




ORIGINAL RESEARCH

Associations of B-Type Natriuretic Peptide and Its Coding Gene Promoter Methylation With Functional Outcome of Acute Ischemic Stroke: A Mediation Analysis

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BACKGROUND: The prognostic role of B-type natriuretic peptide (BNP) in stroke has been suggested, but limited studies have shown mixed results and unknown underlying mechanisms. DNA methylation, a molecular modification that alters gene expression, may represent a candidate mechanism for this purpose. We aimed to examine the associations of BNP and methylation of its coding gene (natriuretic peptide B [*NPPB*]) with the functional outcome in a large sample of patients with acute ischemic stroke from CATIS (China Antihypertensive Trial in Acute Ischemic Stroke).

METHODS AND RESULTS: Leveraging participants from CATIS with available specimens, serum proBNP (equimolarly produced with BNP) was measured in 3216 patients (mean age, 62 years; 64% men), and peripheral blood DNA methylation of the *NPPB* promoter was quantified by targeted bisulfite sequencing in 806 patients (mean age, 62 years; 54% men). The functional outcome was defined as an ordered modified Rankin Scale score assessed at 14 days or hospital discharge after stroke onset. Mediation analysis was conducted to test the potential mediating effect of proBNP on the relationship between *NPPB* methylation and functional outcome. The results showed that a higher level of proBNP was significantly associated with a higher risk of having a poorer functional outcome (odds ratio [OR], 1.14; $P=0.006$). Every 5% of hypermethylation at 2 (Chr1:11919160 [OR, 0.93; $P=0.022$] and Chr1:11918989 [OR, 0.92; $P=0.032$]) of 11 CpG loci assayed was associated with 7% and 8% lower risk, respectively, of having a poor functional outcome. In addition, proBNP was negatively correlated to hypermethylation at 1 CpG (Chr1:11918989 [$\beta=-0.029$; $P=0.009$]) and mediated approximately 7.69% (95% CI, 2.50%–13.82%) of the association between this CpG methylation and the functional outcome.

CONCLUSIONS: Hypermethylation at the *NPPB* promoter is associated with the functional outcome after ischemic stroke, at least partially by suppressing BNP expression or excretion.

Key Words: B-type natriuretic peptide ■ DNA methylation ■ functional outcome ■ ischemic stroke

B-type natriuretic peptide (BNP) is a circulating cardiac hormone synthesized and released as a precursor protein, mainly from the ventricles, in response to increased myocardial wall stress related to volume or pressure overload.¹ It has been globally

endorsed in clinical guidelines as a biomarker to aid in the diagnosis of heart failure and to monitor disease progression.^{2–4} In addition to heart diseases, BNP was elevated in patients with acute ischemic stroke (AIS)^{5,6} and associated with the poor functional

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CLINICAL PERSPECTIVE

What Is New?

- Hypermethylation at the natriuretic peptide B (*NPPB*) promoter is associated with functional outcome after ischemic stroke.
- Hypermethylation at the *NPPB* promoter is associated with lower circulating levels of pro-B-type natriuretic peptide.

What Are the Clinical Implications?

- Hypermethylation at the *NPPB* promoter may be a marker of poor functional outcome after ischemic stroke.
- Hypermethylation at the *NPPB* promoter may be potential drug target for ischemic stroke.

Nonstandard Abbreviations and Acronyms

AIS	acute ischemic stroke
BNP	B-type natriuretic peptide
CATIS	China Antihypertensive Trial in Acute Ischemic Stroke
KEGG	Kyoto Encyclopedia of Genes and Genomes
mRS	modified Rankin Scale
NIHSS	National Institutes of Health Stroke Scale
NPPB	natriuretic peptide B

outcome of AIS,^{7–11} although not consistently.^{12,13} Its expression in the brain implies a possible role for BNP in neurologic function.¹⁴ Administration of BNP has been demonstrated to improve cerebral blood flow and to reduce inflammation in brain injury models of mice, as manifested by reduced neurodegeneration and improved functional outcome.¹⁵ BNP seems to be implicated in neuroprotection following brain injury from AIS, but the underlying molecular mechanisms are not clear.

Genetic polymorphisms in the coding gene of BNP (natriuretic peptide B [*NPPB*]) have been associated not only with blood BNP concentrations^{16,17} but also with determinants of AIS pathogenesis and prognosis, for example, hypertension^{18,19} and diabetes mellitus.²⁰ As an interface between the fixed genome and dynamic environment, epigenetic factors such as DNA methylation in the *NPPB* gene may affect its function and subsequent BNP synthesis and excretion. DNA methylation status can change dramatically, and dysregulated DNA methylation has previously been related to poor tissue outcome after cerebral ischemic injury in mice.^{21,22} However, the epigenetic markers

of functional outcome of AIS, including DNA methylation of the *NPPB* gene, have scarcely been studied in humans.

The objectives of this study were to examine whether BNP and its coding gene promoter methylation levels at the acute phase could predict functional outcome and whether BNP mediated the relationship between *NPPB* promoter methylation and functional outcome in patients with AIS, using data from CATIS (China Antihypertensive Trial in Acute Ischemic Stroke).²³

METHODS

The data that support the findings of this study are available from the corresponding author on reasonable request.

Study Patients

CATIS is a multicenter randomized clinical trial (ClinicalTrials.gov identifier NCT01840072) designed to test whether moderate lowering of blood pressure in the acute phase after the onset of an AIS reduces the risks of death and major disability at 14 days or hospital discharge. The study design, intervention, and survey methods of CATIS have been described previously.²³ In brief, 4071 patients with first-ever ischemic stroke were recruited into CATIS. All surviving patients were reexamined at 14 days or hospital discharge after stroke onset. The study protocols were approved by the institutional review boards at Soochow University in China and Tulane University in the United States and by the ethics committees of all participating hospitals. Written informed consent was obtained from all study participants or their immediate family members.

Figure 1 describes the selection of study participants in the current analysis. After excluding 855 patients because of lack of data on proBNP, 3216 patients were included in the analysis of the association between proBNP and functional outcome of AIS. In total, 806 patients with eligible DNA samples for DNA methylation quantification were included in the analysis of the association between *NPPB* promoter methylation and functional outcome of AIS. Of those, 704 patients with available data on both proBNP and DNA methylation were included in the analysis of the mediating effect of proBNP on the association between *NPPB* promoter methylation and functional outcome of AIS.

Measurements of proBNP

Despite being equimolarly cleaved from its precursor protein (1–108 amino acids), the biologically inert proBNP (1–76 amino acids; ie, N-terminal proBNP) has a longer half-life and is more stable in the

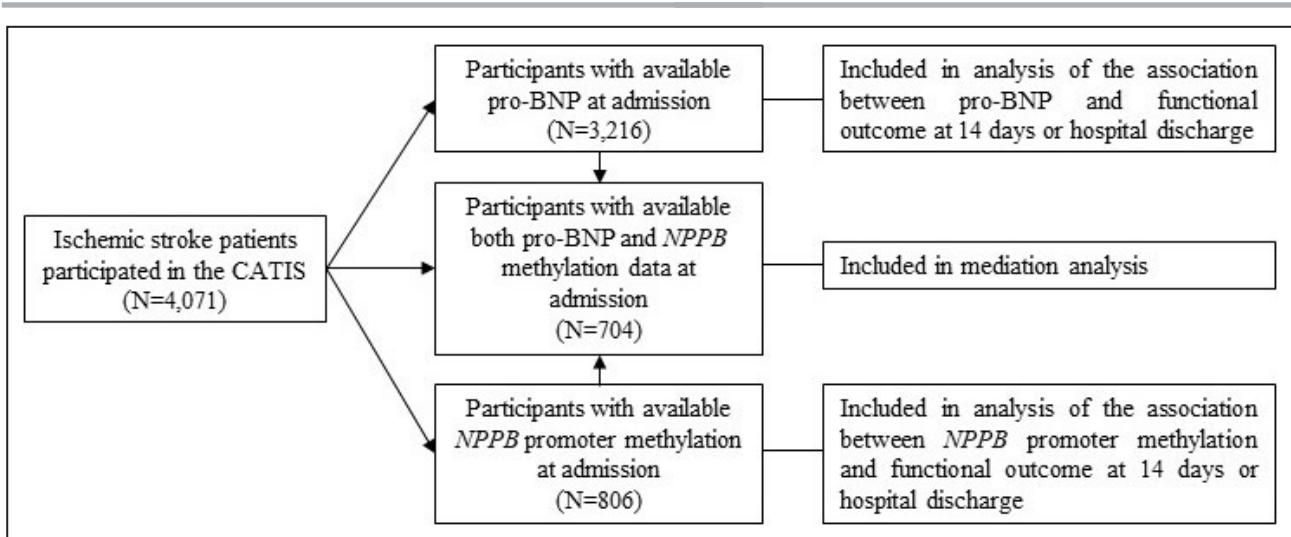


Figure 1. Flowchart of selection of study participants.

BNP indicates B-type natriuretic peptide; CATIS, China Antihypertensive Trial in Acute Ischemic Stroke; and NPPB, natriuretic peptide B.

circulation than BNP (77–108 amino acids).²⁴ Therefore, we used proBNP concentrations to approximately reflect BNP excretion in this study. Serum proBNP concentrations were measured using ELISA (Biomedica Medizinprodukte), according to the manufacturer's guidelines, in fasting blood samples drawn within 24 hours of hospital admission. Intra- and interassay coefficients of variation were <4% and <3%, respectively. All samples were processed at the Jiangsu Key Laboratory of Preventive and Translational Medicine for Geriatric Diseases in a duplicate assay by laboratory technicians blinded to the clinical characteristics of the study participants.

Quantification of *NPPB* Promoter Methylation

DNA methylation levels in the promoter region of *NPPB* were quantified by targeted bisulfite sequencing, as described previously,²⁵ using genomic DNA isolated from peripheral blood mononuclear cells. Briefly, based on the genomic coordinates of the *NPPB* promoter in Genome Reference Consortium Human Build 37, we carefully designed the primers to detect the maximum CpG loci within the CpG islands. The targeted sequence (Chr1:11918953–11919190, reverse strand) and primers for sequencing are schematically illustrated in Figure 2. Following primer validation, genomic DNA was treated with bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research), according to the manufacturer's protocol, which converts unmethylated cytosine into uracil and leaves methylated cytosine unchanged. The treated samples were amplified, bar-coded, and sequenced by Illumina HiSeq 2000 (Illumina) using the paired-end sequencing protocol, according to the manufacturer's guidelines.

Methylation level at each CpG locus was calculated as the percentage of the methylated alleles over the sum of methylated and unmethylated alleles. For quality control, the samples with a bisulfite conversion rate <98% and the cytosine sites with average coverage <20× were filtered out.

Assessment of Functional Outcome of AIS

For patients with AIS, the modified Rankin Scale (mRS) was recommended to assess functional outcome after stroke.²⁶ It ranged from 0 to 6, with 0 indicating no symptoms, 5 indicating severe disability (ie, bedridden, incontinent, or requiring constant nursing care and attention), and 6 indicating death. The mRS evaluation at 14 days after stroke onset or at hospital discharge was administered by trained neurologic physicians blinded to baseline characteristics. An ordered 7-level categorical score of the mRS at 14 days or hospital discharge was the functional outcome of AIS in this study.

Assessment of Potential Covariates at Admission

Data on demographic characteristics (age, sex), lifestyle risk factors (current smoking, current drinking), and medical history (coronary heart disease, hypertension, diabetes mellitus, hyperlipidemia) were collected at admission using a standard questionnaire. Fasting glucose and lipids at admission were examined at every participating hospital. Ischemic stroke was classified as thrombotic, embolic, and lacunar subtypes according to the symptoms and imaging data.²⁷ Stroke severity at baseline was evaluated by trained neurologists using the National Institutes of Health Stroke Scale (NIHSS). Trained research staff measured

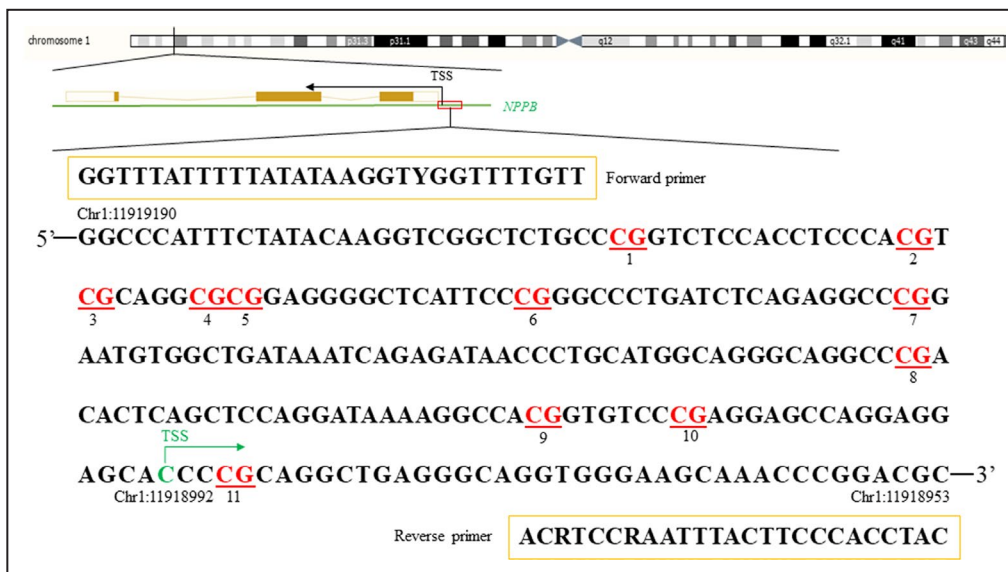


Figure 2. Schematic illustration of the targeted sequence and primers for targeted bisulfite sequencing. Red represents the CpG loci assayed in the *NPPB* gene promoter (–198 to +39 bp from TSS). *NPPB* indicates natriuretic peptide B; and TSS, transcriptional start site

participants’ body weight (in kilograms) and height (in centimeters) with participants wearing light clothes and no shoes. Body mass index was calculated by dividing weight in kilograms by the square of height in meters (kg/m²). Three blood pressure measurements were performed at admission by trained nurses, according to a common protocol adapted from procedures recommended by the American Heart Association.²⁸

Statistical Analysis

To carefully evaluate the roles of BNP and its coding gene methylation in the neurologic recovery of AIS, we examined the associations of proBNP and *NPPB* promoter methylation at admission with functional outcome, followed by a mediation analysis among them. Log₁₀ transformation was applied to maximize normal distribution of proBNP, and the generated values (log₁₀-proBNP) were used in downstream analyses. All statistical analyses were performed using SAS statistical software (v9.4; SAS Institute).

Association Analysis

To examine the association between proBNP at admission and functional outcome of AIS, we constructed an ordered logistic regression model in which mRS score was the dependent variable and proBNP (continuous log₁₀-proBNP or categorical quartiles) was the independent variable, adjusting for potential covariates at admission including age, sex, stroke subtype, NIHSS score, hours from onset to hospitalization, current smoking, current drinking,

body mass index, systolic blood pressure, disease history (hypertension, diabetes mellitus, dyslipidemia, coronary heart disease), and treatment group. The model fit was assessed using a Hosmer–Lemeshow goodness-of-fit test. To examine the association between *NPPB* promoter methylation at admission and functional outcome of AIS, we constructed similar ordered logistic regression models with DNA methylation at each CpG locus as the independent variable. Multiple testing was controlled by adjusting for the total number of CpG loci tested, and a false discovery rate–adjusted *P* value (ie, *q* value) of <0.2 was considered nominally significant.

Mediation Analysis

This analysis focuses on CpG sites at which DNA methylation showed nominally significant associations with both proBNP and functional outcome of AIS. To test whether proBNP mediates the association between *NPPB* promoter methylation and functional outcome, we constructed a causal mediation model by fitting a series of conditional regression models. Specifically, we tested the following conditions:

- The relationship between DNA methylation (*X*) and mRS (*Y*) (ordered logistic regression model $Y = \beta_{Tot}X$, β_{Tot} : total effect);
- The relationship between DNA methylation (*X*) and log₁₀-proBNP (*M*) (quantile regression model $M = \beta_1X$);
- The relationship between log₁₀-proBNP (*M*) and

mRS (Y) after controlling for DNA methylation (X) ($Y = \beta_2 M + \beta_{Dir} X, \beta_{Dir}$: direct effect);

We then calculated the mediating effect ($\beta_{Med} = \beta_1 \times \beta_2$) and the proportion of mediation ($\beta_{Med} / \beta_{Tot} \times 100\%$). Mediation analysis was performed using R package “mediation,”²⁹ adjusted for the covariates listed above. The 95% CI of the mediating effect was estimated by Monte Carlo CIs.

Secondary Analysis

Based on the associations of each single CpG methylation with functional outcome, we tested the joint association of DNA methylation at multiple CpG probes in the *NPPB* gene with functional outcome using the weighted truncated product method, as described previously.³⁰ This method combines P values of all CpGs that reach a preselected threshold (eg, raw $P < 0.1$ in this study). The regression coefficient of each individual CpG methylation was included as weight in the weighted truncated product method statistic. Gene Ontology function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were conducted for the *NPPB* gene by DAVID online analysis (<https://david.ncifcrf.gov/>). The regulatory network involving the *NPPB* gene was also constructed by GeneMANIA (<http://genemania.org/>).

RESULTS

Baseline Characteristics

Of the 4071 patients with AIS in CATIS, 3216 patients (mean age, 62 years; 64% male) with available proBNP and 806 patients (mean age, 62 years; 54% male) with measurements of *NPPB* promoter methylation were included in the current study. Their clinical characteristics at admission are shown in Table 1. In both subsets of study participants, approximately 79%, 18%, 7%, and 10% of patients had hypertension, diabetes mellitus, hyperlipidemia, and coronary heart disease, respectively.

Association Between proBNP at Admission and the Functional Outcome of AIS

About 58% of the 3216 patients with available proBNP experienced an unfavorable functional outcome including disability that was slight ($n=773$, 24.04%), moderate ($n=462$, 14.37%), moderately severe ($n=437$, 13.59%), or severe ($n=156$, 4.85%) or death ($n=30$, 0.93%) at 14 days or hospital discharge (Table 2). Table 3 shows the association between proBNP at admission and the functional outcome. Ordered logistic regression using \log_{10} -proBNP as the independent

Table 1. Clinical Characteristics of Acute Ischemic Stroke Patients at Admission

Characteristics	Patients With Available proBNP	Patients With Available <i>NPPB</i> Methylation
No. of participants	3216	806
Age, y	62.5±10.8	62.6±12.2
Sex, male (%)	2048 (63.68)	432 (53.60)
Current smoking, n (%)	1187 (36.91)	278 (34.49)
Current drinking, n (%)	986 (30.66)	211 (26.18)
Disease history, n (%)		
Hypertension	2541 (79.01)	629 (78.04)
Diabetes mellitus	562 (17.48)	153 (18.98)
Hyperlipidemia	226 (7.03)	54 (6.70)
Coronary heart disease	341 (10.60)	84 (10.42)
Ischemic stroke subtype, n (%)		
Thrombotic	2448 (76.12)	766 (95.04)
Embolic	165 (5.13)	40 (4.96)
Lacunar	686 (21.33)	...
Body mass index, kg/m ²	24.90±3.13	25.12±3.39
Systolic blood pressure, mm Hg	166.5±17.0	168.3±16.8
Diastolic blood pressure, mm Hg	96.6±11.0	97.0±10.7
Fasting glucose, mmol/L	6.69±2.73	6.79±2.84
Total cholesterol, mmol/L	5.08±1.15	5.12±1.17
Triglycerides, mmol/L	1.84±2.82	1.92±5.10
LDL cholesterol, mmol/L	2.95±0.95	2.94±1.00
HDL cholesterol, mmol/L	1.30±0.45	1.30±0.41
Hours from onset to hospitalization	15.10±13.00	13.95±12.93
NIHSS score, points	5.8±4.8	7.1±5.2
proBNP, pg/mL, median (IQR)	142.4 (67.1–327.3)	156.9 (71.4–374.5)
Log ₁₀ -transformed proBNP	2.17±0.55	2.20±0.57

All results are expressed as mean±SD unless otherwise noted. BNP indicates B-type natriuretic peptide; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; NIHSS, National Institutes of Health Stroke Scale; and *NPPB*, natriuretic peptide B.

variable showed that a higher level of proBNP at admission was significantly associated with a higher risk of having a higher mRS score at 14 days or hospital discharge (odds ratio [OR], 1.14; $P=0.006$), independent of age, sex, stroke subtype, NIHSS score, hours from onset to hospitalization, current smoking, current drinking, body mass index, blood pressure, disease history (hypertension, diabetes mellitus, dyslipidemia, coronary heart disease), and treatment group at admission. Regression using categorical proBNP quartiles as the independent variable revealed a similar result with the same direction. Compared with patients with the lowest level of proBNP, those with the highest level of proBNP had a 32% higher risk of having a higher mRS score (OR, 1.32; $P=0.004$).

Table 2. Distribution of Functional Outcome at 14 days or Hospital Discharge After Stroke Onset

mRS Score	Patients With Available proBNP (n=3216)		Patients With Available NPPB Methylation (n=806)	
	n	%	n	%
0 (No symptoms)	288	8.96	43	5.33
1 (No significant disability despite symptoms)	1070	33.27	215	26.67
2 (Slight disability)	773	24.04	203	25.19
3 (Moderate disability)	462	14.37	123	15.26
4 (Moderately severe disability)	437	13.59	143	17.74
5 (Severe disability)	156	4.85	67	8.31
6 (Death)	30	0.93	12	1.49

BNP indicates B-type natriuretic peptide; and NPPB indicates natriuretic peptide B.

Association Between NPPB Promoter Methylation and the Functional Outcome of AIS

About 68% of the 806 patients with available NPPB methylation data experienced an unfavorable functional outcome including disability that was slight (n=203, 25.19%), moderate (n=123, 15.26%), moderately severe (n=143, 17.74%), or severe disability (n=67, 8.31%) or death (n=12, 1.49%) at 14 days or hospital discharge (Table 2). DNA methylation levels at almost all CpG loci seemed to decrease with increased mRS score, but DNA methylation at only 2 CpG sites (CpG1 located in Chr1:11919160 [$\beta=-0.524$, $P=0.027$] and CpG11 located in Chr1:11918989 [$\beta=-0.606$, $P=0.032$]) preserved a statistically significant trend (Figure 3). The ordered logistic regression using mRS score as the dependent variable revealed similar results (Table 4). After multivariate adjustment, almost all CpGs methylation presented a negative association with odds of having a higher mRS score. Of these, every 5% of hypermethylation at the same CpG sites (CpG1: OR, 0.93

[$P=0.022$]; CpG11: OR, 0.92 [$P=0.032$]) was associated with 7% and 8% lower risk of having a higher mRS score, which indicates poorer functional outcome at 14 days or hospital discharge. These associations persisted as nominally significant after adjusting for multiple testing (all false discovery rate-adjusted $P<0.2$).

Correlation Between NPPB Promoter Methylation and proBNP

Figure 4 illustrates the correlation between \log_{10} -proBNP and each CpG methylation in the promoter region of the NPPB gene. DNA methylation at almost all 11 CpG loci assayed appeared to be negatively correlated with proBNP, but only 1 CpG methylation reached statistical significance, with a correlation coefficient of -0.09 (CpG11, $P=0.016$). These results indicated that hypermethylation of the NPPB gene promoter may be associated with a lower level of proBNP.

Mediating Effect of proBNP on the Association Between NPPB Promoter Methylation and the Functional Outcome of AIS

Our mediation analysis focused on the only CpG locus (CpG11 located in Chr1:11918989) at which DNA methylation was identified as significantly associated with both proBNP and functional outcome. The mediating effect of proBNP on the association between this CpG methylation and functional outcome is schematically illustrated in Figure 5. We found that that proBNP accounted for $\approx 7.69\%$ (95% CI, 2.50%–13.82%) of the association between DNA methylation at this CpG site and functional outcome.

Results of Secondary Analysis

The gene-based association analysis revealed that NPPB promoter methylation as a whole was also significantly associated with the functional outcome of AIS ($P=0.022$).

Table 3. Association of proBNP at Admission With the Functional Outcome at 14 Days or Hospital Discharge After Stroke Onset (n=3216)

proBNP, pg/mL	Unadjusted		Adjusted [†]	
	OR (95% CI)*	P Value	OR (95% CI)*	P Value
Log ₁₀ -transformed proBNP	1.18 (1.05–1.32)	0.004	1.14 (1.01–1.33)	0.006
Quartile 1 (≤ 67.1)	1.00 (reference)	—	1.00 (reference)	—
Quartile 2 (67.2–142.4)	1.16 (0.97–1.38)	0.093	1.16 (0.95–1.40)	0.118
Quartile 3 (142.5–327.2)	1.28 (1.07–1.52)	0.006	1.30 (1.06–1.54)	0.004
Quartile 4 (≥ 327.3)	1.32 (1.11–1.57)	0.002	1.32 (1.11–1.59)	0.004

BNP indicates B-type natriuretic peptide; and OR, odds ratio.

*Odds of having a higher mRS score at 14 d or hospital discharge.

[†]Adjusted for age, sex, stroke subtype, National Institutes of Health Stroke Scale score, hours from onset to hospitalization, current smoking, current drinking, body mass index, systolic blood pressure, disease history (hypertension, diabetes mellitus, dyslipidemia, coronary heart disease), and treatment group at admission. A Hosmer–Lemeshow goodness-of-fit test was applied to examine the model fit ($\chi^2=68.30$, $P=0.077$ for continuous \log_{10} -transformed proBNP).

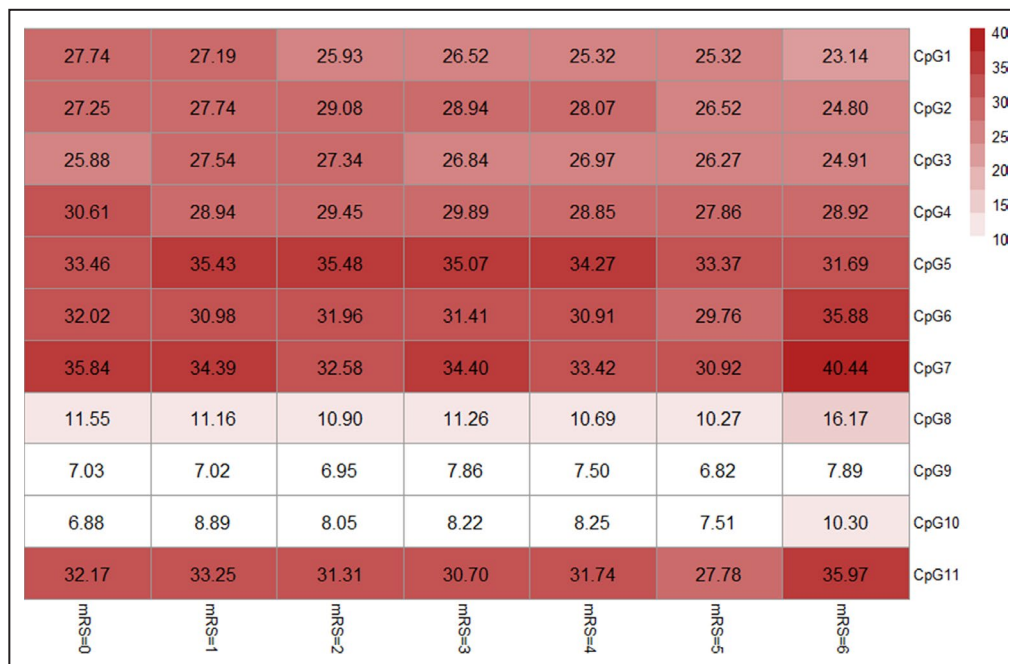


Figure 3. Heat map illustrating the average methylation levels at all CpG loci in the *NPPB* promoter according to mRS score at 14 days or hospital discharge. Color depth shows the value of the corresponding methylation level. BNP, B-type natriuretic peptide; mRS indicates the modified Rankin Scale; and *NPPB*, natriuretic peptide B.

Bioinformatic analysis found that the *NPPB* gene was highly expressed in extracellular space³¹ and participated in 3 KEGG pathways including vascular smooth muscle contraction, the cGMP-PKG signaling pathway, thermogenesis, and 17 biological processes (Table 5), all of which are involved in the pathogenesis of AIS and its

recovery. Gene network analysis showed similar results that, interacting with other natriuretic peptides (*NPPA* [natriuretic peptide A], *NPPC* [natriuretic peptide C]) and receptors (*NPR1* [natriuretic peptide receptor 1], *NPR2* [natriuretic peptide receptor 2], *NPR3* [natriuretic peptide receptor 3]) and their activator (*CORIN* [corin]), *NPPB*

Table 4. Association of CpG Methylation in *NPPB* Promoter With the Functional Outcome at 14 days or Hospital Discharge After Stroke Onset (n=806)

CpG Loci	Genomic Position (GRCh37)	Relative to TSS, bp	Methylation Level, %	Unadjusted		Adjusted [†]	
				OR (95% CI)*	P Value	OR (95% CI)*	P Value
CpG1	Chr1:11919160	-168	26.25±9.80	0.93 (0.87–0.99) [‡]	0.027 [‡]	0.93 (0.86–0.98) [‡]	0.022 [‡]
CpG2	Chr1:11919144	-152	28.15±9.94	0.99 (0.93–1.05)	0.788	0.98 (0.93–1.07)	0.684
CpG3	Chr1:11919141	-149	27.05±9.50	0.98 (0.92–1.04)	0.466	0.96 (0.90–1.05)	0.439
CpG4	Chr1:11919135	-143	29.19±10.27	0.98 (0.92–1.04)	0.476	0.97 (0.92–1.04)	0.330
CpG5	Chr1:11919133	-141	34.85±10.38	0.96 (0.91–1.02)	0.228	0.97 (0.92–1.03)	0.126
CpG6	Chr1:11919117	-125	31.31±11.98	0.99 (0.94–1.04)	0.719	1.00 (0.96–1.08)	0.848
CpG7	Chr1:11919096	-104	33.65±12.66	0.97 (0.92–1.02)	0.231	0.99 (0.93–1.06)	0.747
CpG8	Chr1:11919047	-55	11.05±7.65	0.99 (0.91–1.07)	0.729	0.98 (0.90–1.05)	0.851
CpG9	Chr1:11919019	-27	7.21±5.43	1.05 (0.94–1.17)	0.413	1.05 (0.94–1.18)	0.376
CpG10	Chr1:11919011	-19	8.26±6.51	0.98 (0.89–1.08)	0.644	0.99 (0.89–1.08)	0.787
CpG11	Chr1:11918989	3	31.63±11.75	0.94 (0.89–0.99) [‡]	0.023 [‡]	0.92 (0.88–0.99) [‡]	0.032 [‡]

GRCh37 indicates Genome Reference Consortium Human Build 37; *NPPB*, natriuretic peptide B; OR, odds ratio; and TSS, transcriptional start site.

*Odds of having a higher modified Rankin Scale score at 14 d or hospital discharge associated with per5% increment of corresponding CpG methylation.

[†]Adjusted for age, sex, stroke subtype, National Institutes of Health Stroke Scale score, hours from onset to hospitalization, current smoking, current drinking, body mass index, systolic blood pressure, disease history (hypertension, diabetes mellitus, dyslipidemia, coronary heart disease), and treatment group at admission. A Hosmer–Lemeshow goodness-of-fit test was applied to examine the model fit ($\chi^2=88.28$, $P=0.002$ for CpG1; $\chi^2=51.46$, $P=0.534$ for CpG11).

[‡]Values in bold indicate a false discovery rate–adjusted $P<0.2$.

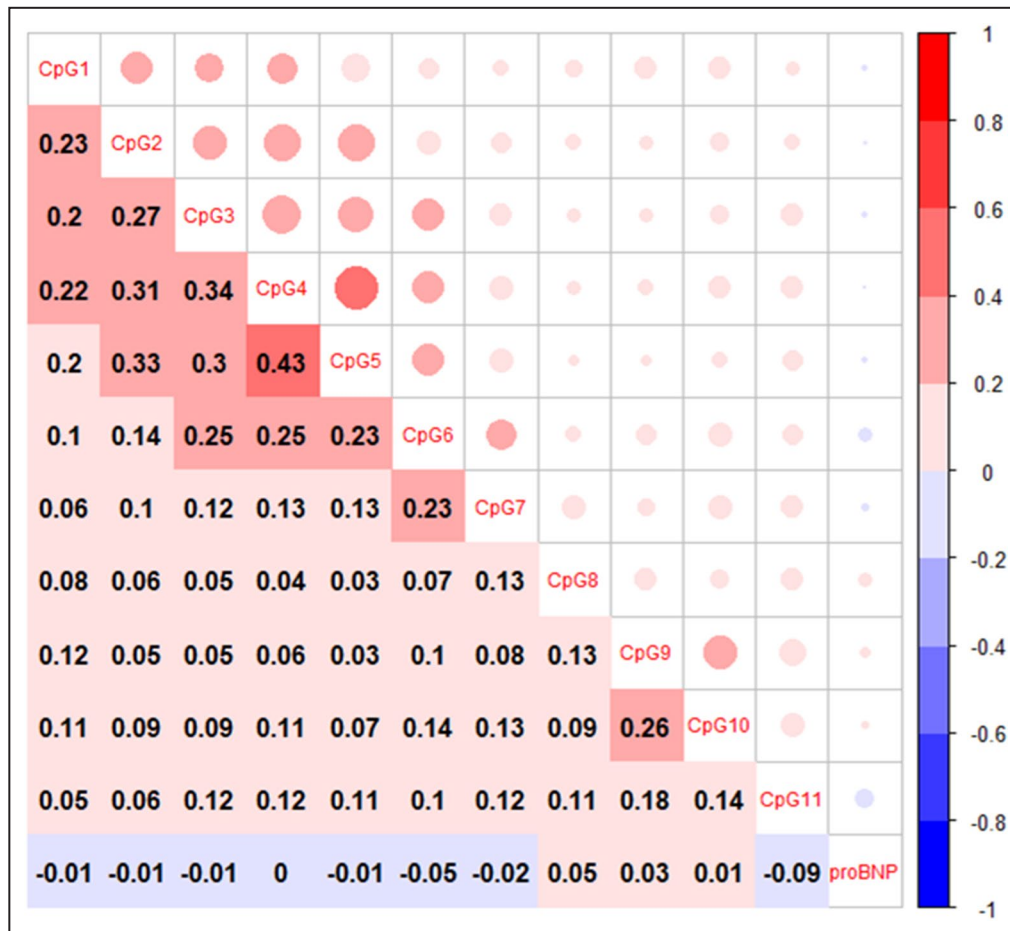


Figure 4. Spearman correlation matrix of DNA methylation of all CpG loci assayed in the *NPPB* promoter and proBNP (after log₁₀-transformation) at admission. The areas of circles and color depth show the absolute value of corresponding correlation coefficients. BNP indicates B-type natriuretic peptide; and *NPPB*, natriuretic peptide B.

played a critical role in above biological processes, for example, receptor guanylyl cyclase signaling pathway, cGMP biosynthetic process, blood circulation, regulation of blood pressure, circulatory system process, and cGMP metabolic process (Figure 6).

DISCUSSION

In a large sample of patients with AIS included in CATIS, we demonstrated for the first time that hypermethylation of the *NPPB* gene promoter at symptom onset was significantly associated with favorable neurologic recovery at 14 days or hospital discharge after stroke, and this association was partially mediated through proBNP concentrations, independent of conventional risk factors. Our findings suggest that *NPPB* gene methylation may be involved in the molecular mechanisms underlining the association between proBNP and functional outcomes of AIS.

The prognostic role of proBNP in ischemic stroke identified in our study is in line with prior studies. For example, a small sampled clinical study including 231 patients with AIS found that proBNP levels were higher in patients with a higher mRS score at discharge.³² This phenomenon was also observed in another study including 217 patients with AIS.⁷ In a larger sample of 615 patients with AIS, proBNP was positively associated with poor functional outcome defined as an mRS score ≥ 3 at 3 months after stroke.¹¹ In contrast, other studies found inconsistent results that proBNP was either negatively¹² or insignificantly¹³ associated with the functional outcome of ischemic stroke. These heterogeneous results may be due to small sample sizes of limited power. Well-designed prospective studies with large sample sizes are needed to further examine the potential relationship between proBNP and functional outcomes of ischemic stroke. Our previous study with a large sample size of >3000 patients with AIS found

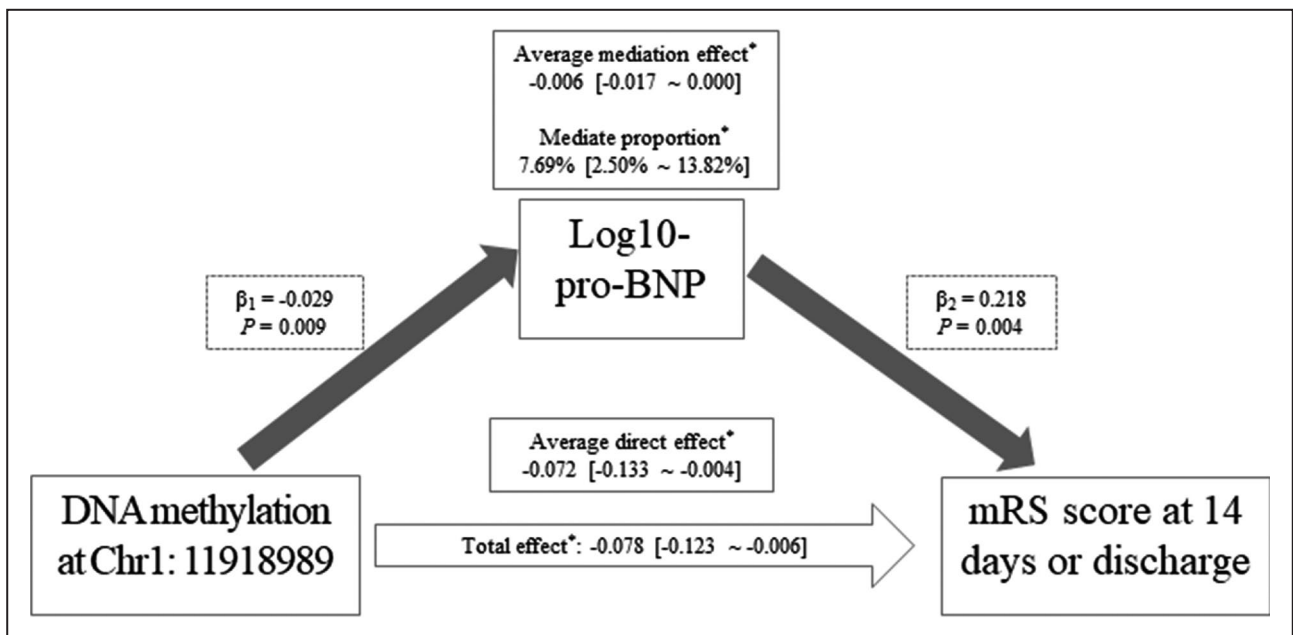


Figure 5. Schematic illustration of the mediating effect of proBNP on the association between DNA methylation at CpG11 (located in Chr:11918989) in the *NPPB* promoter and the functional outcome at 14 days or hospital discharge in 704 patients with ischemic stroke.

In this model, DNA methylation at CpG11 (per 5% increment) was the exposure variable, proBNP (after \log_{10} -transformation) was the mediator variable, and mRS was the outcome variable, adjusting for age, sex, stroke subtype, National Institutes of Health Stroke Scale score, hours from onset to hospitalization, current smoking, current drinking, body mass index, systolic blood pressure, disease history (hypertension, diabetes mellitus, dyslipidemia, coronary heart disease), and treatment group at admission. $P < 0.05$. BNP indicates B-type natriuretic peptide; mRS, the modified Rankin Scale; and *NPPB*, natriuretic peptide B.

that a higher level of proBNP could predict a long-term poor functional outcome at 1 year after stroke onset.³³ Together with these studies, the present

study provided further evidence that proBNP may be a validated biomarker for short- and long-term tissue damage of ischemic stroke and thus could be

Table 5. Biological Processes for the *NPPB* Gene Identified by GO

GO ID	Qualified GO Term	Evidence	PubMed IDs
GO:0003085	Negative regulation of systemic arterial blood pressure	IBA	21873635
GO:0006182	cGMP biosynthetic process	IDA, IBA	1672777
GO:0006457	Protein folding	IDA	16870210
GO:0007166	Cell surface receptor signaling pathway	NAS	12727915
GO:0007168	Receptor guanylyl cyclase signaling pathway	IDA	1672777
GO:0007218	Neuropeptide signaling pathway	IBA	21873635
GO:0007589	Body fluid secretion	TAS	14960748
GO:0008217	Regulation of blood pressure	NAS	12727915
GO:0010469	Regulation of signaling receptor activity	IEA	
GO:0016525	Negative regulation of angiogenesis	TAS	14737067
GO:0019934	cGMP-mediated signaling	IBA	21873635
GO:0030308	Negative regulation of cell growth	NAS	12727915
GO:0035810	Positive regulation of urine volume	TAS	14737067
GO:0035815	Positive regulation of renal sodium excretion	TAS	14960748
GO:0043114	Regulation of vascular permeability	TAS	14737067
GO:0050880	Regulation of blood vessel size	IEA	
GO:0097746	Regulation of blood vessel diameter	NAS	7601467

GO indicates Gene Ontology; IBA, inferred from biological aspect of ancestor; IDA, inferred from direct assay; IEA, inferred from electronic annotation; NAS, nontraceable author statement; *NPPB*, natriuretic peptide B; and TAS, traceable author statement.

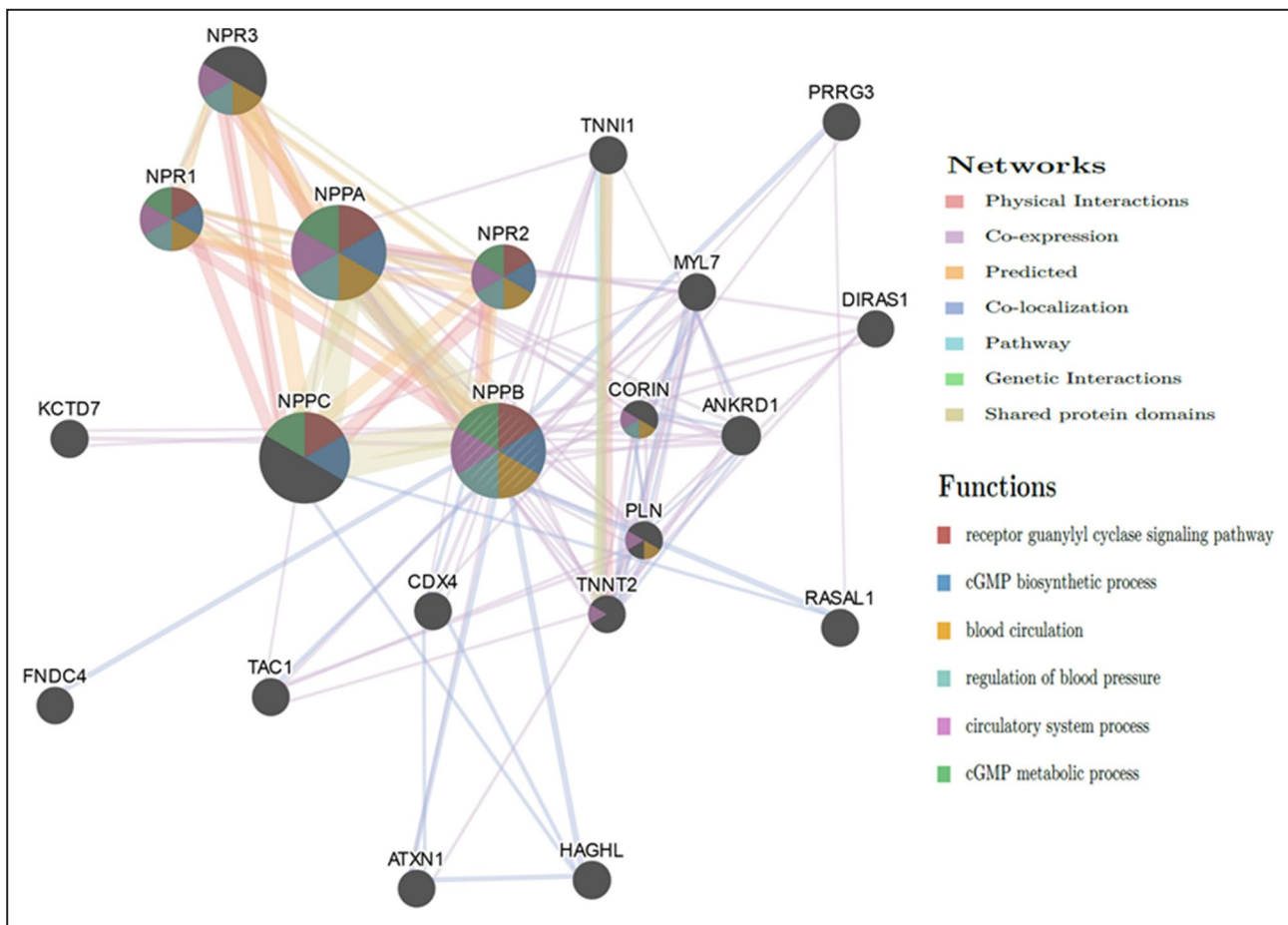


Figure 6. Schematic illustration of the gene network and biological functions involving the *NPPB* gene.

ANKRD1 indicates ankyrin repeat domain 1; *ATXN1*, Ataxin 1; *CDX4*, Caudal type homeobox 4; *CORIN*, corin; *DIRAS1*, *DIRAS* family GTPase 1; *FNDC4*, Fibronectin type III domain containing 4; *HAGHL*, Hydroxyacylglutathione hydrolase like; *KCTD7*, Potassium channel tetramerization domain containing 7; *MYL7*, myosin light chain 7; *NPR1*, natriuretic peptide receptor 1; *NPR2*, natriuretic peptide receptor 2; *NPR3*, natriuretic peptide receptor 3; *NPPA*, natriuretic peptide A; *NPPB*, natriuretic peptide B; *NPPC*, natriuretic peptide C; *PLN*, phospholamban; *PRRG3*, proline rich and Gla domain 3; *RASAL1*, RAS protein activator like 1; *TAC1*, Tachykinin precursor 1; *TNNI1*, troponin I1; and *TNNT2*, troponin T2.

applied in risk stratification among patients with AIS. However, the underlined molecular mechanisms are still unclear. Better understanding of the mechanisms would help us identify more potential therapeutic targets for ischemic stroke.

The protein of BNP is a circulating cardiac hormone synthesized and released as a precursor protein (1–108 amino acids), which is subsequently cleaved by furin into equimolar amounts of the active peptide BNP (77–108 amino acids) and the biologically inert proBNP (1–76 amino acids; ie, N-terminal proBNP). Despite being produced equimolarly, proBNP has a longer half-life and is more stable in the circulation than BNP.²⁴ Consequently, it is widely studied and used as a biomarker for heart failure and ischemic stroke.^{2–4,7–10,12,13} Concentrations of proBNP in the circulation could reflect the levels of BNP synthesis and excretion. As a result, factors influencing genetic function and expression of the coding gene of BNP

(*NPPB*) may be involved in the mechanisms underlying the association between proBNP and functional outcomes of ischemic stroke. As an interface between the fixed genome and the dynamic environment, epigenetic factors such as DNA methylation in the *NPPB* gene may affect its function and subsequent BNP synthesis and excretion. This hypothesis has been validated in our study where hypermethylation at the promoter region of the *NPPB* gene was not only correlated to lower levels of proBNP, indicating suppressed BNP synthesis and excretion, but also associated with favorable neurologic recovery after ischemic stroke. In line with our epigenetic study, animal studies found a similar phenomenon in which DNA methylation status changed dramatically after cerebral ischemic injury, and dysregulated DNA methylation was related to poor tissue outcome in mice.^{21,22} Although DNA methylation of the *NPPB* gene has been scarcely studied in association with

functional prognosis of ischemic stroke in humans, some epigenetic studies have linked it to some other related phenotypes. For example, an epigenome-wide association study including 41 cases of heart failure and 31 healthy controls found that DNA methylation of a CpG locus (cg00831929) located in the *NPPB* promoter was significantly decreased in heart failure.³⁴ We are the first, to the best of our knowledge, to examine and find that hypermethylation of the *NPPB* promoter could predict favorable neurologic recovery after ischemic stroke and, therefore, may be a candidate molecular mechanism explaining the prognostic role of proBNP in ischemic stroke.

We further examined whether hypermethylation of the *NPPB* promoter identified in our study conserves biological functions on regulating gene expression, which is of critical importance in subsequent clinical translation. We linked DNA methylation to proBNP concentrations and performed a causal mediation analysis to validate the pathway of *NPPB* methylation to functional outcome. The results showed that $\approx 8\%$ of the association between hypermethylation at 1 CpG locus (located in Chr1:11918989) and functional outcome was mediated through proBNP. Hypermethylation at this locus of the *NPPB* gene may predict favorable functional outcome after AIS, at least partially by suppressing BNP synthesis or excretion.

Considering the prospective associations of proBNP and *NPPB* methylation in the blood with the functional outcome of AIS observed in populations, BNP is either a causal factor or a surrogate biomarker for neurologic function with AIS. BNP and its receptors have been found in different cerebral regions such as hypothalamus or cerebral cortex,¹⁴ whereas BNP-mRNA has not been detected in the brain, suggesting a peripheral origin of this peptide.^{35,36} In animal models of cerebral ischemia, administration of BNP improved cerebral blood flow and reduced inflammation in brain injury.¹⁵ The observed neuroprotective effect seemed to be mediated through the cGMP pathway, which reduces the sodium and water content in the brain and reduces the neurotoxicity caused by overexcitation of N-methyl-D-aspartate receptors and inflammation.^{37,38} However, it is not clear to date whether the neuroprotective effect of BNP is mediated by a direct effect on cerebral receptors or an indirect effect on the systemic system. Although the causality is uncertain, the predictive role of proBNP in the functional outcome of AIS is true. The paradoxical phenomenon of higher proBNP levels in the blood with the poorer functional outcome of AIS in populations may suggest that BNP is more likely to be a surrogate biomarker rather than a pathological cause of neurologic recovery.

Several strengths in our study deserved to be mentioned. First, we used innovative statistical methods to

test the joint association of DNA methylation at multiple CpG sites in the *NPPB* gene with the functional outcome. Our results provide initial evidence that altered DNA methylation of the *NPPB* gene may be jointly associated with functional outcome after ischemic stroke. Second, although previous studies have reported the relationship between proBNP and the functional prognosis of ischemic stroke,^{7,11,32,33} the noted molecular mechanisms are still unclear. Our mediation analysis demonstrated that proBNP mediated the association between DNA methylation of the *NPPB* gene and the functional outcome. This finding may unravel a molecular mechanism of *NPPB* promoter hypermethylation underlying the association between proBNP and the functional outcome of ischemic stroke.

Our study also has some limitations. First, we assayed DNA methylation only in peripheral blood, but because DNA methylation is tissue-specific, it is unclear whether or to what extent our results could reflect DNA methylation changes in the brain, the target organ of functional recovery of ischemic stroke. According to the algorithm suggested by Hannon and colleagues,³⁹ we found significant correlations in DNA methylation of the *NPPB* gene between white blood cells and the brain tissue. Moreover, accumulating evidence indicated that epimutations might not be limited to the affected tissue but also could be detected in peripheral blood.⁴⁰ Second, although our statistical analyses adjusted for many covariates, we cannot entirely exclude residual confounding by unknown and/or unmeasured factors. The causality of BNP and its coding gene methylation in the neurologic recovery of ischemic stroke is uncertain. Our results could not be overinterpreted. Third, all participants were Chinese, and their cardiovascular health profiles might be different from those with other racial/ethnic backgrounds. Therefore, the generalizability of our results to other populations is uncertain. Fourth, some authors suggested that proBNP was associated with a higher chance of cardioembolic stroke etiology, which is also known to be associated with unfavorable outcome. However, we did not have data on atrial fibrillation, which may influence our results, although stroke subtype was adjusted for in our statistical analysis. Furthermore, we did not find an interaction between DNA methylation of the *NPPB* promoter and embolism subtype in the unfavorable outcome of ischemic stroke. Fifth, data on proportions of white blood cell type were not available in CATIS, so we cannot eliminate the influence of cell type on our results. Last, the calibration of the regression model with CpG1 appears poor given that the *P* value of the Hosmer-Lemeshow goodness-of-fit test is 0.002. Caution should be used in interpreting the results regarding this CpG site.

In summary, we demonstrated that hypermethylation in the *NPPB* promoter at the acute phase of

ischemic stroke is associated with functional outcome at 14 days or hospital discharge, at least in part by suppression of BNP expression or excretion, indicated by a lower level of proBNP in the circulation. Our results may unravel a molecular mechanism underlying the association between proBNP and the progression of ischemic stroke.

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Disclosures

None.

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