

Original Article

Hypermethylation of *SCNN1A* gene-body increases the risk of essential hypertension

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Abstract: The goal of this study was to investigate the contribution of nonvoltage-gated 1 alpha subunit (*SCNN1A*) gene-body methylation of essential hypertension (EH), and to explore whether the methylation level could be altered by antihypertensive therapy. Our study performed methylation analysis of peripheral blood DNA using bisulphite pyrosequencing technology among 60 non-EH controls, 60 incident EH cases and 60 prevalent EH cases. Our results reported that the incident cases had a higher *SCNN1A* methylation level than the non-EH controls (16.15 ± 4.51 versus 13.66 ± 4.08 , $P=0.041$), and prevalent cases (16.15 ± 4.51 versus 13.77 ± 3.90 , $P=0.002$). Logistic regression analysis results showed that *SCNN1A* hypermethylation was the risk factor of EH in incident cases compared with non-EH ($OR=1.157$, $P=0.01$), and in incident cases compared with prevalent cases ($OR=1.149$, $P=0.013$). The *SCNN1A* methylation level was found to be positively correlated with age in non-EH ($r=0.343$, $P=0.007$). Significantly higher methylation level was identified in female than male in non-EH ($t=3.878$, $P=2.71 \times 10^{-4}$). Receiver operating characteristic (ROC) curve analysis revealed that 10.833% methylation level is an appropriate cut-off value for the *SCNN1A* gene-body methylation to predict the onset of EH between non-EH and incident cases (area under curve: 0.638, $P<0.009$, 95% CI: 0.539~0.736). In conclusion, our study indicated hypermethylation of *SCNN1A* gene-body was associated with the risk of EH, *SCNN1A* gene-body methylation has an important diagnostic value to EH, and *SCNN1A* gene-body methylation level could be modified by age, gender and antihypertensive therapy.

Keywords: Epigenetic, DNA methylation, *SCNN1A* gene, essential hypertension

Introduction

Essential hypertension (EH), one of the leading causes of death worldwide, is a multifactorial disease. Hosts of environmental factors, such as high sodium, low potassium diet, overweight and obesity, alcohol consumption, and long-term psychological stress, were documented to be the risk factors of EH in epidemiology studies [1, 2]. In addition, the important role of genetic components in EH was indicated in genome-wide studies [3-6]. However, the pathogenesis of EH remains unclear.

Recently, DNA methylation, a critical way of epigenetic mechanisms, was reported to play a significant regulatory role in gene expression and be associated with EH. Promoter DNA methylation could silence the transcription of

the genes [7, 8], and gene-body methylation was positively correlated with gene activity [9-11]. In previous studies, aberrant promoter DNA methylation of *ADD1* [2], *11 β -HSD2* [12], *NKCC1* [13] and gene-body methylation of *GCK* were indicated to be made in association with EH [14]. Furthermore, DNA methylation is both stable and changeable compared with the stable DNA sequence and rapidly changing fate of mRNA, DNA methylation levels could be altered by the drugs therapy [15].

Epithelial sodium channel (ENaC), the nonvoltage-gated sodium channel, is made of three homologous subunits (α , β , and γ), and alpha subunit (*SCNN1A*) is absolutely required for the sodium channel activity. ENaC plays a critical role in maintaining the extracellular fluid volume, blood pressure, and sodium homeostatic.

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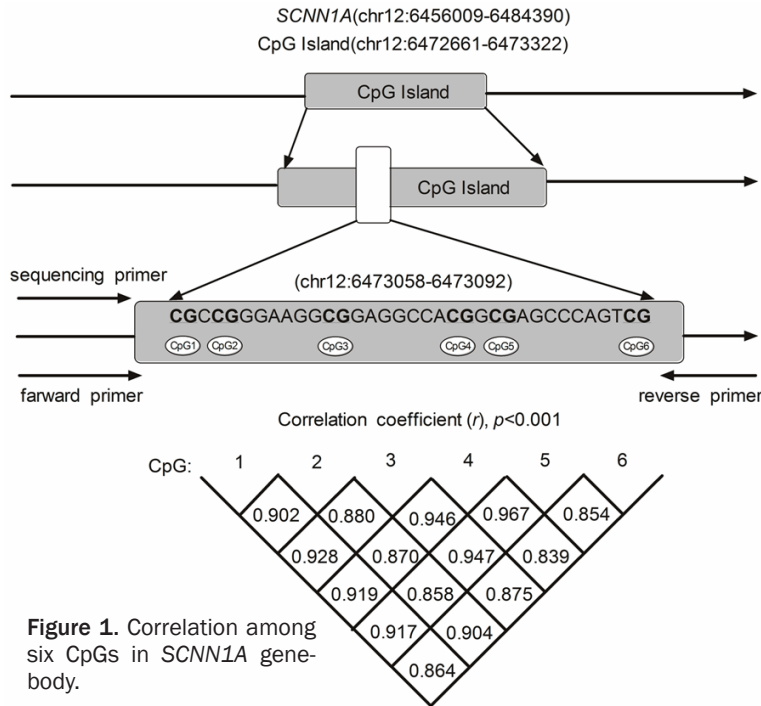


Figure 1. Correlation among six CpGs in SCNN1A gene-body.

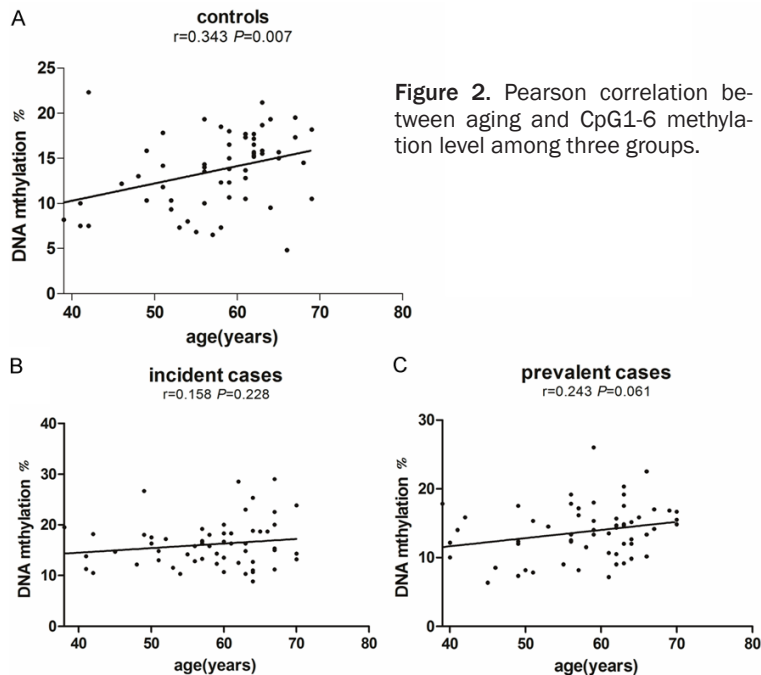


Figure 2. Pearson correlation between aging and CpG1-6 methylation level among three groups.

association between SCNN1A methylation and EH.

In the present research, we tried to study whether SCNN1A gene-body methylation was associated with EH, investigate the difference of SCNN1A methylation levels and clinical biochemical indicators among the non-EH controls, incident EH cases (In-EH), and prevalent EH cases (Pre-EH). And, we evaluated the diagnostic value of SCNN1A gene-body methylation by ROC curve analysis. We also accessed the relationship of SCNN1A methylation with gender, drinking, and smoking. DNA methylation was regarded to be modified by drugs in some researches. So our study also attempted to investigate the effects of antihypertensive therapy to SCNN1A methylation.

Materials and methods

Sample collection

This study included 60 non-EH controls, 60 incident EH cases and 60 prevalent EH cases collected from the Ningbo Seventh Hospital in Ningbo city of Zhejiang province, China. All of the people were Han Chinese residing in Ningbo city for at least three generations. Gender and age were well matched among three groups. Hypertensive patients were determined by the golden standard [18]. All the hypertensive patients had received antihypertensive medication for >3 months or had at least three consecutive records of systolic blood pressures (SBP) >140 mmHg and/or diastolic blood pressure (DBP) >90 mmHg. Incident EH cases (In-EH) were hypertensive patients who had never received antihypertensive drugs therapy. Prevalent EH cases (Pre-EH) were hypertensive

And ENaC gene was deemed to be an important candidate gene of EH. Recent studies documented the mutation of SCNN1A gene (α ENaC gene) were linked to pseudohypoaldosteronism type 1 (PHA1) [16] and could influence the lung diffusing capacity in cystic fibrosis [17]. However, no studies were carried out to explain the

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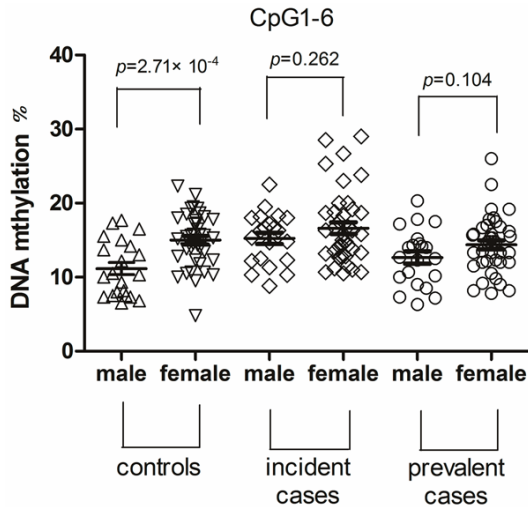


Figure 3. Comparison of methylation level between males and females in three groups respectively.

patients who had received antihypertensive drugs therapy. In addition, the non-EH controls were those individuals who exhibited SBP <120 mm Hg and DBP <80 mmHg, and had never taken any antihypertensive therapy and had no family history of hypertension. All the people didn't have a history of secondary hypertension, diabetes mellitus, renal failure, myocardial infarction, drug abuse, stroke and other serious diseases. The clinical indicators data was acquired from Ningbo Seventh Hospital and acquired the patient's agreement. Blood samples were obtained after a 12 h overnight fast from the antecubital vein using vacutainer tubed containing EDTA and then stored at -80°C for DNA extraction. The study protocol was approved by the Ningbo Seventh Hospital. Informed written consent was obtained from all subjects.

Biochemical analyses

Plasma levels of triglyceride, alanine transaminase, total cholesterol; glucose were measured by an Olympus AU2700 automatic analyzer (Olympus, Tokyo, Japan). Nucleic acid extraction analyzer (Lab-Aid 820; Zeesan Biotech, Xiamen, China) was used to extract genomic DNA from the blood samples. The concentration of DNA was measured by an ultramicro nucleic acid ultraviolet tester (Nano Drop 1000; Thermo Fisher Scientific, Wilmington, Del, USA). Genomic DNA was prepared from peripheral blood samples using the nucleic acid extraction automatic analyzer (Lab-Aid 820, Xiamen City, China). DNA was quantified using the double

strand DNA (dsDNA) Quantification Kit (Molecular Probes, Inc. Eugene, USA). Six CpG dinucleotides methylation levels were detected by bisulphate pyrosequencing technology in the SCNN1A gene-body (**Figure 1**). Sodium bisulfite DNA conversion chemistry combines pyrosequencing assays. The advantage of the bisulphite pyrosequencing technology was that it was highly efficient, accurate and quantitative, and the results could be repeated. Polymerase chain reaction (PCR) amplification and sequencing of the target sequence were performed by synthesis assay (PyroMark Glod Q96 Reagents, Qiagen #972804). PCR amplification was performed in reaction mixtures containing 12 μl ZymoTaq™ Premix (Zymo Research Corporation, Irvine, California, USA), 1.5 μl forward primer, 1.5 μl reverse primer, 2 μl converted DNA and 8 μl DNAase/RNAase free waters, conducted using an Eppendorf Mastercycler Nexus Gradient (Eppendorf, Hamburg, Germany) under the following conditions: 95°C for 10 min, 40 cycles of 95°C for 30 secs, then 57.2°C for 40 secs and 72°C for 50 secs, followed by one cycle of 72°C for 7 min. Primers were designed using PyroMark Assay Design software v2.0.1.15. Primers were as follows: Forward primer: 5'-GGTGAAGGGTAAATAGGTG-TG-3'; Reverse primer: 5'-Biotin-TCTCCTCAATAACAATCCACAATATC-3'; Sequencing primer: 5'-GGGTAAATAGGTGTGT-3.

Statistics analysis

Statistics analysis was performed with PASW Statistics 19.0 software (SPSS, Inc., Somers, NY, USA). The mean of continuous variables (including age, BMI, total cholesterol, triglyceride, glucose, ALT, and DNA methylation levels) among the three groups were tested by one-way ANOVA. Either Pearson chi-square or Fisher exact test was used for the association of EH with categorical variables including gender, smoking and drinking. The relationship of CpG1 to CpG6 was analyzed by Bivariate correlation analysis. Logistic regression analysis was used to evaluate the relative risk or odds of SCNN1A gene methylation and clinical index variables. Logistic regression analysis was also utilized to adjust for confounding factors. A two sample t-test analyses was performed to analyze the differences of SCNN1A methylation level in gender among three groups. Receiver operating characteristic (ROC) curves were used to explore the sensitivity of SCNN1A methylation

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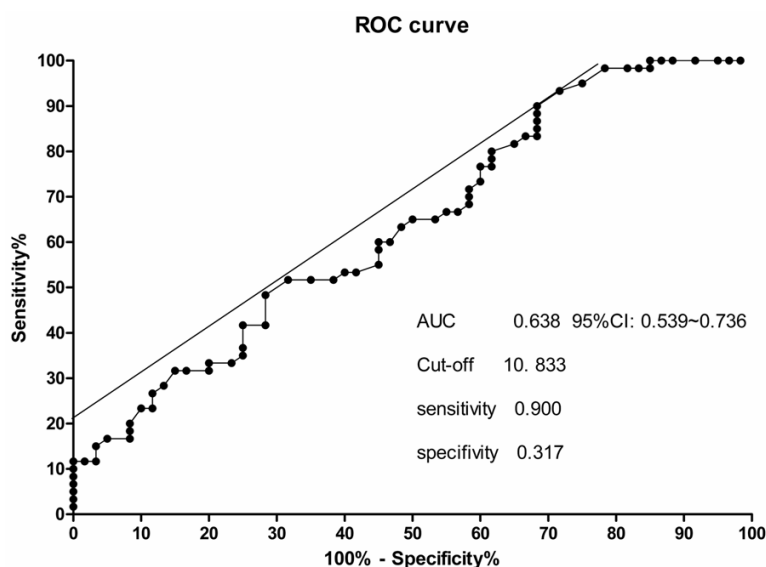


Figure 4. Receiver operating characteristic (ROC) curve of *SCNN1A* gene-body methylation in EH.

as a predictor of EH. Two-sided $P < 0.05$ was considered statistically significant.

Results

Methylation differences of SCNN1A gene-body among three groups

In this research, we could not find specific CpG islands in *SCNN1A* gene promoter, so six CpGs in *SCNN1A* gene-body were selected to explore the relationship between *SCNN1A* methylation and EH (**Figure 1**). As **Figure 1** showed, six CpGs in *SCNN1A* were highly correlated ($r > 0.86$, $P < 0.01$). So we regarded the six CpG sites as one CpG site using the average of the six CpG sites methylation levels from each individual. We also presented the **Figure 5** to explain methylation level of six individual CpGs comparing Control, In-EH, Pre-EH groups. The PCR primers were designed for the amplification of the CpG islands region of *SCNN1A* gene-body. Results of One-Way ANOVA showed that CpG1-6 methylation level was different among three groups ($F = 6.799$, $P = 0.001$, **Table 1**). So we took multiple comparisons to examine the difference between every two groups (**Table 2**). Significantly higher *SCNN1A* methylation level was observed in incident cases than non-EH (16.15 ± 4.51 versus 13.66 ± 4.08 , $t = 3.26$, $P = 0.001$), and prevalent cases (16.15 ± 4.51 versus 13.77 ± 3.90 , $t = 3.11$, $P = 0.002$). But no difference in *SCNN1A* gene-body methylation level between non-EH and prevalent cases ($t = 0.15$, $P = 0.911$) was discovered.

Drug impact to SCNN1A gene-body methylation

Binary Logistic regression analysis was used to analyze the impact of anti-hypertensive drugs therapy to *SCNN1A* gene-body methylation (**Table 3**). The results indicated that CpG1-6 methylation level was considerably higher in incident cases than non-EH ($OR = 1.157$, $P = 0.010$), and prevalent cases ($OR = 1.149$, $P = 0.013$). *SCNN1A* gene-body methylation was important risk factor of EH. But there were not any differences between non-EH and prevalent cases ($OR = 1.005$, $P = 0.934$).

Correlation between age and gene-body methylation of SCNN1A

Recent researches have documented the aging is linked to the DNA methylation [19, 20]. We accessed the correlation between aging and CpG1-6 methylation level among three groups (non-EH: $r = 0.343$, $P = 0.007$; incidence cases: $r = 0.158$, $P = 0.228$; prevalent cases: $r = 0.243$, $P = 0.061$, **Figure 2**).

Gender difference of SCNN1A gene-body methylation

As shown in **Figure 3** higher CpG1-6 methylation level was observed in female than male in non-EH ($t = -3.878$, $P = 2.71E-4$, **Figure 3**), not in incident cases ($t = -1.133$, $P = 0.262$, **Figure 3**) and prevalent cases ($t = -1.661$, $P = 0.104$, **Figure 3**). In male, we found higher CpG1-6 methylation level in incident cases than non-EH (15.25 ± 3.50 versus 11.16 ± 3.77 , $t = 3.58$, $P = 0.001$, **Table 4**) and prevalent cases (15.25 ± 3.50 versus 12.65 ± 3.84 , $t = 2.28$, $P = 0.026$, **Table 4**). In female, we found higher CpG1-6 methylation level in incident cases than prevalent cases (16.63 ± 4.94 versus 14.38 ± 3.85 , $t = 2.38$, $P = 0.019$, **Table 4**).

Diagnostic value of SCNN1A gene-body methylation

As shown in **Figure 4**, receiver operating characteristic (ROC) curve analysis revealed that 10.833% CpG1-6 methylation level was an appropriate cut-off value for the *SCNN1A* gene-

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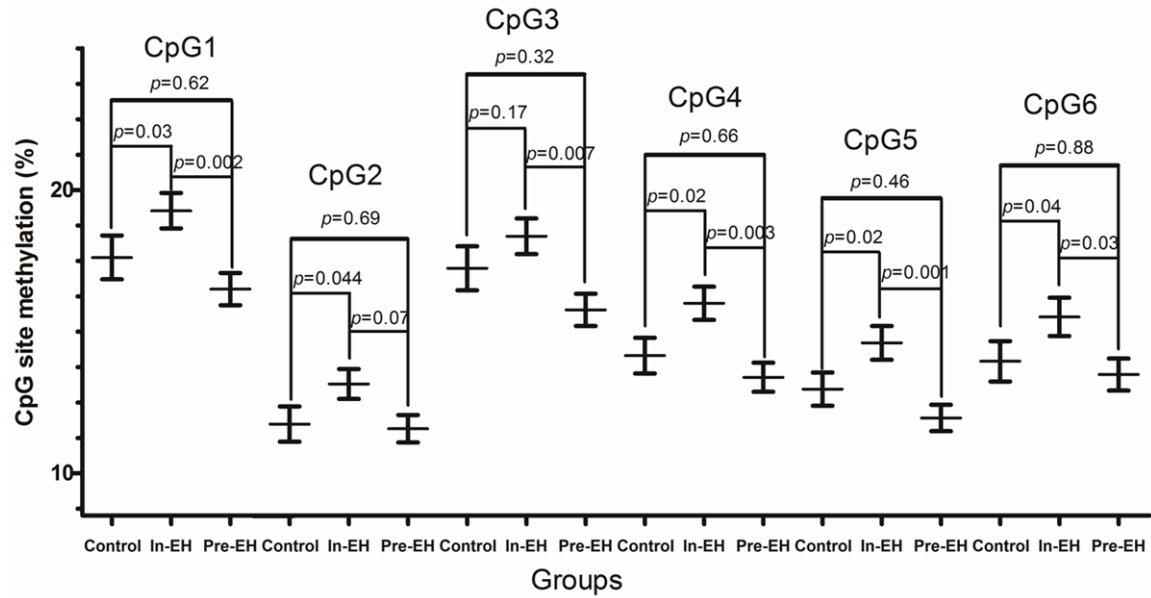


Figure 5. Methylation level of six individual CpGs comparing Control, In-EH, Pre-EH groups.

Table 1. Characteristics of three groups

Characteristics	Controls (Mean ± sd)	In-EH (Mean ± sd)	Pre-EH (Mean ± sd)	F/ χ^2	P
Age	57.52±7.29	58.05±7.89	57.92±8.03	0.077	0.926
Gender (M/F)	21/39	21/39	21/39	0.000	1.000
Smoking (Y/N)	11/49	12/48	15/45	0.867	0.648
Drinking (Y/N)	5/55	13/47	13/47	4.988	0.083
BMI	22.43±3.02	24.46±2.68	24.60±3.03	10.431	5.22×10 ⁻⁵
TC (mmol/L)	5.14±1.02	5.37±1.11	5.40±1.10	1.075	0.343
TG (mmol/L)	1.59±1.00	1.92±1.19	1.48±0.59	3.418	0.035
Glucose (mmol/L)	5.25±0.55	5.40±0.61	5.62±0.89	4.272	0.015
ALT (IU/L)	22.30±27.68	26.63±19.49	22.73±13.83	0.767	0.466
CpG1-6 Methylation (%)	13.66±4.08	16.15±4.51	13.77±3.90	6.799	0.001

BMI, body mass index; TC, total cholesterol; TG, triglycerides; ALT, alanine transaminase; In-EH, Incidence EH cases; Pre-EH, Prevalent EH cases.

Table 2. Characters' multiple comparisons LSD (least significant difference t test)

Characters	Controls vs. In-EH		In-EH vs. Pre-EH		Controls vs. Pre-EH	
	t/ χ^2	P	t/ χ^2	P	t/ χ^2	P
BMI	3.82	1.84×10 ⁻⁴	0.26	0.797	4.08	6.86×10 ⁻⁵
TG (mmol/L)	1.88	0.062	2.51	0.013	0.63	0.527
Glucose (mmol/L)	1.17	0.246	1.73	0.084	2.90	0.004
CpG1-6 Methylation (%)	3.26	0.001	3.11	0.002	0.15	0.911
Drinking	4.18	0.041	0.00	1.000	4.18	0.041

BMI, body mass index; TG, triglycerides; In-EH, Incidence EH cases; Pre-EH, Prevalent EH cases.

body methylation to predict the onset of EH between the non-EH and incident (area under curve: 0.638, $P < 0.009$, 95% CI: 0.539-0.736).

Difference of clinical biochemical indicators among three groups

In addition, we compared the risk factors (BMI, total cholesterol, triglyceride, Glucose) us-

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Table 3. Variables in the logistic regression model

Variables	Controls vs. In-EH			In-EH vs. Pre-EH			Controls vs. Pre-EH		
	β	OR	P	β	OR	P	β	OR	P
Gender	-0.280	0.756	0.640	0.036	1.037	0.946	-0.168	0.845	0.760
Age	0.003	1.003	0.919	0.008	1.008	0.795	-0.031	0.970	0.340
BMI	0.221	1.247	0.004	0.059	1.061	0.465	0.246	1.279	0.002
TC (mmol/L)	0.178	1.195	0.411	0.153	1.165	0.431	0.347	1.414	0.130
TG (mmol/L)	0.155	1.167	0.443	-0.555	0.574	0.041	-0.281	0.755	0.296
Glucose (mmol/L)	0.231	1.260	0.553	0.577	1.781	0.114	1.061	2.888	0.012
ALT (IU/L)	-0.004	0.996	0.645	-0.004	0.996	0.766	0.001	1.001	0.880
CpG1-6	0.146	1.157	0.010 ^a	-0.139	0.870	0.013 ^a	0.005	1.005	0.934 ^a
Smoking	-0.284	0.753	0.677	0.968	2.633	0.174	-0.082	0.921	0.894
Drinking	1.512	4.535	0.056	-0.568	0.567	0.402	1.015	2.758	0.161
Constant	-7.774	6.86×10 ⁻⁵	0.003	-2.944	0.053	0.322	-11.085	1.53×10 ⁻⁵	1.92×10 ⁻⁵

BMI, body mass index; TC, total cholesterol; TG, triglycerides; ALT, alanine transaminase; In-EH, Incidence EH cases; Pre-EH, Prevalent EH cases; ^aP-values were adjusted for age, sex, gender, BMI, TC, TG, Glucose, ALT, Smoking and Drinking.

Table 4. Comparison of methylation level among three groups stratified by gender

Gender	Controls (Mean ± sd)	In-EH (Mean ± sd)	Pre-EH (Mean ± sd)	Controls vs. In-EH		In-EH vs. Pre-EH		Controls vs. Pre-EH	
				t	P	t	P	t	P
				Males Methylation (%)	11.16±3.77	15.25±3.50	12.65±3.84	3.58	0.001
Females Methylation (%)	15.01±3.62	16.63±4.94	14.38±3.85	1.71	0.089	2.38	0.019	0.67	0.506

In-EH, Incidence EH cases; Pre-EH, Prevalent EH cases.

ing variance analysis. The levels of BMI ($F=10.341$, $P=5.22E-5$, **Table 1**) and triglyceride ($F=3.418$, $P=0.035$, **Table 1**) were quite different among three groups. The level of BMI of non-EH was lower than incident cases (22.43 ± 3.02 versus 24.46 ± 2.68 , $t=3.82$, $P=1.84\times10^{-4}$, **Table 2**) and Prevalent cases (22.43 ± 3.02 versus 24.60 ± 3.03 , $t=4.08$, $P=6.86\times10^{-5}$, **Table 2**). Logistic regression analysis indicated that BMI was risk factor of EH (OR=1.247, $P^a=0.004$, **Table 3**; OR=1.279, $P=0.002$, **Table 3**). The level of triglyceride was lower in prevalent cases than incident cases (1.48 ± 0.59 versus 1.92 ± 1.19 , $t=2.524$, $P=0.013$).

Alcohol consumption and hypertension

We found a significant difference in the ratio of alcohol consumption between cases and non-EH using Chi-square test ($\chi^2=4.183$, $P=0.041$, **Table 2**). We could not find the difference of the ratio of smoking consumption among three groups ($\chi^2=0.867$, $P=0.648$, **Table 2**).

Discussion

ENaC gene is an important candidate gene of essential hypertension. Our results indicated

that the incident cases had a hypermethylation level of *SCNN1A* gene-body than non-EH controls, and prevalent cases. *SCNN1A* gene-body methylation not only was a significant important risk factor but also was a new diagnostic index of EH. In addition, we found *SCNN1A* gene-body methylation level was positively correlated with age in non-EH controls, and *SCNN1A* gene-body methylation level in female was higher than male.

As is shown in recent studies, positive correlation between gene-body methylation and gene expression was observed in human cells [10, 21, 22]. Gene-body methylation was correlated with hosts of diseases through regulating gene expressions [23-25]. Consequently, the *SCNN1A* gene-body methylation may be associated with ENaC gene mRNA expression. In addition, global DNA methylation analysis of human atherosclerotic plaques was also revealed that hypomethylation of promoter and hypermethylation of gene-body was associated with stimulated mRNA expression of imprint locus *14p32* [26]. Glucokinase (*GCK*) gene-body methylation was reported to increase the risk of EH and coronary heart disease [27]. So we speculate

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that the increase of *SCNN1A* gene-body methylation may influence the transcription and gene expression. Furthermore, ENaC played a notable role in the regulation of extracellular fluid volume and blood pressure [28, 29]. The mice model showed that α ENaC was liable to be critical to the overall salt balance, compared with β ENaC and γ ENaC [30]. For this reason, in our study, the increase of *SCNN1A* gene-body methylation might impact *SCNN1A* gene transcription and expression, and increase Na⁺ absorption, water retention largely, finally lead to EH. Furthermore, Receiver operating characteristic (ROC) curve analysis revealed that *SCNN1A* gene-body methylation level is an appropriate cut-off value to predict the onset of EH. This result was in agreement with our previous research about *ADD1* gene methylation [31]. So we think the gene methylation may become a useful diagnostic criteria to predict the EH. However, the exact mechanism is not clear, ongoing study is required.

Previously, glucocorticoid was reported to have an impact on *AT1b* angiotensin receptor gene methylation levels and expression [32]. Recent colorectal cancer cells experiment also showed that the combination of DNA methyltransferase inhibitor (5-azacytidine, decitabine) and standard chemotherapeutics (oxaliplatin, 5-fluorouracil) could be a novel therapeutic approach to improve the curative effect of patients with colorectal cancer [15]. In addition, the DNA methyltransferase inhibitor 5-azacytidine, 5-aza-2'-deoxycytidine (decitabine) have been approved for the cure of hematological malignancies as epigenetic drugs by the American Food and Drug Administration (FDA). All the above researches suggest that gene methylation could be changed by drugs treatment of EH. Similarly, our study revealed the difference of methylation level among three groups, suggesting the anti-hypertension drugs therapy might change *SCNN1A* gene-body methylation level. Notably, the main difference between In-EH group and Pre-EH group was the patients whether had received antihypertensive drugs therapy. Furthermore, In-EH group showed differential methylation against control whereas there was no change between control and Pre-EH group, which further evidenced antihypertensive drugs therapy might reduce methylation level of Pre-EH and eventually lead to no difference between Pre-EH and control. However, different

antihypertensive drugs have different mechanisms of action. In the present, although we found antihypertensive drugs likely decreased the gene-body methylation of *SCNN1A*, we did not know what kind of antihypertensive drug therapy caused this alteration. Therefore, ongoing studies with the specified information of antihypertensive drug are needed to confirm our finding.

It is well known that age is a risk factor in the progressing of hypertension [33-35]. Gene methylation level may be changed over time by the hereditary factors, environmental factors, and their interactions with each other. Aberrant DNA methylation was associated with the age-related diseases such as cancer [36-38], cardiovascular disease [39, 40], diabetes mellitus [41]. It's reported that *ADD1* and *GCK* gene hypermethylation was associated with rising age in EH [2, 14]. The results we have observed the age-related increase in *SCNN1A* gene-body methylation in non-EH controls were consistent with these studies. In addition, gender difference of *SCNN1A* gene-body methylation was identified in our study. The methylation level in females was higher than males. Consistent with our study, gender differences of the gene methylation level were explored in human tissues in other studies [42-45]. A genome-wide DNA methylation analysis showed that the females had a higher methylation level compared with males on the X chromosome in human pancreatic islets, and the altered methylation level could induce the pancreatic islets differential gene expression [46].

There are a few limitations of our research. For example, ENaC gene was expressed in many human tissues, but we only tested the gene methylation in human peripheral blood, although many evidence indicated that CpG methylation patterns were similar between peripheral blood and other tissues [47-49]. On the other hand, we didn't have gene expression or protein data to demonstrate the transcriptional regulation now. Ongoing expression analysis is required to confirm our findings. The last but not the least, six CpG sites might not entirely reflect the situation of the whole gene.

In summary, we found *SCNN1A* gene-body hypermethylation level was associated with EH, and the methylation level could be altered by

anti-hypertension drugs, age and gender. Our study was likely to bring new clues to elaborate the pathogenesis of the EH from the view of epigenetics.

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Disclosure of conflict of interest

None.

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