A genome-wide methylation study on essential hypertension in young african american males.

Xiaoling Wang  
*Georgia Health Sciences University*

Bonita Falkner  
*Thomas Jefferson University*

Haidong Zhu  
*Georgia Health Sciences University*

Huidong Shi  
*Georgia Health Sciences University*

Shaoyong Su  
*Georgia Health Sciences University*

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Authors
Xiaoling Wang, Bonita Falkner, Haidong Zhu, Huidong Shi, Shaoyong Su, Xiaojing Xu, Ashok Kumar Sharma, Yanbin Dong, Frank Treiber, Bernard Gutin, Gregory Harshfield, and Harold Snieder

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A Genome-Wide Methylation Study on Essential Hypertension in Young African American Males

Xiaoling Wang1*, Bonita Falkner2, Haidong Zhu1, Huidong Shi3, Shaoyong Su1, Xiaojing Xu1, Ashok Kumar Sharma4, Yanbin Dong1, Frank Treiber5, Bernard Gutin1, Gregory Harshfield1, Harold Snieder6

1 Georgia Prevention Institute, Department of Pediatrics, Georgia Health Sciences University, Augusta, Georgia, United States of America, 2 Department of Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania, United States of America, 3 The Cancer Research Center, School of Medicine, Georgia Health Sciences University, Augusta, Georgia, United States of America, 4 Center for Biotechnology & Genomic Medicine, School of Medicine, Georgia Health Sciences University, Augusta, Georgia, United States of America, 5 Technology Applications Center for Healthful Lifestyles, Colleges Of Nursing and Medicine, Medical University of South Carolina, Charleston, South Carolina, United States of America, 6 Unit of Genetic Epidemiology and Bioinformatics, Department of Epidemiology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

Abstract

Objective: There is emerging evidence from animal studies suggesting a key role for methylation in the pathogenesis of essential hypertension. However, to date, very few studies have investigated the role of methylation in the development of human hypertension, and none has taken a genome-wide approach. Based on the recent studies that highlight the involvement of inflammation in the development of hypertension, we hypothesize that changes in DNA methylation of leukocytes are involved in the pathogenesis of hypertension.

Method & Results: We conducted a genome-wide methylation analysis on 8 hypertensive cases and 8 normotensive age-matched controls aged 14–23 years and performed validation of the most significant CpG sites in 2 genes in an independent sample of 36 hypertensive cases and 60 normotensive controls aged 14–30 years. Validation of the CpG sites in the SULF1 gene was further conducted in a second replication sample of 36 hypertensive cases and 34 controls aged 15.8–40 years. A CpG site in the SULF1 gene showed higher methylation levels in cases than in healthy controls in the genome-wide approach (p = 6.2 × 10⁻⁷), which was confirmed in the validation step (p = 0.011) for subjects ≥30 years old but was not significant for subjects of all ages combined (p = 0.095).

Conclusion: The identification of a difference in a blood leukocyte DNA methylation site between hypertensive cases and normotensive controls suggests that changes in DNA methylation may play an important role in the pathogenesis of hypertension. The age dependency of the effect further suggests complexity of epigenetic regulation in this age-related disease.

Introduction

Essential hypertension (EH) is a major health problem worldwide with approximately one in three adults suffering from the disease. Although twin and family studies highlight a clear inherited component to EH [1], the current purely sequence-based approach only accounts for a fraction of the genetic risk of the disease as evidenced by the recent genome-wide association studies in which the identified genetic variants explain less than 1% of the blood pressure (BP) variation in the population [2]. Several epidemiological and clinical peculiarities of EH such as the incomplete concordance between monozygotic (MZ) twins (ranges from 38% to 52%) [3,4] and its late onset and progressive nature, are difficult to explain with traditional DNA sequence-based approaches. These observations may indicate the involvement of epigenetic factors in EH development. Epigenetics refers to all meiotically and mitotically heritable changes in gene expression that are not coded in the DNA sequence itself. DNA methylation is an important epigenetic modification and can play a significant regulatory role in both normal and pathological cellular processes. Emerging evidence from animal studies [5,6,7,8] suggests a key role for methylation in the pathogenesis of EH. However, to date, very few studies [9,10] have investigated the role of methylation in the development of human EH, and none has taken a genome-wide approach. Based on recent studies [11,12] that highlight the involvement of inflammation in the development of EH, we hypothesized that changes in the DNA methylation of leukocytes are involved in the pathogenesis of EH. The goal of this study was to characterize the DNA methylation profile in peripheral blood leukocytes in EH cases versus normotensive controls using a 3-stage genome-wide approach.
Methods

Subects
A total of 44 EH cases and 68 age (±2 years) matched controls were selected from 4 existing cohorts in Georgia Prevention Institute, Georgia Health Sciences University using the following inclusion criteria: (1) African American (AA) ancestry; (2) male; (3) having leukocyte DNA available; (4) EH cases have age, sex, and height adjusted systolic BP (SBP ≥95th percentile if the age of the subject is less than 20 years), or SBP ≥140 mmHg; while controls have age, sex and height adjusted SBP ≤20th percentile, or have SBP levels <120 mmHg. These 4 cohorts include the BP stress study (n = 603) [13], the Georgia Cardiovascular twin study (n = 1183) [14], the Lifestyle, Adiposity, and Cardiovascular Health in Youth (LACHY) study (n = 740) [15], and the Prevention of Hypertension in African American Teens (PHAT) study (n = 262) [16]. Both the BP Stress study and the twin study are ongoing longitudinal studies which have followed the subjects for more than 10 years. Both studies included relatively equal numbers of AA and European Americans (EA) or males and females. The BP stress study was established in 1989 with subjects aged 7–16 years at baseline [13] and the Georgia Cardiovascular Twin study was established in 1996 with subjects aged 7–25 years at baseline [14]. LACHY and PHAT are cross-sectional studies. The LACHY study [15] consisted of roughly equal numbers of AA and EA adolescents aged 14–18 years of both sexes and the PHAT study [16] consisted of AA males and females aged 14–20 years. For the subjects from the BP Stress and the Georgia Cardiovascular Twin study, if multiple visits (with multiple leukocyte DNA) were available for a subject, the leukocyte DNA collected at the visit when the subject had the highest (for cases) or lowest (for controls) SBP was used. For the subjects from the twin study, only one twin from a pair was selected if both twin and co-twin met the criteria.

Subjects in all the 4 studies were recruited from Augusta, GA area. For all four cohorts self identification by self-reports of each subject or by a parent if the subject was under 18 years of age was used to classify ethnicity according to previously described criteria [17]. Subjects in all the 4 studies were overtly healthy, free of any acute or chronic illness on the basis of parental reports and were not on anti-hypertensive, lipid lowering, anti-diabetic and anti-inflammatory medications. For the young adult cohort, the exclusion criteria included secondary HBP, history of diabetes, renal disease, heart failure, autoimmune disease, sickle cell anemia, or endocrine disorders.

Measurements
For all the four Georgia cohorts, height and weight were measured by standard methods using a wall-mounted stadiometer and a scale, respectively. Body mass index (BMI) was calculated as weight/height^2. SBP and diastolic BP (DBP) were measured with Dinamap monitors, using an appropriately sized BP cuff placed on the subject’s right arm. BP measurements were taken at 11, 13, and 15 minutes, during a 15-minute supine relaxation period. The average of the last 2 readings was used to represent SBP and DBP values [13,14,15,16].

Fasting peripheral blood samples in the LACHY cohort and non-fasting peripheral blood samples in the other three cohorts were collected. The buffy coat and plasma samples were separated and stored at −80°C. DNA was extracted from the buffy coat.

For the two cohorts in Pennsylvania, BP was measured, in the seated position, by auscultation. The average of eight separate BP measurements obtained at two separate visits (four measurements at each visit) was used to represent BP values. Fasting peripheral blood samples were collected and DNA was extracted from the buffy coat [18,19].

Genome-wide Methylation Chip
The HumanMethylation27 BeadChip from Illumina (Illumina, San Diego, CA, USA) was used. This chip can quantitatively measure 27,000 CpG sites, covering more than 14,000 well-annotated genes at single-CpG resolution. Each chip can accommodate 12 samples. After bisulfite treatment, 200 ng of the converted DNA was whole genome amplified (WGA) and enzymatically fragmented. The bisulfite-converted WGA-DNA samples were purified and applied to the BeadChips. Image processing and intensity data extraction were performed according to Illumina’s instructions (www.illumina.com/products/infinium_humanmethylation27_beadchip_kits.ilmn). Each methylation data point is represented by fluorescent signals from the methylated and unmethylated alleles. DNA methylation beta values are continuous variables between 0 (completely unmethylated) and 1 (completely methylated), representing the ratio of the intensity of the methylated bead type to the combined locus intensity. Initial array processing and quality control were performed with BeadStudio software. The microarray data discussed in this paper have been deposited in NCBI's Gene
Pyrosequencing

The methylation levels of the top CpG sites from the 2 genes selected for replication were determined by pyrosequencing technology, a rapid and robust method for quantitative methylation analysis. After bisulfite treatment, 10 ng of the converted DNA was used in a PCR reaction to amplify the target region. One of the PCR primers was biotin labeled. Single-stranded biotinylated PCR products were prepared for sequencing by use of the Pyrosequencing Vacuum Prep Tool according to the manufacturer’s instructions. The PCR products (each 10 μl) were sequenced by Pyrosequencing PSQ96 HS System (Pyrosequencing-Qiagen) following the manufacturer’s instructions. The methylation status of each locus was analyzed individually as a T/C SNP using Q CpG software (Biotage, Kungsgatan, Sweden). PCR primers and sequencing primers for the 2 genes selected for replication are available upon request.

Statistical Analysis

To identify genome wide methylation differences between EH cases and controls, the LIMMA (Linear Models for Microarray Analysis) package from the Bioconductor project [20] was used. LIMMA uses an empirical Bayes approach that uses the variability in all genes for testing for significant differences, which results in more stable inferences for a relatively small number of arrays. We used a design matrix of a paired test to analyze each CpG site for differential methylation. Each CpG site was assigned a raw p-value based on a moderated t statistic. To correct for multiple testing, the set of raw p-values were converted to false discovery rates (FDR) according to Benjamini and Hochberg [21]. For the replication cohort, the methylation levels of the CpG sites were square root-transformed to obtain a better approximation of the normal distribution prior to analysis. A Student’s t-test was used to investigate whether their methylation levels differed between cases and controls. Linear regression was further used to adjust for the potential effect of age and BMI. We combined the replication steps as well as the genome wide step on the CpG sites carried to the validation in the replication cohort. The general characteristics of the first replication cohort are displayed in Table 3. Although the pyrosequencing assays were designed to target one specific CpG site for each gene [Illumina ID cg04845379 for SULF1 and cg00772827 for PRCP], both assays covered several surrounding CpG sites. For the SULF1 gene, methylation levels on 4 CpG sites were obtained with CpG2 as the target CpG site. All these 4 CpG sites were observed only in the replication cohort. The significant result of the first replication cohort was further validated in the meta-analysis on the discovery panel. The significant result of the first replication which only comprised subjects ≤30 years old, we further split the sample by age (≤30 years or >30 years). The general characteristics of the split samples were also listed in Table 5. The differences in methylation status of these 4 CpG sites in the SULF1 gene between cases and controls in the replication cohort are shown in Table 4. None of these CpG sites showed a significant difference in their methylation levels between cases and controls in the overall sample or in the samples split by age. Meta-analysis on the CpG1 and CpG2 with the two replication panels was conducted and the results are shown in Table 7. Significant higher methylation levels of CpG1 & CpG2 were observed in cases (p = 0.014 and p = 0.011, respectively) in the meta-analysis on the first replication cohort and the young age group of the second replication cohort. The significant result remained after adjustment of age (p = 0.017 and p = 0.015, respectively) or age and BMI (p = 0.030 and p = 0.037, respectively). Further meta-analysis with the discovery panel on CpG2 showed a p value of 0.0051 (with p = 0.0027 after adjustment of age and BMI) in both the subjects and a p value of 0.0004 (with p = 0.0004 after adjustment of age and BMI) in subjects younger or equal to 30 years old.

Gene Ontology analysis was performed to test whether some common functional trends in molecular functions and biological processes were associated with the genes exhibiting differences between cases and controls for the genome-wide methylation analyses. We included those genes with a raw p-value ≤0.01 in the first list (n = 226) and included all the other genes in the second list. As expected from a pilot study in 8 cases and 8 controls, we did not observe any CpG sites survive multiple testing corrections with the most significant CpG site showing a FDR of 0.75 and a raw p value of 6.2 × 10⁻³. Table 2 lists the top 10 most significant CpG sites. Out of the 10 CpG sites, we selected the top 2 CpG sites (one CpG site in the sulfatase 1 gene [SULF1] and one CpG site in the prolylcarboxypeptidase gene [PRCP]) for validation in the replication cohort. The general characteristics of the first replication cohort are displayed in Table 3. Although the pyrosequencing assays were designed to target one specific CpG site for each gene [Illumina ID cg04845379 for SULF1 and cg00772827 for PRCP], both assays covered several surrounding CpG sites. For the SULF1 gene, methylation levels on 4 CpG sites were obtained with CpG2 as the target CpG site. All these 4 CpG sites were observed in cases (p = 0.040 and 0.046, respectively). The results remained significant after adjustment for age (p = 0.041 and 0.038, respectively) but became non-significant after further adjustment for BMI (p = 0.074 and 0.081, respectively). For the PRCP gene, methylation levels on 8 CpG sites were obtained with CpG6 as the target CpG site. All these 8 CpG sites were observed in cases (p = 0.040 and 0.046, respectively). The results remained significant after adjustment for age (p = 0.041 and 0.038, respectively) but became non-significant after further adjustment for BMI (p = 0.074 and 0.081, respectively). For the PRCP gene, methylation levels on 8 CpG sites were obtained with CpG6 as the target CpG site. All these 8 CpG sites were observed in cases (p = 0.040 and 0.046, respectively). The results remained significant after adjustment for age (p = 0.041 and 0.038, respectively) but became non-significant after further adjustment for BMI (p = 0.074 and 0.081, respectively). For the PRCP gene, methylation levels on 8 CpG sites were obtained with CpG6 as the target CpG site. All these 8 CpG sites were observed in cases (p = 0.040 and 0.046, respectively). The results remained significant after adjustment for age (p = 0.041 and 0.038, respectively) but became non-significant after further adjustment for BMI (p = 0.074 and 0.081, respectively). For the PRCP gene, methylation levels on 8 CpG sites were obtained with CpG6 as the target CpG site. All these 8 CpG sites were observed in cases (p = 0.040 and 0.046, respectively). The results remained significant after adjustment for age (p = 0.041 and 0.038, respectively) but became non-significant after further adjustment for BMI (p = 0.074 and 0.081, respectively).
observe any GO categories survive multiple testing. Table S5 list the GO categories with raw P value less than 0.05. Interestingly, we observed enriched functional processes that are potentially relevant for inflammation with response to biotic stimulus (GO:0009607), response to other organism (GO:0051707), interleukin-1 production (GO:0032612), and interleukin-13 production (GO:0032616) among the top GO categories. The results are consistent with the involvement of inflammation and oxidative stress in the development of EH.

Discussion

In this study we aimed to identify methylation differences in peripheral blood leukocytes between EH cases and controls using a genome-wide approach in male AA youth and young adults. We observed increased methylation levels at two CpG sites in the SULF1 gene in EH cases in comparison with normotensive controls in subjects equal or younger than 30 years.

Our study is the first genome wide methylation study on EH. In fact, there are very few human studies that explored the role of epigenetics on the risk of EH. In one study, Friso et al [9] measured promoter methylation of the HSD11B2 gene in peripheral blood mononuclear cells from patients with EH and 32 subjects on prednisone therapy. Elevated HSD11B2 promoter methylation was associated with decreased HSD11B2 activity and EH development in glucocorticoid-treated patients. In a recent study by Smolarek et al [10] global DNA methylation level indexed by the genome level of 5-methylcytosine was significantly lower in patients with EH in comparison with controls.

The protein encoded by the SULF1 gene is Sulfatase 1 (Sulf1). It is a cell surface polypeptide that can rapidly modify the sulfation status of heparin sulfate proteoglycans (HSPGs), resulting in changes in HSPG-related signal transduction pathways [24]. Sulf1 has been reported to be down-regulated in several human cancers [25,26]. Absence of Sulf1 in cancer cell lines is associated with increased cell growth, proliferation and reduced cell apoptosis [25]. In addition to cancer, Sulf1 was also studied with respect to normal development including neural, muscular, vascular and skeletal development. However, there is no direct study on Sulf1

Table 1. General characteristics of cases and controls for genome-wide methylation analysis.

|                  | Cases                  | Controls               |
|------------------|------------------------|------------------------|
| N                | 7                      | 7                      |
| Age, years       | 18.0±3.1 [14.8–23.3]   | 18.3±3.2 [14.8–23.0]   |
| BMI, kg/m²       | 27.3±3.5 [21.2–31.5]   | 23.8±5.5 [17.8–33.2]   |
| SBP, mmHg        | 145.8±4.7 [137.3–149.5]| 107.6±3.3 [103.3–114]  |
| SBP percentile   | 0.99±0.01 [0.96–1.0]    | 0.16±0.04 [0.10–0.19]  |
| DBP, mmHg        | 69.1±6.8 [62.0–78.3]   | 55.4±5.4 [49.5–63.3]   |
| DBP percentile   | 0.45±0.22 [0.25–0.89]   | 0.14±0.10 [0.01–0.26]  |

Means±SD [Range].
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and EH. SULF1 and SULF2 (another protein in this family) double knockout mice show impairment in skeletal development [27], but whether they display high blood pressure has never been explored.

In this study, we observed that the methylation levels of 2 CpG sites in the promoter region of the SULF1 gene were higher in EH cases than in controls. This is in line with the previous studies [28,29] in cancers in which epigenetic silencing is involved in the down-regulation of Sulf1. The SULF1 gene spans a ~211 kb genomic fragment on chromosome 8q13.3 with 23 exons [25]. Staub et al. [29] observed that methylation of 12 CpG sites within SULF1 exon 1A was associated with ovarian cancer cells and primary ovarian cancer tissues lacking Sulf1 expression. This region is about 10 kb downstream of the promoter region we targeted. The relationship between the methylation level of this region and the promoter region is unknown. No CpG island exists targeted. The relationship between the methylation level of this region is about 10 kb downstream of the promoter region we found to high BP earlier [27].

Table 2. Top 10 differentially methylated CpG sites.

| Gene  | Illumina ID | Distance to TSS | CpG island | Methylation, % | P      | FDR  |
|-------|-------------|-----------------|------------|----------------|--------|------|
| SULF1 | cg0485579   | 186             | NO         | 29.49          | 22.79  | 6.70 | 0.000062 0.75 |
| PRCP  | cg09772827  | 346             | YES        | 13.85          | 17.87  | -4.03 | 0.000098 0.75 |
| NEUROG1 | cg14958635 | -               | YES        | 7.82           | 11.24  | -3.41 | 0.000134 0.75 |
| PITPN1 | cg11719157  | 630             | YES        | 61.38          | 57.51  | 3.87 | 0.000182 0.75 |
| SLC26A10 | cg14377150 | 222             | NO         | 22.04          | 27.33  | -5.30 | 0.000209 0.75 |
| CDC34 | cg27431859  | 691             | YES        | 10.43          | 13.45  | -3.02 | 0.000315 0.75 |
| C9orf95 | cg07962315  | 1375            | YES        | 37.50          | 42.83  | -5.33 | 0.000321 0.75 |
| YWHAQ | cg06701500  | 565             | YES        | 13.79          | 20.26  | -6.47 | 0.000334 0.75 |
| SIRT7 | cg15118204  | 191             | YES        | 19.48          | 21.89  | -2.41 | 0.000348 0.75 |
| CLDN5 | cg0443638   | 148             | NO         | 75.20          | 72.27  | 2.93 | 0.000483 0.75 |

Table 3. General characteristics of the subjects in GA cohort (1st replication panel).

|         | Case          | Control        |
|---------|---------------|----------------|
| N       | 36            | 60             |
| Age, years | 20.6±5.3 [14.3–30.7] | 19.6±4.5 [14.1–30.9] |
| BMI, kg/m² | 29.9±6.7 [21.0–52.4] | 24.7±8.0 [16.5–59.9] |
| SBP, mmHg | 143.6±7.5 [133.3–175] | 106.9±5.2 [93.7–117] |
| SBP percentile | 0.98±0.01 [0.96–1.00] | 0.12±0.06 [0.02–0.20] |
| DBP, mmHg | 71.9±10.4 [56.5–96.5] | 58.6±5.6 [46–74.5] |
| DBP percentile | 0.50±0.23 [0.18–0.98] | 0.20±0.13 [0.03–0.67] |

Table 4. Pyrosequencing results of SULF1 gene in GA cohort.

|         | Case          | Control        |
|---------|---------------|----------------|
| P       | 0.040         | 0.041          |
| P, adjusted * | 0.074         | 0.074          |
| Cpg1    | 16.8±9.0      | 13.4±7.5       |
| Cpg2    | 22.7±9.4      | 19.0±8.6       |
| Cpg3    | 5.9±3.04      | 4.8±3.22       |
| Cpg4    | 20.6±9.1      | 17.8±7.9       |

*Adjusted for age.

Means ± SD [Range].

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cellular RNA is not available for the samples used in the current study, which were selected from several existing cohorts. We searched the GEO database and identified a dataset [37] (GSE3846) which included genome wide gene expression data in peripheral blood samples in six healthy volunteers tested by Affymetrix microarrays. In all the 6 samples, the expression of SULF1 was detectable. The average expression value of SULF1 is 4.38 at baseline. The average rank order of expression measure-

Table 5. General characteristics of the subjects in PA cohort (2nd replication panel).

|                | All subjects | Subjects older than 30 | Subjects younger or equal to 30 |
|----------------|--------------|------------------------|-------------------------------|
|                | Case         | Control                | Case                         | Control            |
| Age, years     | 39.3±7.6     | 35.6±9.7               | 41.8±3.8                     | 40.8±3.7          |
| Age range, years| 16.8-49      | 15.8-47                | 33-49                        | 34-47             |
| BMI, kg/m²     | 29.5±6.2     | 28.5±5.7               | 29.4±6.2                     | 28.4±5.6          |
| SBP, mmHg      | 149.5±16.3   | 108.3±6.4              | 149.8±17.4                   | 108±6.7           |
| DBP, mmHg      | 94.6±12.3    | 68.1±6.1               | 96.8±10.3                    | 69±6.1            |
| Antihypertensive| 25%          | 0%                     | 29%                          | 0%                |
| Medication     |              |                        |                               |                   |

Means±SD.

Table 6. Pyrosequencing results of SULF1 gene in the PA cohort.

|                | Case        | Control     | P     | P, adjusted* | P, adjustedb |
|----------------|-------------|-------------|-------|--------------|--------------|
| Overall        |             |             |       |              |              |
| CpG1           | 12.5±6.2    | 12.1±5.6    | 0.77  | 0.61         | 0.65         |
| CpG2           | 19.7±7.0    | 18.9±6.7    | 0.64  | 0.51         | 0.53         |
| CpG3           | 5.3±2.1     | 5.6±1.9     | 0.59  | 0.77         | 0.69         |
| CpG4           | 20.9±7.3    | 21.0±7.9    | 0.97  | 0.94         | 0.96         |
| Older than 30  |             |             |       |              |              |
| CpG1           | 11.9±6.4    | 12.0±5.9    | 0.81  | 0.88         | 0.80         |
| CpG2           | 19.1±7.3    | 18.6±7.3    | 0.85  | 0.81         | 0.86         |
| CpG3           | 5.1±2.1     | 5.6±2.2     | 0.39  | 0.41         | 0.35         |
| CpG4           | 20.3±7.3    | 21.1±7.9    | 0.70  | 0.70         | 0.66         |
| Younger or equal to 30 |             |             |       |              |              |
| CpG1           | 16.3±2.7    | 12.5±4.9    | 0.13  | 0.18         | 0.16         |
| CpG2           | 23.6±3.3    | 19.7±4.6    | 0.12  | 0.20         | 0.25         |
| CpG3           | 6.9±1.9     | 5.7±1.0     | 0.13  | 0.17         | 0.22         |
| CpG4           | 24.9±6.4    | 20.8±4.2    | 0.17  | 0.23         | 0.21         |

*Adjusted for age.

Table 7. Meta -analysis on two CpG sites in the SULF1 gene.

|                | P          | P, adjusted* |
|----------------|------------|--------------|
| CpG1 GA cohort+PA cohort | 0.080 | 0.059 |
| GA cohort+PA cohort (age ≤30) | 0.014 | 0.017 |
| CpG2 GA cohort+PA cohort | 0.095 | 0.058 |
| GA cohort+PA cohort (age ≤30) | 0.011 | 0.015 |
| Discovery+Replication | 0.0051 | 0.0027 |
| Discovery+Replication (age ≤30) | 0.0004 | 0.0004 |

*Adjusted for age.

In this study, we used the DNA from leukocytes, which represent different cell populations with distinct epigenetic profiles [38]. A recent study by Kelsey's group [39] indicated that shifts in leukocyte subpopulations may account for a considerable proportion of variability in peripheral blood DNA methylation of diseases such as head and neck squamous cell carcinoma and ovarian cancer. This study also provided a list of the top 50 differentially methylated CpG sites among 6 leukocyte subtypes including CD4+ T cells, CD8+ T cells, CD56+ NK cells, B cells, monocytes and granulocytes. The SULF1 gene CpG site is not within the list. Another study [40] from the same group developed an algorithm which predicts the distributions of the 6 leukocyte subtypes using illumina 27 k methylation data from peripheral blood DNA. We applied this algorithm to our data and did not observe difference in the distributions of these 6 cell types between EH cases and controls (Table S6). Therefore, it is highly unlikely that our significant finding on the SULF1 gene is caused by shifts in these 6 leukocyte subpopulations. On the other hand, it is plausible that only the change in the epigenetic profile of one specific cell type is related to EH. In this case, the actual epigenetic differences might be more substantial than reported here but only present in this specific blood leukocyte cell type. Future studies on epigenetic profiling of various types of cell populations of leukocytes are warranted to gain a greater understanding of the epigenetic dysregulation in EH.

Two strengths of our study deserve mentioning. First, we selected controls with low blood pressure, which maximizes the power to make discoveries. Second, a hypothesis free genome-wide approach was used. This approach supersedes the limitations...
imposed by candidate gene methylation studies and allows searching the whole genome in an unbiased manner.

Interpretation of these data is also limited by several additional constraints. First, in this study we aimed to identify EH related methylation changes. However, our study design cannot determine whether the identified methylation changes are the cause or the consequence of EH. Future studies on subjects with baseline DNA and follow-up for de novo development of EH will be needed to resolve causality [41]. Second, because obesity is an important risk factor for EH, patients often have higher BMI levels than normotensive controls. Obesity might be a confounder explaining the relation between methylation levels of the SULT1E1 gene and EH. In the first replication cohort, the association of the SULT1E1 gene CpG1 and CpG2 with EH attenuated and became nonsignificant after adjustment of BMI. Therefore, in the second replication cohort, controls were selected to match with cases on obesity status (normal weight/overweight/obese). We could not replicate the findings from the first 2 stages in the overall analysis. In addition to BMI, this discrepancy might also be due to the age difference between the second replication cohort and the cohorts used in the first 2 stages. Moreover, it is also possible that SULT1E1 gene methylation is a mediator of obesity related EH. In this case, including BMI as a covariate in the analysis or matching cases and controls on obesity status will lead to over-adjustment. Future studies on hypertensive subjects with normal weight are needed to clarify whether SULT1E1 gene methylation independently affects EH. Third, in the current study, the Infinium HumanMethylation27 Beadchip was selected because of its quantitative measure at each CpG site. However, the limited coverage of this genome-wide chip will restrict the findings to certain CpG sites within certain genes. Future studies should use chips with more complete coverage of the genome such as the recently released 450 K Infinium Methylation BeadChip from Illumina. Fourth, the current study is a pilot study with the genome-wide step conducted only in 7 EH cases and 7 normotensive controls and one MZ pair discordant for EH. Future studies with much larger sample size are warranted to discover a more complete profile of EH related methylation changes.

In conclusion, we identified a reproducible change in DNA methylation of peripheral blood leukocytes between EH cases and controls in subjects ≤50 years. It provides preliminary evidence that DNA methylation may play an important role in the pathogenesis of EH. Further studies are warranted to determine the causal direction of this relationship.

Supporting Information

Table S1 General characteristics of the MZ pair discordant for EH. (DOCX)

Table S2 Replication results for PRCP gene. (DOCX)

Table S3 Correlation among the CpG sites in the SULT1E1 gene. (DOCX)

Table S4 Correlation among the CpG sites in the PRCP gene. (DOCX)

Table S5 Gene-Ontology analysis. (DOCX)

Table S6 Cell population estimates in cases vs. controls. (DOCX)

Author Contributions

Conceived and designed the experiments: XW BF SS H. Snieder. Performed the experiments: HZ H. Shi. Analyzed the data: XW SS AS XX. Contributed reagents/materials/analysis tools: FT BG GH YD XX. Wrote the paper: XW BF SS H. Snieder XX.

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