

Association of *SCNN1B* promoter methylation with essential hypertension

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Abstract. The amiloride-sensitive sodium channel beta subunit (*SCNN1B*) gene encodes the beta subunit of the epithelial sodium channel, which is involved in blood pressure homeostasis. The aim of the present study was to investigate the association between *SCNN1B* gene promoter methylation and essential hypertension (EH), and to explore whether *SCNN1B* methylation was altered by antihypertensive therapy. The present study recruited 282 individuals: 94 controls, 94 incident cases and 94 prevalent cases. Subsequently, the methylation status of six CpG sites in the *SCNN1B* promoter region was measured using bisulfite pyrosequencing technology. Among the six CpG sites, a significant difference in CpG1 and CpG2 methylation levels were detected between controls and incident cases (CpG1: β -standardized=0.17, adjusted $P=0.015$; CpG2: β -standardized=-0.41, adjusted $P=0.001$). In addition, a significant difference was detected in CpG1 methylation levels between incident cases and prevalent cases (β -standardized=-0.252, adjusted $P=3.77E-04$). The present study also demonstrated that CpG1 and CpG2 methylation levels were significantly lower in males compared with in females (CpG1: $t=-3.180$, $P=0.002$; CpG2: $t=-2.148$, $P=0.033$).

CpG1 methylation was also shown to be positively correlated with age (controls: $r=0.285$, $P=0.008$; incident cases: $r=0.401$, $P=0.0001$; prevalent cases: $r=0.367$, $P=0.001$). These results indicated a significant association between EH and *SCNN1B* methylation, which was affected by age, gender and antihypertensive therapy.

Introduction

Blood pressure regulation is a complex, multifactorial process, which is associated with physiological, biochemical and molecular mechanisms. An increase in Na^+ and water retention is required for the development of most forms of hypertension (1). The epithelial sodium channel (ENaC) mediates the initial step of active sodium reabsorption, which is essential for the maintenance of body salt and water homeostasis (2). The ENaC is composed of three different subunits (3), one of which is the amiloride-sensitive sodium channel beta subunit (SCNN1B). Mutations in *SCNN1B* may cause Liddle's syndrome (4-6), which is an autosomal dominant disorder that is characterized by early, and frequently severe, hypertension (7). Essential hypertension (EH), the form of hypertension that by definition has no identifiable cause, tends to be familial and may be due to an interaction between environmental and genetic factors.

DNA methylation refers to the addition of a methyl group to the cytosine or adenine DNA nucleotides in mammalian cells, and usually occurs at CpG islands, which contain clusters of CpG dinucleotides. Promoter hypermethylation often silences gene transcription and is an important event during disease progression (8). Our previous study demonstrated that reduced α -adducin gene promoter methylation increased the risk of essential hypertension (EH) in Chinese men and women (9). Furthermore, hypo/hypermethylation of the sulfatase 1 gene may serve an important role in the pathogenesis of hypertension in young African American men (10). Altered gene methylation has also been observed in other cardiovascular diseases (11,12), as well as in type 2 diabetes (13-16).

DNA methylation has been reported to mediate some of the effects of environmental exposure and lifestyle factors on disease risk (17). DNA methylation is heritable, but can also

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Abbreviations: ALT, alanine aminotransferase; BD, Behçet's disease; BMI, body mass index; EH, essential hypertension; ENaC, epithelial sodium channel; SCNN1B, amiloride-sensitive sodium channel beta subunit; TC, total cholesterol; TG, triglyceride

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be altered by medical therapy. It has previously been reported that drugs can alter the gene methylation status in patients with cancer and schizophrenia (18). In light of these previous studies, the present study aimed to investigate whether *SCNN1B* promoter DNA methylation was associated with the risk of EH, and whether antihypertensive drug treatment would alter the *SCNN1B* methylation status in patients with EH.

Materials and methods

Sample collection. A total of 282 individuals (94 incident cases, 94 prevalent cases and 94 normotensive controls) were recruited to the present study from the Ningbo Seventh Hospital (Ningbo, China). All individuals selected were Han Chinese that had resided in Ningbo for ≥ 3 generations. Incident cases were hypertensive patients who had previously never received any antihypertensive drug treatment. Prevalent cases were hypertensive patients who had previously received antihypertensive drug treatment. The diagnosis of hypertension was made when the average of ≥ 2 diastolic blood pressure (DBP) measurements on ≥ 2 subsequent visits was ≥ 90 mmHg, or when the average of several systolic blood pressure (SBP) readings on ≥ 2 subsequent visits was consistently ≥ 140 mmHg. Isolated systolic hypertension is defined as SBP ≥ 140 mmHg and DBP < 90 mmHg. Individuals with SBP < 120 mmHg and DBP < 80 mmHg were recruited as controls (19). None of the control group had received antihypertensive therapy. Furthermore, none of the recruited individuals suffered from other diseases, including diabetes mellitus, secondary hypertension, myocardial infarction, stroke, renal failure, and drug abuse. Blood samples were collected in 3.2% citrate sodium-treated tubes and were then stored at -80°C for DNA extraction. The protocol of the present study was approved by the ethical committee of the Ningbo Seventh Hospital. Written informed was obtained from all subjects.

DNA isolation and bisulfite treatment. Genomic DNA was extracted and underwent bisulfite conversion for subsequent polymerase chain reaction (PCR) amplification and pyrosequencing, as described previously (11,13-15,20). Genomic DNA was extracted from peripheral blood samples using a nucleic acid extraction analyzer (Lab-Aid 820; Xiamen Zeesun Biotech Co., Ltd., Xiamen, China). The NanoDrop 1000 spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, DE, USA) was used to measure the concentrations of extracted DNA. Subsequently, ~ 500 ng genomic DNA isolated from whole blood cells was bisulfite-treated using the EZ DNA Methylation-Gold™ kit (Zymo Research Corp, Irvine, CA, USA), according to the manufacturer's protocol. This treatment involves converting unmethylated cytosines into uracil, whereas methylated cytosines remain unchanged. Once converted, the methylation profile of the DNA can be determined by PCR amplification followed by DNA sequencing.

DNA methylation assay. Ten primer sets (including forward, reverse and sequencing primers) were designed by PyroMark Assay Design software v2.0.1.1 (Qiagen, Inc., Valencia, CA, USA) to amplify the CpG island region in overlapping

fragments and to sequence target DNA fragments. Each primer set was given a score and these primer sets were ranked from high to low. According to the rank, the top three primer sets were synthesized and purified by high-performance liquid chromatography by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China) for PCR. Subsequently, 2% agarose gel electrophoresis was used to analyze the PCR products. The results indicated that primer set one exhibited the best amplification effects. Detailed information regarding this primer set is presented in Table I. PCR was conducted in a final volume of 20 μl containing 10 μl Zymo Taq™ PreMix, 1.5 μl forward primer (10 μM), 1.5 μl reverse primer (10 μM) and 40 ng DNA and water was used to raise volume to 20 μl . The PCR process began with an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 40 sec and extension at 72°C for 50 sec. The final extension step was performed at 72°C for 7 min. Following amplification, PCR products were maintained at 4°C for ≥ 4 min. The target sequence was finally sequenced by synthesis assay (Pyromark Gold Q24 Reagents; #970802; Qiagen, Inc.).

Statistical analysis. Statistical analyses were performed using PASW Statistics 18.0 software (SPSS, Inc., Chicago, IL, USA). The mean of continuous variables [including age, body mass index (BMI), DNA methylation level, total cholesterol (TC), triglycerides (TG), glucose and alanine aminotransferase (ALT)] between case and control groups was analyzed using two-tailed unpaired t test or one-way analysis of variance followed by least significant difference multiple comparison tests. Either Pearson χ^2 test or Fisher exact test were used to analyze the association between EH and categorical variables including gender, and smoking and drinking habits. Partial correlative test was used to analyze the relationship between continuous variables. Logistic regression was implemented for controlling the possible confounding factors to analyze the correlation between independent variables and dependent variables. Meanwhile, R software and GraphPad Prism (version 5.01; GraphPad Software, Inc., La Jolla, CA, USA) were used for statistical computing and graphical representation. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

As presented in Fig. 1, PCR primers were designed for amplification of the CpG island region of *SCNN1B*, whereas the sequencing primer was set to sequence the fragment containing seven CpG dinucleotides (Fig. 1 and Table I). Since the last CpG dinucleotide (CpG7) was not well sequenced, the remaining six CpG sites (CpG1-6) were analyzed in the present study (Fig. 1). Significant correlations were found among CpG1-6 (Fig. 1; $r > 0.30$; $P < 0.01$) in DNA methylation levels.

A series of EH risk factors (including BMI, TC, TG and glucose) were compared among the three groups using the variance analysis. As shown in Table II, there were significant differences among the three groups in the mean levels of BMI ($F = 12.478$, $P = 6.45\text{E-}06$), TG ($F = 4.631$, $P = 0.011$) and glucose ($F = 3.982$, $P = 0.02$). In addition, alcohol and smoking

Table I. Oligonucleotides for bisulfite sequencing.

| Variable | Sequence | Nucleotides (bp) |
|---------------------|---|------------------|
| Forward primer | 5'-GGATGAGGGGTTTGTGGATA-3' | -227 to -207 |
| Reverse primer | 5'- ACCTCCCTCCCCCT CCAATAAACT-3' | -66 to -42 |
| Amplicon sequence | 5'-GGATGAGGGGTTTGTGGATA TATT <u>CGT</u> TGG <u>CGT</u> TATGTGGGTAT <u>CGT</u> TGGTGT <u>TTT</u> <u>CG</u> AGGTG GGGAGGGAGAATG <u>CGG</u> AG <u>CGCGT</u> G <u>CGT</u> G <u>CG</u> GGGGG <u>CGT</u> TTAGTGT <u>TTTT</u> TGAATTTGG <u>CGT</u> GT GGGGGTTGGAGTTTATTGGGAGGGGAGGGAGGT-3' | -227 to -42 |
| Sequencing primer | 5'-GGTGGGGAGGGAGAA-3' | -172 to -158 |
| Sequence to analyze | 5'-TG <u>CGG</u> AG <u>CGCGT</u> G <u>CGT</u> G <u>CGG</u> GGGG <u>CGT</u> TTA GTGTTTTTGAATTTGG <u>CG</u> -3' | -156 to -107 |

Bold and underlined font indicates the CpG sites.

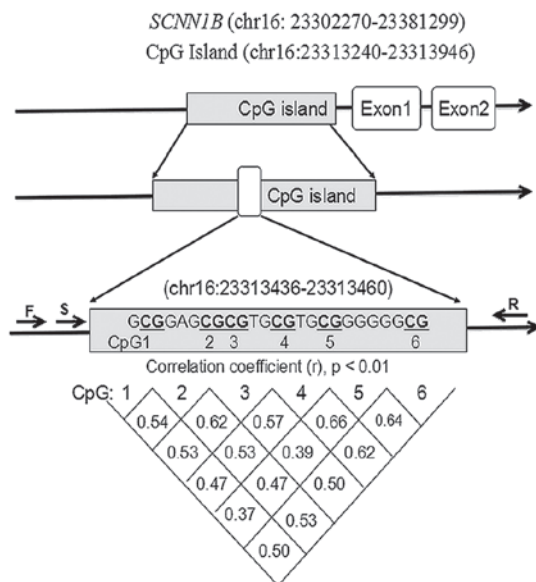


Figure 1. Correlations among the six CpGs in the *SCNN1B* gene promoter. *SCNN1B*, amiloride-sensitive sodium channel beta subunit.

consumption were compared among the three groups, since they are known to be associated with hypertension (21). The results demonstrated that the ratio of alcohol drinking ($\chi^2=9.189$; $P=0.011$) was inconsistent among the three groups using the χ^2 test (Table II).

Among the six CpG sites, CpG1 ($F=3.555$, $P=0.03$) and CpG2 ($F=4.952$, $P=0.008$) methylation levels exhibited a significant difference among the three groups. Increased methylation levels of CpG1 were detected in the incident cases compared with in the other two groups. Conversely, lower methylation levels of CpG2 were detected in the cases compared with in the controls (Table II). In addition, higher methylation levels of the two CpG sites were detected in women compared with in men for controls (CpG1: $t=-2.283$, $P=0.025$; CpG2: $t=-2.568$, $P=0.012$, Fig. 2) and incident cases (CpG1: $t=-2.694$, $P=0.009$; CpG2: $t=-2.583$, $P=0.011$, Fig. 2). Notably, for the two CpG sites no significant difference was observed between males and females in the

prevalent cases group (CpG1: $t=0.409$, $P=0.068$; CpG2: $t=0.621$, $P=0.536$), thus suggesting a role for antihypertensive therapy in the modification of DNA methylation.

Since aging is able to alter levels of DNA methylation (22), the present study further explored the association of *SCNN1B* CpG methylation with age using the partial correlative test. Significant correlations were found between age and CpG1 (Controls: $r=0.285$, $P=0.008$; Incident cases: $r=0.401$, $P=0.0001$; Prevalent cases $r=0.367$, $P=0.001$, Fig. 3) with an adjustment for other metabolic phenotypes (including BMI, TC, TG, glucose, ALT, and smoking and alcohol drinking habits). In addition, no correlations were detected between *SCNN1B* methylation and these aforementioned metabolic phenotypes.

In the present study, EH cases were separated into two groups: Incident cases without antihypertensive therapy and prevalent cases with antihypertensive therapy, since drug treatment may influence DNA methylation (23-26). The current study compared the two case groups to explore whether antihypertensive therapy may affect *SCNN1B* CpG methylation status using a logistic regression test.

As shown in Table III, the P-values and odds ratio (OR) values were all adjusted by other parameters. When analyzing the association between a specific independent variable and dependent variable, the other independent variables are controlled in a logistic regression model. When the P-value is <0.05 , this suggests that the variable has an effect on the dependent variable. When the OR value in a logistic regression model is >1 , this variable acts as a risk factor for the dependent variable. Conversely, when the OR value is <1 , this variable acts as a protective factor. As determined using this analytical model, methylation of CpG1 (β -standardized=0.17, OR=1.185, adjusted $P=0.015$) and CpG2 (β -standardized=-0.41, OR=0.663, adjusted $P=0.001$) were significantly associated with EH, since both P-values were <0.05 in the logistic regression analysis of controls and incident cases. In this model, the OR of CpG1 was 1.185, which suggests that hypermethylation of CpG1 is associated with EH; however, the OR of CpG2 was 0.663, which suggests that hypomethylation of CpG2 may increase the probability of EH. In addition, according to this statistical model,

Table II. Comparison of characteristics among the three groups (n=282).

| Characteristic | Non-EH (Mean ± SD) | Incident cases (Mean ± SD) | Prevalent cases (Mean ± SD) | F/ χ^2 | P |
|--------------------------|-----------------------|-------------------------------|--------------------------------|-------------|-----------------------------|
| Age (years) | 58.36±7.53 | 56.31±7.73 | 58.37±7.95 | 2.217 | 0.111 |
| Gender (M/F) | 29/65 | 34/60 | 33/61 | 0.537 | 0.642 |
| Smoking (Y/N) | 17/77 | 21/73 | 20/74 | 0.586 | 0.714 |
| Drinking (Y/N) | 7/87 | 21/73 | 20/74 | 0.007 | 0.011 |
| BMI (kg/m ²) | 22.752±3.721 | 24.331±2.543 | 24.962±3.021 | 12.478 | 6.45×10⁻⁶ |
| TC (mmol/l) | 5.231±1.002 | 5.381±1.043 | 5.491±1.032 | 1.456 | 0.235 |
| TG (mmol/l) | 1.521±0.892 | 1.893±1.071 | 1.591±0.702 | 4.631 | 0.011 |
| Glu (mmol/l) | 5.301±0.642 | 5.371±0.591 | 5.562±0.760 | 3.982 | 0.020 |
| ALT (IU/l) | 24.490±40.231 | 24.862±16.781 | 25.181±25.261 | 0.013 | 0.987 |
| CpG1 (%) | 16.211±4.082 | 17.371±4.090 | 16.072±2.628 | 3.555 | 0.030 |
| CpG2 (%) | 10.021±2.778 | 9.123±2.252 | 9.121±1.652 | 4.952 | 0.008 |
| CpG3 (%) | 6.391±1.904 | 6.550±2.040 | 6.361±1.490 | 0.298 | 0.743 |
| CpG4 (%) | 8.841±2.192 | 9.140±2.671 | 9.430±3.281 | 1.065 | 0.346 |
| CpG5 (%) | 11.104±4.250 | 10.961±2.870 | 11.060±4.041 | 0.035 | 0.966 |
| CpG6 (%) | 6.061±2.493 | 6.402±1.601 | 6.432±1.464 | 1.068 | 0.345 |

Values in bold represent significant differences. EH, essential hypertension; SD, standard deviation; M, male; F, female; Y, yes; N, no; BMI, body mass index; TC, total cholesterol; TG, triglyceride; Glu, glucose; ALT, alanine aminotransferase.

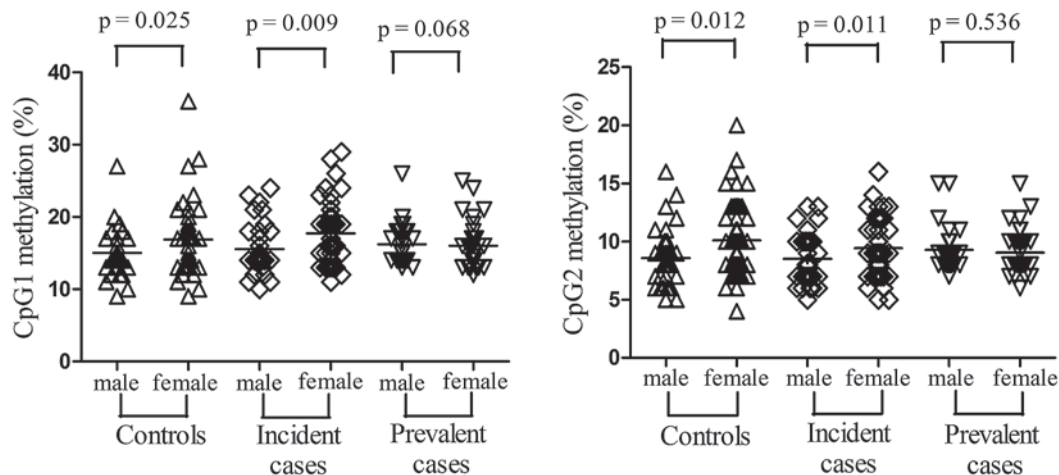


Figure 2. Comparison of DNA methylation between men and women.

the methylation level of CpG1 (β -standardized=-0.252, OR=0.777, adjusted P=3.77E-04, Table III) was higher in the incident cases compared with in the prevalent cases in the regression model of the two cases. This result suggests that antihypertensive therapy may lower the methylation level of CpG1. No significant associations were detected in the remaining CpG sites.

Discussion

The present study observed opposite results in the association of CpG1 and CpG2 methylation with EH. CpG2 methylation was significantly lower in incident cases and prevalent cases

compared with in the controls. In addition, CpG2 methylation was observed to be inversely correlated with blood pressure in controls and incident cases. Notably, CpG2 methylation was not significantly different between the incident cases and the prevalent cases, thus suggesting that the antihypertensive therapy of EH did not affect CpG2 methylation levels.

Previous studies have revealed the correlation between DNA methylation and blood pressure (27-29). The present study hypothesized that CpG1 hypermethylation and CpG2 hypomethylation may increase blood pressure by upregulating the protein expression of *SCNN1B*, thus amplifying the function and activity of the ENaC and leading to increased sodium reabsorption and water retention. However, to what extent this

Table III. Variables in the logistic regression model.

| Variable | β^a | OR ^a | P ^a | β^b | OR ^b | P ^b | β^c | OR ^c | P ^c |
|----------|-----------|-----------------|----------------|-----------|-----------------|-----------------------------|-----------|-----------------|-----------------------------|
| Gender | 0.146 | 1.157 | 0.732 | 0.242 | 1.274 | 0.559 | 0.414 | 1.513 | 0.340 |
| Age | -0.066 | 0.936 | 0.019 | 0.062 | 1.064 | 0.014 | -0.044 | 0.957 | 0.136 |
| BMI | 0.136 | 1.146 | 0.028 | 0.062 | 1.064 | 0.321 | 0.246 | 1.279 | 2.89x10⁻⁴ |
| TC | 0.248 | 1.281 | 0.191 | 0.114 | 1.121 | 0.508 | 0.131 | 1.140 | 0.539 |
| TG | 0.500 | 1.649 | 0.012 | -0.558 | 0.573 | 0.008 | 0.029 | 1.030 | 0.905 |
| Glu | 0.338 | 1.403 | 0.275 | 0.479 | 1.615 | 0.103 | 1.320 | 3.743 | 0.001 |
| ALT | -0.004 | 0.996 | 0.554 | 0.002 | 1.002 | 0.845 | 0.004 | 1.004 | 0.442 |
| CpG1 | 0.170 | 1.185 | 0.015 | -0.252 | 0.777 | 3.77x10⁻⁴ | 0.005 | 1.005 | 0.934 |
| CpG2 | -0.410 | 0.663 | 0.001 | 0.183 | 1.201 | 0.176 | -0.530 | 0.589 | 1.66x10⁻⁴ |
| CpG3 | 0.084 | 1.088 | 0.523 | -0.095 | 0.909 | 0.527 | 0.052 | 1.054 | 0.738 |
| CpG4 | 0.075 | 1.078 | 0.526 | 0.137 | 1.147 | 0.191 | 0.397 | 1.487 | 0.080 |
| CpG5 | -0.054 | 0.948 | 0.473 | -0.073 | 0.930 | 0.349 | -0.207 | 0.813 | 0.057 |
| CpG6 | 0.186 | 1.204 | 0.145 | -0.004 | 0.996 | 0.981 | 0.346 | 1.413 | 0.078 |
| Smoking | -0.350 | 0.704 | 0.505 | 0.317 | 1.372 | 0.547 | -0.306 | 0.737 | 0.595 |
| Drinking | 1.345 | 3.838 | 0.034 | -0.064 | 0.938 | 0.897 | 1.291 | 3.638 | 0.048 |
| Constant | -4.423 | 0.012 | 0.067 | -5.152 | 0.006 | 0.032 | -11.002 | 0.001 | 5.84x10 ⁻⁵ |

^aRepresents the logistic regression analysis of control and incident case groups; ^bRepresents the logistic regression analysis of incident case and prevalent case groups; ^cRepresents the logistic regression analysis of control and prevalent case groups. Values in bold represent significant differences. OR, odds ratio; BMI, body mass index; TC, total cholesterol; TG, triglyceride; Glu, glucose; ALT, alanine aminotransferase.

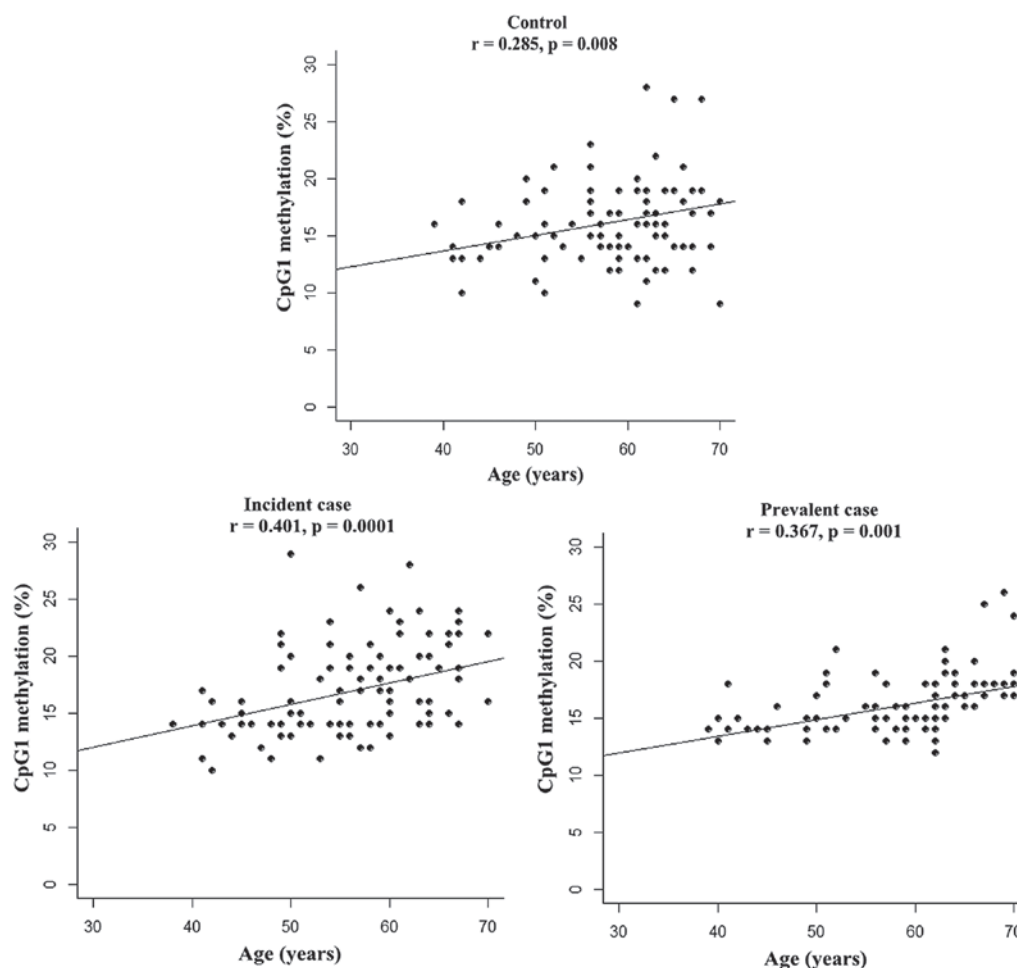


Figure 3. Correlation between age and CpG1 methylation. Correlation coefficients and P-values are all adjusted by gender, body mass index, total cholesterol, triglycerides, glucose, alanine aminotransferase, and smoking and drinking habits.

change will alter ENaC expression, and to what extent this change will affect sodium reabsorption requires follow-up research. In our subsequent studies, we aim to focus on functional research regarding the effects of DNA methylation on ENaC expression.

Drug treatment may affect DNA methylation levels (23,24,26). Several CpG sites have been reported to be differentially methylated between patients with Behçet's disease (BD) prior to and following treatment (23), providing strong evidence that DNA methylation was modified by BD treatment. Furthermore, bisphosphonate treatment in 68 patients with hypocalcaemia altered the DNA methylation levels of excision repair cross-complementation group 8, prolyl 3-hydroxylase 2 and syndecan 2 genes, thus affecting the cumulative bisphosphonate exposure levels (24). Glucocorticoid treatment has also been reported to induce acute and long-term effects on DNA methylation states in the fetus and offspring (26). Similarly, the present study indicated that CpG1 methylation, rather than CpG2 methylation, was likely to be altered by antihypertensive drug treatment. These results may provide novel information regarding pharmaco-epigenomic research of EH.

Alterations in DNA methylation patterns are a hallmark of aging (22). Previous studies have demonstrated that DNA methylation levels are altered alongside aging in several tissue types in mice and humans (30-34). Aging is also known to be a risk factor in the progression of hypertension (35,36). The results of the present study revealed that a positive correlation existed between aging and *SCNN1B* CpG methylation levels. Furthermore, gender differences in DNA methylation levels are frequently reported (37-44). In the present study, DNA methylation levels of CpG1 and CpG2 in *SCNN1B* were higher in women compared with in men. Epigenetic changes associated with aging, and gender differences in DNA methylation, may provide clues to elucidate the mechanisms underlying hypertension.

In conclusion, the present study was the first, to the best of our knowledge, to demonstrate a drug-, age-, and gender-dependent association between *SCNN1B* promoter methylation and EH.

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