chromosome is randomly inactivated, a process termed X-inactivation (27). The inactive female X chromosome has higher methylation levels compared with the active female X chromosome in promoter CpG islands (28). However, the *ACE2* gene location on Xp22 encompasses an area where genes are reported to escape from X-inactivation (29), which may lead to the methylation differences of *ACE2* CpG2 and CpG5 between the two sexes observed in the current study. In addition, sex-specific hormones that modify DNA methylation (30) and sex differences in non-heritable risk factors for EH, including alcohol consumption, smoking, physical activity and a high-sodium diet, may also alter *ACE2* methylation levels (31-34). Additionally, it is possible that site-specific differences, as observed between males and females in CpG2 and CpG5 methylation, may be due to heterogeneity in methylation of different CpG sites in the same promoter (35-38). This heterogeneity is biologically relevant; however, the mechanisms

Table II. GMDR models of high-order interaction among the five CpG sites in angiotensin I converting enzyme 2 promoter on essential hypertension risk.

Model	Training balanced accuracy	Testing balanced accuracy	Sign test (P-value)	Cross-validation consistency
CpG5	0.62	0.62	9 (P=0.011[a])	10/10
CpG3, CpG5	0.63	0.57	7 (P=0.172)	6/10
CpG2, CpG3, CpG5	0.67	0.58	7 (P=0.172)	6/10
CpG1, CpG3, CpG4, CpG5	0.69	0.60	8 (P=0.055)	7/10
CpG1, CpG2, CpG3, CpG4, CpG5	0.72	0.65	9 (P=0.011[a])	10/10

P-values were adjusted for age, sex, smoking, drinking, body mass index, triglycerides, high-density lipoprotein, uric acid, homocysteine using logistic regression in GMDR analysis. [a]P<0.05 vs. control group. GMDR, generalized multifactor dimensionality reduction.

Table III. Angiotensin I converting enzyme 2 CpG methylation in healthy males (n=38) and females (n=58).

Characteristic	Males	Females	t/χ^2	P-value
Age (years)	55.55±10.79	56.83±6.06	-0.66	0.51
Smoking (Y/N)	17/21	0/58	31.53	$1.96x10^{-8}$[b]
Drinking (Y/N)	21/17	10/48	15.18	$9.77x10^{-5}$[b]
BMI (kg/m^2)	23.11±2.35	21.54±2.05	3.46	0.001[b]
Total cholesterol (mmol/l)	5.07±1.07	5.27±0.76	-1.06	0.293
Triglycerides (mmol/l)	1.44±0.87	1.06±0.47	2.46	0.017[b]
Glucose (mmol/l)	4.86±1.20	4.94±0.33	0.43	0.669
ALT (IU/l)	27.05±14.29	25.98±17.29	0.32	0.752
HDL (mmol/l)	5.16±6.58	9.85±5.44	-3.79	$2.62x10^{-4}$[b]
LDL (mmol/l)	3.20±1.05	3.21±0.73	-0.07	0.942
Uric acid (mmol/l)	352.36±2.18	266.23±50.48	6.88	$6.52x10^{-10}$[b]
Hcy (μmol/l)	12.07±8.18	9.21±1.17	2.14	0.039[b]
CpG1 methylation (%)	70.92±5.51	67.86±4.44	2.87	0.190[a]
CpG2 methylation (%)	36.21±2.21	34.71±1.40	3.73	0.018[a,b]
CpG3 methylation (%)	23.42±3.06	23.02±4.89	0.45	0.202[a]
CpG4 methylation (%)	97.39±7.09	97.67±4.52	-0.24	0.124[a]
CpG5 methylation (%)	10.97±4.28	13.91±3.66	-3.60	0.032[a,b]

Data are presented as the mean ± standard deviation. [a]P-values were adjusted by logistic regression for age, smoking, drinking, body mass index, triglycerides, HDL, uric acid and Hcy. [b]P<0.05 vs. control group. Y, yes; N, no; ALT, alanine transaminase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Hcy, homocysteine.

that drive site-specific methylation remain to be elucidated. It is of note that no association between *ACE2* methylation and other clinical variables such as age and BMI was observed, therefore further investigation is required to confirm this result.

The present study had numerous strengths, and was able to draw conclusions by adjusting for confounding factors through the use of logistic and multiple linear regression and by overcoming the 'curse of dimensionality' through GMDR models. However, the following limitations have been identified: i) Cause-effect association between methylation of the *ACE2* promoter and EH remains to be determined, as the survey was a case-control study; ii) only a fragment of the CpG island in the *ACE2* promoter was analyzed; iii) the statistical analysis controlled for certain confounding factors, however, it is possible that other confounding factors that influence *ACE2* methylation may have not been accounted for; iv) peripheral blood is a surrogate tissue for epigenetic studies, although previous studies have indicated that CpG methylation patterns are similar between peripheral blood and other tissues (39,40), as DNA methylation, may vary across tissues, similar analysis of *ACE2* methylation in other tissues may be required; and v) no expression analysis was performed in the present study. Therefore, the observations of the current study can only be regarded as correlative. Ongoing expression analysis is required to confirm the results of the present study.

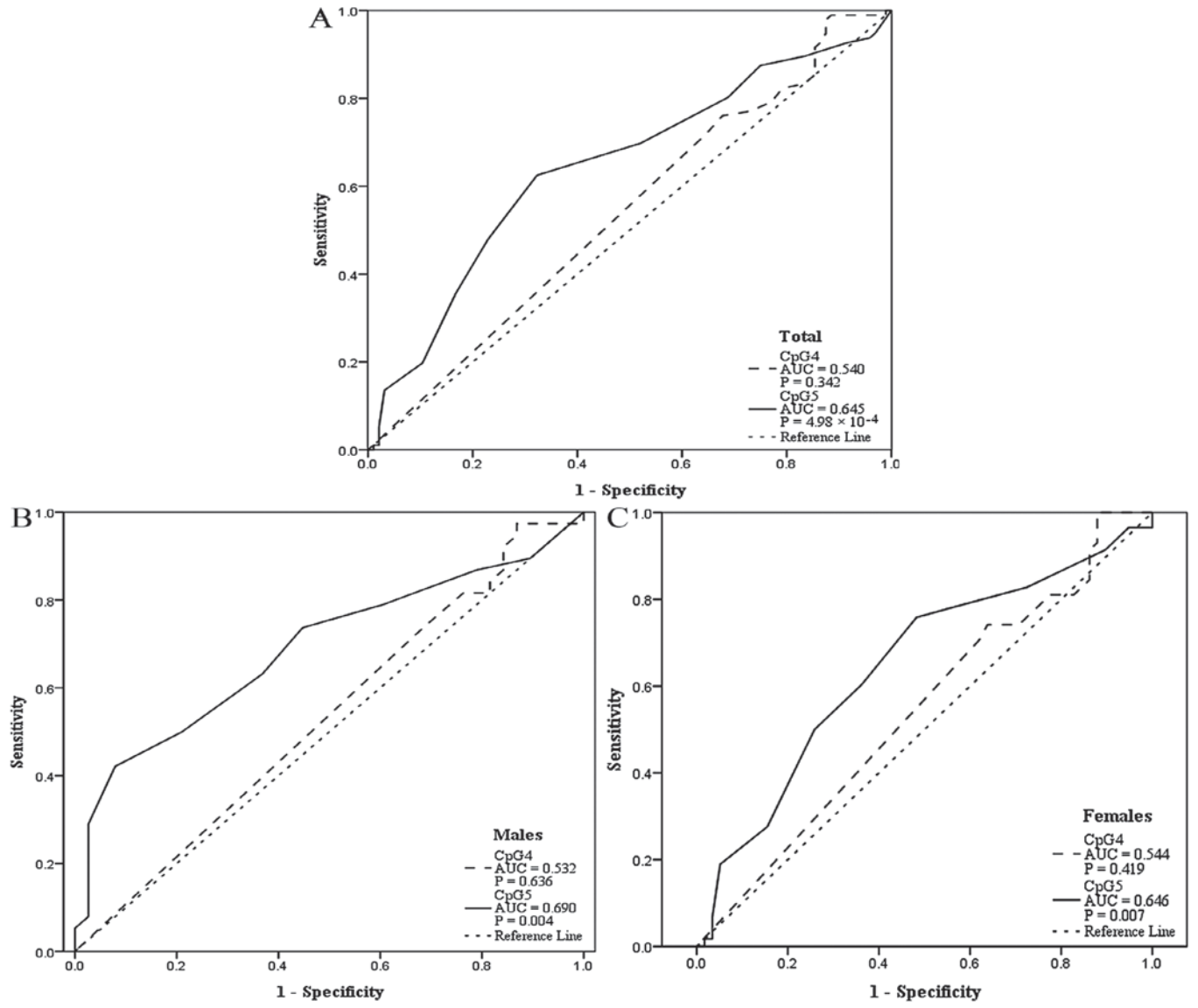

Figure 3. Receiver operating characteristic curves of angiotensin I converting enzyme 2 methylation in (A) total, (B) males and (C) females with essential hypertension. AUC, area under the curve.

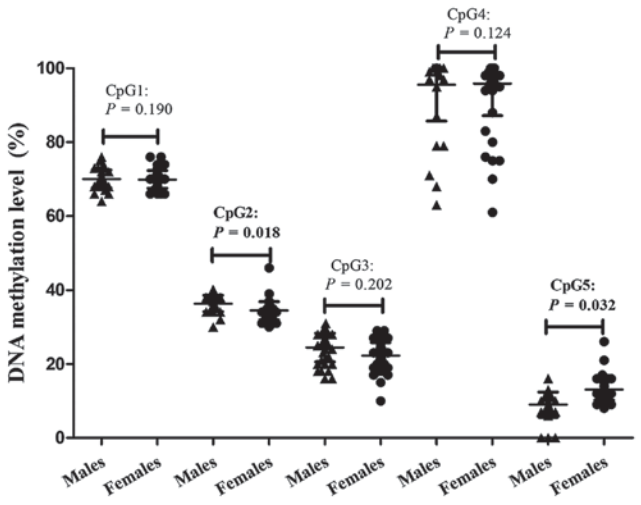

Figure 4. Difference in angiotensin I converting enzyme 2 methylation between healthy males (n=38) and healthy females (n=58). P-values were adjusted by logistical regression for age, smoking, drinking, body mass index, triglycerides, high-density lipoprotein, uric acid and homocysteine.

In conclusion, the observations of the present study provided evidence of the association between EH and hypermethylation of CpG4 and CpG5 in the *ACE2* promoter and the interactions among CpG1-CpG5. It is of note, that methylation of *ACE2* CpG5 may have predictive potential as a tool to estimate risk of EH in patients. Additionally, sex may affect *ACE2* methylation. These observations further understanding of the pathogenesis of EH and may aid in the improvement of the diagnosis and treatment of patients with EH.

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