Lower ADD1 Gene Promoter DNA Methylation Increases the Risk of Essential Hypertension

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Abstract

The goal of our study is to investigate the contribution of promoter DNA methylation of α-adducin (ADD1) gene to the risk of essential hypertension (EH). Using the bisulphite pyrosequencing technology, DNA methylation levels of five CpG dinucleotides on ADD1 promoter were measured among 33 EH cases and 28 healthy controls. Significantly higher ADD1 DNA methylation levels were observed in the females than in the males (CpG1: \( P = 0.016 \); CpG2-5: \( P = 0.021 \)). A breakdown analysis by gender showed that lower CpG1 methylation was associated with an increased risk of EH in females (adjusted \( P = 0.042 \)). A much more significant association between lower CpG2-5 methylation levels and the increased risk of EH was found in males (adjusted \( P = 0.008 \)). CpG1 methylation was inversely correlated with age in females (\( r = -0.407, P = 0.019 \)) but not in males. ADD1 CpG1 and CpG2-5 methylation levels were significantly lower in post-menopausal (>50 years) women than pre-menopausal (<50 years) women (CpG1: \( P = 0.006 \); CpG2-5: \( P = 0.034 \)). A significant interaction between CpG1 methylation and age was found in females (CpG1*age: \( P = 0.029 \)). CpG2-5 methylation was shown as a significant predictor of EH in males [area under curve (AUC) = 0.855, \( P = 0.001 \)], in contrast that CpG1 methylation was a trend toward indicator in females (AUC = 0.699, \( P = 0.054 \)). In addition, significant differences were observed between males and females for alanine aminotransferase (ALT, \( P = 0.001 \)), aspartate aminotransferase (AST, \( P = 0.005 \)) and uric acid (\( P < 0.001 \)). The concentration of AST was inversely correlated with ADD1 CpG2-5 methylation levels in female controls (\( r = -0.644, P = 0.024 \)). These observations may bring new hints to elaborate the pathogenesis of EH.

Introduction

Essential hypertension (EH) is one of the most important causes of premature death worldwide. EH is a complex disorder resulting from both genetic and environmental factors [1,2]. Approximately 20–60% of the blood pressure variability in general population is heritable [3]. Epidemiological studies have documented environmental factors such as physical inactivity, obesity, high sodium and low potassium diet, and alcohol consumption are associated with hypertension risk [4,5]. Disorders in the metabolism of high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) play a key role in EH progression [6,7].

A sexual dimorphism exists in the developmental origins of EH [8,9]. Males are reported to be more susceptible to hypertension than females [10]. Gender difference in the risk of hypertension was observed to be associated with altered expression of hormone receptors such as renal alpha2-adrenergic receptors [11] and angiotensin receptors [12]. In addition, evidence has shown a gender dimorphism in the expression of renin [13,14] and urinary angiotensinogen excretion [15] which are important risk factors for EH. The sexual dimorphism in mammalian gene expression have been observed to be linked to the gender differences in the amounts of sex hormones [16] (e.g., estrogens, androgens).

ADD1 gene encodes one of adducin subunits (α-adducin) [17]. Adducin modulates the surface expression of multiple transporters and ion pumps, and thus regulates cellular signal transduction and cytolemma ion transport [18]. Human and animal model studies have found that ADD1 gene is a candidate gene for EH [18,19]. However, epidemiological studies have shown that the contribution of ADD1 Gly460Trp mutation (rs4961) to hypertension varies among different ethnic groups [20–24]. Meta-analyses were unable to reach a consensus for this mutation [25–28]. Moreover, gender dimorphism of the association between hypertension and ADD1 Gly460Trp was observed in Caucasians [29].

DNA methylation is a stable epigenetic mark and usually occurs at cytosine residues in the context of cytosine-phosphate-guanine dinucleotide (CpG) in mammalian cells [30]. Promoter DNA methylation is linked to transcriptional silencing of protein-coding genes [31] and thus regulates the function of protein. Aberrant methylation is shown to play important roles in the occurrence and
development of diseases including colorectal cancer [32,33], breast cancer [34,35], coronary artery disease [36] and schizophrenia [37,39]. The evidence of the association between DNA methylation and the risk of EH was scarce. A significant decline in global DNA methylation level is observed in EH patients and the trend continues along with the progression of hypertension [39]. Altered global DNA methylation in pre-eclampsia placentas was shown to be associated with maternal hypertension [40]. Aberrant DNA methylation of 11beta-HSD2 and Add1 genes were found to be associated with EH [41] and the outcome of medications [42], respectively.

We hypothesize that ADD1 promoter DNA methylation contributes to EH. Our goal is to study whether promoter DNA methylation of ADD1 gene is associated with EH, and to explore the interaction of promoter DNA methylation with gender and clinical indicators of lipid and amino acid metabolism.

**Materials and Methods**

**Sample Collection**

This study comprised 33 cases (14 males, 50.1 ± 4.9 years; 19 females, 51.3 ± 4.7 years) and 28 controls (14 males, 51.2 ± 6.3 years; 14 females, 47.9 ± 5.0 years) collected from the community residents in Zhenhai district of Ningbo city in Zhejiang province, China. All individuals are Han Chinese living in Ningbo city for at least three generations. Hypertensive patients were defined according to the golden standard [43]. All hypertensives have received antihypertensive medications for more than three months or have at least three consecutive records of systolic blood pressure (SBP) >140 mmHg and/or diastolic blood pressure (DBP) >90 mmHg (European Society of Hypertension-European Society of Cardiology Guidelines, 2003). Patients had SBP <120 mmHg and DBP <80 mmHg and had no family history of hypertension in the first degree relatives were recruited as controls. None of the controls has received antihypertensive therapy. The gender and age of controls were well matched with EH cases. All the individuals don’t have a history of diabetes mellitus, secondary hypertension, myocardial infarction, stroke, renal failure, drug abuse and other serious diseases. A calibrated mercury sphygmomanometer with appropriate adult cuff size was applied to measure blood pressures according to a standard protocol recommended by the American Heart Association [44]. Blood pressures were measured in supine position by two trained observers at an interval of at least 10 minutes. Blood samples were collected in 3.2% citrate sodium-treated tubes and then stored at −80°C for DNA extraction. The study protocol was approved by the ethical committee of Ningbo University. The informed written consent was obtained from all subjects.

**Phenotypes Collection**

Blood samples were obtained after a 12 h overnight fast from the antecubital vein using vacutainer tubes containing EDTA. Plasma levels of cholesterol, TG, ALT, AST, uric acid and glucose concentrations were enzymatically measured using CX7 biochemistry analyzer (Beckman, Fullerton, CA).

**DNA Methylation Assay**

Human 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 PicoGreen® double strand DNA (dsDNA) Quantification Kit (Molecular Probes, Inc. Eugene, USA). Bisulfite pyrosequencing technology was used to determine the 5 CpG dinucleotides methylation levels on the fragment within ADD1 promoter (Figure 1). Pyrosequencing assays combine sodium bisulfite DNA conversion chemistry (EpiTech Bisulfite Kits; Qiagen; #39104), polymerase chain reaction (PCR) amplification (Pyromark PCR Kit; Qiagen; #978703) and sequencing by synthesis assay (Pyromark Gold Q24 Reagents; Qiagen; #978802) of the target sequence. Sodium bisulfite preferentially deaminates unmethylated cytosine residues to thymines (after PCR amplification), whereas methyl-cytosines remain unmodified. PCR primers were selected using PyroMark Assay Design software v2.0.1.15. The PCR and pyrosequencing primers for ADD1 gene promoter amplification were described in Table S1.

**Statistical Analysis**

Statistical analyses were performed to investigate the association among ADD1 DNA methylation, metabolic profile and EH. Either Pearson chi-square or Fisher exact test was used for the association of EH with categorical variables including gender, smoking, and drinking. Two sample t-test was applied for the association of EH with continuous variables including age, body mass index (BMI), total cholesterol, total triglycerides, glucose, ALT, AST, and uric acid. Pearson correlation was used to determine the association between the ADD1 DNA methylation and metabolic characteristics. Receiver operating characteristic (ROC) curve was used to analyze the sensitivity of ADD1 DNA methylation in EH diagnosis. Logistic regression was implemented for the interaction of ADD1 methylation and age. A two-sided p-value < 0.05 was considered statistically significant. All the above statistical analyses were performed with PASW Statistics 18.0 software (SPSS, Inc., Somers, NY, USA). Meanwhile, Power and Sample Size Calculation software (v3.0.43) was used to estimate the power of the study [45].

**Results**

A total of 33 cases and 28 age- and gender-matched controls were recruited in the current association study. As shown in Table 1, mean levels of body mass index (BMI) and all metabolic phenotypes were within normal ranges. The mean levels of age and BMI were well paired between males and females (Table 1).

In this study, we selected a locus containing 5 CpG dinucleotides to explore the association of ADD1 gene promoter DNA methylation with EH (Figure 1). We found DNA methylation levels were closely correlated among CpG2-5 (Figure 1, r = 0.133). As shown in Table 2 and Figure S1, lower levels of ADD1 methylation levels (including CpG1 and CpG2-5) and EH. As shown in Table 2 and Figure S1, our results showed that ADD1 CpG2-5 methylation levels were significantly associated with EH (cases versus controls (%): 27.54 ± 7.48 versus 31.44 ± 5.30, adjusted P = 0.026). Moreover, significantly higher ADD1 DNA methylation levels were observed in females than in males (CpG1: P = 0.016; CpG2-5: P = 0.021). CpG1 methylation was inversely correlated with age in females (Figure 2, r = −0.407, P = 0.019). ADD1 methylation levels were significantly higher in pre-menopausal (<50 years) women than post-menopausal (>50 years) women (Figure 3, CpG1: P = 0.006; CpG2-5: P = 0.034).

A breakdown association test by gender was also performed to explore the association between ADD1 methylation levels (including CpG1 and CpG2-5) and EH. As shown in Table 2 and Figure S1, ADD1 CpG1 methylation level was significantly associated with EH in females (cases versus controls (%): 10.00 ± 1.41 versus 11.36 ± 3.63, adjusted P = 0.042) but not in males (adjusted P = 0.133). As shown in Table 2 and Figure S1, lower levels of ADD1 CpG2-5 methylation were associated with increased risk of EH in males (cases versus controls: 22.48% versus 31.86%, adjusted P = 0.008). In contrast, no association of CpG2-5 methylation levels with EH was found in females (adjusted...
Furthermore, significant interaction of CpG1 methylation and age was found to influence EH status in females (CpG1*age: $P = 0.029$). Prediction potential of EH for ADD1 CpG1 and CpG2-5 methylation levels was assessed by the ROC curves. CpG2-5 methylation was shown as a significant predictor of EH in males [Figure 4a, area under curve (AUC) = 0.855, $P = 0.001$]. CpG1 methylation was also shown with a trend toward indicator in females (Figure 4b, AUC = 0.699, $P = 0.054$).

As shown in Table 1, significant differences were observed between males and females for ALT ($P = 0.001$), AST ($P = 0.005$) and uric acid ($P < 0.001$). Subsequently, we performed correlation tests between ADD1 DNA methylation levels and metabolic

| Characteristics | Mean ± s.e. | Men (Mean ± s.e.) | Women (Mean ± s.e.) | EH (Mean ± s.e.) | Non-EH (Mean ± s.e.) | P_EH |
|-----------------|------------|-------------------|---------------------|-----------------|---------------------|------|
| Age (years)     | 50.2 ± 5.3 | 50.7 ± 5.5        | 49.8 ± 5.1          | 50.8 ± 4.7      | 49.6 ± 5.8          | 0.361|
| Gender (M/F)    | 28/33      | NA                | NA                  | 14/19           | 14/14               | 0.554|
| BMI (kg/m²)a    | 22.50 ± 2.55 | 22.63 ± 1.77    | 22.41 ± 3.01        | 22.95 ± 2.72   | 21.84 ± 2.19        | 0.159|
| Smoking (Y/N)   | NA^4       | 9/19              | 0/33                | 5/28            | 3/25                | 0.896|
| Drinking (Y/N)  | NA^4       | 6/22              | 0/33                | 4/29            | 2/26                | 0.826|
| Total cholesterol (mmol/L) | 5.05 ± 0.93 | 5.01 ± 0.85     | 5.08 ± 1.01         | 5.09 ± 0.81    | 4.99 ± 1.07         | 0.681|
| Total triglycerides (mmol/L) | 1.50 ± 0.91 | 1.44 ± 0.84      | 1.56 ± 0.97         | 1.69 ± 1.08    | 1.28 ± 0.58         | 0.055|
| Glucose (mmol/L) | 5.18 ± 0.58 | 5.15 ± 0.59      | 5.20 ± 0.58         | 5.31 ± 0.59    | 5.02 ± 0.53         | 0.055|
| ALT (IU/L)      | 21.4 ± 15.5 | 28.6 ± 17.9      | 15.3 ± 9.9          | 23.7 ± 13.5    | 18.8 ± 17.5         | 0.228|
| AST (IU/L)^b    | 23.9 ± 7.0  | 26.6 ± 7.7       | 21.5 ± 5.4          | 25.3 ± 5.7     | 22.2 ± 8.3          | 0.089|
| Uric Acid (μmol/L) | 298.8 ± 88.8 | 351.5 ± 91.0    | 254.0 ± 57.3        | 312.0 ± 96.4   | 283.2 ± 77.7        | 0.210|
| CpG1 methylation (%) | 9.97 ± 2.24 | 9.25 ± 1.40      | 10.58 ± 2.63        | 9.52 ± 1.46    | 10.50 ± 2.85        | 0.091^d|
| CpG2-5 methylation (%) | 29.33 ± 6.81 | 27.17 ± 7.56    | 31.16 ± 5.58        | 27.54 ± 7.48   | 31.44 ± 5.30        | 0.026^d|

^an = 44 (18 men versus 26 women, 26 EH versus 18 Non-EH);
^bn = 59 (28 men versus 31 women, 33 EH versus 26 Non-EH);
^NA denotes not applicable.
^The p-values were adjusted for age, gender, smoking and drinking using a logistic regression analysis.
phenotypes including uric acid, ALT, and AST in controls. However, we didn’t find any correlations in controls between ADD1 DNA methylation levels and these metabolic phenotypes ($P > 0.05$, Figure S2a–c). A post hoc analysis was performed for the correlation between ADD1 CpG1 and CpG2-5 methylation levels and metabolic phenotypes (uric acid, ALT, and AST) in females and males. Other than a significant correlation between CpG2-5 and AST in females ($r = 0.644$, $P = 0.024$, Figure 5), no significant results observed in the rest of correlation tests (Figure S3a–e).

**Discussion**

The goal of the current study is to evaluate the contribution of ADD1 gene promoter DNA methylation to EH. We found that DNA methylation of ADD1 gene was significantly higher in females than in males. In addition, DNA methylation was shown to be a risk factor of EH in males (CpG2-5) and females (CpG1). To our knowledge, this is the first study showing the association of ADD1 gene promoter DNA methylation with EH. Our comprehensive analysis on the role of ADD1 methylation in the risk of EH may provide new hints to clarify the pathogenesis of EH in future.

Adducin was implicated in the pathogenesis of EH by modulating Na$^+$-K$^+$-ATPase activity [46–48]. Evidence indicated

![Figure 2. Pearson correlation between ADD1 methylation and age in males and in females.](doi:10.1371/journal.pone.0063455.g002)

![Figure 3. Significant ADD1 methylation difference between pre-menopausal and post-menopausal females.](doi:10.1371/journal.pone.0063455.g003)

**Table 2. Characteristics comparison between EH and Non-EH stratified by gender.**

| Characteristics                           | EH Mean ± s.e. | Non-EH Mean ± s.e. | p value |
|-------------------------------------------|----------------|--------------------|---------|
| **Men (28)**                              |                |                    |         |
| Age (years)                               | 50.1 ± 4.9     | 51.3 ± 6.3         | 0.595   |
| BMI (kg/m$^2$)$^b$                        | 22.70 ± 2.09   | 22.53 ± 1.41       | 0.846   |
| Smoking (Y/N)                             | 6/8            | 3/11               | 0.420   |
| Drinking (Y/N)                            | 4/10           | 2/12               | 0.648   |
| Total cholesterol (mmol/L)                | 5.09 ± 0.80    | 4.93 ± 0.92        | 0.630   |
| Total triglycerides (mmol/L)              | 1.54 ± 0.98    | 1.34 ± 0.71        | 0.546   |
| Glucose (mmol/L)                          | 5.31 ± 0.60    | 4.99 ± 0.55        | 0.146   |
| ALT (IU/L)                                | 30.4 ± 13.9    | 26.9 ± 21.6        | 0.607   |
| AST (IU/L)                                | 28.0 ± 4.6     | 25.2 ± 9.9         | 0.351   |
| Uric Acid (μmol/L)                        | 375.0 ± 107.3  | 328.1 ± 67.3       | 0.178   |
| CpG1 methylation (%)                      | 8.86 ± 1.29    | 9.64 ± 1.45        | 0.133$^d$|
| CpG2-5 methylation (%)                    | 22.48 ± 6.29   | 31.86 ± 5.65       | 0.008$^d$|
| **Women (n = 33)**                        |                |                    |         |
| Age (years)                               | 51.3 ± 4.7     | 47.9 ± 5.0         | 0.051   |
| BMI (kg/m$^2$)$^b$                        | 23.11 ± 3.11   | 21.29 ± 2.60       | 0.137   |
| Smoking (Y/N)                             | 0/19           | 0/14               |         |
| Drinking (Y/N)                            | 0/19           | 0/14               |         |
| Total cholesterol (mmol/L)                | 5.09 ± 0.85    | 5.06 ± 1.23        | 0.914   |
| Total triglycerides (mmol/L)              | 1.81 ± 1.17    | 1.22 ± 0.44        | 0.058   |
| Glucose (mmol/L)                          | 5.31 ± 0.60    | 5.06 ± 0.54        | 0.235   |
| ALT (IU/L)                                | 18.7 ± 11.2    | 10.8 ± 5.2         | 0.012   |
| AST (IU/L)$^c$                            | 23.4 ± 5.6     | 18.7 ± 3.6         | 0.008   |
| Uric Acid (μmol/L)                        | 265.6 ± 53.4   | 238.2 ± 60.6       | 0.180   |
| CpG1 methylation (%)                      | 10.00 ± 1.41   | 11.36 ± 3.63       | 0.042$^a$|
| CpG2-5 methylation (%)                    | 31.26 ± 6.04   | 31.02 ± 5.11       | 0.557$^e$|

$^a$n = 18 (10 EH versus 8 Non-EH);  
$^b$n = 26 (16 EH versus 10 Non-EH);  
$^c$n = 31 (19 EH versus 12 Non-EH);  
$^d$The p-values were adjusted for age, smoking and drinking using logistic-regression analysis.  
$^e$The p-values were adjusted for age, smoking, drinking, ALT and AST using logistic-regression analysis.  

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that adducin might be a candidate protein to explain genetic alterations in ion transport associated with EH [47]. A previous study reported that hypertensive rat had an increased activity and expression of Na\(^{+}\)-K\(^{+}\)-pump [48]. In this study, we hypothesized that the aberrant ADD1 methylation may cause hypertension. We speculated that lower ADD1 methylation led to higher expression of \(\alpha\)-adducin which resulted in an increased activity and expression of Na\(^{+}\)-K\(^{+}\)-pump and eventually caused high Na\(^{+}\)-reabsorption and hypertension. In accordance with the speculation, we did observe a significant lower level of ADD1 gene promoter methylation in EH cases than in controls.

Interestingly, CpG1 methylation was associated with EH in females, while the CpG2-5 methylation was significantly associated with EH in males. We also found ADD1 methylation was significantly higher in females than in males. In the present study, all subjects were recruited from Han Chinese residents in Ningbo city for at least three generations and diagnosed by generally recognized protocol. The gender and age were well matched between EH cases and Non-EH controls. The power of our association test of EH was 86.5% for ADD1 CpG2-5 in all samples, 66.7% for ADD1 CpG1 in females, and 84.4% for ADD1 CpG2-5 in males. Additionally the power of the analysis stratified by menopausal status was 71% for ADD1 CpG1 and 61.7% for ADD1 CpG2-5. However, our study was only involved with 61 samples and we could not exclude a possibility of spurious association due to the hidden structures in the tested samples. Future replication study with larger size of samples is warranted to confirm our findings.

Sexual dimorphism was found in the whole genome analysis for the risk loci of hypertension in rats [49,50] and humans [51]. Gender dimorphism was also observed in the association studies of hypertension. These studies addressed ADD1 Gly460Trp polymorphism in female Caucasians [29], CYP19A1 polymorphisms (rs700518, rs10046 and rs4646) in male Japanese [52], SELE T1559C polymorphism [53] and PNMT G390A polymorphism [54] in male Chinese, and other P2RY2 polymorphism (rs4944831) [55]. In the current study, we found that ALT and AST were associated with EH only in females, and that ADD1 CpG1 and CpG2-5 methylation levels were associated with EH in females and males, respectively. Moreover, CpG2-5 was correlated with AST in females, but not in males. These results may be associated with the difference of sex hormones. The role for gender (and/or sex hormones) has been described in mediating differential epigenetic effects on cardiomyocytes [56], effects of radiation [57,58] and the endocrine system [59]. Significant higher ADD1 methylation levels were found in pre-menopausal women than in post-menopausal ones. Our results provide new clues to explain the sexual dimorphism of EH.

DNA methylation level in humans is reported to alter along with changes of environmental factors such as nutrients [60] and drugs [61,62]. Therefore, major risk factors for hypertension including physical inactivity, high sodium diet, alcohol consumption and obesity [4,5] may alter the DNA methylation levels of EH risk genes and cause EH over time. Since lifestyle factors such as smoking, drinking, and physical activity are different between males and females, our findings of gender-dependent ADD1
methylation may reflect the difference in these non-heritable risk factors of EH.

Uric acid was reported positively correlated to the incidence of EH [63]. Previous study also showed uric acid was the risk factor associated with the mortality in hypertensive stroke patients [64]. In addition, uric acid has been reported associated with the prevalence of chronic kidney disease [65]. Raised plasma ALT level was associated with hypertension in Hong Kong Chinese [66]. Methodology also reported that elevated ALT and AST were associated with increased risk of pulmonary arterial hypertension [67]. However, ALT and AST have mostly been reported to be associated with hepatic disease [68–70], and they were seldom reported to related with EH. In the current study, we didn’t find the associations of uric acid and ALT with ADD1 DNA methylation levels in controls, whereas CpG2-5 was associated with AST in females. Future study is needed to investigate the mechanism underlying this association.

In summary, this study presents a lower ADD1 promoter methylation increases EH risk. ADD1 CpG2-5 methylation is able to predict EH risk in males with higher fidelity than CpG1 methylation does in females. The loss of power for CpG1 may be due to a significant interaction between CpG1 and age in females. Our findings are likely to bring new hints to elaborate the pathogenesis of this complex disease.

Supporting Information

Figure S1 Subgroup analysis in ADD1 promoter DNA methylation*, a: Triangles and circles stand for males and females respectively; blue and red stand for cases and controls, respectively.

Figure S2 Pearson correlation between ADD1 methylation and metabolic phenotypes in controls (A–C), (TIF)

Figure S3 Pearson correlation between ADD1 methylation and metabolic phenotypes in males (A–C) and in females (D–F).

Table S1 Primers for ADD1 gene CpG island loci analysis.

Author Contributions

Conceived and designed the experiments: LZ, SD, MY. Performed the experiments: PL, FY. Analyzed the data: PL, SD. Contributed reagents/materials/analysis tools: LW Leiting Xu YN, LP QZ LH XQ YH Limin Xu YL. Wrote the paper: PL SD LZ. Proofread the manuscript: PL, SD.

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