RESEARCH ARTICLE OPEN ACCESS

DNA Methylation of the Natriuretic Peptide System Genes and Ischemic Stroke

Gene-Based and Gene Set Analyses

Hao Peng, PhD,* Yiming Fan, MBBS,* Jing Li, MD, Xiaowei Zheng, PhD, Chongke Zhong, PhD, Zhengbao Zhu, PhD, Yan He, PhD, Mingzhi Zhang, PhD, and Yonghong Zhang, PhD

Correspondence
Dr. Zhang
yhzhang@suda.edu.cn

Neurol Genet 2022;8:e679. doi:10.1212/NXG.0000000000000679

Abstract

Background and Objectives

The natriuretic peptide (NP) system has been considered an important regulator for ischemic stroke (IS) with a limited clinical implication. A better understanding of the underlying molecular mechanisms is urgent. Here, we aimed to examine the role of DNA methylation of NP system genes in IS.

Methods

DNA methylation at promoter regions of 4 core NP system genes, e.g., *CORIN*, *FURIN*, *NPPA*, and *NPPB*, was measured by targeted bisulfite sequencing in 853 patients with IS and 918 controls. We first examined the association between DNA methylation at each single CpG and IS, followed by gene-based and gene set analyses to examine the joint associations of DNA methylation at multiple CpGs in a gene or all 4 genes as a pathway with IS.

Results

After control of covariates and multiple testing, DNA methylation at 19 of the 36 assayed CpGs was individually associated with IS at $q < 0.05$. Higher average methylation levels at the targeted regions of *CORIN* (odds ratio [OR] = 0.64, 95% confidence interval [CI]: 0.56–0.73), *FURIN* (OR = 0.78, 95% CI: 0.69–0.88), and *NPPA* (OR = 0.78, 95% CI: 0.69–0.88) were associated with a lower odds of IS (all $q < 0.05$). The truncated product method revealed the same gene-based associations (all $q < 0.05$) and found that DNA methylation at all 4 NP system genes together was jointly associated with IS ($p = 0.0001$).

Discussion

DNA methylation at NP system genes was downregulated in patients with IS. Our results may unravel a molecular mechanism underlying the regulating effect of the NP system on IS and highlight the relevance of testing the joint effect of multiple CpGs in the epigenetic analysis.

*These authors contributed equally to this work and should be considered as co–first authors.

From the Department of Epidemiology (H.P., J.L., X.Z., C.Z., Z.Z., Y.H., M.Z., Y.Z.), School of Public Health, Medical College of Soochow University; Jiangsu Key Laboratory of Preventive and Translational Medicine for Geriatric Diseases (H.P.); and Medical College of Soochow University (Y.F.), Suzhou, China.

Go to Neurology.org/NG for full disclosures. Funding information is provided at the end of the article.

The Article Processing Charge was funded by the authors.

Glossary

ANP = atrial natriuretic peptide; **BMI** = body mass index; **BNP** = brain natriuretic peptide; **CATIS** = China Antihypertensive Trial in Acute Ischemic Stroke; **CI** = confidence interval; **CNP** = C-type natriuretic peptide; **CVD** = cardiovascular disease; **DBP** = diastolic blood pressure; **EWAS** = epigenome-wide association study; **FDR** = false discovery rate; **GTEx** = Genotype-Tissue Expression; **HDL-C** = high-density lipoprotein cholesterol; **IS** = ischemic stroke; **LDL-C** = low-density lipoprotein cholesterol; **NP** = natriuretic peptide; **OR** = odds ratio; **PMMS** = Prevention of Metabolic syndrome and Multi-metabolic disorders Study; **SBP** = systolic blood pressure; **wTPM** = weighted truncated product method.

Cardiac natriuretic peptides (NPs) play relevant hemodynamic and antiremodeling actions in the cardiocerebral vascular system due to their biological properties of natriuresis, diuresis, vasodilation, energy homeostasis, antiproliferation, antihypertrophy, and anti-inflammation.[1-3] These hormones have been demonstrated to be useful predictors for risks of stroke[4,5] and other cardiovascular diseases (CVD)[6-8] in various populations. Admittedly, some drugs based on the NP system, e.g., carperitide, have been produced and used in clinical settings to treat acute heart failure,[9,10] but could cause some unfavorable side reactions, e.g., severe hypotension[10-12] and in-hospital death.[13-15] Furthermore, the clinical translation of the NP system to stroke, a main constituent of CVD, is also limited. The molecular mechanisms underneath the association between NPs and stroke are still incompletely understood, which would undoubtedly assist their clinical development as drug agents or targets against ischemic stroke (IS), the most prevalent cerebral vascular disorder and a leading cause of long-term disability worldwide.

The NP family includes 3 well-characterized hormones—atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), C-type natriuretic peptide (CNP),[16] and the related protease convertases (furin and corin).[17,18] Sequence variants in their coding genes, e.g., *NPPA* (the gene coding ANP),[19,20] *NPPB* (the gene coding BNP),[21] *CORIN* (the gene coding corin),[22,23] and *FURIN* (the gene coding furin)[24,25] but not *NPPC* (the gene coding CNP), have previously been related to susceptibility to IS and its main risk factors, e.g., hypertension and atherosclerosis. Although these studies highlight the contributions of genetic variants in the NP system to the risks of IS, additional underlying molecular mechanisms remain to be elucidated. The transcripts for *NPPA*,[26] *CORIN*,[27] and *FURIN*[28] have been associated with cardiac disease, although the transcription of NP system genes in IS is limited studied. As a mediator between genome and environment, epigenetic factors such as DNA methylation in the NP system may affect its function and subsequent biological activities, thereby representing a candidate molecular mechanism underlying the role of NPs in IS. In fact, dysregulation of DNA methylation has been associated with IS.[29,30] For example, global DNA methylation[31] and promoter methylation at some candidate genes, e.g., major histocompatibility complex,[32] methylenetetrahydrofolate reductase,[33] apolipoprotein E,[34] and matrix metalloproteinase-2,[35] were previously associated with IS in small sampled clinical studies. Therefore, we hypothesized that DNA methylation of the NP system genes may be associated with IS, but this has not been studied in humans.

Furthermore, epigenetic studies typically examined the individual effect of a single CpG methylation, which may be very small. As such, only testing the effect of a single CpG methylation on a complex phenotype such as IS may be insufficient. Examination of the joint effects of DNA methylation at multiple CpG sites in variate genes in an identical biological pathway could be welcome in uncovering the epigenetic basis of IS. Applying a gene set approach, this study examined the joint association of DNA methylation at 36 CpG loci in 4 core NP system genes (*NPPA*, *NPPB*, *CORIN*, and *FURIN*) with IS in 853 patients with IS and 918 age- and sex-matched healthy controls. As a modifiable molecular modification that regulates gene expression, DNA methylation at the loci identified may provide the basis for the development of innovative approaches in the prevention and treatment of IS.

Methods

Study Participants

Selection of Cases

The China Antihypertensive Trial in Acute Ischemic Stroke (CATIS) is designed to test whether blood pressure reduction within the first 48 hours after the onset of an acute IS would reduce death and major disability at 14 days or hospital discharge.[36] After providing written informed consent, 4,071 patients aged over 22 years with first-ever IS were recruited. Among them, 3,013 patients provided their blood samples and agreed to participate in the subsequent genetic studies with the voluntary principles. Of the 3,013 patients with available DNA samples, 1,000 patients were randomly selected as cases of the present study.

Selection of Controls

A number of 1,000 controls were selected by frequency matching on age and sex from the 3,999 community individuals free of CVD and with available DNA samples participating in the Prevention of Metabolic syndrome and Multi-metabolic disorders Study (PMMS),[37] a community-based prospective cohort study of CVD and its risk factors in Chinese adults.

The selection of study participants of our study was illustrated in Figure 1. After excluding 229 samples (147 cases and 82 controls) failed in methylation quantification, a total of 1,771 participants including 853 IS cases and 918 age- and sex-matched controls were included in the final analysis.

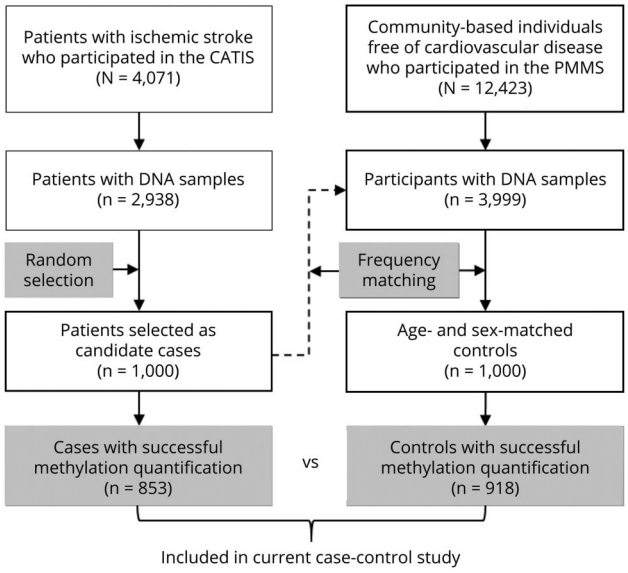
Standard Protocol Approvals, Registrations, and Patient Consents

The study protocols of CATIS were approved by the institutional review boards at Soochow University in China and Tulane University in the United States. The protocols of the PMMS were approved by the Soochow University Ethics Committee. Written informed consent was obtained from all study participants.

Quantification of DNA Methylation

Using genomic DNA isolated from peripheral blood, DNA methylation levels of 4 core NP system genes including *NPPA*, *NPPB*, *CORIN*, and *FURIN* were quantified by targeted bisulfite sequencing.[38,39] This method has been used in validation of DNA methylation status identified by reduced representation bisulfite sequencing [40] and the Illumina HumanMethylation 450K BeadChip.[41] The genomic coordinates, targeted sequences, and primers for sequencing were shown in eTable 1 (links.lww.com/NXG/A524). DNA samples were bisulfite treated using the EZ DNA Methylation-Gold Kit (Zymo Research, Inc., Irvine, CA) following the manufacturer's protocol. The treated samples were then sequenced by Illumina HiSeq 2000 (Illumina, Inc., San Diego, CA). The bisulfite-treated reads were mapped to the genome, and methylation calling was processed by using BS-Seeker2.[42] For quality control, non-CpG cytosines were applied as internal control to monitor the efficiency of bisulfite conversion. The percentage of the converted non-CpG cytosines was calculated as conversion rate of each DNA sample. The samples with a bisulfite conversion rate <98% were excluded. We then calculated the average coverage as well as the missing rate for each CpG site and those with average coverage less than 20× were further filtered out. A total of 36 CpG sites in the *CORIN* (9 CpGs), *FURIN* (7 CpGs), *NPPA* (9 CpGs), and *NPPB* (11 CpGs) genes were finally assayed and included in analysis.

Figure 1 Flowchart Illustrating the Selection of Study Participants



CATIS = China Antihypertensive Trial in Acute Ischemic Stroke; PMMS = Prevention of Metabolic syndrome and Multi-metabolic disorders Study.

Assessment of Risk Factors

As detailed in eMethods (links.lww.com/NXG/A524) demographic data, lifestyle factors, body mass index (BMI), blood pressure, fasting glucose, and blood lipids were collected.

Statistical Analysis

Clinical characteristics of study participants were presented in patients with IS and healthy controls, respectively. All statistical analyses were performed using SAS statistical software (version 9.4; SAS Institute, Cary, NC).

Single CpG Association Analysis

The Student *t* test was applied to examine the difference in methylation levels at CpG sites between IS cases and healthy controls. To examine the association between DNA methylation at each CpG locus and IS, we constructed a logistic regression model in which prevalent IS (y/n) was the dependent variable and DNA methylation at each CpG site was the independent variable, adjusting for age, sex, education level, cigarette smoking, alcohol consumption, BMI, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), hypertension, and diabetes. Multiple testing was controlled by adjusting for the total number of CpG loci tested using false discovery rate (FDR) approach, and an FDR-adjusted p value (i.e., q value) of less than 0.05 was considered statistically significant.

Gene-Based Association Analysis

To examine whether DNA methylation at multiple CpG sites was jointly associated with IS, we treated the average methylation level of multiple CpG sites in a gene as a substitute for the methylation level of the targeted region and similarly examined its association with prevalent IS. The rationale of using the average methylation level to present the regional methylation is the highly correlation among neighboring CpG sites as shown in eFigure 1 (links.lww.com/NXG/A524). We also used the weighted truncated product method (wTPM) as previously described,[43] based on the results of the single CpG association analyses. This method combines p values of all CpGs in a chosen gene that reaches a preselected threshold (e.g., raw $p < 0.1$ in this study). The regression coefficient of each individual CpG methylation was included as weights in the wTPM statistic. Multiple testing for gene-based analysis was controlled by adjusting for the total number of genes.

Gene Set Association Analysis

To test whether DNA methylation of the NP system genes affects IS, we similarly performed the wTPM by including all genes with a raw gene-based p value of less than 0.1. The weight was set as 1 for every gene in the model. The wTPM has been evaluated by simulation studies[44] and applied to epigenetic analysis.[39,45] The Genotype-Tissue Expression (GTEx) database was applied to examine whether the *CORIN*, *FURIN*, *NPPA*, and *NPPB* genes were expressed in white blood cells and target organs of IS, e.g., artery and heart. The integrative DNA methylation (iMethyl) database was applied to examine whether the CpG sites assayed presented in white blood cells and regulated gene expression.[46]

Data Availability

The data sets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Dryad doi: 10.5061/dryad.tqjq2bw15, datadryad.org/stash/share/FhZdX3QGYvUwoSS3C2jnrl088vJhhkDrwkMSknJQBcc.

Results

Clinical Characteristics

A total of 853 patients with prevalent IS (mean age 62 years, 53% men) and 918 age- and sex-matched healthy controls (mean age 61 years, 55% men) were included in the current study. Their clinical characteristics were presented in Table 1. As expected, cases of IS had more metabolic risk factors, such as hypertension, diabetes, dyslipidemia, and obesity than their healthy controls (all $p < 0.05$). We did not find any significant differences in cigarette smoking and alcohol consumption between the 2 groups.

Single CpG Association Between DNA Methylation of NP System Genes and IS

Table 2 presents the DNA methylation levels of 4 core NP system genes in patients with IS and their healthy controls. Of the 36 CpG sites assayed, DNA methylation levels at 25 CpG sites seemed to be lower in patients with IS than that in healthy controls (all $p < 0.05$). After the correction of multiple testing, the group differences persisted at 23 CpG sites including 9 in the *CORIN* gene, 6 in the *FURIN* gene, and 8 in the *NPPA* gene (all $q < 0.05$). The average methylation levels of the targeted regions at these 3 genes were also significantly decreased in patients with IS compared with controls (all $p < 0.05$).

Figure 2 shows the association between DNA methylation level at each CpG site and IS, after adjusting for age, sex, education level, cigarette smoking, alcohol consumption, BMI, LDL-C, HDL-C, hypertension, and diabetes. DNA methylation levels at 22 of 36 CpG sites assayed were

Table 1 Clinical Characteristics of Ischemic Stroke Cases and Healthy Controls

Characteristics	Healthy controls	Ischemic stroke cases	*p* Value
No. of participants	918	853	—
Age, y	61.2 ± 12.2	62.5 ± 12.1	0.260
Sex, men (%)	503 (54.79)	453 (53.11)	0.507
Education level, high school or above (%)	580 (63.18)	732 (85.81)	<0.001
Current smoking, n (%)	348 (37.91)	294 (34.47)	0.145
Current drinking, n (%)	253 (27.56)	222 (26.03)	0.500
Hypertension, n (%)	325 (35.40)	670 (78.55)	<0.001
Diabetes, n (%)	27 (2.94)	165 (19.34)	<0.001
Body mass index, kg/m^2	22.36 ± 3.37	25.09 ± 3.39	<0.001
Systolic blood pressure, mm Hg	134.6 ± 20.7	168.1 ± 16.8	<0.001
Diastolic blood pressure, mm Hg	80.0 ± 10.8	97.0 ± 10.7	<0.001
Fasting glucose, mmol/L	5.04 ± 1.18	6.78 ± 2.82	<0.001
Total cholesterol, mmol/L	4.66 ± 0.96	5.12 ± 1.16	<0.001
Triglycerides, mmol/L	1.56 ± 1.15	1.89 ± 4.96	0.054
LDL cholesterol, mmol/L	3.17 ± 0.97	2.94 ± 0.98	<0.001
HDL cholesterol, mmol/L	1.34 ± 0.33	1.30 ± 0.41	0.032

Abbreviations: HDL = high-density lipoprotein; LDL = low-density lipoprotein.
All results were expressed with mean ± SD, unless otherwise noted.

Table 2 DNA Methylation Level of Each Single CpG in Four Core NP System Genes in Ischemic Stroke Cases and Healthy Controls

CpG loci (GRCh37)	Relative to TSS (bp)	Methylation level (%, mean ± SD)		Raw *p* value	*q*
		Healthy controls	Ischemic stroke cases		
CORIN					
Chr4:47840096	27	39.04 ± 10.46	35.51 ± 8.31	1.91E-14	1.18E-13
Chr4:47840051	72	16.14 ± 8.71	13.40 ± 6.87	8.02E-13	4.24E-12
Chr4:47840038	85	10.76 ± 7.07	9.06 ± 5.20	2.13E-08	5.62E-08
Chr4:47840029	94	12.58 ± 8.16	10.35 ± 6.23	3.55E-10	1.31E-09
Chr4:47840012	111	15.36 ± 8.28	13.22 ± 6.29	2.65E-09	7.53E-09
Chr4:47839981	142	18.91 ± 9.66	15.14 ± 7.10	1.20E-19	1.11E-18
Chr4:47839946	177	14.72 ± 9.98	11.96 ± 7.37	1.12E-10	4.60E-10
Chr4:47839941	182	17.90 ± 11.84	14.53 ± 8.87	3.99E-11	1.85E-10
Chr4:47839933	190	17.11 ± 12.47	14.45 ± 9.37	7.46E-07	1.62E-06
Average		18.06 ± 8.75	15.29 ± 6.49	1.74E-13	—
FURIN					
Chr15:91415964	1,196	49.54 ± 6.32	47.68 ± 6.47	1.55E-09	5.20E-09
Chr15:91415975	1,207	13.63 ± 4.11	10.76 ± 3.17	1.37E-56	5.09E-55
Chr15:91416006	1,238	62.68 ± 6.04	62.19 ± 5.76	7.61E-02	1.08E-01
Chr15:91416008	1,240	41.41 ± 5.92	39.92 ± 5.60	6.51E-08	1.60E-07
Chr15:91416047	1,279	51.73 ± 5.81	50.99 ± 5.72	7.08E-03	1.14E-02
Chr15:91416060	1,292	35.44 ± 5.84	34.68 ± 5.66	5.73E-03	9.63E-03
Chr15:91416118	1,350	40.43 ± 5.97	40.43 ± 5.46	1.00E+00	1.00E+00
Average		42.12 ± 4.50	40.95 ± 4.37	3.32E-08	—
NPPA					
Chr1:11908353	−513	29.44 ± 4.83	26.35 ± 5.12	3.91E-37	7.24E-36
Chr1:11908348	−508	93.14 ± 2.18	92.77 ± 2.94	2.81E-03	4.95E-03
Chr1:11908299	−459	23.19 ± 3.94	22.07 ± 3.91	2.50E-09	7.53E-09
Chr1:11908200	−360	68.62 ± 6.24	67.17 ± 7.30	7.72E-06	1.50E-05
Chr1:11908182	−342	81.77 ± 4.65	80.83 ± 5.57	1.31E-04	2.43E-04
Chr1:11908178	−338	40.83 ± 5.73	38.40 ± 6.47	1.35E-16	9.98E-16
Chr1:11908168	−328	50.61 ± 6.09	49.12 ± 7.05	2.28E-06	4.69E-06
Chr1:11908165	−325	30.89 ± 6.08	29.26 ± 6.83	1.41E-07	3.25E-07
Chr1:11908142	−302	36.37 ± 7.22	36.54 ± 8.46	6.43E-01	7.00E-01
Average		50.54 ± 4.47	49.17 ± 5.33	6.09E-09	—
NPPB					
Chr1:11919160	−168	26.61 ± 9.62	26.25 ± 9.80	4.49E-01	5.53E-01
Chr1:11919144	−152	28.95 ± 10.10	28.15 ± 9.94	1.01E-01	1.39E-01
Chr1:11919141	−149	27.35 ± 9.99	27.05 ± 9.50	5.21E-01	5.84E-01
Chr1:11919135	−143	28.84 ± 10.11	29.19 ± 10.27	4.72E-01	5.63E-01

Continued

Table 2 DNA Methylation Level of Each Single CpG in Four Core NP System Genes in Ischemic Stroke Cases and Healthy Controls *(continued)*

CpG loci (GRCh37)	Relative to TSS (bp)	Methylation level (%, mean ± SD)		Raw *p* value	*q*
		Healthy controls	Ischemic stroke cases		
Chr1:11919133	−141	34.73 ± 10.25	34.85 ± 10.38	8.20E-01	8.67E-01
Chr1:11919117	−125	31.23 ± 11.04	31.31 ± 11.98	8.97E-01	9.22E-01
Chr1:11919096	−104	34.04 ± 11.16	33.65 ± 12.66	4.97E-01	5.74E-01
Chr1:11919047	−55	10.35 ± 6.74	11.05 ± 7.65	4.86E-02	7.20E-02
Chr1:11919019	−27	7.49 ± 5.73	7.21 ± 5.43	3.08E-01	4.06E-01
Chr1:11919011	−19	7.97 ± 6.09	8.26 ± 6.51	3.51E-01	4.47E-01
Chr1:11918989	3	32.78 ± 11.11	31.63 ± 11.75	4.03E-02	6.21E-02
Average		24.57 ± 4.49	24.42 ± 4.61	4.83E-01	—

Abbreviation: TSS = transcription start site.
p Value indicates the significance level of differences in methylation levels between ischemic stroke cases and healthy controls tested by the *t* test. *q* Value indicates the significance level after adjusting for multiple testing by false discovery rate.

negatively associated with risks of having IS (all $p < 0.05$). After further adjusting for multiple testing, 19 single CpG associations persisted ($q < 0.05$), including 9 in *CORIN*, 3 in *FURIN*, and 7 in *NPPA*.

Gene-Based and Gene Set Associations Between DNA Methylation of NP System Genes and IS

The regression using the average level of multiple CpG sites in a targeted region as the independent variable found that hypermethylation at the targeted regions of *CORIN* (odds ratio [OR] = 0.75, 95% confidence interval [CI]: 0.69–0.82), *FURIN* (OR = 0.75, 95% CI: 0.66–0.86), and *NPPA* (OR = 0.78, 95% CI: 0.69–0.88) were significantly associated with a decreased risk of having IS (Figure 2). The wTPM combining the *p* values of single CpG associations revealed similar gene-based associations at the level of $q < 0.05$ (Table 3). Gene set analysis revealed that DNA methylation in all 4 NP system genes as a pathway was jointly associated with the risks of having IS ($p = 0.0001$). The GTEx database showed that the *CORIN*, *FURIN*, *NPPA*, and *NPPB* genes were not only expressed in white blood cells but also expressed in the artery and heart. As illustrated in Figure 3, among the 36 CpG sites assayed, the iMethyl database showed that DNA methylation at 34 CpG sites could occur in white blood cells, and 9 CpG sites (2 at *CORIN*, 5 at *FURIN*, and 2 at *NPPB*) were expression quantitative trait methylations.

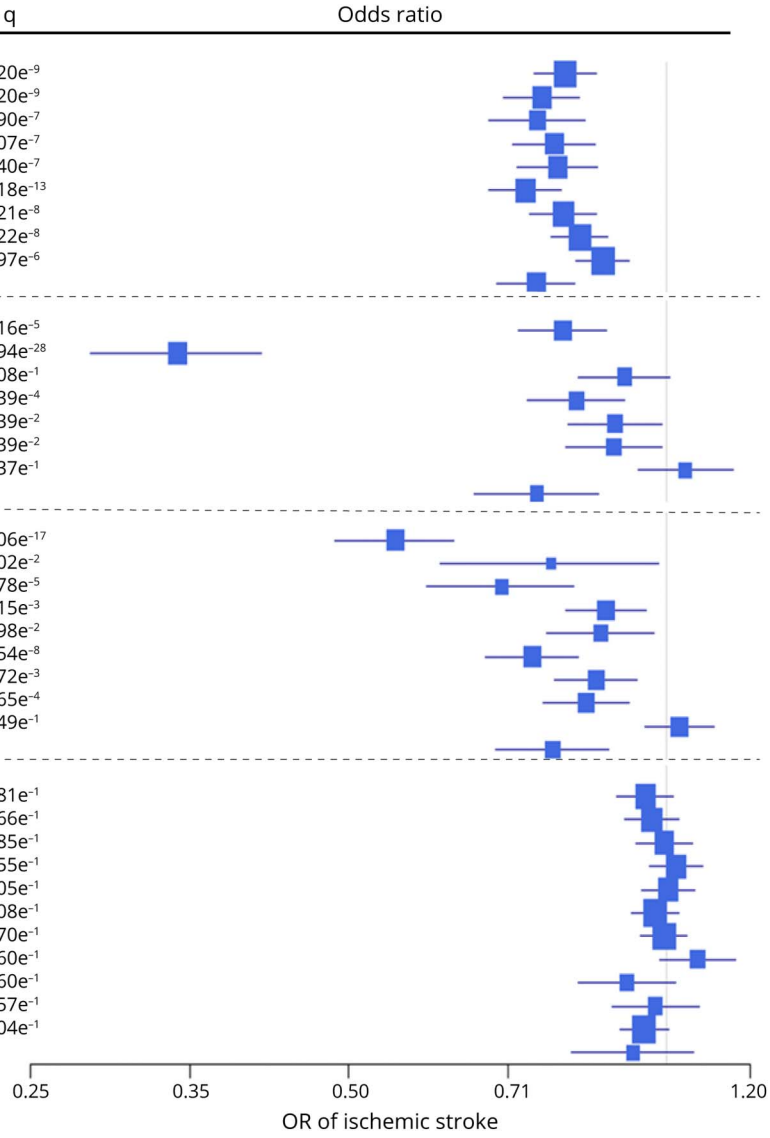
Discussion

In a relatively large sample of patients with IS and well-matched healthy controls, we demonstrated that the promoter regions of the *CORIN*, *FURIN*, and *NPPA* genes were significantly demethylated in patients with IS. Using a gene-family approach, we demonstrated that altered DNA methylation in these NP system genes jointly contributed to IS risks, independent of metabolic and behavioral factors. Our results may unravel a molecular mechanism underneath the potential role of the NP system in the pathogenesis of IS and suggest that simultaneously testing the joint effects of multiple CpG sites in a gene or multiple genes in a pathway is a powerful approach in the epigenetic analysis of human complex traits.

Although mountains of evidence from basic, population, and genetic studies have demonstrated the potential role of natriuretic peptides in IS and other cardiovascular diseases,[4-8] the clinical implications, therapy in particular, of NPs in cardiovascular disease are still limited.[13,15] Our study trying to unraveling the molecular mechanisms underlying the associations between NPs and IS may help translate NPs into clinical practice of IS. In line with our study, accumulating studies suggest the involvement of epigenetic factors such as DNA methylation in the pathogenesis of IS in both humans and animal models. For example, global DNA methylation was upregulated in the brain of mice models of IS.[47] Epigenome-wide association studies (EWASs) have been conducted in multiple populations and found many methylation markers of IS.[48,49] Nevertheless, the assayed CpG loci located at NP system genes including NPs and their receptors and protease convertases failed to pass the genome-wide significant level in previous EWAS studies, probably due to the relatively small effect of a single CpG methylation on a complex trait such as IS. The selection of CpG sites assayed may be a relevant factor too. Of the 36 CpG sites assayed in our study, 13 CpG loci were included in prior EWAS studies, but their methylation levels were not genome-widely associated with IS. As observed in our study, of the 7 CpG loci assayed at *FURIN* promoter, DNA methylation at 5 CpG sites was nominally associated with IS (raw $p <$

Figure 2 Forest Plot Illustrating the Single CpG Associations Between DNA Methylation of Four Core NP System Genes and Ischemic Stroke

CpG loci	OR (95% CI)	Raw P	q
***CORIN* gene**			
Chr4:47840096	0.80 (0.75–0.86)	$1.67e^{-10}$	$1.20e^{-9}$
Chr4:47840051	0.76 (0.70–0.83)	$1.40e^{-10}$	$1.20e^{-9}$
Chr4:47840038	0.75 (0.68–0.84)	$1.08e^{-7}$	$3.90e^{-7}$
Chr4:47840029	0.78 (0.71–0.86)	$1.24e^{-7}$	$4.07e^{-7}$
Chr4:47840012	0.79 (0.72–0.86)	$8.49e^{-8}$	$3.40e^{-7}$
Chr4:47839981	0.74 (0.68–0.79)	$1.82e^{-14}$	$2.18e^{-13}$
Chr4:47839946	0.80 (0.74–0.86)	$2.02e^{-9}$	$1.21e^{-8}$
Chr4:47839941	0.83 (0.78–0.88)	$2.37e^{-9}$	$1.22e^{-8}$
Chr4:47839933	0.87 (0.82–0.92)	$1.99e^{-6}$	$5.97e^{-6}$
Average (per SD)	0.64 (0.56–0.73)	$6.35e^{-11}$	
***FURIN* gene**			
Chr15:91415964	0.80 (0.72–0.88)	$4.19e^{-6}$	$1.16e^{-5}$
Chr15:91415975	0.34 (0.29–0.41)	$1.93e^{-29}$	$6.94e^{-28}$
Chr15:91416006	0.91 (0.82–1.01)	$7.18e^{-2}$	$1.08e^{-1}$
Chr15:91416008	0.82 (0.74–0.91)	$2.84e^{-4}$	$6.39e^{-4}$
Chr15:91416047	0.89 (0.81–0.99)	$3.15e^{-2}$	$5.39e^{-2}$
Chr15:91416060	0.89 (0.80–0.99)	$3.12e^{-2}$	$5.39e^{-2}$
Chr15:91416118	1.04 (0.94–1.15)	$4.48e^{-1}$	$5.37e^{-1}$
Average (per SD)	0.78 (0.69–0.88)	$4.49e^{-5}$	
***NPPA* gene**			
Chr1:11908353	0.55 (0.48–0.63)	$5.89e^{-19}$	$1.06e^{-17}$
Chr1:11908348	0.78 (0.61–0.98)	$3.68e^{-2}$	$6.02e^{-2}$
Chr1:11908299	0.70 (0.59–0.82)	$1.08e^{-5}$	$2.78e^{-5}$
Chr1:11908200	0.88 (0.80–0.96)	$3.08e^{-3}$	$6.15e^{-3}$
Chr1:11908182	0.87 (0.77–0.97)	$1.57e^{-2}$	$2.98e^{-2}$
Chr1:11908178	0.75 (0.67–0.82)	$1.45e^{-8}$	$6.54e^{-8}$
Chr1:11908168	0.86 (0.78–0.94)	$8.14e^{-4}$	$1.72e^{-3}$
Chr1:11908165	0.84 (0.76–0.92)	$2.36e^{-4}$	$5.65e^{-4}$
Chr1:11908142	1.03 (0.95–1.11)	$4.73e^{-1}$	$5.49e^{-1}$
Average (per SD)	0.78 (0.69–0.88)	$6.64e^{-5}$	
***NPPB* gene**			
Chr1:11919160	0.95 (0.90–1.01)	$1.36e^{-1}$	$1.81e^{-1}$
Chr1:11919144	0.97 (0.91–1.03)	$2.85e^{-1}$	$3.66e^{-1}$
Chr1:11919141	0.99 (0.93–1.06)	$8.60e^{-1}$	$8.85e^{-1}$
Chr1:11919135	1.02 (0.96–1.08)	$4.93e^{-1}$	$5.55e^{-1}$
Chr1:11919133	1.00 (0.95–1.06)	$9.05e^{-1}$	$9.05e^{-1}$
Chr1:11919117	0.97 (0.93–1.03)	$3.29e^{-1}$	$4.08e^{-1}$
Chr1:11919096	0.99 (0.95–1.05)	$8.21e^{-1}$	$8.70e^{-1}$
Chr1:11919047	1.07 (0.98–1.16)	$1.16e^{-1}$	$1.60e^{-1}$
Chr1:11919019	0.92 (0.82–1.02)	$1.11e^{-1}$	$1.60e^{-1}$
Chr1:11919011	0.98 (0.89–1.07)	$6.02e^{-1}$	$6.57e^{-1}$
Chr1:11918989	0.95 (0.90–1.00)	$6.65e^{-2}$	$1.04e^{-1}$
Average (per SD)	0.93 (0.83–1.06)	$2.77e^{-1}$	



Odds ratios indicate the risks of having ischemic stroke associated with per 5% increase in DNA methylation levels at each CpG site and with per SD increase in the average of all CpG sites in a gene, adjusting for age, sex, education level, cigarette smoking, alcohol consumption, BMI, LDL-C, HDL-C, hypertension, and diabetes. *q* indicates the significance level after multiple testing correction by false discovery rate approach. BMI = body mass index; CI = confidence interval; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol.

0.05), with only 3 CpG sites survived multiple testing ($q < 0.05$). However, the combined effects of multiple CpG sites could be somewhat larger. For example, although DNA methylation at 4 of 7 CpG loci assayed within the *FURIN* gene lost significant associations with IS in our study after adjustment for covariates and multiple testing, gene-based analysis revealed a significant joint association of all 7 CpG sites in this gene with IS. Such a phenomenon was also observed in previous epigenetic studies.[39,45,50,51] This highlights the importance of testing the joint epigenetic effect of multiple CpG loci on human complex traits.

Similarly, a complex trait like IS is most likely contributed to many genes in one or more biological pathways. Testing the contribution of one individual gene may be insufficient and does not comply with the true biology. Therefore, we examined the joint effect of 4 core NP system genes as a pathway on IS. The result shows that the NP system genes as a pathway were jointly associated with IS, although not all individual genes showed a significant association with IS. Together, our findings suggest that examination of the joint epigenetic effects of DNA methylation at multiple CpG sites in a gene or a pathway may represent a preferred approach for epigenetic analysis of human complex traits.

As an interface between the fixed genome and dynamic environment, DNA methylation represents a mechanism through which our changing environment can affect the

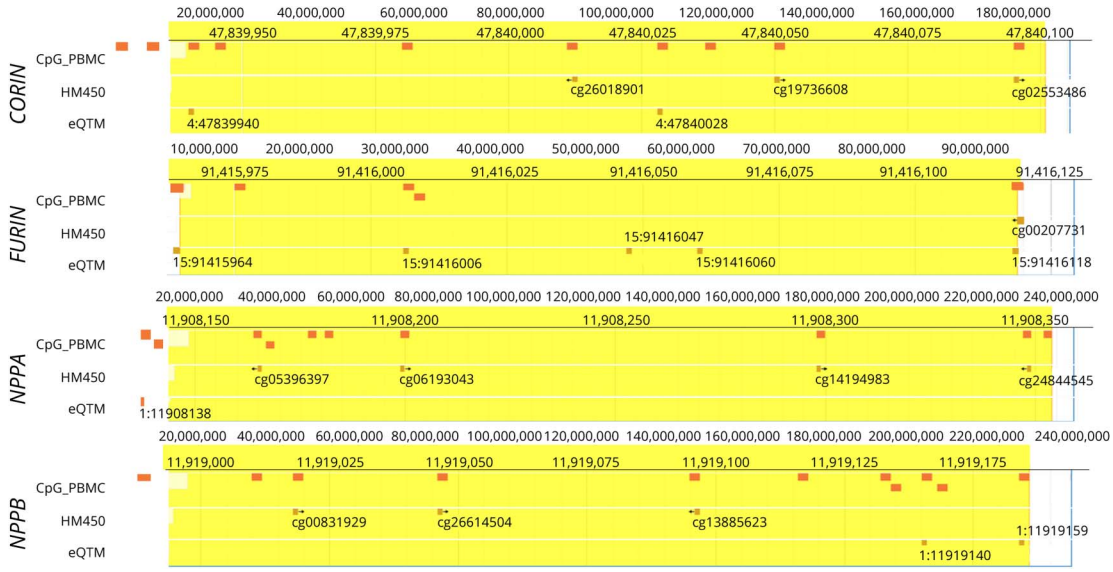
Table 3 Gene-Based and Gene Set Associations of DNA Methylation in 4 Core NP System Genes With Ischemic Stroke

NP system genes	Raw *p* value[a]	*q*
Gene-based association		
CORIN	3.00E-04	0.0004
FURIN	1.00E-04	0.0002
NPPA	1.00E-04	0.0002
NPPB	3.53E-01	0.3530
Gene set association		
NP system	1.00E-04	

Abbreviation: NP = natriuretic peptide.
[a] Combined raw *p* values of the single CpG associations for gene-based analysis and raw *p* values of gene-based associations for gene set analysis by the weighted truncated product method. In this model, the corresponding regression coefficients of single CpG associations presented in eTable 2 (links.lww.com/NXG/A524) were included as weights for testing the gene-based association, and the weights were set as 1 for testing the gene set association.

pathogenesis of IS. However, very few studies, to the best of our knowledge, systemically examined the effect of DNA methylation at genes involved in the NP system, which plays an integral role in the regulation of salt-water balance and volume homeostasis[52] on the risks of IS and other cardiovascular diseases. Our study examined the association between DNA methylation at NP system genes and IS. Our results provide initial evidence that altered DNA methylation of key NP system genes may play an important role in the pathogenesis of IS. The other strength of our study is the application of innovative statistical approaches to test the combined effects of multiple CpG sites in a gene or multiple genes in the NP system pathway. Our study also has some limitations. First, the case-control study design precludes causal inference. It is still unclear whether altered methylation at NP system genes is a risk factor, consequence, or just an accompanying phenomenon of IS. Second, we only studied Chinese adults. Thus, the generalizability of our results to other ethnic populations is of concern. Third, we studied DNA methylation using genomic DNA from peripheral blood but not the target organs of IS, e.g., brain and artery. However, accumulating evidence indicated that epigenetic modifications may not be limited to the affected tissue but could also be detected in peripheral blood.[53] Fourth, we had no data on cell types. The effects of individual variations of cell types on the association between NP system genes methylation and IS cannot be controlled. Fifth, although we found differential methylation of the CpGs in NP system genes associated with IS, the observed differences in DNA methylation at individual CpG sites were so small in terms of magnitude that their utility as potential biomarkers are questionable. Nevertheless, our previous study including 69 monozygotic twin pairs found that a 0.85% of twin-pair difference in methylation at the *BMAL1* gene was significantly associated with insulin resistance.[50] The role of NP system genes methylation in IS is still needed to be further studied. Sixth, we only measured DNA methylation levels at the promoter region of the NP system genes. Whether DNA methylation at gene body of these genes was associated with IS is unknown. Finally, the present study is a secondary data analysis based on genes assayed in a previous candidate

Figure 3 Schematic Illustration of the Regulatory Potential of the CpG Sites Assayed Produced by the Integrative DNA Methylation (iMethyl) Database



The targeted region is yellow highlighted. CpG_PBMC indicates the CpG loci presented in white blood cells. HM450 indicates the CpG loci included in Infinium HumanMethylation450 BeadChip. eQTM indicates the CpG loci whose methylation was associated with gene expression. eQTM = expression quantitative trait methylation; PBMC = peripheral blood mononuclear cell.

gene-based study that did not include all the NP system genes (e.g., the receptors of peptides and NPPC).

In summary, our results demonstrated that DNA methylation of the 4 core NP system genes was jointly associated with IS. These findings also highlight the importance of examination of the effect of DNA methylation jointly rather than separately on human complex traits in the epigenetic analysis.

Acknowledgment

Numerous organizations have provided invaluable assistance in the conduct of this study, including the Center for Disease Prevention and Control of Changshu City, Genesky Biotechnologies Inc, Shanghai, China, and the CATIS investigators. Most importantly, the authors gratefully acknowledge the continued cooperation and participation of the members of the PMMS and the CATIS and their families. Without their contribution, this research would not have been possible.

Study Funding

This study was supported by the National Natural Science Foundation of China (Nos. 81903384, 81872690, and 81673263), the Natural Science Foundation of Jiangsu Province (No. BK20180841), the Suzhou Municipal Science and Technology Bureau (Nos. SS201853 and SYS2020091), Soochow University Undergraduate Training Program for Innovation (2020xj056), and a Project of the Priority Academic Program Development of Jiangsu Higher Education Institutions. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Disclosure

The authors report no disclosures relevant to the manuscript. Go to Neurology.org/NG for full disclosures.

Publication History

Received by *Neurology: Genetics* January 7, 2022. Accepted in final form March 18, 2022. Submitted and externally peer reviewed. The handling editor was Alexandra Durr, MD, PhD.

Appendix Authors

Name	Location	Contribution
Hao Peng, PhD	Department of Epidemiology, School of Public Health, Medical College of Soochow University; Jiangsu Key Laboratory of Preventive and Translational Medicine for Geriatric Diseases, Suzhou, China	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; and analysis or interpretation of data
Yiming Fan, MBBS	Medical College of Soochow University, Suzhou, China	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; and analysis or interpretation of data
Jing Li, MD	Department of Epidemiology, School of Public Health, Medical College of Soochow University, Suzhou, China	Major role in the acquisition of data
Xiaowei Zheng, PhD	Department of Epidemiology, School of Public Health, Medical College of Soochow University, Suzhou, China	Major role in the acquisition of data
Chongke Zhong, PhD	Department of Epidemiology, School of Public Health, Medical College of Soochow University, Suzhou, China	Major role in the acquisition of data
Zhengbao Zhu, PhD	Department of Epidemiology, School of Public Health, Medical College of Soochow University, Suzhou, China	Major role in the acquisition of data
Yan He, PhD	Department of Epidemiology, School of Public Health, Medical College of Soochow University, Suzhou, China	Major role in the acquisition of data
Mingzhi Zhang, PhD	Department of Epidemiology, School of Public Health, Medical College of Soochow University, Suzhou, China	Major role in the acquisition of data and study concept or design
Yonghong Zhang, PhD	Department of Epidemiology, School of Public Health, Medical College of Soochow University, Suzhou, China	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; and study concept or design

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Hao Peng, Yiming Fan, Jing Li, et al.

Neurol Genet 2022;8;
DOI 10.1212/NXG.0000000000000679