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RESEARCH ARTICLE

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Significant association between *DHFR* promoter methylation and ischemic stroke in a Chinese hypertensive population

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Abstract

Objective: *DHFR* encodes dihydrofolate reductase, a major enzyme in the metabolism of folate, and is a candidate gene for ischemic stroke (IS). Therefore, we aimed to investigate the association between *DHFR* promoter methylation and IS in a Chinese population with primary hypertension.

Methods: Quantitative methylation-specific PCR was used to measure the level of *DHFR* promoter methylation. A multivariate logistic regression model was used to investigate the association between *DHFR* promoter methylation and IS. Receiver operating characteristic (ROC) curve analysis was used to evaluate the diagnostic value of *DHFR* promoter methylation for IS.

Results: The level of methylation of the *DHFR* promoter in the IS group was significantly lower than that in the hypertensive group (median [interquartile range]: 9.11 [2.81-16.20] vs 24.94 [7.16-56.45], $P < .001$). *DHFR* promoter methylation and homocysteine (Hcy) levels were both related to IS, with an ORs (95% CI) of 0.976 (0.967-0.984) and 1.057 (1.027-1.108), respectively. The areas under the curve for the diagnosis of *DHFR* promoter hypomethylation in IS were 0.603 (95% CI, 0.527-0.678) in men and 0.754 (95% CI, 0.693-0.815) in women. A dual-luciferase reporter assay revealed that the target sequence in the *DHFR* promoter upregulated gene expression.

Conclusion: There is a significant association between methylation of the *DHFR* promoter and IS in this Chinese hypertensive population. Hypomethylation of the *DHFR* promoter may serve as a novel marker for the diagnosis of IS in women.

KEYWORDS

DHFR, homocysteine, hypertension, ischemic stroke, promoter methylation

Jingcen Hu, Hong Zhu and Guodong Xu contributed equally to this work.

1 | INTRODUCTION

Ischemic stroke (IS), the leading cause of death in China,[1,2] is a major public health burden.[3,4] After adjusting for age, mortality from stroke was approximately 354 per 100 000 person-years in China in 2016.[1]

Ischemic stroke is a multifactorial disorder, influenced by environmental factors, genetic alterations, and gene-environment interactions. Hypertension is recognized as one of the critical risk factors in stroke, contributing to 34.6% of the risk of stroke.[2] Dihydrofolate reductase (DHFR), a key enzyme involved in the metabolism of folate, converts dihydrofolate to tetrahydrofolate, which is then converted to 5-methyltetrahydrofolate under the catalysis of methylenetetrahydrofolate reductase. 5-Methyltetrahydrofolate provides a methyl group to folate and thus influences folate metabolism.[5] Folate is also an important factor influencing stroke, and our previous study indicated that folic acid supplementation can reduce the risk of IS by reducing the level of homocysteine (Hcy).[6,7]

It is known that epigenetic modifications are involved in the pathogenesis of IS.[8] DNA methylation, one of the commonest epigenetic modifications, plays a critical role in controlling gene expression. Hypermethylation can silence gene transcription, whereas hypomethylation may promote transcription.[9] Compelling evidence suggests that aberrant methylation of genes is an important transcriptional regulator in the pathogenesis of stroke, as reported for long interspersed nucleotide element 1 (*LINE-1*),[10] methylenetetrahydrofolate dehydrogenase 1 (*MTHFD1*),[11] ATP-binding cassette sub-family B member 1 (*ABCB1*),[12] and matrix metalloproteinase-2 (*MMP-2*).[13]

Therefore, we investigated the association between *DHFR* promoter methylation and IS in a Chinese population with primary hypertension and evaluated the diagnostic value of *DHFR* promoter methylation for IS risk.

2 | MATERIALS AND METHODS

2.1 | Subjects

The study included 302 hypertensive controls and 158 age- and gender-matched IS cases with primary hypertension, who were registered in the Hypertension Management Information System[6] used by community health service centers in Nanshan district, Shenzhen. A flowchart for the case-control selection procedure is shown in Figure S1. All of the IS patients were re-diagnosed according to clinical evaluation and neuroimaging (CT or MRI) based on the diagnostic criteria of the World Health Organization.[6] Hypertension was confirmed by a systolic blood pressure (SBP) of ≥140 mm Hg or/and a diastolic blood pressure (DBP) of ≥90 mm Hg or self-reported use of antihypertensive medications. Individuals with potentially related diseases (such as CVD and diabetes) were excluded. None of the participants were pregnant, and none had secondary hypertension, liver or kidney failure, or malignancy. Patients with a history of taking vitamin B6, vitamin B12, or folic acid supplements were also excluded. The study was reviewed and approved by the Ethical Committee of Shenzhen Nanshan Center for Chronic Disease Control, and all of the participants provided informed consent.

2.2 | Physical examination

Trained medical physicians recorded each participant's SBP, DBP, body mass index (BMI), waist circumference (WC), and hip circumference (HC). Blood pressure was measured on the right arm after a 5-minute rest via a standard mercury sphygmomanometer. WC and HC were measured using inextensible anthropometric tape with the participants standing straight, feet positioned close together, and arms at their sides.

2.3 | Biochemical measurements

Biochemical measurements included plasma Hcy, uric acid (UA), triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL), and glucose (Glu). The levels of all biological indicators were quantified using a Hitachi 7080 automatic biochemical analyzer (Hitachi).

2.4 | DNA methylation analysis

We chose the target fragment of the *DHFR* gene promoter after searching the UCSC Genome Bioinformatics database (http://genome.ucsc.edu/) to determine whether a CpG island exists in the *DHFR* promoter region. The procedures used for DNA extraction and bisulfite conversion were as described in a previous study.[14] The following primer sequences were used to amplify the *DHFR* gene and beta-actin gene (*ACTB*). The forward primers were 5′-TATTTGAGCGGTGGTTAG-3′ and 5′-TGGTGATGGAGGAGGTTTAGTAAGT-3′, respectively, and the reverse primers were 5′-TCTACTATAACGAACGAACTC-3′ and 5′-AACCAATAAAACCTACTCCTCCCTTAA-3′, respectively. Amplification was conducted under the following conditions: denaturation at 95°C for 600 seconds, followed by 45 cycles at 95°C for 20 seconds, annealing at 56°C for 45 seconds, and a cycle at 72°C for 20 seconds. A melting curve step was performed at 95°C for 15 seconds and 60 seconds at 60°C, with the temperature rising by 0.11°C per second up to 95°C during the measurement of the fluorescence signal.

2.5 | Luciferase reporter gene assay

The amplified promoter DNA fragment was digested and purified by XhoI and NheI (New England Biolabs) and a Cycle Pure Kit (Omega), respectively. The target fragment (Figure 1) was then cloned to a

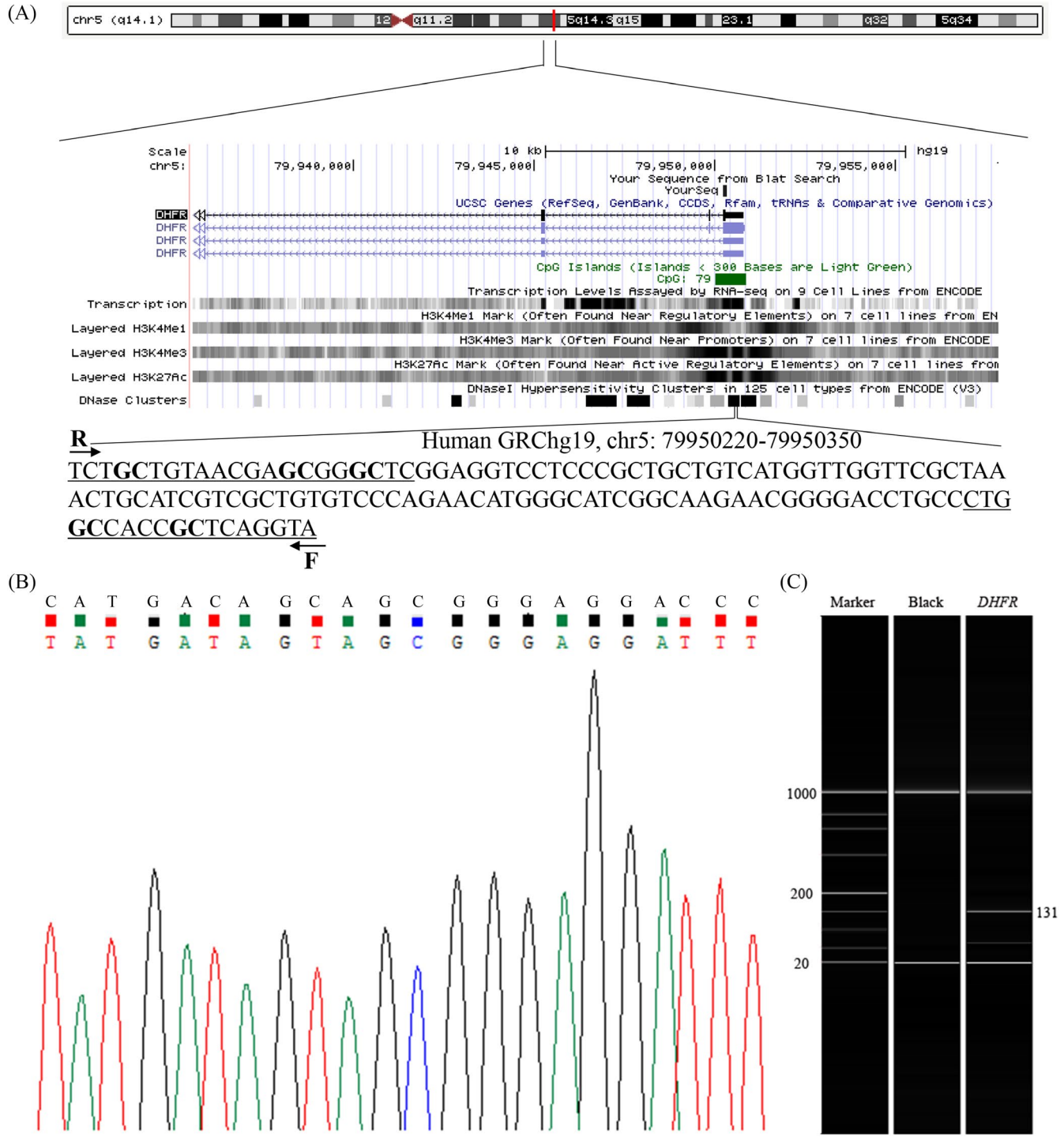

FIGURE 1 Methylation assay of *DHFR* gene and its quality control. A, The target sequence is located on the CpG island of *DHFR* gene (location). F: forward primer; R: reverse primer. B, Sequencing validation of the MSP product. The top row of the sequences represents the original gene sequence, and the second row shows the converted sequence. C, The fragment length of MSP product is 131 bp

pGL3 basic vector (Promega) using a DNA ligation kit (Takara). The pGL3 basic and promoter vectors were used as the negative and positive controls, respectively. We prepared cells using 96-well plates, and the details of plasmid transfection of human embryo kidney 293T (HEK293T) cells were as previously described.[15] Luciferase activity measured with a dual-luciferase reporter assay system (Promega) was used to explore the gene promoter's regulatory function.

2.6 | Statistical analysis

Continuous variables with a normal distribution were presented as mean ± standard deviation (SD) and were analyzed using *t* tests. Skewed distribution data were presented as medians (interquartile range [IQR]) and analyzed using Mann-Whitney *U* tests. Frequencies (percentages) and chi-square tests were used to express and analyze

TABLE 1 Baseline characteristics of hypertensive controls and ischemic stroke cases

	Hypertensive controls	Ischemic stroke cases	t/χ^2	*P*
Age (y)	65.40 ± 9.82	66.77 ± 9.20	1.14	.361
Gender (Men)	149 (49.34%)	78 (49.37%)	0.00	.995
BMI (kg/m^2)	24.29 ± 2.91	24.29 ± 2.97	−0.01	.994
WC (cm)	86.96 ± 8.91	87.40 ± 10.10	−0.49	.625
HC (cm)	95.51 ± 8.03	95.63 ± 9.34	−0.14	.887
SBP (mm Hg)	134.33 ± 16.37	134.80 ± 15.23	−0.30	.765
DBP (mm Hg)	82.50 ± 10.65	81.35 ± 10.90	1.09	.274
Hcy (μmol/L)	15.60 ± 6.86	19.55 ± 17.08	−3.52	.001
TC (mmol/L)	5.18 ± 0.98	4.97 ± 1.09	1.94	.055
TG (mmol/L)	1.96 ± 1.52	1.80 ± 0.92	1.20	.231
UA (μmol/L)	359.33 ± 99.44	358.04 ± 89.20	0.14	.891
LDL (mmol/L)	3.02 ± 0.75	2.88 ± 0.83	1.81	.071
Glu (mmol/L)	5.59 ± 1.14	5.66 ± 1.24	−0.62	.536
Smoking	33 (10.93%)	18 (11.39%)	0.02	.880
Drinking	76 (25.17%)	32 (20.25%)	1.39	.238
Antihypertension	257 (85.10%)	139 (87.97%)	0.72	.397
PMR-DHFR (%)	24.94 (7.16-56.45)	9.11 (2.81-16.20)	38.93	<.001

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; Glu, blood glucose; HC, hip circumference; Hcy, plasma homocysteine; LDL, low-density lipoprotein; PMR, percent of methylated reference; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride; UA, uric acid; WC, waist circumference.

TABLE 2 Baseline characteristics in men and women

	Men	Women	t/χ^2	*P*
Age (y)	66.50 ± 9.69	65.19 ± 9.52	1.49	.138
BMI (kg/m^2)	24.38 ± 3.00	24.20 ± 2.85	0.65	.518
WC (cm)	88.77 ± 9.79	85.28 ± 8.58	4.14	<.001
HC (cm)	95.98 ± 8.74	94.80 ± 8.31	1.52	.130
SBP (mm Hg)	135.85 ± 15.61	133.27 ± 16.22	1.76	.078
DBP (mm Hg)	84.14 ± 10.61	80.07 ± 10.33	4.24	<.001
Hcy (μmol/L)	20.12 ± 15.03	13.68 ± 4.25	6.39	<.001
TC (mmol/L)	4.89 ± 0.97	5.32 ± 1.01	−4.73	<.001
TG (mmol/L)	1.83 ± 1.54	2.00 ± 1.25	−1.28	.202
UA (μmol/L)	392.19 ± 95.71	324.75 ± 83.02	8.21	<.001
LDL (mmol/L)	2.91 ± 0.71	3.04 ± 0.83	−1.84	.066
Glu (mmol/L)	5.58 ± 1.25	5.70 ± 1.16	−1.10	.271
Smoking	48 (21.15%)	5 (2.15%)	40.72	<.001
Drinking	79 (34.80%)	22 (9.44%)	43.03	<.001
Antihypertension	196 (86.34%)	200 (85.84%)	0.03	.875
PMR-DHFR in hypertension (%)	27.06 (1.26-62.05)	21.34 (7.16-43.31)	0.68	.493

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; Glu, blood glucose; HC, hip circumference; Hcy, plasma homocysteine; LDL, low-density lipoprotein; PMR, percent of methylated reference; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride; UA, uric acid; WC, waist circumference.

the categorical variables. Spearman's correlation was performed to explore the relationship between methylation level and age. The relationship between the level of *DHFR* promoter methylation and the risk of IS was analyzed using a multivariate binary logistic regression analysis model. A receiver operating characteristic curve (ROC) was used to evaluate the prognostic value of *DHFR* promoter

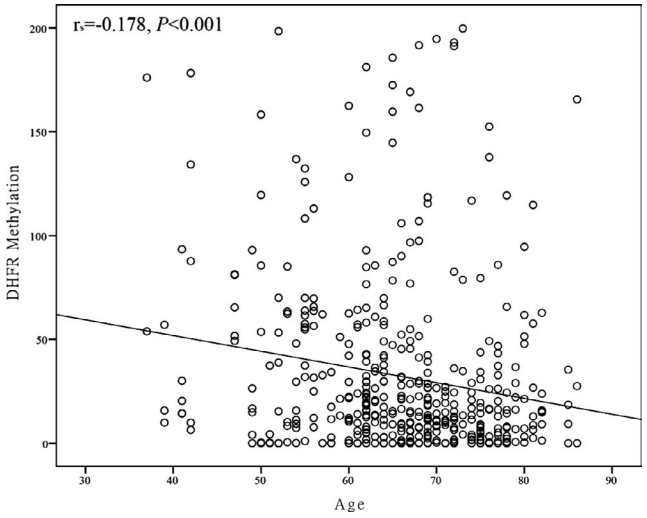

FIGURE 2 Spearman's correlation between age and *DHFR* methylation

methylation for IS. All statistical analyses were performed using SPSS version 18.0 (SPSS, Inc.). A two-sided $P < .05$ was considered statistically significant.

3 | RESULTS

The target fragment of the *DHFR* gene promoter was located in Human GRChg19, chr5: 79950220-79950350 (Figure 1). Five CpG sites were selected from the target fragment. The methylation level of the *DHFR* promoter was assessed from this fragment. Agarose gel electrophoresis confirmed that the fragment length of the methylation-specific PCR product was 131 bp (Figure 1).

The mean level of Hcy was higher in the IS group than in the hypertensive controls (19.55 ± 17.08 μmol/L vs 15.60 ± 6.86 μmol/L, $P = .001$, Table 1). The median percentage of *DHFR* promoter methylation was significantly higher in the hypertensive controls than in the IS cases (24.94% vs 9.11%, respectively, $P < .001$, Table 1). However, in the hypertensive group, there were no significant differences of the median percentage of *DHFR* promoter methylation in men and women (27.06% vs 21.34%, $P = .493$, Table 2).

A significant inverse correlation was found between *DHFR* promoter methylation and age ($r = -.178$, $P < .001$, Figure 2). In multivariate analysis, the OR (95%CI) of Hcy on the risk of IS was 1.057 (1.027-1.108, $P = .001$, Table 3). *DHFR* promoter methylation level was also related to IS, with an OR (95% CI) of 0.976 (0.967-0.984). There was a gender difference, with an OR (95% CI) of 0.986 (0.977-0.995) for men and 0.940 (0.917-0.963) for women (Table 3). The area under the ROC curve (AUC) was 0.677, suggesting that *DHFR* promoter methylation level may play a role in the diagnosis of IS. The AUCs for *DHFR* promoter methylation were 0.603 (95% CI, 0.527-0.678) for men and 0.754 (95%CI, 0.693-0.815) for women (Figure 3).

In the dual-luciferase reporter assay, there was no difference in the transcriptional expression of the recombinant pGL3-*DHFR* plasmid and the pGL3 basic vector control (mean ± SD: 0.011 ± 0.002 vs 0.008 ± 0.002, $P = .168$). However, there was significant positive transcriptional activity of the recombinant pGL3-promoter-*DHFR*

TABLE 3 Multivariable logistic regression analysis of association between *DHFR* methylation and the risk of ischemic stroke in total subjects, men, and women

	Odds ratio (95% CI)		
	Total	Men	Women[#]
PMR-DHFR	0.976 (0.967-0.984)*	0.986 (0.977-0.995)*	0.940 (0.917-0.963)*
Age (y)	0.993 (0.968-1.018)	1.022 (0.985-1.061)	0.998 (0.931-1.007)
Gender(W/M)	1.163 (0.707-1.913)	—	—
BMI (kg/m^2)	1.017 (0.938-1.092)	1.060 (0.939-1.197)	0.966 (0.853-1.094)
WC	0.997 (0.961-1.034)	1.014 (0.959-1.073)	0.992 (0.937-1.051)
HC	0.997 (0.962-1.034)	0.962 (0.909-1.073)	1.009 (0.957-1.064)
SBP (mm Hg)	0.998 (0.984-1.012)	0.994 (0.974-1.015)	0.998 (0.977-1.019)
DBP (mm Hg)	0.991 (0.970-1.011)	0.999 (0.968-1.030)	0.992 (0.960-1.025)
Hcy (μmol/L)	1.057 (1.027-1.108)*	1.045 (1.013-1.077)*	1.074 (0.944-1.159)
TC (mmol/L)	0.851 (0.498-1.452)	0.548 (0.252-1.189)	1.170 (0.551-2.483)
TG (mmol/L)	0.936 (0.762-1.150)	1.193 (0.923-1.543)	0.920 (0.628-1.096)
UA (μmol/L)	0.999 (0.996-1.001)	0.996 (0.993-1.000)	1.003 (0.999-1.008)
LDL (mmol/L)	0.932 (0.487-1.785)	0.977 (0.378-2.522)	0.786 (0.319-1.937)
Glu (mmol/L)	1.090 (0.905-1.313)	0.985 (0.755-1.2285)	1.183 (0.878-1.593)

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; Glu, blood glucose; HC, hip circumference; Hcy, plasma homocysteine; LDL, low-density lipoprotein; PMR, percent of methylated reference; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride; UA, uric acid; WC, waist circumference.

*$P < .05$,

[#]P-interaction < .05.

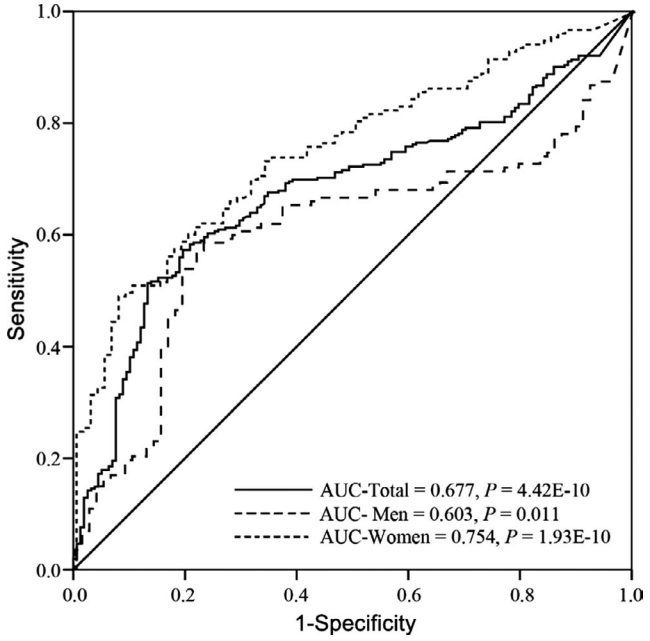

FIGURE 3 Receiver operation characteristics (ROC) curve of *DHFR* methylation and ischemic stroke in hypertensive patients

plasmid when compared with the pGL3-promoter vector (0.515 ± 0.017 vs 0.294 ± 0.015, fold change = 2, P = .004, Figure 4), suggesting an additional significant gene regulatory function of the *DHFR* promoter fragment (Figure 4).

4 | DISCUSSION

In this community-based case-control study, hypomethylation of *DHFR* was observed in patients with IS and verified as a risk factor for IS. We found an inverse correlation between *DHFR* promoter methylation and age, and a gender difference in the risk of IS. We also found an upregulated gene expression function of the target sequence in the *DHFR* promoter. All of these findings indicate that *DHFR* promoter methylation might play an important role in the development of IS.

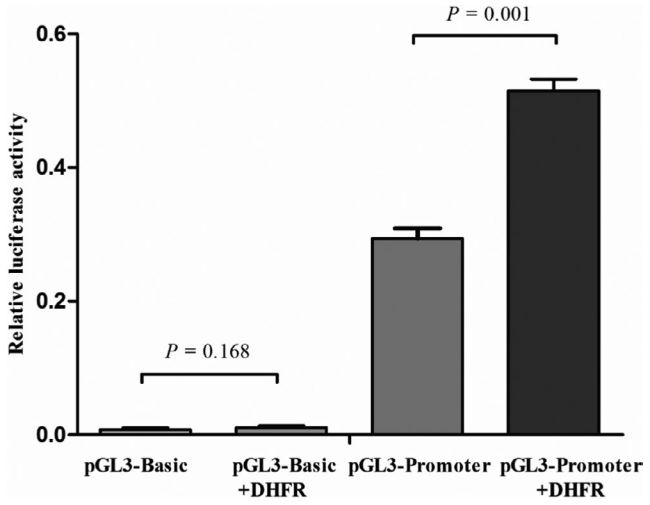

FIGURE 4 Dual-luciferase reporter assay in HEK-293T cell line. The pGL3 basic and promoter vectors were used as control in this study. Relative luciferase activity was performed in triplicates

Compelling evidence suggests that Hcy is a modifiable, independent risk factor for IS.[16] Our previous prospective cohort study showed that hyperhomocysteinemia patients have an 2.18-fold increased risk of IS.[6] We also verified the risk of elevated Hcy on IS in the current study, with the OR (95% CI) being 1.057 (1.027-1.108). Hcy increases the risk of IS via increasing arterial blood pressure,[17] decreasing the release of vascular endothelial cells, and promoting the growth of vascular smooth muscle cells.[18]

Notably, aging can affect DNA methylation by altering the methylation levels of age-related CpG sites. Antihypertensive treatment might influence DNA methylation by accelerating epigenetic biomarkers of age through its potential side effects, such as abnormal glucose and lipid metabolism, and psychological/cognitive disorders.[19] However, no statistical difference in the use of antihypertensive treatment was found between IS cases and controls.

Age is one of the risk factors for IS, and the level of methylation changes as age increases.[20] Previous studies have found positive associations between decreased DNA promoter methylation and older age for *ADD1*,[21] *LINE-1*,[22] and *SHMT1*.[23] Consistent with these studies, we found a weak inverse correlation (r = −.178) between age and *DHFR* promoter methylation level. We speculate that age might play an important role in the level of methylation. Changes in DNA methylation with increasing age may influence gene expression, resulting in age-related diseases.[24] However, aging can promote hypertension via a "vicious cycle," consisting of inflammation, oxidative stress, and endothelial dysfunction, which might not be closely related to age-related DNA methylation changes.[19]

A risk of IS associated with hypomethylation in the hypertensive population was also found in this study. Hypomethylation may contribute to *DHFR* expression activities, resulting in folate metabolism disorders, which are associated with the development of IS.

Women have a significantly higher rate of stroke compared with men.[25] The association between *DHFR* promoter methylation and IS was different in men and women, and we found a novel predictive value of *DHFR* promoter methylation for IS in women, but not in men. Sexual dimorphism has been found in whole-genome analysis of the risk loci of cardiovascular disease in humans.[26] The level of DNA methylation is also influenced by sex hormones.[27-29] It has been proposed that X chromosome inactivation may deplete the resources required for proper methylation of autosomal loci.[30] Another possible hypothesis is that methylation differences may be associated with different levels of dietary folate or other one-carbon nutrients in men and women.[31]

To the best of our knowledge, this is the first study to estimate the association between *DHFR* promoter methylation and IS in a hypertensive population. Regulation of gene expression by the *DHFR* promoter region was confirmed by a luciferase reporter gene assay. The following limitations should be acknowledged. First, as this was a case-control study, causality cannot be

confirmed. Second, the detailed mechanism of *DHFR* promoter hypomethylation in IS remains unclear. Third, we tested the methylation level using a fragment of the *DHFR* promoter, which may not reflect the DNA methylation status of the whole gene. However, the five CpG sites could not be separated using quantitative methylation-specific PCR, so we presented the major results by using the mean methylation level of the five CpG sites. Furthermore, mRNA and protein levels were not tested.

5 | CONCLUSION

In conclusion, the present study suggests that *DHFR* promoter hypomethylation is a potential biomarker for IS in Chinese hypertensive populations, especially in women.

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REFERENCES

1. Wang W, Jiang B, Sun H, et al. Prevalence, incidence, and mortality of stroke in China: results from a nationwide population-based survey of 480 687 adults. *Circulation*. 2017;135(8):759-771.
2. Liu L, Wang D, Wong KS, Wang Y. Stroke and stroke care in China: huge burden, significant workload, and a national priority. *Stroke*. 2011;42(12):3651-3654.
3. Ng M, Fleming T, Robinson M, et al. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet*. 2014;384(9945):766-781.
4. Feigin VL, Krishnamurthi RV, Parmar P, et al. Update on the global burden of ischemic and hemorrhagic stroke in 1990–2013: the GBD 2013 study. *Neuroepidemiology*. 2015;45(3):161-176.
5. Sun S, Gui Y, Wang Y, et al. Effects of methotrexate on the developments of heart and vessel in zebrafish. *Acta Biochim Biophys Sin*. 2009;41(1):86-96.
6. Han L, Wu Q, Wang C, et al. Homocysteine, ischemic stroke, and coronary heart disease in hypertensive patients: a population-based, prospective cohort study. *Stroke*. 2015;46(7):1777-1786.
7. Gellekink H, Blom HJ, van der Linden IJ, den Heijer M. Molecular genetic analysis of the human dihydrofolate reductase gene: relation with plasma total homocysteine, serum and red blood cell folate levels. *Eur J Human Genet*. 2007;15(1):103-109.
8. Feinberg AP. Epigenomics reveals a functional genome anatomy and a new approach to common disease. *Nat Biotechnol*. 2010;28(10):1049-1052.
9. Zhong J, Agha G, Baccarelli AA. The role of DNA methylation in cardiovascular risk and disease: methodological aspects, study design, and data analysis for epidemiological studies. *Circ Res*. 2016;118(1):119-131.
10. Lin RT, Hsi E, Lin HF, Liao YC, Wang YS, Juo SH. Line-1 methylation is associated with an increased risk of ischemic stroke in men. *Curr Neurovasc Res*. 2014;11(1):4-9.
11. Wang C, Xu G, Wen Q, et al. Significant association of promoter hypomethylation with stroke in a Chinese population with primary hypertension. *Ann Clin Lab Sci*. 2019;49(1):112-118.
12. Yang J, Zhou JS, Zhao YX, et al. Abcb1 hypomethylation is associated with decreased antiplatelet effects of Clopidogrel in Chinese ischemic stroke patients. *Pharmazie*. 2015;70(2):97-102.
13. Lin HF, Hsi E, Huang LC, Liao YC, Juo SH, Lin RT. Methylation in the matrix metalloproteinase-2 gene is associated with cerebral ischemic stroke. *J Investig Med*. 2017;65(4):794-799.
14. Yang Y, Chen X, Hu H, et al. Elevated UMOD methylation level in peripheral blood is associated with gout risk. *Sci Rep*. 2017;7(1):11196.
15. Ji H, Wang Y, Liu G, et al. OPRK1 promoter hypermethylation increases the risk of Alzheimer's disease. *Neurosci Lett*. 2015;606:24-29.
16. Giusti B, Saracini C, Bolli P, et al. Early-onset ischaemic stroke: analysis of 58 polymorphisms in 17 genes involved in methionine metabolism. *Thromb Haemost*. 2010;104(2):231-242.
17. Cui R, Moriyama Y, Koike KA, et al. Serum total homocysteine concentrations and risk of mortality from stroke and coronary heart disease in Japanese: the JACC study. *Atherosclerosis*. 2008;198(2):412-418.
18. Dardik R, Varon D, Tamarin I, et al. Homocysteine and oxidized low density lipoprotein enhanced platelet adhesion to endothelial cells under flow conditions: distinct mechanisms of thrombogenic modulation. *Thromb Haemost*. 2000;83(2):338-344.
19. Gao XU, Colicino E, Shen J, et al. Accelerated DNA methylation age and the use of antihypertensive medication among older adults. *Aging (Albany NY)*. 2018;10(11):3210-3228.
20. Fuke C, Shimabukuro M, Petronis A, et al. Age related changes in 5-methylcytosine content in human peripheral leukocytes and placentas: an HPLC-based study. *Ann Hum Genet*. 2004;68(3):196-204.
21. Bayoumy NMK, El-Shabrawi MM, Leheta OF, Omar HH. A-adducin gene promoter DNA methylation and the risk of essential hypertension. *Clin Exp Hypertens*. 2017;39(8):764-768.
22. Wei L, Liu S, Su Z, Cheng R, Bai X, Li X. Line-1 hypomethylation is associated with the risk of coronary heart disease in Chinese population. *Arq Bras Cardiol*. 2014;102(5):481-488.
23. Xu G, Wang C, Ying X, et al. Serine hydroxymethyltransferase 1 promoter hypermethylation increases the risk of essential hypertension. *J Clin Lab Anal*. 2019;33(3):e22712.
24. Christensen BC, Houseman EA, Marsit CJ, et al. Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CpG Island context. *PLoS Genet*. 2009;5(8):e1000602.
25. Dagres N, Nieuwlaat R, Vardas PE, et al. Gender-related differences in presentation, treatment, and outcome of patients with atrial fibrillation in Europe: a report from the euro heart survey on atrial fibrillation. *J Am Coll Cardiol*. 2007;49(5):572-577.
26. Seda O, Tremblay J, Gaudet D, et al. Systematic, genome-wide, sex-specific linkage of cardiovascular traits in French Canadians. *Hypertension*. 2008;51(4):1156-1162.
27. Sebag IA, Gillis M-A, Calderone A, et al. Sex hormone control of left ventricular structure/function: mechanistic insights using echocardiography, expression, and DNA methylation analyses in adult mice. *Am J Physiol Heart Circ Physiol*. 2011;301(4):H1706-H1715.
28. Cheng J, Wang Y, Zhou K, et al. Male-specific association between dopamine receptor D4 gene methylation and schizophrenia. *PLoS ONE*. 2014;9(2):e89128.
29. Gao S, Cheng J, Li G, et al. Catechol-O-methyltransferase gene promoter methylation as a peripheral biomarker in male schizophrenia. *Eur Psychiatry*. 2017;44:39-46.

30. El-Maarri O, Becker T, Junen J, et al. Gender specific differences in levels of DNA methylation at selected loci from human total blood: a tendency toward higher methylation levels in males. *Hum Genet*. 2007;122(5):505-514.
31. Zhang FF, Cardarelli R, Carroll J, et al. Significant differences in global genomic DNA methylation by gender and race/ethnicity in peripheral blood. *Epigenetics*. 2011;6(5):623-629.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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