



Association between BHMT and CBS gene promoter methylation with the efficacy of folic acid therapy in patients with hyperhomocysteinemia

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Abstract

Both betaine homocysteine methyltransferase (BHMT) and cystathionine β -synthase (CBS) are major enzymes in the metabolism of plasma homocysteine (Hcy). Abnormal methylation levels of BHMT and CBS are positively associated with Hcy levels. The present study is performed to explore the association between the methylation levels in the promoter regions of the BHMT and CBS genes and the efficacy of folic acid therapy in patient with hyperhomocysteinemia (HHcy). A prospective cohort study recruiting HHcy (Hcy $\geq 15 \mu\text{mol/L}$) patients was performed. The subjects were treated with oral folic acid (5 mg/d) for 90 days, and the patients were divided into the success group (Hcy $< 15 \mu\text{mol/L}$) and the failure group (Hcy $\geq 15 \mu\text{mol/L}$) according to their Hcy levels after treatment. In the logistic regression model with adjusted covariates, the patients with lower total methylation levels in the BHMT and CBS promoter regions exhibited 1.627-fold and 1.671-fold increased risk of treatment failure compared with higher methylation individuals, respectively. Similarly, subjects who had lower methylation levels ($<$ methylation mean) in BHMT CpG1 had 1.792 times higher risks. Stratified analysis by sex found that lower CBS methylation levels were associated with a 2.128-fold increased risk for treatment failure in males with HHcy. Lower levels of BHMT or CBS promoter total methylation might be associated with increased the risk of treatment failure. These studies suggest that lower levels of BHMT and CBS methylation are all predictors of failure in folic acid therapy for HHcy. However, due to some limitations of this study, such as the small number of the loci tested, further large-scale studies are necessary to verify our observations.

Introduction

Homocysteine (Hcy), a nonessential and sulfur-containing amino acid, is produced as an important intermediary of methionine metabolism [1]. The fasting total plasma Hcy levels in healthy adults are 5–15 $\mu\text{mol/L}$, and higher than 15 $\mu\text{mol/L}$ is defined as hyperhomocysteinemia (HHcy). Yang and Fan performed a systematic review of the prevalence of HHcy in China and the results of these studies

suggested that the overall pooled prevalence of HHcy was 27.5% and the prevalence increased with age was significantly higher in men than in women [2]. Furthermore, numerous studies have supported that HHcy could be a strong risk factor for neural tube defects, and it also has been significantly associated with many noncommunicable diseases, including cardiovascular and cerebrovascular diseases, type 2 diabetes, and other cancers [3–7]. Therefore, it is significant in sanitary science to reduce the total plasma Hcy levels for decreasing the risk of stroke and other cardiovascular diseases.

In the past two decades, many studies have suggested that the total Hcy levels can be effectively reduced after folic acid supplementation [8–10]. A meta-analysis had also indicated that folic acid supplementation can effectively reduce the risk of stroke in primary prevention [11]. However, our previous research results showed that after 90 days of folic acid (5 mg/d) therapy for HHcy patients, the total plasma Hcy levels of 43.59% of the patients failed to

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recover the normal range [12]. Anderson's research also had consistent results [13]. Currently, studies on the efficacy of folic acid therapy on HHcy mainly focus on the polymorphism of genes related to Hcy metabolic enzyme and their interaction with environmental factors, but the results can only explain part of the reasons for treatment failure [14–16]. Therefore, in order to further investigate the causes of this phenomenon, we were inspired by a previous review which indicated that Hcy is a byproduct of methyltransferase reaction, so DNA methylation may be one of the underlying mechanisms of HHcy-associated disorders [17]. Therefore, we need to analyze the failure of folic acid intervention more comprehensively from the perspective of epigenetics, especially the changes of DNA methylation.

So far, there is few reports on the relationship between gene methylation and the efficacy of folic acid therapy in patients with HHcy. Recently, DNA methylation has been discovered as an important modification pathway of epigenetics. Several studies have demonstrated that DNA methylation might lead to a series of diseases related to HHcy [17–19]. The concentrations of Hcy are maintained by two main routes; namely: the remethylation pathway, where Hcy is converted back to methionine by betaine homocysteine methyltransferase (BHMT), and the transsulfuration pathway, where Hcy is converted to cystathionine to form cysteine by cystathionine β -synthase (CBS) [20]. However, the two key genes, which are related to Hcy metabolism, promoter regions abnormal methylation shall affect their own activity and abnormal expression, resulting in the abnormal metabolism of Hcy. Thus, the purpose of this study is to explore the association of both BHMT and CBS genes promoter methylation with the efficacy of folic acid therapy in patients with HHcy, so as to provide a new theoretical foundation for treatment of HHcy.

Materials and methods

Study design and subjects

A prospective cohort study of HHcy patients (Hcy $\geq 15 \mu\text{mol/L}$) was performed, who had plasma Hcy levels measured in the Department of Neurology in the Fifth Affiliated Hospital of Zhengzhou University from July to December 2014 in a central Han Chinese population. The subjects included and excluded in our study met the following criteria from a previously described database. Inclusion criteria: (1) measured the plasma Hcy level, (2) diagnosed with HHcy (total plasma Hcy $\geq 15 \mu\text{mol/L}$), (3) >18 years of age, and (4) voluntarily participated in this study and received 90 days folic acid supplementation. Exclusion criteria: (1) a history of serious infection, hepatic

or kidney diseases, hematologic disorders, or cancer and (2) used vitamin B or folic acid supplements or use medications that interfere with folic acid metabolism (methotrexate, phenytoin, etc.) in the prior 2 weeks. Fasting plasma Hcy levels were measured on the first day. Then, the recruited patients were treated with oral folic acid (5 mg/d) for 90 days. The compliance with oral folic acid was assessed by telephone interview at 45 day (first compliance) and 90 day (second compliance) of follow-up. Criteria for compliance were as follows: (1) perfect compliance, following doctor's prescription on time; (2) intermediate compliance, missing a folic acid dose no more than 3 times per week; and (3) poor compliance, missing a folic acid dose 3 times or more per week. Finally, plasma Hcy levels were obtained on the 90th day (second follow-up).

The study was approved by the Ethics Review Committee of the Life Science of Zhengzhou University. All subjects or relatives signed informed consent.

Efficacy criteria and methylation grouping

Among 1071 patients were enrolled in this study. However, 253 patients with poor compliance and 180 subjects with loss to follow-up were excluded. Thus, a total of 638 subjects were ultimately included in our study.

If the patients' Hcy levels dropped to $15 \mu\text{mol/L}$ or less, then the treatment was effective and they were included in the success group ($N = 325$). If the patients' Hcy levels were $\geq 15 \mu\text{mol/L}$, then the therapy was unsuccessful and they were included in the failure group ($N = 313$).

There were 152 cases of patients with the best efficacy (Hcy levels decrease maximum) in the success group and 152 cases of patients with the worst efficacy (Hcy levels decrease minimum) in the failure group whose key genes promoter methylation level was detected, and five cases of patients in the success group whose methylation level was not detected for some reason were excluded. Thus, a total of 299 cases of patients in the oral folic acid therapy group were included.

Blood collection and DNA extraction

At baseline, overnight-fasting blood samples were drawn, plasma and cells were separated and stored, and DNA extraction was performed. The methods in this part are consistent with the previous research done by the research group. Please refer to this literature for details.

DNA quantification and purity determination

The methods in this part are consistent with the previous research done by the research group. Please refer to this literature for details.

PCR amplification and MethylTarget™ analysis gene promoter methylation

DNA methylation level was analysis by MethylTarget™ (Genesky Biotechnologies Inc., Shanghai, China), an NGS-based multiple Targeted CpG methylation analysis method. First, Genomic DNA (400 ng) was subjected to sodium bisulfite treatment using the EZ DNA Methylation™ kit (Zymo Research) according to manufacturer's protocols. Multiplex PCR was performed with optimized primer sets combination. A 20 µl PCR reaction mixture was prepared for each reaction and included 1× reaction buffer (Takara), 3 mM Mg²⁺, 0.2 mM dNTP, 0.1 µM of each primer, 1U HotStarTaq polymerase (Takara) and 2 µl template DNA. The cycling program was 95 °C for 2 min; 11 cycles of 94 °C for 20 s, 63 °C for 40 s with a decreasing temperature step of 0.5 °C per cycle, 72 °C for 1 min; then followed by 24 cycles of 94 °C for 20 s, 65 °C for 30 s, 72 °C for 1 min; 72 °C for 2 min. Then, PCR amplicons were diluted and amplified using indexed primers. Finally, sequencing: libraries from different samples were quantified and pooled together, followed by sequencing on the Illumina MiSeq platform according to manufacturer's protocols. Sequencing was performed with a 2 × 300 bp paired-end mode. Quality control of sequencing reads was performed by FastQC. Filtered reads were mapped to genome by Blast. After reads recalibration with USEARCH, methylation was analyzed using Perl script. The total methylation level was calculated as (CpG1 + CpG2 + CpG3, ..., CpGn)/n. The primer was designed online (<http://primer3.ut.ee/>). The sequences of the primers were presented in Table 1.

Statistical analysis

The statistical analyses were done with the SPSS software package (version 21.0 for windows). The clinical characteristics of the subjects were described as the mean ± S.D. or number (%) and the methylation data were expressed as

the mean and range. The *t* test and χ^2 test were used to compare clinical parameters between success group and failure group.

A logistic regression model was applied to estimate the association between BHMT and CBS promoter methylation and two groups with and without adjustment for different variables, including age, sex, smoking, drinking, body mass index (BMI), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), and vitamin B12 (VitB12), which were significantly different in baseline characteristics. The methylation level in the regression model was divided into a binary variable (\geq and $<$ methylation mean). We performed unconditional logistic regression analyses in two groups to estimate the odds ratio (OR), with confidence interval (CI) of 95%. All reported *P* values were two-sides, and all associations were considered significant when *P* values were less than 0.05.

Results

Population characteristics

The baseline characteristics of all participants were shown in Tables 2 and 3. Compared with success group, the patients in the failure group had a higher BMI and baseline plasma Hcy levels, but lower TC, LDL-c, HDL-c, and VitB12 (Table 3). Furthermore, both smoking status and second compliance all had effect on the efficacy of folic acid therapy in patient with HHcy.

Lower methylation level in BHMT and CBS promoter were associated with an increased risk of folic acid therapy failure

A significant difference was found in the total methylation level of the BHMT promoter region between failure group

Table 1 BHMT and CBS primer regions information

Gene-CpGn	Primer	Product/bp
BHMT_1	Forward primer 5'-GGGTTGGTGGGYGTGTTT-3'	229
	Reverse primer 5'-AAAACCTCACCTTCTTAACCTTTTACC-3'	
BHMT_2	Forward primer 5'-GGYGYGTTTAGAATAGGGAGGAG-3'	241
	Reverse primer 5'-TCTTACAACCACTCTACCCATTTT-3'	
CBS_1	Forward primer 5'-GGTGTTTATTAGTTTTGGGTAGAGG-3'	227
	Reverse primer 5'-CCTATCCCCTCCCCACATC-3'	
CBS_2	Forward primer 5'-GYGGTTTAYGGTTAGGGGTTGT-3'	157
	Reverse primer 5'-TCAACCTCTAAAAATCCAAAACAC-3'	
CBS_3	Forward primer 5'-GGGGTTTTGGGAGATTTTGTT-3'	175
	Reverse primer 5'-AACCRCTCRCCCCTCTTTTC-3'	

Table 2 General characteristics of success group and failure group

Variables	Success group (n = 325) ^a	Failure group (n = 313) ^b	χ^2/t	P
Age (years)	64.80 ± 16.70	66.30 ± 13.40	-1.19	0.234
BMI (kg/m ²)	23.60 ± 2.00	24.20 ± 2.10	-3.26	<0.001
TG (mmol/L)	1.55 ± 1.08	1.63 ± 1.21	-0.95	0.345
TC (mmol/L)	4.45 ± 0.99	4.21 ± 1.02	3.00	0.003
LDL-c (mmol/L)	2.64 ± 0.75	2.44 ± 0.74	3.36	<0.001
HDL-c (mmol/L)	1.15 ± 0.32	1.06 ± 0.27	3.39	<0.001
Baseline plasma Hcy levels (μmol/L)	20.14 ± 6.39	24.30 ± 9.71	-6.41	<0.001
Sex (male)	183 (56%)	199 (64%)	3.51*	0.061
Smoking (yes)	98 (30%)	121 (39%)	5.12*	0.024
Alcohol consumption (yes)	42 (13%)	51 (16%)	1.46*	0.228
Compliance ^c				
First compliance (perfect)	202 (62%)	194 (62%)	0.02*	0.515
Second compliance (perfect)	276 (85%)	49 (16%)	284.49*	<0.001

BMI body mass index, TG triglyceride, TC total cholesterol, LDL-c low-density lipoprotein cholesterol, HDL-c high-density lipoprotein cholesterol, Hcy homocysteine

* χ^2 value; P < 0.05 was considered statistically significant

^aSuccess group: Hcy < 15 μmol/L

^bfailure group: Hcy < 15 μmol/L or Hcy = 15 μmol/L

^cCompliance is divided into perfect compliance and intermediate compliance categories

Table 3 General characteristics of success group and failure group

Variables	Success group (n = 147)	Failure group (n = 152)	t	P-value
BHMT (%)	19.32 ± 2.76	18.66 ± 2.69	2.09	0.038*
BHMT_1 (%)	19.96 ± 4.27	19.02 ± 4.08	1.95	0.052
BHMT_2 (%)	18.68 ± 1.77	18.31 ± 1.77	1.82	0.070
CBS (%)	6.37 ± 1.35	6.10 ± 1.30	1.81	0.072
CBS_1 (%)	11.89 ± 2.92	11.37 ± 2.71	1.60	0.112
CBS_2 (%)	4.16 ± 1.10	3.90 ± 1.01	2.14	0.033*
CBS_3 (%)	3.07 ± 0.71	2.97 ± 0.75	1.10	0.273
FA (nmol/L)	35.05 ± 6.52	34.34 ± 6.93	0.92	0.360
VitB6 (nmol/L)	35.20 ± 6.80	34.45 ± 7.21	0.92	0.357
VitB12 (nmol/L)	228.42 ± 43.26	217.27 ± 44.43	2.20	0.029*

FA folic acid, VitB6 vitamin B6, VitB12 vitamin B12

*P < 0.05

and success group, whereas no significant difference was observed in BHMT CpG1 and CpG2 (Fig. 1a, c). The total methylation and CBS CpG1, CpG3 methylation in CBS promoter were not significant difference in two groups. However, CBS CpG2 methylation in the CBS promoter was statistically lower in failure group than success group (Fig. 1b, d).

In the logistic regression model with adjusted age, sex, smoking, drinking status BMI, vitB12, HDL-c, and LDL-c

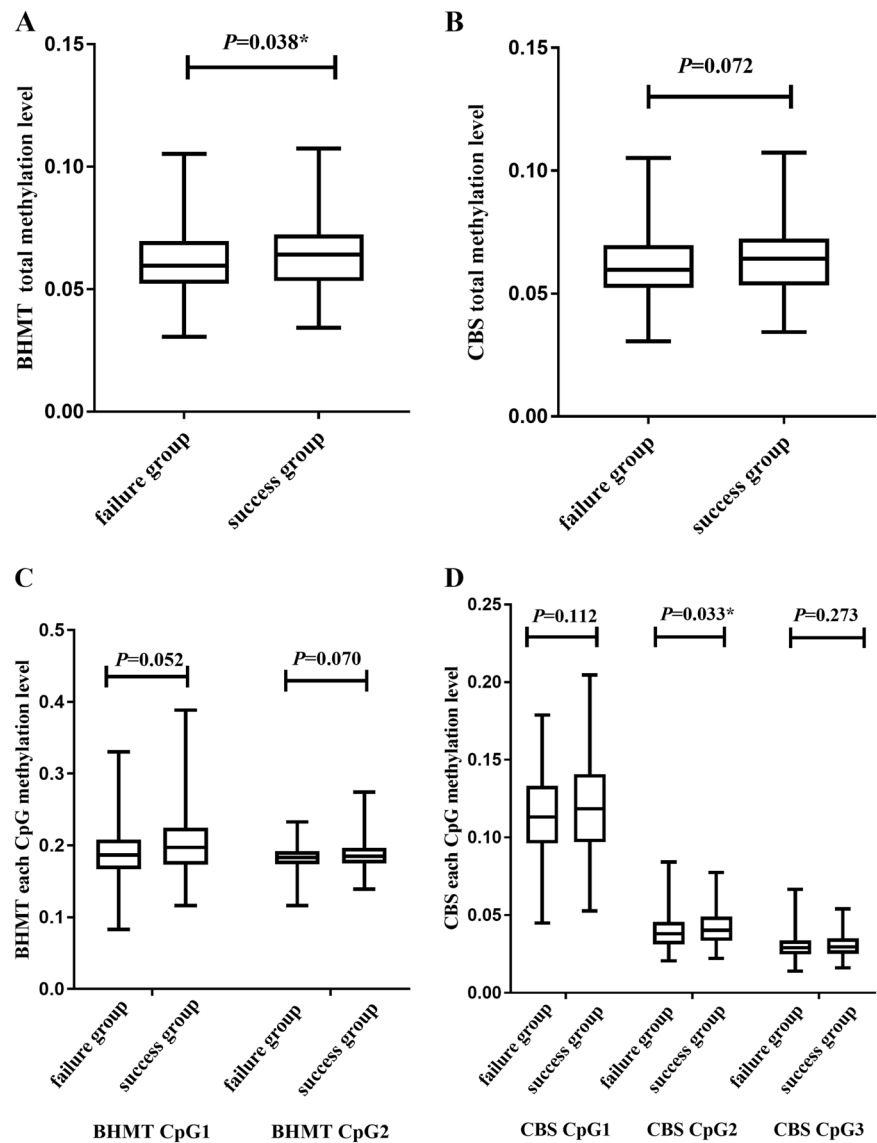
(Table 4), a significant difference was observed in BHMT and CBS methylation levels (both total methylation) between failure group and success group and the patients in the failure group with lower methylation levels. Furthermore, the patients with lower BHMT total methylation, BHMT CpG1 methylation level, and CBS total methylation level (<methylation mean) exhibited increased risks of therapy failure compared with those with higher methylation levels (≥methylation mean), respectively. In the CBS gene, the decrease of per standard deviation of CpG methylation was associated with increased the risk of therapy failure.

A negative correlation existed for CBS CpG1 and CpG2 methylation level and treatment effect, respectively. However, no significant difference was observed in CBS CpG3 and BHMT methylation level (both total methylation and each CpG methylation) between two groups.

Association between gender and CBS methylation level with risk of HHcy treatment failure

A highly significant interaction between gene methylation and sex was seen for CBS promoter methylation (P for interaction < 0.05, Table 5). The OR for treatment failure in relation to male with the lower levels of CBS promoter methylation was 1.950 when nothing is adjusted, 2.153 when adjusted for age, sex, smoking and drinking status,

Fig. 1 The methylation levels of promoter regions in BHMT and CBS genes in failure group and success group. **a** Total methylation level in BHMT gene; **b** total methylation level in CBS gene; **c** each CpG methylation level in BHMT gene; **d** each CpG methylation level in CBS gene; the Mann–Whitney test, * $P < 0.05$



and 2.128 when further adjusted for other potentially confounding variables (see Table 5).

Discussion

The purpose of the prospective cohort study was to examine the association between BHMT and CBS gene promoter methylation with the efficacy of folic acid therapy in patients with HHcy. The results revealed that both BHMT and CBS promoter hypomethylation were predictive factors of failure in folic acid therapy for HHcy. Stratified analysis by sex also suggested that the lower levels CBS promoter methylation was a predictive factor in male subjects for treatment failure.

Accumulating evidence, as well as our group's previous research findings, indicates that HHcy may be associated with variants in genes related to Hcy metabolism. However,

epigenetics, especially DNA methylation, were rarely studied [15, 21–23]. Both BHMT and CBS enzymes, two of three predominant enzymes, are involved in two metabolic pathways of Hcy conversion to methionine and cysteine, which contribute approximately half of the methionine necessary for AdoMet synthesis [24] and nearly 40–50% of cysteine production [25]. CBS deficiency was identified as a genetic factor that resulted in elevated levels of Hcy or HHcy [25]. However, in our study, we found that the total methylation level of the BHMT and CBS promoter in failure group was lower than that in success group. These discrepant results may be caused by the fact that this study is a study of drug efficacy. A previous study also found that HHcy might be caused by DNA Hypomethylation [26]. However, the exact molecular mechanism of the association between increased Hcy plasma concentration and changes in DNA methylation remains unclear. In addition, Hcy

Table 4 Association of BHMT and CBS genes promoter methylation with failure group by unconditional logistic regression

Gene	CpGs	Methylation level*	failure group vs. success group					
			OR (95% CI) ^a	P-value ^a	OR (95% CI) ^b	P-value ^b	OR (95% CI) ^c	P-value ^c
BHMT	Total methylation	≥0.190	Ref.		Ref.		Ref.	
		<0.190	1.597 (1.010–2.525)	0.450	1.656 (1.041–2.634)	0.033**	1.627 (1.012–2.614)	0.044**
		Per SD less methylation	1.226 (0.955–1.574)	0.110	1.251 (0.970–1.613)	0.084	1.250 (0.963–1.623)	0.094
CpG1		≥0.195	Ref.		Ref.		Ref.	
		<0.195	1.735 (1.095–2.749)	0.019**	1.790 (1.121–2.857)	0.015**	1.792 (1.108–2.899)	0.017**
		Per SD less methylation	1.271(0.982–1.643)	0.068	1.291 (0.993–1.677)	0.056	1.306 (0.997–1.710)	0.052
CpG2		≥0.185	Ref.		Ref.		Ref.	
		<0.185	0.843 (0.535–1.327)	0.460	1.250 (0.787–1.983)	0.345	1.218 (0.760–1.953)	0.413
		Per SD less methylation	1.166 (0.899–1.513)	0.247	1.209 (0.927–1.577)	0.162	1.190 (0.907–1.562)	0.210
CBS	Total methylation	≥0.062	Ref.		Ref.		Ref.	
		<0.062	0.661 (0.419–1.044)	0.076	1.625 (1.017–2.598)	0.042**	1.671 (1.026–2.722)	0.039**
		Per SD less methylation	0.852 (0.669–1.085)	0.193	1.202 (0.940–1.537)	0.143	1.221 (0.947–1.574)	0.124
CpG1		≥0.116	Ref.		Ref.		Ref.	
		<0.116	0.678 (0.430–1.069)	0.095	1.562 (0.981–2.488)	0.060	1.632 (1.001–2.661)	0.050
		Per SD less methylation	1.235 (0.975–1.564)	0.080	1.248 (0.982–1.586)	0.070	1.292 (1.007–1.657)	0.044**
CpG2		≥0.040	Ref.		Ref.		Ref.	
		<0.040	0.698 (0.442–1.103)	0.123	1.536 (0.963–2.448)	0.072	1.459 (0.904–2.354)	0.122
		Per SD less methylation	1.276 (0.988–1.648)	0.062	1.316 (1.015–1.708)	0.039**	1.312 (1.005–1.714)	0.046**
CpG3		≥0.030	Ref.		Ref.		Ref.	
		<0.030	0.832 (0.526–1.316)	0.431	1.335 (0.830–2.147)	0.233	1.213 (0.744–1.977)	0.439
		Per SD less methylation	1.194 (0.934–1.528)	0.157	1.232 (0.958–1.584)	0.104	1.173 (0.907–1.518)	0.224

The bold in the table means statistically significant. ($P < 0.05$) Per SD less methylation, the decrease of per standard deviation of CpG methylation, the change of OR value.

** $P < 0.05$

Asterisk indicates mean of total or each CpG methylation in all subjects

^aOR, P -values did not adjust for other factors

^bOR, P -values were from logistic regression analyses controlling for age, sex, smoking, and drinking

^cOR, P -values were from logistic regression analyses controlling for age, sex, smoking, drinking, BMI, vitB12, HDL-c, and LDL-c

Table 5 Association between gender and CBS methylation level with Risk of HHcy treatment failure by Unconditional Logistic Regression

Gender	CBS methylation level	failure group/N (%)	success group/N (%)	OR (95% CI) ^a	P-value ^a	OR (95% CI) ^b	P-value ^b	OR (95% CI) ^c	P-value ^c
Male (N = 190)	≥6.2%	38/100 (38%)	49/90 (54%)	Ref.		Ref.		Ref.	
	<6.2%	62/100 (62%)	41/90 (46%)	1.950 (1.093–3.479)	0.024**	2.153 (1.179–3.930)	0.013**	2.128 (1.122–4.036)	0.021**
Female (N = 109)	≥6.2%	26/52 (50%)	28/57 (49%)	Ref.		Ref.		Ref.	
	<6.2%	26/52 (50%)	29/57 (51%)	0.966 (0.455–2.048)	0.927	1.004 (0.460–2.192)	0.993	1.024 (0.448–2.343)	0.955

The bold in the table means statistically significant. (*P* < 0.05)

***P* < 0.05

^aOR, *P*-values did not adjust for other factors

^bOR, *P*-values were from logistic regression analyses controlling for age, sex, smoking, and drinking

^cOR, *P*-values were from logistic regression analyses controlling for age, sex, smoking, drinking, BMI, vitB12, HDL-c, and LDL-c

concentration also affects the methylation levels. Cacciapuoli [27] found that Hcy concentration was negatively correlated with the levels of genomic DNA methylation. Hcy can play an important biological role by affecting the methylation levels, thus affecting the occurrence and development of diseases. High concentration of Hcy can promote the activity of methyltransferase by inhibiting the metabolism of s-adenosine homocysteine, which result in the reduction of gene methylation levels and promote a compensatory mechanism of Hcy metabolism [28].

In addition, the risk in the treatment failure group was statistically significant only in males with the lower levels of CBS promoter methylation, and gender differences were observed in several genes [29, 30]. Sexual dimorphism in mammalian gene expression is thought to result in different sex hormone (testosterone, androgens) levels in males and females [31]. Testosterone has a distinct impact on renal CBS enzyme levels, and when female mice were injected with androgen, the renal CBS activity increased two-fold [32]. However, the specific mechanism of this gender difference is still unclear.

As far as we are aware, the present work is the first study to assess the association between BHMT and CBS gene promoter methylation with the efficacy of folic acid therapy in patients with HHcy. In addition, as this study is a prospective cohort study, it had strict population inclusion criteria, sample collection, laboratory testing, and data analysis. A face-to-face questionnaire survey was conducted to collect the data from study population, and all the investigators were trained uniformly for quality control. However, there are some limitations to this study. First, the genetic link between the ancestors of different RACES is different, and it has an important effect on DNA methylation level, leading to different susceptibility to diseases [33, 34]. Since the subjects recruited in this study were only Chinese Han individuals from Zhengzhou, the conclusion might not be suitable for other ethnic groups. Second, due to the lack of research funding, the number of methylated genes tested is limited. Therefore, further studies with larger sample size are needed to support this finding.

In conclusion, patients with HHcy had decreased BHMT and CBS promoter methylation levels. In a central Han Chinese population, BHMT and CBS promoter hypomethylation in the blood might serve as predictors of failure in folic acid therapy for HHcy. A further large-scale study in different races with gene methylation changes should be performed to verify our findings.

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Author contributions The authors' responsibilities were as follows: Xiaowen Huang contributed equally to the writing of this paper. Binghui Du and Opolot Godfrey were involved in the study concept and design. Bingnan Ren, Dankang Li, and Qinglin Zhao acquired data of this study. Xiliang Wang, Chengda Zhang, and Limin Yue carried out data analysis and interpretation. Weidong Zhang was in charge of study supervision. All authors read and approved the final paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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