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RESEARCH ARTICLE

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Serine hydroxymethyltransferase 1 promoter hypermethylation increases the risk of essential hypertension

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Background: Serine hydroxymethyltransferase 1 (SHMT1) is an enzyme involved in folic acid metabolism and is known to contribute to the development of hypertension. We evaluated the relationship between *SHMT1* promoter methylation and essential hypertension (EH).

Methods: Quantitative methylation-specific polymerase chain reaction was used to measure the *SHMT1* promoter methylation level in 241 EH patients and 288 age- and gender-matched healthy individuals. The diagnostic value of *SHMT1* promoter hypermethylation was analyzed using a receiver operating characteristic (ROC) curve. The Gene Expression Omnibus (GEO) database and dual-luciferase reporter assay were used to validate our findings.

Results: Compared with the control group, significant differences in *SHMT1* promoter methylation were found in both EH and hyperhomocysteinemia groups ($P < 0.001$ and $P = 0.029$, respectively). The area under the curve of the diagnosis of *SHMT1* promoter hypermethylation for EH was 0.808, with a sensitivity and specificity of 73.9% and 77.8%, respectively. The risk of *SHMT1* promoter hypermethylation was significantly higher in the >65-year group than in the ≤65-year group (odds ratio = 3.925; 95% confidence interval = 2.141-7.196). In addition, GEO database analysis showed that 5-aza-deoxycytidine increased gene expression in several carotid endothelial cell lines. A dual-luciferase reporter assay revealed that the target sequence in the *SHMT1* promoter upregulated gene expression.

Conclusion: Our findings indicate that *SHMT1* promoter hypermethylation increases the risk of EH and may be a promising biomarker for EH.

KEYWORDS
essential hypertension, methylation, promoter, *SHMT1*

1 | INTRODUCTION

Essential hypertension (EH), the second largest risk factor accounting for 12.0% of disability-adjusted life years and 24.6% of deaths,[1] is a major public health burden.

There are more than 1.5 billion hypertensive patients worldwide, and responsible for approximately 9.4 million deaths.[2,3]

However, the pathogenesis of EH is not fully understood. Currently, it is known that epigenetic modifications play an important role in the pathogenesis of EH. As one of the most common epigenetic modifications, DNA methylation affects gene expression.[4] Increasing the methylation level of a promoter region will silence the transcription of the corresponding gene, whereas decreasing it may promote gene transcription.[5] Aberrant gene methylation has been identified as an

important contributor to the pathogenesis of EH, for example, hypomethylation of interleukin-6 (*IL-6*),[6] angiotensin II type 1 receptor (*AGTR1*),[7] angiotensin-converting enzyme (*ACE*),[8] Na+/K+/Cl− cotransporter protein 1 (*NKCC1*),[9] and α-adducin (*ADD1*),[10] and the hypermethylation of 11β-hydroxysteroid dehydrogenase type II (*11β-HSD-2*).[11]

Serine hydroxymethyltransferase 1 (SHMT1), located on chromosome 17p11.2, is a key enzyme involved in folic acid metabolism. SHMT1 reversibly catalyzes the conversion of serine and tetrahydrofolate to glycine and 5, 10-methylenetetrahydrofolate (5, 10-methyleneTHF).[12] Besides, it also plays a key role in inducing gene methylation and DNA synthesis by providing one-carbon units for purine, thymidylate, and methionine.[12] The one-carbon moiety of 5, 10-methyleneTHF is in turn directed to the synthesis of purines or thymidylate or to the methionine cycle, where it remethylates homocysteine (Hcy) to synthesize methionine and S-adenosyl methionine. Low levels of SHMT1 enzyme resulted in decreased levels of 5-methylTHF, which is a cosubstrate for the conversion of Hcy to methionine,[13] and low levels of 5-methylTHF in turn resulted in increased levels of Hcy, which is a well-known risk factor for hypertension.[14] Based on these results taken together, we hypothesized that *SHMT1* promoter hypermethylation might contribute to the development of hypertension.

Therefore, here, we aimed to investigate the association between *SHMT1* promoter hypermethylation and EH in an age- and gender-matched case-control study. We also performed a data mining analysis to verify the relationship between *SHMT1* promoter hypermethylation and *SHMT1* gene expression.

2 | MATERIALS AND METHODS

2.1 | Study participants

We enrolled 241 EH patients and 288 age- and gender-matched non-EH individuals who were all Chinese, aged either 18 years or older, and lived in the Nanshan District (Shenzhen, China) community for at least 6 months. The diagnosis of EH was based on the WHO diagnostic criteria, with systolic blood pressure (SBP) ≥140 mm Hg and/or diastolic blood pressure (DBP) ≥90 mm Hg or self-reporting a history of antihypertensive drug use.[15] All individuals provided informed consent. We excluded participants with a history of secondary hypertension; malignancy; liver and kidney failure; pregnancy; or vitamin B6, vitamin B12, or folate intake. This study was approved by the Ethics Committees of the School of Medicine at Ningbo University and Nanshan Chronic Disease Prevention Center.

2.2 | Biochemical measurements

Glucose (Glu), total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), uric acid (UA), and plasma homocysteine (Hcy) levels were measured. Uric enzyme method was performed for quantitative determination of uric acid levels. TC, TG, LDL, and Glu levels were determined by the enzymatic method, and Hcy level was determined by the circulating enzyme method. All these biological indicators were measured using an automatic biochemical analyzer (HITACHI 7080; Tokyo, Japan).

2.3 | DNA methylation analysis

The isolation and quantification of genomic DNA were performed using a Nucleic Acid Extraction Kit (TIANLONG NP968, Xi'an, China) and a NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA). Sodium bisulfite modification was performed using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA). DNA methylation was measured by quantitative methylation-specific polymerase chain reaction (PCR) (qMSP), using a LightCycler 480 machine (Roche Diagnostics, Mannheim, Germany), as described in previous studies.[16,17] The qMSP primer sequences for *SHMT1* are shown in Table 1. The reaction conditions were as follows: initial denaturation at 95°C for 60 seconds, followed by 45 cycles of denaturation at 95°C for 20 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 20 seconds. The melting curve step was performed at 95°C for 15 seconds and 60°C for 60 seconds, with the temperature being increased at 0.11°C per second to 95°C to measure the fluorescence signal. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative change in *SHMT1* promoter methylation.

2.4 | Public databases to analyze the correlation between *SHMT1* gene methylation and expression

Expression values of the *SHMT1* gene in the carotid artery endothelial cell lines (left carotid artery: LCA; right carotid artery: RCA) treated with 5-aza-deoxycytidine (5-AZA; 0.5 μmol/L, 7 days) or left untreated were obtained from the GEO database (Accession No. GSE56143).

2.5 | Luciferase reporter gene assay

The amplified promoter DNA fragment was digested with XhoI and NheI (New England Biolabs, Ipswich, MA, USA). After being purified using a Cycle-Pure Kit (Omega, Norcross, GA, USA), the target fragment was cloned into the pGL3 basic vector (Promega, Madison

TABLE 1 Primer sequences for quantitative methylation-specific PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product (bp)	Tm (°C)
SHMT1	CGAGTTTAGGAAGGTGTATT	CCATACTTAACTACGCTCTC	88	58
ACTB	TGGTGATGGAGGAGGTTTAGTAAGT	AACCAATAAAACCTACTCCTCCCTTAA	133	58

city, WI, USA), using a DNA Ligation Kit (Takara, Kusatsu, Japan). Luciferase activity was determined using the dual-luciferase reporter assay system (Dual-Luciferase® Reporter Assay Systems, Promega).

2.6 | Statistical analysis

The continuous variables with normal distribution are expressed as mean ± standard deviation (SD) and were analyzed by the *t* test. The frequency (percent) and chi-square test were used to express and analyze categorical variables, respectively. The methylation level of *SHMT1* promoter is expressed as the median and interquartile range, and the difference in *SHMT1* methylation levels between groups was analyzed by the Mann-Whitney *U* test. The chi-square test was conducted to assess *SHMT1* promoter methylation and clinical characteristics of the EH patients, including gender (male or female), age (<65 or ≥65 years), body mass index (BMI; <24 or ≥24 kg/m^2), and homocysteinemia (<15 or ≥15 μmol/L). A receiver operating characteristic (ROC) curve was generated to analyze the cutoff levels of *SHMT1* promoter methylation with the maximum Youden index, and the area under the ROC curve (AUC) was used to evaluate the diagnostic value of *SHMT1* methylation for hypertension. The cutoff value was used to distinguish hypermethylation from hypomethylation. Bilateral $P < 0.05$ was considered to indicate statistically significant differences. All statistical analyses were performed using the SPSS 18.0 software (SPSS, Inc, Somers, NY, USA).

3 | RESULTS

A fragment in the *SHMT1* promoter island (hg19, Chr17: 18 266 824-18 266 911) was selected for the methylation assay (Figure 1A). Sequencing of the qMSP product showed successful bisulfite conversion of the template DNA (Figure 1B). Further, capillary electrophoresis confirmed that the qMSP-amplified fragment was 88 bp in length (Figure 1C). We observed statistically significant differences in LDL and Glu levels, BMI, SBP, DBP, and alcohol consumption between the cases and controls (Table 2), and the average Hcy level was 15.33 μmol/L in the case group, which was higher than 13.90 μmol/L in the control group ($P = 0.005$). There were no significant differences in age; gender; UA, TG, TC, and waist-hip rate (WHR) levels; and smoking status between the cases and controls.

As shown in Figure 2, *SHMT1* promoter methylation levels were significantly higher in the 241 EH patients than in the 288 age- and gender-matched controls (median [interquartile range]: 26.39 [14.81,

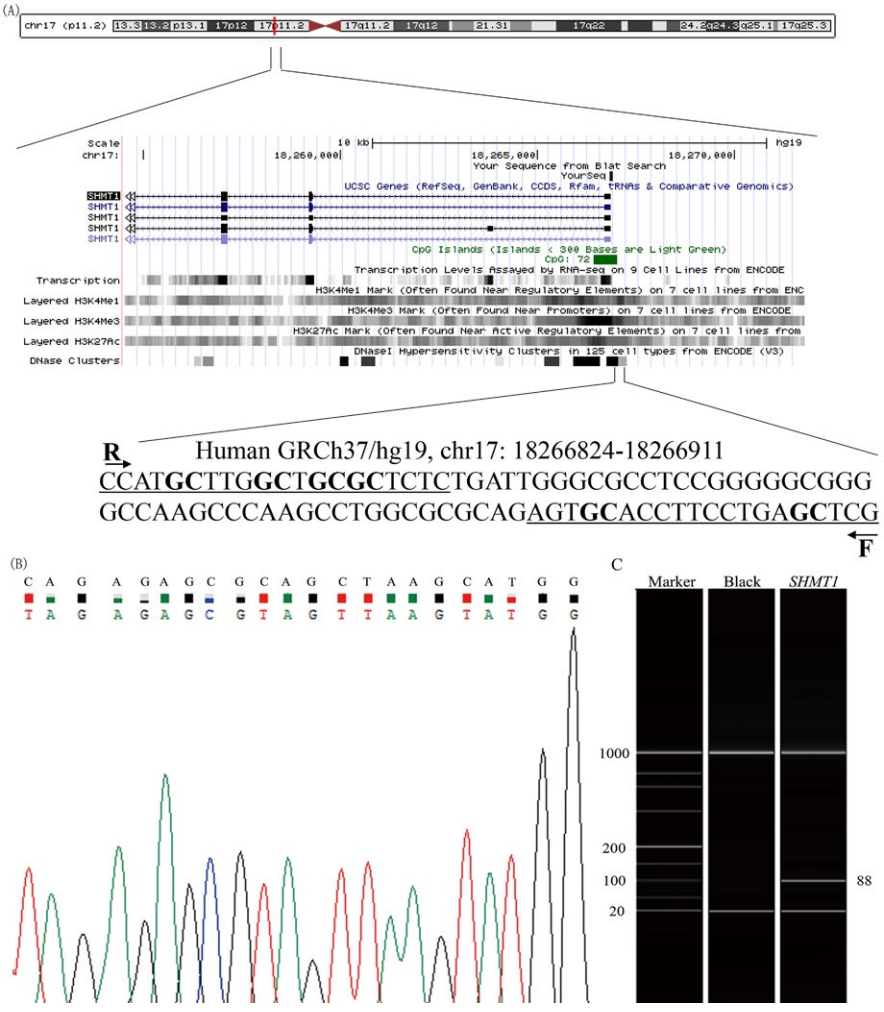

FIGURE 1 Characteristics of target sequences in *SHMT1* gene. A, The location of *SHMT1* on the chromosome and the sequences of primer for target gene. F stands for forward primer, and R stands for reverse primer. B, Sequencing validation of the PCR product. The top row of sequencing validation result represents the original gene sequence, and the second row shows the converted one. C, The electrophoresis experiments reveal the results of methylated sample

	Controls	Cases	t/χ^2	*P*-value
Age (y)	65.12 ± 8.84	66.15 ± 8.81	−1.34	0.181
Gender (M/F)	140/148	120/121	0.07	0.787
Hcy (μmol/L)	13.90 ± 5.37	15.33 ± 6.21	−2.85	0.005
UA (μmol/L)	356.74 ± 89.93	354.02 ± 91.78	0.34	0.732
TG (mmol/L)	1.89 ± 1.56	1.96 ± 1.61	−0.50	0.614
TC (mmol/L)	5.18 ± 1.00	5.20 ± 1.03	−0.30	0.762
LDL (mmol/L)	3.23 ± 0.78	3.02 ± 0.77	3.06	0.002
Glu (mmol/L)	5.05 ± 1.33	5.65 ± 1.27	20.56	<0.0001
BMI (kg/m^2)	23.45 ± 3.17	24.42 ± 2.86	−3.65	<0.001
WHR	0.90 ± 0.10	0.91 ± 0.07	−0.97	0.334
SBP (mm Hg)	123.81 ± 14.11	133.30 ± 16.78	−7.07	<0.001
DBP (mm Hg)	77.34 ± 8.66	82.78 ± 11.34	−6.25	<0.001
Drinking (No/Yes)	194/94	184/57	5.20	0.023
Smoking (No/Yes)	247/41	215/26	1.41	0.235

BMI, body mass index; DBP, diastolic blood pressure; Glu, blood glucose; Hcy, plasma homocysteine; LDL, low-density lipoprotein; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride; UA, uric acid; WHR, Waist-hip ratio.

TABLE 2 Baseline characteristics between cases and controls

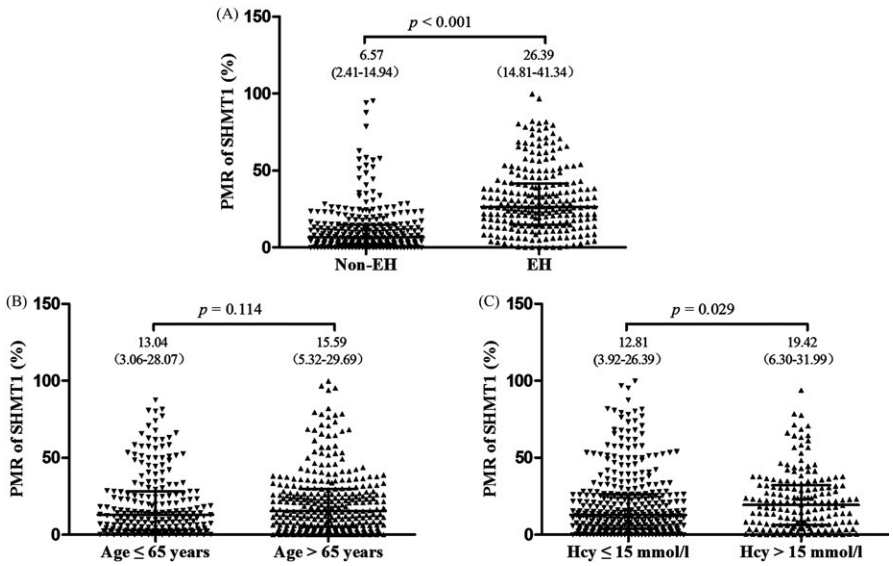

FIGURE 2 Comparison of methylation level of *SHMT1* between different subgroups. The methylation levels of *SHMT1* are represented as median and interquartile range

41.34] vs 6.57 [2.41, 14.94], $P < 0.001$). *SHMT1* promoter methylation was also significantly higher in patients with hyperhomocysteinemia than in patients with low homocysteinemia (19.42 [6.30, 31.99] vs 12.81 [3.92, 26.39], $P = 0.029$). As shown in Table 3, the risk of *SHMT1* promoter hypermethylation was significantly higher in EH cases than in healthy controls (odds ratio = 10.104; 95% CI = 6.769-15.086). Besides, older EH patients (odds ratio [OR] = 3.925, 95% confidence interval [CI] = 2.141-7.196) and EH patients with hyperhomocysteinemia (OR = 2.250, 95% CI = 1.237-4.091) had higher levels of *SHMT1* promoter methylation than their counterparts. In addition, *SHMT1* promoter methylation was not associated with gender, BMI, and LDL and Glu levels (Table 3). The diagnostic value of *SHMT1* promoter methylation was shown in Figure 3, with AUC and 95% CI being 0.808 (0.771-0.846), and sensitivity and specificity being 73.9% and 77.8%, respectively.

We further analyzed *SHMT1* expression in the carotid endothelium (LCA and RCA) with and without 5-AZA treatment (Accession No. GSE56143). The expression level of *SHMT1* in the RCA was significantly increased after demethylation by 5-AZA ($P = 0.028$, Figure 4A). The data indicated that higher levels of *SHMT1* promoter methylation reduced *SHMT1* expression. Subsequently, we also performed dual-luciferase reporter assays to check whether the *SHMT1* CpG island region could regulate gene expression. Our results showed that the transcriptional activity of the recombinant

TABLE 3 Association between promoter methylation of *SHMT1* and baseline characteristics in EH cases

	N	*SHMT1* hypomethylation	*SHMT1* hypermethylation	OR (95% CI)	*P*-value
Case	241	62	179	10.104 (6.769-15.086)	<0.001
Control	288	224	64		
Gender					
Male	144	39	105	1.195 (0.659-2.167)	0.557
Female	97	23	74	1	
Age (y)					
>65	93	39	54	3.925 (2.141-7.196)	<0.001
≤65	148	23	125	1	
BMI (kg/m^2)					
>24	110	30	80	1.160 (0.650-2.069)	0.615
≤24	131	32	99	1	
Hcy (mmol/L)					
>15	120	40	80	2.250 (1.237-4.091)	0.007
≤15	121	22	99	1	
LDL (mmol/L)					
>3.12	110	26	84	1.224 (0.683-2.195)	0.496
≤3.12	131	36	95	1	
Glu (mmol/L)					
>6.18	47	15	32	0.682 (0.340-1.368)	0.352
≤6.18	194	47	147	1	

The methylation level of *SHMT1* ≥15.67% was considered as hypermethylation and <15.67% was considered as hypomethylation.
BMI, body mass index; EH, essential hypertension; Glu, blood glucose; Hcy, plasma homocysteine; LDL, low-density lipoprotein.

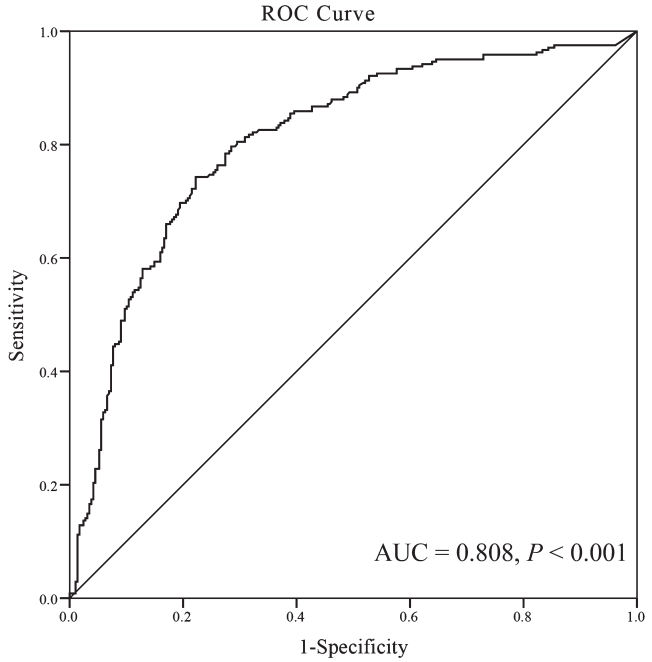

FIGURE 3 Receiver operating characteristic (ROC) curve for the diagnostic value of *SHMT1* methylation. The area under the curve (AUC) of *SHMT1* methylation in patients with hypertension was 0.808 (0.771, 0.846), with sensitivity and specificity being 73.9% and 77.8%, respectively

pGL3-SHMT1 plasmid was higher than that of the empty vector pGL3 basic (mean: 1.39 vs 0.02, fold change = 70, $P = 0.003$, Figure 4B).

4 | DISCUSSION

In this age- and gender-matched case-control study, *SHMT1* promoter methylation levels were higher in EH patients than in healthy controls. The risk of *SHMT1* promoter hypermethylation was significantly higher in both hyperhomocysteinemia and older groups. The AUC was 0.808, with a sensitivity and specificity of 73.9% and 77.8%, respectively, suggesting that *SHMT1* promoter hypermethylation is a potential biomarker for EH. In addition, data from our GEO analysis showed an inverse regulatory association between *SHMT1* promoter methylation and *SHMT1* expression. Dual-luciferase reporter assays indicated that the *SHMT1* promoter plays an important regulatory role in its gene expression.

It is known that SHMT1 plays a key role in inducing gene methylation and DNA synthesis.[12] Our study demonstrated significantly increased *SHMT1* promoter methylation levels in the EH patients. The higher *SHMT1* promoter methylation might have led to lower expression of the SHMT1 enzyme, which resulted in the decreased glycine levels, further restricting the production of glutathione.[18] As an estimator of oxidative stress, glutathione accelerates the development of hypertension.[18] Besides, low expression of SHMT1 might

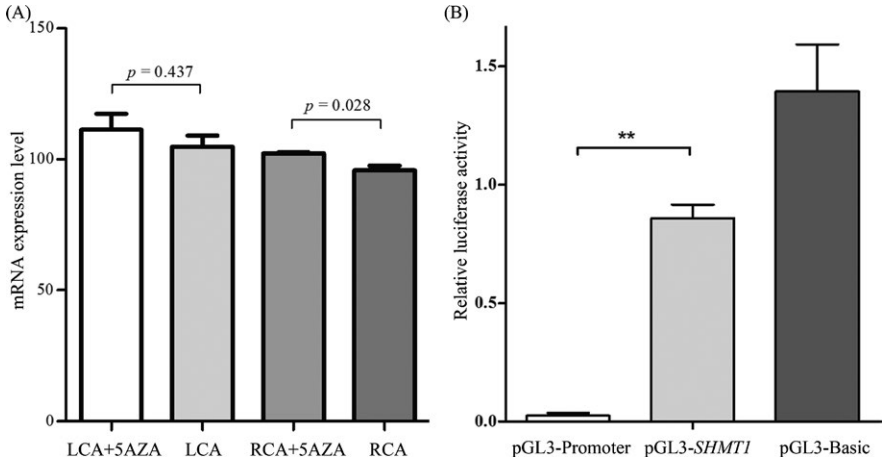

FIGURE 4 A, *SHMT1* expression level with and without 5-aza-deoxycytidine treatment in carotid artery endothelium lines (LCA and RCA) derived from Gene Expression Omnibus database (GSE56143). 5AZA, 5-aza-deoxycytidine treatment; LCA, left carotid artery; RCA, right carotid artery. B, Dual-luciferase reporter assay in HEK293T cell line. The pGL3 basic and promoter vectors were used as negative control and positive control, respectively. Relative luciferase activity was performed in triplicates. Bars represent the means ± standard deviation of three independent experiments. ** $P < 0.01$, pGL3-*SHMT1* promoter vector (51-350 bp) vs pGL3 basic vector.

result in high levels of Hcy, thus contributing to the development of hypertension.[19]

Hyperhomocysteinemia is a well-known risk factor for EH.[19] A previous meta-analysis suggested that elevated Hcy levels increased the risk of EH by about an additional 36%.[20] In a prospective cohort study, the highest tertile of Hcy levels showed a 2.31-fold increased risk of hypertension.[21] Further, significant positive association between *SHMT1* promoter methylation and high Hcy level was observed; higher Hcy level showed a 1.90-fold increased risk of higher *SHMT1* promoter methylation. Therefore, *SHMT1* promoter hypermethylation may exert its effects on the risk of EH via regulating the levels of Hcy.[14]

In our study, increased *SHMT1* promoter methylation was associated with older age. Participants older than 65 years had a 1.46-fold increased risk of higher *SHMT1* promoter methylation. Consistent with our study, previous studies also indicated a positive association between increased DNA promoter methylation, such as that for *ADD1*[22] and *LINE-1*,[23] and older age. Changes in DNA methylation may be induced by aging and environmental exposures.[24,25]

Notably, smoking and drinking are also important risk factors for hypertension,[27,28] and both interact with DNA methylation of genes to affect the risk of EH.[7,30,31] However, we did not observe any significant interactions in our study, although future studies with larger sample size are needed to confirm our findings.

The following limitations of our study should be acknowledged. First, we did not measure *SHMT1* expression in our study participants. Alternatively, we used data from GEO and found an inverse correlation between *SHMT1* methylation and expression. Second, owing to the nature of our case-control study, we could not determine the causality between *SHMT1* promoter hypermethylation and EH. Third, the underlying mechanism of *SHMT1* promoter hypermethylation in EH remains unclear. In addition, we cannot completely exclude the influence of other genes on folic acid metabolism. Finally, we also did not measure the folic acid level; the relationship between *SHMT1* promoter methylation and folic acid was therefore not explored in our study. To the best of our knowledge, this is the first age- and gender-matched case-control study to investigate the relationship between *SHMT1* promoter methylation and EH.

In summary, *SHMT1* promoter methylation is significantly correlated with homocysteinemia and age, and its hypermethylation increases the risk of EH *SHMT1* promoter hypermethylation may therefore be a promising biomarker for diagnosing EH.

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AUTHORS' CONTRIBUTIONS

LL conceived and designed the experiments. GX, XY, FK, HJ, and CW performed the experiments. GX, CW, LZ, XZ, JZ, SD, and LH analyzed the data and wrote this manuscript.

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