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RESEARCH ARTICLE

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A comprehensive association analysis between homocysteine metabolic pathway gene methylation and ischemic stroke in a Chinese hypertensive population

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Abstract

Background: Ischemic stroke (IS) is a serious global health burden. In order to improve our understanding of the risk factors associated with IS, we investigated the combined effect of the methylation of five genes related to the metabolism of homocysteine on developing IS.

Methods: Quantitative methylation-specific PCR was used to measure the levels of promoter methylation in hypertensive and stroke patients. The cutoff value calculated by the maximum Youden index was used to classify the levels of gene methylation as hypomethylation and hypermethylation. Logistic regression was used to explore the relationship between gene methylation and IS.

Results: The methylation levels of the genes encoding methylenetetrahydrofolate dehydrogenase 1 [*MTHFD1*], cystathionine β-synthase [*CBS*], and dihydrofolate reductase [*DHFR*] in hypertensive patients were higher than those in stroke patients (all $p < 0.01$). *MTHFD1* hypermethylation, *CBS* hypermethylation, and *DHFR* hypermethylation were protective factors for stroke after adjustment for confounding factors. Compared with individuals carrying none of the biomarkers, the ORs [95% CIs] for stroke of those with 1 and 2 elevated biomarkers were 4.068 [1.670–9.913] and 15.345 [6.198–37.994] after adjustment for confounding factors. The participants with a larger number of biomarkers had an increased risk of stroke (p for trend <0.001). For the combination biomarkers, the area under the curve of the receiver operating characteristic was 0.716.

Conclusion: A significant linear relationship between the number of elevated biomarkers and the risk of stroke has been observed, suggesting that elevations of these biomarkers could be used for potentially predicting the disease.

KEYWORDS
association, DNA methylation, hyperhomocysteine, hypertension, ischemic stroke

Bo Li and Yuying Li these authors equally contributed.

1 | INTRODUCTION

Ischemic stroke (IS) is a serious disease burden and the third leading cause of death worldwide.[1,2] Actually, this health condition has become the leading cause of death in China.[3] Stroke is known to be a complex syndrome involving many biological pathways,[4] including folic acid and homocysteine metabolism.[5] However, the currently established pathway does not fully explain the risks associated with IS.

Exploring the relationship between stroke and the level of DNA methylation has attracted an increasing interest in recent years.[6,7] The methylation of DNA sequences is a heritable and reversible epigenetic mechanism for regulating gene expression.[8] Hyperhomocysteinemia and hypertension are both considered as the important risk factors for developing stroke and have a synergistic effect on it.[9] Studies have shown that abnormal methylation of genes related to the metabolism of homocysteine is associated with stroke in hypertensive patients.[10–14] The incremental usefulness of multiple biomarkers for predicting the outcomes due to IS has been well assessed.[4,5] In the current study, we aimed to evaluate the combined value of methylation of the five genes related to the metabolism of homocysteine (methylenetetrahydrofolate dehydrogenase 1 [*MTHFD1*], cystathionine β-synthase [*CBS*], dihydrofolate reductase [*DHFR*], serine hydroxymethyltransferase 1 [*SHMT1*], and S-adenosylhomocysteine hydrolase [*AHCY*]) for predicting the risk to develop stroke in patients with hypertension.

2 | MATERIALS AND METHODS

2.1 | Study population

This was a community-based cross-sectional study involving 216 hypertensive patients without stroke and 136 hypertensive patients with stroke recruited from patients attending the Community Health Service Center of Nanshan District in, Shenzhen, China, during the period of October 2018 to October 2019. The inclusion criteria were as follows: (1) Han ethnicity and aged ≥20 years, (2) integrated health and hypertension records at the community health service center, and (3) residence in Shenzhen for >6 months. The diagnostic criteria for hypertension were a diastolic blood pressure (DBP) ≥140 mmHg and/or systolic blood pressure (SBP) ≥90 mmHg, or a self-reported medication history for hypertension. Stroke was diagnosed by two neurologists according to WHO diagnostic criteria, based on the ICD-10. The following exclusion criteria were applied: (1) patients with secondary hypertension, (2) patients with regular intaking of folic acid or vitamin B6 or vitamin B12, (3) patients with coronary heart disease and other cardiovascular and cerebrovascular diseases, (4) patients with malignant tumor, (5) patients with severe liver and kidney diseases, and (6) patients who are pregnancy. All subjects were apprised of the purpose and method of the experiment before the study, and signed informed consent voluntarily. This study has been approved by the Ethics Committee of Shenzhen Nanshan Hospital for Chronic Disease Prevention and Control (No: 1120170008).

2.2 | Demographic and laboratory assays

Questionnaires, physical examinations, and laboratory analyses were conducted by investigators who had been specially trained. The questionnaires were designed to obtain demographic information, such as age, sex, smoking status, and alcohol status. Physical examinations included blood pressure, height, weight, and waist/hip ratio (WHR). Laboratory indicators included homocysteine (Hcy), triglycerides (TGs), total cholesterol (TC), low-density lipoprotein (LDL), blood glucose (Glu), and uric acid (UA). An automatic biochemical analyzer (HITACH7080) was used to detect biochemical indexes, and a standard quantitative analysis was used to detect UA.

2.3 | DNA methylation analysis

After 12 h of fasting, an ethylenediaminetetraacetic acid (EDTA) anticoagulation tube was used to collect 5 mL venous blood samples from the enrolled participants. The samples were then immediately analyzed in the laboratory or stored at −80°C. DNA was extracted from blood using a Lab-Aid 820 Nucleic Acid Extractor (Xiamen Zhishan Biotechnology Co., Ltd.). An EZ DNA Methylation-Gold Kit (Zymo Research Corporation, USA) was used for the conversion of DNA into bisulfite. The specific reaction conditions were 1.5 μl sulfite-converted DNA, 0.5 μl forward and reverse primers, 10 μl Zymo Taq™ PreMix, and 7.5 μl DNase/RNase-free water. The specific primer design is shown in Table A1. The conditions of polymerase chain reaction (PCR) amplification were the same as in a previous study.[11] After PCR amplification of the DNA sequences of the samples to be tested, the methylation level was detected using a Qsep100 DNA Analyzer (BiOptic Inc., China). The experiments were carried out in strict accordance with the manufacturers' instructions.

2.4 | Statistical analysis

All statistical analyses were performed using SPSS version 18.0 (SPSS, Inc., Somers, NY, USA). Effects with a two-sided $p < 0.05$ were considered statistically significant. Normally distributed data were analyzed using Student's *t* test, and skewed data were analyzed using the Mann-Whitney *U* test. Spearman's correlation coefficients (r) were calculated to explore the relationships between the effects of different gene methylations. Multivariate logistic regression analysis was applied to explore the association of gene methylation with suffering from stroke to prove adjusted odds ratio (OR) and its 95% confidence interval (CI). The cutoff value calculated by the maximum Youden index was used to divide gene methylation

levels into hypomethylation and hypermethylation, and genes with a methylation level less than the cutoff value were considered as the biomarkers. The areas under the receiver operating characteristic (ROC) and the area under the curves (AUC) were calculated to evaluate the diagnostic value of the elevated biomarkers.

3 | RESULTS

Between the control group (216 hypertensive patients without stroke) and the case group (136 patients with stroke), there were no statistically significant differences in age, sex, BMI, WHR, SBP, DBP, smoking status, UA, TG, or Glu (all $p > 0.05$, Table 1). The TC and LDL levels were both higher in the control group than in the case group (5.21 ± 0.99 mmol/L vs. 4.92 ± 1.09 mmol/L and 3.04 ± 0.76 mmol/L vs. 2.84 ± 0.81 mmol/L, both $p < 0.05$, Table 1). The level of Hcy was 15.60 μmol/L in the case group, compared with 14.00 μmol/L in the control group ($p < 0.05$, Table 1). In addition, there was a statistically significant difference in the alcohol status between the two groups ($p < 0.05$, Table 1).

Data on promoter methylation of the five investigated genes present a skewed distribution in the two groups. As shown in Table 1, the methylation levels of the *MTHFD1*, *CBS*, and *DHFR* genes were higher in the control group than in the case group (13.28; 95% CI [8.49–18.89] vs. 5.36 [2.72–7.26], 51.85 [27.90–72.82] vs. 36.08 [19.46–52.67], 30.7 [11.34–62.45] vs. 9.71 [3.2–16.13], all $p < 0.01$).

Methylenetetrahydrofolate dehydrogenase 1 methylation, *CBS* methylation, and *DHFR* methylation were risk factors for stroke after adjusting for age, sex, BMI, WHR, SBP, DBP, smoking status, alcohol status, UA, TC, TG, Glu, LDL, and Hcy (OR [95% CI] =0.873 [0.823–0.926]; OR [95% CI] =0.990 [0.980–0.999]; OR [95% CI] =0.955 [0.935–0.975], Table 2). As shown in Figure 1, *MTHFD1* methylation was also correlated with both *CBS* methylation and *DHFR* methylation (r_{CBS} =0.42, r_{DHFR} =0.33, both $p < 0.05$). Therefore, the *MTHFD1* gene was not considered to be a candidate combination biomarker for stroke in patients with hypertension.

Using the ROC curves to determine the optimal cutoff values for predicting stroke, the cutoff values for *DHFR* methylation and *CBS* methylation were 19.65 and 48.33, and their corresponding AUC values were 0.661 and 0.581 (Table 3). The prevalence of stroke in individuals simultaneously with these two biomarkers was 60.7%. Participants who occupied more number of elevated biomarkers had an increased risk of suffering from stroke (p for trend <0.001, Table 4). Compared with individuals who carried neither biomarker, the ORs [95% CIs] for stroke of those with 1 and 2 elevated biomarkers were 4.068 [1.670–9.913] and 15.345 [6.198–37.994] after adjustment for relevant risk factors. As shown in Figure 2, the AUC of the combined biomarkers (*DHFR* and *CBS*) was 0.716, suggesting a high potential diagnostic value for predicting stroke.

TABLE 1 Characteristics of participants by study group

	Controls (n = 216)	Cases (n = 136)	t/χ^2	p value
Age (yrs)	64.55 ± 10.51	66.43 ± 9.52	−1.57	0.12
Gender (men)	94	65	0.28	0.59
BMI (kg/m^2)	24.42 ± 2.81	24.53 ± 2.85	−0.34	0.73
WHR	0.91 ± 0.07	0.92 ± 0.10	−1.53	0.13
SBP (mmHg)	134.17 ± 16.79	136.02 ± 15.80	−0.95	0.34
DBP (mmHg)	82.59 ± 11.08	82.00 ± 1.31	0.45	0.65
Smoking (yes)	23	14	0.05	0.83
Drinking (yes)	49	17	6.66	0.01
UA (μmol/L)	365.65 ± 102.93	366.17 ± 88.75	−0.04	0.96
TG (mmol/L)	2.06 ± 1.79	1.81 ± 0.94	1.40	0.16
TC (mmol/L)	5.21 ± 0.99	4.92 ± 1.09	2.35	0.02
Glu (mmol/L)	5.59 ± 1.17	5.63 ± 1.08	−0.32	0.75
LDL (mmol/L)	3.04 ± 0.76	2.84 ± 0.81	2.16	0.03
Hcy (μmol/L)	14.00 (11.80–17.60)	15.60 (12.50–21.70)	8.91	<0.01
MTHFD1	13.28 (8.49–18.89)	5.36 (2.72–7.26)	9871	<0.01
CBS	51.85 (27.90–72.82)	36.08 (19.46–52.67)	14.99	<0.01
DHFR	30.70 (11.34–62.45)	9.71 (3.20–16.13)	40.91	<0.01
SHMT1	33.68 (15.72–68.42)	27.07 (20.92–42.19)	1.11	0.29
AHCY	0.02 (0–0.19)	0.01 (0–0.06)	1.78	0.18

Abbreviations: *AHCY*, S-adenosylhomocysteine hydrolase; BMI, body mass index; *CBS*, cystathionine β-synthase; DBP, diastolic blood pressure; *DHFR*, dihydrofolate reductase; Glu, blood glucose; Hcy, total homocysteine; LDL, low-density lipoprotein; *MTHFD1*, methylenetetrahydrofolate dehydrogenase 1; SBP, systolic blood pressure; *SHMT1*, serine hydroxymethyltransferase 1; TC: total cholesterol; TG, triglyceride; UA, uric acid; WHR, waist-hip ratio.

TABLE 2 Univariate and multivariable analysis of factors associated with suffering from stroke

	Model 1		Model 2	
Biomarkers	**OR (95% CI)**	***p* value**	**OR (95% CI)**	***p* value**
MTHFD1	0.883 (0.838–0.931)	<0.001	0.873 (0.823–0.926)	<0.001
CBS	0.995 (0.956–1.003)	0.222	0.990 (0.980–0.999)	0.038
DHFR	0.953 (0.935–0.972)	<0.001	0.955 (0.935–0.975)	<0.001
SHMT1	0.993 (0.981–1.006)	0.301	0.989 (0.975–1.004)	0.152
AHCY	0.613 (0.123–3.058)	0.551	0.436 (0.078–2.447)	0.346

Note: Model 1: univariate regression model unadjusted for potential confounders, Model 2: multivariate regression model adjusted for age, sex, BMI, WHR, SBP, DBP, smoking and drinking status, UA, TC, TG, Glu, LDL, and Hcy.

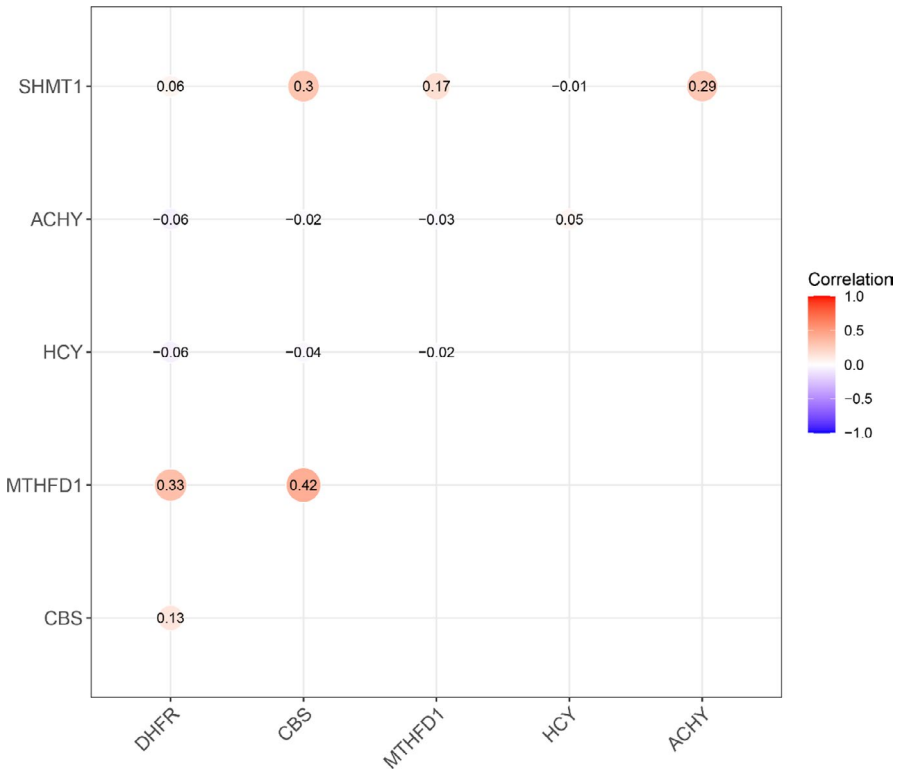

FIGURE 1 Spearman's correction of five biomarkers and Hcy

TABLE 3 Elevated biomarkers and risk for suffering from stroke in patients with hypertension

Elevated biomarkers	OR (95% CI)	AUC	Sensitivity, %	Specificity, %
DHFR, <19.65	6.060 (3.493–10.513)	0.661	0.517	0.934
CBS, <48.33	2.822 (1.680–4.738)	0.583	0.483	0.708

4 | DISCUSSION

Stroke is a multifactorial and polygenic disorder with some common environmental factors.[15] However, the genetic risk factors, especially the interactions of genes, remain to be elucidated. In this study, we performed a comprehensive genetic association analysis of five candidate genes involved in the folic acid and homocysteine metabolic pathway in a Chinese population with IS. Individually, we observed that elevated *CBS*, *DHFR*, and *MTHFD1* methylation levels were associated with a decreased risk of stroke in hypertension, independently of the established covariates. In addition, when *CBS* hypomethylation and *DHFR* hypomethylation were combined as biomarkers, there was a clear trend toward an increasing risk of stroke with increasing number of elevated biomarkers from 0 to 2. Participants with both elevated biomarkers had a 15-fold increased risk of stroke.

Cystathionine β-synthase and *DHFR* are both related to stroke through their regulatory roles in the levels of homocysteine and folic acid.[16] Hyperhomocysteinemia may involve in the mechanism

TABLE 4 Multivariable regression analysis number of biomarkers associated with ischemic stroke

No. of biomarkers[a]	Events, *n* (%)	OR (95% CI)	*p* trend
0 (*n* = 69)	7 (10.1)	Reference	<0.001
1 (*n* = 148)	47 (31.8)	4.068 (1.670–9.913)	
2 (*n* = 135)	82 (60.7)	15.345 (6.198–37.994)	

[a]The number of elevated biomarkers.

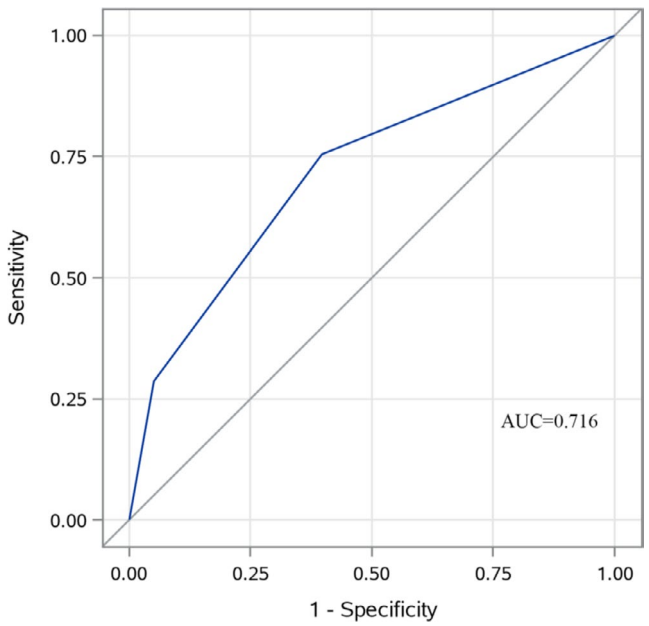

FIGURE 2 Receiver operating characteristic curve of combined biomarkers (*DHFR* and *CBS*) for predicting stroke in patients with hypertension

of stroke by increasing the incidence of atherogenesis[17] through coagulation abnormalities and endothelial damage.[18] Homocysteine can also directly affect nitric oxide synthesis[19] and prohibit endothelial cell proliferation by inhibiting enzymatic activity. *CBS* methylation is the main mechanism used to control the expression of this gene. Methylated *CBS* transcribes an enzyme that participates in the process of transforming homocysteine to cystathionine with the assistance of vitamin B6.[20] The transcription of *CBS* is positively correlated with the level of Hcy.[21] Meanwhile, *DHFR* can convert folate derivatives to dihydrofolate and then to tetrahydrofolate.[22] In the previous studies, the levels of *CBS*[11] and *DHFR*[12] methylation were both found to be independently related to stroke and to play an important role in the incidence of stroke. Moreover, because *CBS* methylation and *DHFR* methylation have no significant association with each other, they can both be used as biomarkers for predicting stroke.

The cumulative effects of single-nucleotide polymorphisms (SNPs) in genes related to homocysteine metabolism have been demonstrated in previous studies.[5,23] The results suggested that the joint effect of several small-to-moderate risk variants could confer an increase in the risk of IS. In our study, when *CBS* methylation and *DHFR* methylation were combined, the diagnostic value for IS was improved. Furthermore, the risk of IS was higher with both biomarkers than with either methylated gene alone. As we know, plasma Hcy has a positive association with the risk of stroke.[9] This observation may lead to a deeper explanation of the potential pathophysiological mechanism of stroke and was thought to be an efficient tool for understanding the effect of Hcy on stroke. From which, in order to prevent the incidence of stroke, we should pay more attention on the level of Hcy.

In this study, real-time quantitative methylation-specific PCR (qMSP) was used to determine the methylation level of genes in a large sample population. qMSP combines the advantages of methylation-specific PCR and real-time quantitative PCR with high sensitivity,[24] and is less costly and time-consuming than pyrophosphate sequencing.[25] To the best of our knowledge, this was the first study to evaluate the associations between gene promoter methylation interactions and stroke, particularly in hypertension.

It should be noted that there are some potential limitations to the current study. First, as cross-sectional studies cannot determine cause-and-effect relationships, but can make inferences about possible relationships to support further research, a well-designed prospective cohort study may be needed to validate the causal association of gene promoter methylation interactions with developing stroke. Second, some of the data collected from questionnaire are subject to self-reporting and recall bias, which may be inevitable in this case-control study. Third, this study did not explore the underlying mechanism. Finally, we focused only on five genes in the homocysteine and folic acid metabolic pathway. Additional genes in this pathway should be considered in further studies in the future.

5 | CONCLUSION

In summary, our results suggest that the methylation of *CBS*, *DHFR*, and *MTHFD1* genes was strongly associated with the development of stroke and that the combination of *CBS* methylation and *DHFR* methylation can serve as a biomarker of stroke. There was a clear linear association between the risk of stroke and increasing numbers of elevated novel biomarkers. The incorporation of a combination of both biomarkers into a predictive model could improve the diagnostic value for IS in patients.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also form part of an ongoing study.

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APPENDIX

TABLE A1 Primer sequences of five homocysteine metabolism key enzyme genes

	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product (bp)	Tm (°C)
AHCY	GGTCGTAATCGGTTGTAG	CAATTCCTATTCCCAAACTAAA	124	58
CBS	GGATGGAGTTATATTATGAAGGT	AACAATCTCGCTCAATCG	93	56
DHFR	TATTTGAGCGGTGGTTAG	TCTACTATAACGAACGAACTC	131	58
MTHFD1	AAGGTTATGGCGTTAGTAGAA	CCACACTCAACAACAATATCAA	77	58
SHMT1	CGAGTTTAGGAAGGTGTATT	CCATACTTAACTACGCTCTC	88	58
ATCB1	GTGATGGAGGAGGTTTAGTAAGTT	CCAATAAAACCTACTCCTCCCTTAA	129	56
ACTB2	TGGTGATGGAGGAGGTTTAGTAAGT	AACCAATAAAACCTACTCCTCCCTTAA	133	58

Abbreviations: *ACTB*, β-actin; *AHCY*, S-adenosylhomocysteine hydrolase; *CBS*, cystathionine β-synthase; *DHFR*, dihydrofolate reductase; *MTHFD1*, methylenetetrahydrofolate dehydrogenase 1; *SHMT1*, serine hydroxymethyltransferase 1.