Genetic and epigenetic regulation of BHMT is associated with folate therapy efficacy in hyperhomocysteinaemia

doi: 10.6133/apjcn.201909/PP.0009
Published online: September 2019

Running title: BHMT and folate therapy in hyperhomocysteinaemia

Dankang Li MS[1], Jiao Yang MS[2], Qinglin Zhao MS[1], Chengda Zhang PhD[3], Bingnan Ren MS[1], Limin Yue MD[1], Binghui Du MS[1], Opolot Godfrey MS[1], Xiaowen Huang MS[1], Weidong Zhang PhD[1]

[1]Department of Epidemiology, School of Public Health, Zhengzhou University, Henan, China
[2]Department of Nutrition and Food Hygiene School of Public health, Peking University, Beijing, China
[3]Department of International Medicine, Beaumont Health System, MI, United States of America

Author contributions
Weidong Zhang and Dankang Li conceived the study. Dankang Li did the experiments and researched data. Qinglin Zhao, Bingnan Ren, Limin Yue, Binghui Du, Chengda Zhang, Xiaowen Huang and Jiao Yang contributed to discussion and reviewed the manuscript. Opolot Godfrey contributed to discussion. Dankang Li wrote the manuscript. All authors read and approved the final manuscript

Corresponding Author: Dr Weidong Zhang, Department of Epidemiology, School of Public Health, Zhengzhou University, Zhengzhou, Henan, 450001, China. Tel: +86-0371-67781964. Email: imooni@163.com

ABSTRACT

Background and Objectives: Hyperhomocysteinaemia (HHcy) is an independent risk factors for several disorders, including cardiovascular disease. The understanding of the relationship among genetic, epigenetic and the efficacy of folate therapy for HHcy remain unclear. This study aim to investigate whether betaine-homocysteine methyltransferase (BHMT) single-nucleotide polymorphisms (SNPs) and DNA methylation are related to the efficacy of folate therapy for HHcy and whether BHMT DNA methylation mediates the SNP–folate therapy efficacy association. **Methods and Study Design:** A total of 638 patients with HHcy were involved in this prospective cohort study. Logistic and linear regression was used to explore associations among SNPs, DNA methylation, and folate therapy efficacy. Finally, mediation analysis was performed to investigate whether DNA methylation of BHMT mediates the association between SNPs and folate therapy efficacy. **Results:** BHMT rs3733890 was significantly associated with folate therapy efficacy (p<0.05). BHMT and BHMT_1 DNA methylation level was significantly associated with folate therapy efficacy (p=0.017 and p=0.028). DNA methylation of BHMT and BHMT_1 mediated 34.84% and 33.06% of the effect of rs3733890 on folate therapy efficacy, respectively. **Conclusions:** There has a consistent interrelationship among BHMT genetic variants, methylation levels of BHMT, and folate therapy efficacy. BHMT and BHMT_1 DNA methylation proportionally mediated the effects of rs3733890 SNPs on the efficacy of folate therapy for HHcy.

Key Words: hyperhomocysteinaemia, BHMT, gene polymorphisms, epigenetic, mediation analysis

INTRODUCTION

Hyperhomocysteinaemia (HHcy) is a common disease caused by the abnormal elevation of plasma homocysteine (Hcy) levels.[1] HHcy is an independent risk factor for several disorders, including cardiovascular disease.[2] HHcy prevalence in China is 27.5%, significantly higher than that in developed countries.[3,4] Therefore, controlling Hcy concentrations for HHcy prevention is a significant public health issue.

Folate supplementation intervention is a mainstream approach for reducing Hcy concentrations. It lowers plasma Hcy concentrations by 20%~30%.[5] However, The field of HHcy research is beset with findings of irrelevancy and unresponsiveness to interventions. For instance, a recent survey reported that B-group vitamins supplementation could not explain the mortality mitigation in HHcy.[6] Moreover, some clinical studies have reported that

still a certain proportion of invalid patients whose Hcy concentrations cannot be reduced to those within the normal range when folate supplementation to control Hcy concentrations for preventing HHcy.[7] However, the relevant reasons underlying this failure remains unclear. Hcy metabolism is affected by genetic factors and various environmental factors (B-group vitamins and n-3 fatty acids).[8,9] Further research is required to explore the risk factors and causes of invalid intervention in the field of HHcy research.

Single-nucleotide polymorphisms (SNPs) in genes encoding enzymes, such as methylenetetrahydrofolate reductase (MTHFR) and cystathionine β-synthase (CBS), are crucial Hcy concentration determinants.[10] Hcy can be also catabolized trough remethylated into methionine via betaine-homocysteine methyltransferase (BHMT).[11] BHMT is highly involved in Hcy metabolism and thus in HHcy.[12] However, the mechanisms linking BHMT SNPs with the efficacy of folate therapy for HHcy remain unknown. The identification of the mechanisms by which gene variation in BHMT affects folate therapy efficacy might implicate our understanding of potential effect of this gene on the folate therapy efficacy.

A genetic study reported that SNPs demonstrate limited predictive value in explain disease risk factors.[13] This suggests the presence of other mechanism influencing the efficacy of folate therapy for HHcy. In a context in which traditional explanations are not sufficient to account for the link between genetics and a disease, epigenetics emerges as a framework providing insight into the underlying mechanisms of that disease.[14,15] DNA methylation, a major epigenetic mechanism, is the process via which methyl groups are added to DNA,[16,17] typically regulating gene expression without changes in the DNA sequence,[18] abnormal methylation has been associated with various adverse health outcomes.[19] In fact, several investigations have demonstrated that DNA methylation is associated with changes in Hcy concentrations.[20,21] Therefore, such epigenetic modifications may provide a possible biological link between genetic variations and folate therapy efficacy. Moreover, accumulating evidence suggests that epigenetic modification, which can be controlled by the DNA sequence, can be a mediator of genetic risk in common diseases.[22] This raises the question of whether DNA methylation plays an important mediating role between genetic variation and the efficacy of folate therapy for HHcy.

Yet, to date, no study has addressed DNA methylation as a possible intermediary mechanism of the relationship between BHMT SNPs and the efficacy of folate therapy for HHcy. In addition, the effects of interventions on the field of HHcy research remain controversial. Based on these observations, in this study, we administered folate supplements to patients with HHcy and divided patients into the success and failure groups according to

their plasma Hcy concentrations after the intervention. The current study was designed to assess the association between the BHMT SNPs, methylation levels, and the efficacy of folate therapy for HHcy, and to further determine whether DNA methylation levels of BHMT mediated the association of BHMT genetic variation with the efficacy of the folate therapy. In order to characterize possible mechanisms linking SNP and folate therapy efficacy, and provide a scientific basis for effective prevention and treatment of HHcy.

MATERIALS AND METHODS

Patients

This prospective cohort study was conducted at the Department of Neurology in the Fifth Affiliated Hospital of Zhengzhou University from July to December in 2014. A total of 858 patients with HHcy (≥18 years old) were included. Inclusion criteria were as follows: (1) diagnosis of HHcy, based on high plasma Hcy concentrations (defined as total plasma Hcy concentrations ≥15 μmol/L)[23] and (2) voluntarily participation and receipt 90-day folate supplementation. Exclusion criteria were as follows: (1) use of vitamin B, folate supplements or medications interfering with folate metabolism (such as methotrexate and phenytoin) at last 2 weeks prior to enrollment and (2) history of serious infection, hepatic or kidney diseases, haematologic disorders, or cancer.

The Ethics Review Committee of the Life Science of Zhengzhou University approved the study (No. 132102310431). All participants and their relatives provided written informed consent before participation.

Study design

Biochemical indices [triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), apolipoprotein (Apo) A1, Apo B, folate, Vitamin B6, and Vitamin B12 concentrations] and fasting plasma Hcy concentrations were measured, and a questionnaire was used to collect data pertaining to personal characteristics, life habits, and disease history on day 1. The enrolled patients were then treated with oral folate (5 mg/day) for 90 days. Compliance with oral folate was assessed through telephone interview at days 45 and 90 of follow-up. Plasma Hcy concentrations were obtained at the day 90. Patients with Hcy concentrations <15 and ≥15 μmol/L were then divided into success and failure groups, respectively.

Measurements

Blood collection and DNA extraction

At baseline, blood samples were drawn after overnight fasting for the clinical chemistry test and for measuring plasma Hcy concentrations. The remaining samples were immediately placed on ice, transported to the molecular biology laboratory, and centrifuged at 12,000 rpm for 5 min. Genomic DNA was extracted using a whole-blood genomic DNA extraction kit (Bioteke, Beijing, China). The purity of the extracted DNA solution was measured using an ultramicroscopic UV-Vis spectrophotometer.

Biochemical analysis

Fasting plasma Hcy concentrations were measured using a fluorescence polarization immunoassay with an automated chemistry analyser (Hitachi 7180, Hitachi High-Tech Science Systems Corporation, Japan). Lipid profile levels (TG, TC, LDL, HDL, Apo A1, and Apo B) were assessed using colorimetric-enzymatic methods (Siemens Advia 1800 System, Deerfield, USA). Folate, vitamins B-6, and B-12 were measured in the serum of the study participants by automated random-access immunoassay system (Siemens, ADVIA Centaur Chemistry Analyser, Bohemia, USA).

Genotyping and DNA methylation

BHMT genotyping was detected by a Mass Array time-of-flight (MALDI-TOF) mass spectrometry biochip system (MassArray Analyzer, Sequenom, San Diego, American).

We determined the DNA methylation levels of BHMT and its fragments BHMT_1 and BHMT_2 by using MethylTarget (Genesky Biotechnologies Inc., Shanghai, China), bisulfite treatment, and polymerase chain reaction (PCR). Specifically， the genomic regions of interest were analysed and transformed to bisulfite-converted sequences using geneCpG software. PCR primer sets were designed with the Methylation Primer software from bisulfate converted DNA. Genomic DNA (400ng) was subjected to sodium bisulfite treatment using an EZ DNA Methylation-GOLD Kit (Zymo Research) according to manufacturer's protocol. Multiplex PCR was performed using optimised primer sets combination. PCR amplicons were diluted and amplified using indexed primers. PCR amplicons (170-270bp) were separated by agarose electrophoresis and purified using QIAquick Gel Extraction kit (QIAGEN). Libraries from different samples were quantified and pooled together, followed by sequencing on the Illumina MiSeq platform according to manufacturer's protocol. Sequencing was performed

with a 2 × 300-bp paired-end mode. Data were analysed using FastQC. After reads were recalibrated using USEARCH, methylation and haplotype were analysed using Perl script.

Statistical analysis

Independent-sample t and chi-square tests were used to test the difference between the success and failure groups with regard to demographic and biochemical variables. Similarly, an independent samples t-test was used to test the association between SNPs, efficacy, and methylation status. We performed unconditional logistic regression analyses to estimate the odds ratios (ORs), with their 95% confidence intervals (95% CI). Adjustments were made for age, sex, smoking, alcohol consumption, history of diabetes, stroke, hypertension, coronary heart disease (CHD), nephropathy and biochemical indicators when appropriate. We performed linear regression analyses to estimate the relationship between SNPs and methylation status. All statistical tests were two-tailed, and results were considered significant when $p<0.05$. Tests were conducted using IBM SPSS (version 25.0).

Mediation analysis

Mediation analyses were used to evaluate the relationship among genotype (predictor), DNA methylation (mediator), and group of efficacy (outcome). We used the mediation package in R (version 4.4.6)[24] to estimate the effect size and its 95% CI of the direct effect (DE) and indirect effect (IDE) using the quasi-Bayesian Monte Carlo method with 1000 times simulations. The mediation effect was then calculated using the equation [mediation percentage (%) = (IDE × 100%) / (DE + IDE)].

RESULTS

Descriptive statistics

Of the 858 patients, 215 patients who were lost to follow-up or had poor compliance were excluded; five patients were also excluded because we could not detect a genotype. A total of 638 patients were ultimately accepted for genotype detection, and 299 of them were included for methylation level analysis. Table 1 displays the distribution of demographic features and baseline Hcy levels for each group.

After folate therapy for 90 days, 325 patients whose plasma Hcy concentration ≤15.0 μmol/L, corresponding to an efficacy rate of 50.94%. Table 2 presents the general characteristics, disease history, and biochemical indices of the success and failure groups. The success group had lower body mass index (BMI), baseline plasma Hcy concentration, and

prevalence of past disorders (diabetes, hypertension, CHD) compared with the failure group. The difference was significant (p<0.05). The difference of biochemical indices (TC, LDL, HDL, Apo A1, Apo B, and vitamin B-12) between two groups was also significant (p<0.05).

Relationship among BHMT SNPs, DNA methylation, and folate therapy efficacy

BHMT SNPs and folate therapy efficacy

The frequency distributions of the genotypes and alleles of the BHMT rs3733890 and rs585800 in the success and failure groups are listed in Table 3. The frequency distributions of rs3733890 genotypes and alleles significantly differed between the two groups after adjustment for age, sex, smoking, alcohol consumption, history of diabetes, stroke, hypertension, CHD, nephropathy and biochemical indicators in a binary logistic regression analysis (p<0.05). The difference in rs585800 genotype or allele frequency between the success and failure groups was nonsignificant. This indicates that rs3733890 was associated with the efficacy of folate therapy for HHcy, but rs585800 was not. Moreover, for rs3733890, compared with the GG genotype, the risk of treatment failure was 1.43-fold (p=0.034, OR=1.43; 95% CI=1.02, 2.06) and 2.03-fold (p=0.008, OR=2.03; 95% CI=1.21, 3.43) for individuals carrying the GA genotype and AA genotypes, respectively. With the G allele as a control, the risk of treatment failure was 1.38-fold (p=0.008, OR=1.38; 95 % CI=1.09, 1.75) for individuals carrying the A allele.

DNA methylation and folate therapy efficacy

In the logistic regression model (Table 4), patients with higher BHMT and BHMT_1 methylation level (≥methylation mean) exhibited 0.485-fold (p=0.017, OR=0.485; 95% CI=0.268, 0.879) and 0.515-fold (p=0.028, OR=0.515; 95% CI=0.285, 0.930) decreased risks of treatment failure compared with those with lower methylation levels (<methylation mean), respectively. This indicates that promoter methylation of BHMT and BHMT_1 was associated with folate therapy efficacy. No significant difference was observed in the BHMT_2 methylation levels between the success and failure groups (p>0.05). A significant difference was observed in BHMT DNA methylation levels (p=0.038; Figure 1A), whereas no significant difference was observed in BHMT_1 and BHMT_2 DNA methylation levels between the success and failure groups (p=0.052 and 0.07, respectively; Figure 1B and 1C, respectively).

BHMT SNPs and DNA methylation

Among patients with different rs3733890 genotypes, we observed differences in methylation levels of BHMT, BHMT_1, and BHMT_2. In the linear regression model, we observed a significant association between DNA methylation of BHMT and BHMT_1 with rs3733890 (p = 0.002 and 0.001, respectively; Figure 2A and 2B, respectively). However, the association between DNA methylation of BHMT_2 and rs3733890 was nonsignificant (p=0.074; Figure 2C).

Mediation analysis

We performed mediation analyses to explore the role, and estimated mediation effect (%), of DNA methylation levels (BHMT and BHMT_1) as a mediator in the relationship between BHMT genotype rs3733890 and folate therapy efficacy. We observed that the methylation levels of BHMT mediated an estimated 34.84% of the effect of rs3733890 on folate therapy efficacy (p=0.03; Figure 3A), and BHMT_1 methylation levels mediate 33.06% of the association between rs3733890 and folate therapy efficacy (p=0.044; Figure 3B).

DISCUSSION

This study has investigated the genetic and epigenetic regulation of the efficacy of folate therapy for HHcy. We observed consistent interrelationships between BHMT SNPs, and DNA methylation levels, and the efficacy of folate therapy. Considered in conjunction with our mediation analysis, these data indicate that methylation levels can mediate part of the known effects of SNPs on folate therapy efficacy.

This study has examined the association between BHMT SNPs and folate therapy efficacy. In our study we selected two most common mutation sites of BHMT: G/A mutation (rs3733890) and T/A mutation (rs585800). Our findings indicated the relevance of BHMT rs3733890 in the efficacy of folate therapy, and indicated that the GA genotype, AA genotype, and A allele of BHMT rs3733890 increases folate therapy failure risk. Our results are consistent with those of Qin et al.: folate metabolic gene SNPs not only affect plasma Hcy concentrations,[25] but also the therapeutic effect of folate intervention. Some studies have observed a significant association between gene SNPs of folate metabolic enzyme and folate therapy efficacy.[26] such as, the decreased level of plasma Hcy in patients with the MTHFR TT genotype significant higher than that patients with the MTHFR CC/CT genotype, and Tian et al. suggested that this situation may be related to higher baseline plasma Hcy concentrations in patients with MTHFR TT genotype.[27] In addition, the association of Hcy and adverse

outcomes was certified by Xiu et al, they suggested that there is a linear and threshold relationship between Hcy status with adverse outcomes when there're is evidence that both low and high hcy may be problematic.[28] Based on these observations, we think Hcy plays an important role in the relationship between SNPs and the treatment of HHcy due to abnormally high Hcy. In the current study, we find the baseline plasma Hcy concentrations in patients with the BHMT GA and AA genotypes were 22.56±9.15 and 22.37±9.16 μmol/L, respectively, which were higher than 21.79±7.6 μmol/L in patients with the GG genotype (p<0.05). Thus, we speculate that the baseline Hcy concentrations in patients with the BHMT GA and AA genotypes is more higher, which needs to be reduced to a greater extent before to the normal level, and thus reducing folate therapy efficacy.

Current studies on what affects the efficacy of folate therapy for HHcy focused on the gene SNPs of Hcy metabolic enzymes, but they could not give a detailed and comprehensive explanation for the reasons of the failure intervention.[13] Therefore, on the basis of previous studies, we extended our research to the field of epigenetics, and found that DNA methylation level of the BHMT in the success group was significantly higher than that in the failure group (p=0.038). Furthermore, our logistic regression analysis demonstrated an association of BHMT DNA methylation levels with folate therapy efficacy (OR=0.485, p=0.017). Some studies have demonstrated a strong relationship between changes of DNA methylation and Hcy concentrations. For example, Wei et al. suggested a positive correlation between Hcy concentrations and DNA methylation levels.[29] Our results also reflect the possible mechanism that how methylation affects the efficacy of folate therapy for HHcy, that is, BHMT DNA methylation may affects folate therapy efficacy via causing abnormal changes of Hcy concentrations. This observation underscores the relevance of the epigenetic mechanism for HHcy. But, the specific mechanism requires further investigation. In addition, Kim et al. suggested that BHMT rs3733890 might be related to the methylation levels of BHMT gene.30 This suggestion is corroborated by our current results.

DNA methylation, one of the most common epigenetic mechanisms, plays a critical mediation role in the pathogenic pathway,[31,32] For instance, some studies confirmed that DNA methylation mediates the effects of lower vitamins B (folate and vitamin B-12) levels on cardiovascular diseases.[33] Our study found that BHMT and BHMT_1 methylation mediated 34.84% and 33.06% of rs3733890's effect on folate therapy efficacy, respectively. Our findings are consistent with accumulating evidence indicating significant effects of SNPs on disease might be mediated by DNA methylation.[34] There is a close correlation of BHMT gene SNPs and methylation with Hcy metabolism.[35] For instance, Hcy can be involved in DNA

methylation, it alters gene expression through the methionine cycle and transfer methylation course, where in turn, HHcy can also induces aberrant methylation of global DNA and some specific genes.[36,37] Our mediation analysis further confirms the interconnected pathway of genetics, epigenetic and the efficacy of folate therapy for HHcy at BHMT. However， additional studies are warranted, to localise and validate this mediating process.

Hcy metabolism has many determinants being genetic and epigenetic, related to B-group vitamins.[9] Some B group vitamins intake are associated with plasma Hcy concentration.[38] So, we explored the folate therapy efficacy and found that B group vitamin supplementation decreased plasma Hcy concentration. It is well know that the role of B group vitamin in the regulation of plasma Hcy and the prevention of HHcy. However, except B group vitamin, Huang et al demonstrated that n-3 polyunsaturated fatty acids (PUFA) supplementation also decreases plasma Hcy and corrects HHcy.[39] Although, B group vitamin and n-3 PUFA could decrease Hcy concentrations, we paid little attention to the role of n-3 PUFA and did not collect dietary information of n-3 PUFA. This might be partly because the nutrients involved in Hcy metabolism we have been incompletely recognized. Thus, further research is needed to explore the role of n-3 PUFA on therapy for HHcy.

The major strengths of our study include that we used BHMT as the target gene and innovatively used the mediation analyses to indicate causality of gene SNPs and folate therapy efficacy for HHcy. Despite these strengths, however, several limitations merit discussion. First, our small sample size was relatively small because of financial constraints. We measured DNA methylation levels in only 299 patients, which may have limited the statistical power for some analysis, particularly those related to the analysis of methylation. In the future, additional cohort study studies with a larger sample size are thus warranted. Second, Hcy metabolism involves multiple metabolic pathways and multiple enzymes,[40] but we only analysed the associations between genetics and epigenetics of BHMT in the Hcy metabolism pathway and their association with the folate therapy efficacy. Thus, the association of other genes in different pathways with folate therapy efficacy may be investigated in the future. Finally, the current study did not collect background data about dietary information, thus we could not assess folate intakes from dietary food and the possibility of other confounders from unmeasured intake of other residual nutrients cannot be completely ruled out. Requires estimate the intake of dietary food and considered the individual differences in folate bioavailability and dietary intake in further research.

Conclusions

Our results indicated that the gene SNPs and DNA methylation levels of BHMT are associated with folate therapy efficacy for HHcy. DNA methylation of BHMT and BHMT_1 mediated 34.84% and 33.06% of the genotype rs3733890's effect on the efficacy of folate therapy, respectively. Out results provide essential guidance for additional studies on the integrated analysis of the causal relationship between gene SNPs and efficacy of folate therapy for HHcy.

ACKNOWLEDGEMENTS

We appreciated all the members to achieve such results for their truly magnificent efforts.

CONFLICTS OF INTEREST AND FUNDING DISCLOSURE

The authors confirm that content of this article has no conflicts of interest. Department of Science and Technology of Henan Province provide the funding (no.132102310431).

REFERENCES

1. Rosi J, Morais BA, Pecorino LS, Oliveira AR, Solla DJF, Teixeira MJ, Figueiredo EG. Hyperhomocysteinemia as a risk factor for intracranial aneurysms: a case-control study. World Neurosurg. 2018;119:e272-e5. doi: 10.1016/j.wneu.2018.07.132.
2. Fu Y, Wang X, Kong W. Hyperhomocysteinaemia and vascular injury: advances in mechanisms and drug targets. Br J Pharmacol. 2018;175:1173-89. doi: 10.1111/bph.13988.
3. Yang B, Fan S, Zhi X, Wang Y, Wang Y, Zheng Q, Sun G. Prevalence of hyperhomocysteinemia in China: a systematic review and meta-analysis. Nutrients. 2014;7:74-90. doi: 10.3390/nu7010074.
4. Liu XD, Gao B, Sun D, Shi M, Ma YY, Liu ZR et al. Prevalence of hyperhomocysteinaemia and some of its major determinants in Shaanxi Province, China: a cross-sectional study. Br J Nutr. 2015;113:691-8. doi: 10.1017/S0007114514004218.
5. Tighe P, Ward M, McNulty H, Finnegan O, Dunne A, Strain J, Molloy AM, Duffy M, Pentieva K, Scott JM. A dose-finding trial of the effect of long-term folic acid intervention: implications for food fortification policy. Am J Clin Nutr. 2011;93:11-8. doi: 10.3945/ajcn.2010.29427.
6. Wahlqvist ML, Xiu L, Lee MS, Chen RC, Chen KJ, Li D. Dietary diversity no longer offsets the mortality risk of hyperhomocysteinaemia in older adults with diabetes: a prospective cohort study. Asia Pac J Clin Nutr. 2016;25:414-23. doi: 10.6133/apjcn.112015.06.
7. Anderson JL, Jensen KR, Carlquist JF, Bair TL, Horne BD, Muhlestein JB. Effect of folic acid fortification of food on homocysteine-related mortality. Am J Med. 2004;116:158-64. doi: 10.1016/j.amjmed.2003.10.024.

8. Huang T, Zheng J, Chen Y, Yang B, Wahlqvist ML, Li D. High consumption of Omega-3 polyunsaturated fatty acids decrease plasma homocysteine: a meta-analysis of randomized, placebo-controlled trials. Nutrition. 2011;27:863-7. doi: 10.1016/j.nut.2010.12.011.
9. Smith AD, Smith SM, de Jager CA, Whitbread P, Johnston C, Agacinski G, Oulhaj A, Bradley KM, Jacoby R, Refsum H. Homocysteine-lowering by B vitamins slows the rate of accelerated brain atrophy in mild cognitive impairment: a randomized controlled trial. PLoS One. 2010;5:e12244. doi: 10.1371/journal.pone.0012244.
10. van Meurs JB, Pare G, Schwartz SM, Hazra A, Tanaka T, Vermeulen SH et al. Common genetic loci influencing plasma homocysteine concentrations and their effect on risk of coronary artery disease. Am J Clin Nutr. 2013;98:668-76. doi: 10.3945/ajcn.112.044545.
11. Teng YW, Mehedint MG, Garrow TA, Zeisel SH. Deletion of betaine-homocysteine S-methyltransferase in mice perturbs choline and 1-carbon metabolism, resulting in fatty liver and hepatocellular carcinomas. J Biol Chem. 2011;286:36258-67. doi: 10.1074/jbc.M111.265348.
12. Sun K, Song J, Liu K, Fang K, Wang L, Wang X et al. Associations between homocysteine metabolism related SNPs and carotid intima-media thickness: a Chinese sib pair study. J Thromb Thrombolysis. 2017;43:401-10. doi: 10.1007/s11239-016-1449-x.
13. Whitfield JB. Genetic insights into cardiometabolic risk factors. Clin Biochem Rev. 2014;35:15-36.
14. Ladd-Acosta C, Fallin MD. The role of epigenetics in genetic and environmental epidemiology. Epigenomics. 2016;8:271-83. doi: 10.2217/epi.15.102.
15. Waddington CH. Canalization of development and genetic assimilation of acquired characters. Nature. 1959;183:1654-5. doi: 10.1038/1831654a0.
16. Bird AP. CpG-rich islands and the function of DNA methylation. Nature. 1986;321:209-13. doi: 10.1038/321209a0.
17. Rakyan VK, Preis J, Morgan HD, Whitelaw E. The marks, mechanisms and memory of epigenetic states in mammals. Biochem J. 2001;356:1-10. doi: 10.1042/0264-6021:3560001.
18. Bird A. Perceptions of epigenetics. Nature. 2007;447:396-8. doi: 10.1038/nature05913.
19. Kader F, Ghai M, Maharaj L. The effects of DNA methylation on human psychology. Behav Brain Res. 2018;346:47-65. doi: 10.1016/j.bbr.2017.12.004.
20. Ramos RB, Fabris V, Lecke SB, Maturana MA, Spritzer PM. Association between global leukocyte DNA methylation and cardiovascular risk in postmenopausal women. BMC Med Genet. 2016;17:71. doi: 10.1186/s12881-016-0335-x.
21. Stirzaker C, Zotenko E, Clark SJ. Genome-wide DNA methylation profiling in triple-negative breast cancer reveals epigenetic signatures with important clinical value. Mol Cell Oncol. 2016;3:e1038424. doi: 10.1080/23723556.2015.1038424.
22. Grant CD, Jafari N, Hou L, Li Y, Stewart JD, Zhang G et al. A longitudinal study of DNA methylation as a potential mediator of age-related diabetes risk. Geroscience. 2017;39:475-89. doi: 10.1007/s11357-017-0001-z.

23. Baszczuk A, Kopczynski Z. [Hyperhomocysteinemia in patients with cardiovascular disease]. Postepy Hig Med Dosw (Online). 2014;68:579-89. doi: 10.5604/17322693.1102340.
24. Imai K, Keele L, Tingley D, Yamamoto T. Causal Mediation Analysis Using R2010; New York, NY.
25. Qin X, Li J, Cui Y, Liu Z, Zhao Z, Ge J et al. MTHFR C677T and MTR A2756G polymorphisms and the homocysteine lowering efficacy of different doses of folic acid in hypertensive Chinese adults. Nutr J. 2012;11:2. doi: 10.1186/1475-2891-11-2.
26. Du B, Tian H, Tian D, Zhang C, Wang W, Wang L, Ge M, Hou Q, Zhang W. Genetic polymorphisms of key enzymes in folate metabolism affect the efficacy of folate therapy in patients with hyperhomocysteinaemia. Br J Nutr. 2018;119:887-95. doi: 10.1017/S0007114518000508.
27. Tian H, Tian D, Zhang C, Wang W, Wang L, Ge M, Hou Q, Zhang W. Efficacy of folic acid therapy in patients with hyperhomocysteinemia. J Am Coll Nutr. 2017;36:528-32. doi: 10.1080/07315724.2017.1330162.
28. Xiu LL, Lee MS, Wahlqvist ML, Chen RC, Huang YC, Chen KJ, Li D. Low and high homocysteine are associated with mortality independent of B group vitamins but interactive with cognitive status in a free-living elderly cohort. Nutr Res. 2012;32:928-39. doi: 10.1016/j.nutres.2012.09.005.
29. Wei LH, Chao NX, Gao S, Yu YT, Shi L, Ma XB et al. Homocysteine induces vascular inflammatory response via SMAD7 hypermethylation in human umbilical vein smooth muscle cells. Microvasc Res. 2018;120:8-12. doi: 10.1016/j.mvr.2018.05.003.
30. Kim S, Parks CG, Xu Z, Carswell G, DeRoo LA, Sandler DP, Taylor JA. Association between genetic variants in DNA and histone methylation and telomere length. PLoS One. 2012;7:e40504. doi: 10.1371/journal.pone.0040504.
31. Meng W, Zhu Z, Jiang X, Too CL, Uebe S, Jagodic M et al. DNA methylation mediates genotype and smoking interaction in the development of anti-citrullinated peptide antibody-positive rheumatoid arthritis. Arthritis Res Ther. 2017;19:71. doi: 10.1186/s13075-017-1276-2.
32. Zhou F, Shen C, Xu J, Gao J, Zheng X, Ko R et al. Epigenome-wide association data implicates DNA methylation-mediated genetic risk in psoriasis. Clin Epigenetics. 2016;8:131. doi: 10.1186/s13148-016-0297-z.
33. Anderson OS, Sant KE, Dolinoy DC. Nutrition and epigenetics: an interplay of dietary methyl donors, one-carbon metabolism and DNA methylation. J Nutr Biochem. 2012;23:853-9. doi: 10.1016/j.jnutbio.2012.03.003.
34. Guerra S, Melen E, Sunyer J, Xu CJ, Lavi I, Benet M et al. Genetic and epigenetic regulation of YKL-40 in childhood. J Allergy Clin Immunol. 2018;141:1105-14. doi: 10.1016/j.jaci.2017.06.030.
35. Castro C, Gratson AA, Evans JC, Jiracek J, Collinsova M, Ludwig ML, Garrow TA. Dissecting the catalytic mechanism of betaine-homocysteine S-methyltransferase by use of intrinsic tryptophan fluorescence and site-directed mutagenesis. Biochemistry. 2004;43:5341-51. doi: 10.1021/bi049821x.
36. He M, Fan J, Jiang R, Tang WX, Wang ZW. Expression of DNMTs and genomic DNA methylation in gastric signet ring cell carcinoma. Mol Med Rep. 2013;8:942-8. doi: 10.3892/mmr.2013.1566.

37. Finkelstein JD, Martin JJ. Methionine metabolism in mammals. Adaptation to methionine excess. J Biol Chem. 1986;261:1582-7. doi: 10.1093/nar/14.3.1542.
38. de Bree A, Verschuren WM, Blom HJ, Kromhout D. Association between B vitamin intake and plasma homocysteine concentration in the general Dutch population aged 20-65 y. Am J Clin Nutr. 2001;73:1027-33. doi: 10.1093/ajcn/73.6.1027.
39. Huang T, Li K, Asimi S, Chen Q, Li D. Effect of vitamin B-12 and n-3 polyunsaturated fatty acids on plasma homocysteine, ferritin, C-reaction protein, and other cardiovascular risk factors: a randomized controlled trial. Asia Pac J Clin Nutr. 2015;24:403-11. doi: 10.6133/apjcn.2015.24.3.19.
40. Mandaviya PR, Stolk L, Heil SG. Homocysteine and DNA methylation: a review of animal and human literature. Mol Genet Metab. 2014;113:243-52. doi: 10.1016/j.ymgme.2014.10.006.

Table 1. Distribution of demographic features and baseline plasma Hcy

Characteristics	Number of individuals		
	Total enrolled (n=858)	Genotype detection (n=638)	Methylation analysis (n=299)
Age, year (mean±SD)	62.79±13.26	65.38±14.69	64.61±14.61
Female, N (%)	322 (37.5)	235 (36.8)	109 (36.5)
Disease, N (%)			
Stroke	444 (51.7)	377 (52.8)	148 (49.5)
Transient ischemic attack	22 (2.6)	19 (3.0)	10 (3.3)
Vertebral-basilar artery insufficiency	151 (17.6)	108 (16.9)	48 (16.1)
Poeterior circular ischemia	159 (18.5)	121 (9.0)	65 (21.7)
Other disease	82 (9.6)	53 (8.3)	28 (9.4)
Baseline Plasma Hcy, μmol/L (mean±SD)	22.10±8.55	22.17±8.44	21.05±7.02

Hcy: homocysteine.
Data are presented as means±SD or n (%).

Table 2. General characteristics of the success and failure groups

Variables	Success group n=325	Failure group n=313	t/χ^2	*p*
Age, year (mean±SD)	64.57±15.82	66.23±13.38	−1.43†	0.152
Sex, N (%)				
Male	183 (56.31)	199 (63.58)		0.061
Female	142 (43.69)	114 (36.42)		
Smoking, N (%)	98 (30.15)	121 (38.66)	5.12	0.024*
Alcohol, N (%)	42 (12.92)	51 (16.29)	1.46	0.228
BMI	23.69±2.03	24.20±2.08	−3.16†	0.002*
Baseline plasma Hcy	20.2±6.4	24.3±9.7	−6.3†	<0.001*
Disease history				
Diabetes, N (%)	51 (15.69)	110 (35.14)	31.98	<0.001*
Hypertension, N (%)	145 (44.62)	208 (66.45)	30.77	<0.001*
Stroke N (%)	127 (39.00)	139 (44.41)	1.87	0.172
Hyperlipidaemia, N (%)	5 (1.54)	12 (3.83)	3.24	0.072
CHD, N (%)	42 (12.92)	122 (38.98)	56.68	<0.001*
Hepatopathy, N (%)	4 (1.2)	8 (2.6)	1.54	0.214
Nephropathy, N (%)	4 (1.2)	18 (5.8)	9.88	0.002*
Biochemical Indices				
TG	1.53±1.04	1.63±1.21	-1.13†	0.261
Apo A1	1.12±0.55	1.01±0.20	2.94†	0.003*
Apo B	0.86±0.24	0.80±0.31	2.57†	0.01*
TC	4.23±1.00	4.47±0.99	-3.01†	0.003*
LDL	2.45±0.73	2.65±0.75	-3.51†	<0.001*
HDL	1.16±0.32	1.07±0.27	3.47†	0.001*
Folate	35.05±6.52	34.58±6.24	0.64†	0.525
Vitamin B-12	228.42±43.26	218.75±40.67	1.99†	0.047*
Vitamin B-6	35.20±6.79	34.69±6.62	0.66†	0.508

Hcy: homocysteine; CHD: coronary artery heart disease; TG: triglyceride; Apo: apolipoprotein; TC: total cholesterol; LDL: low density lipoprotein; HDL: high density lipoprotein.
Data are presented as means±SD or n (%), with the significance of differences between groups evaluated using t-tests or the χ2 test, respectively.
†t value, not χ^2
*p value<0.05 was considered statistically significant.

Table 3. The correlation between BHMT gene polymorphisms and folate therapy efficacy

Gene	Success group N=325	Failure group N=313	Crude OR (95%CI)	*p*	Adjusted OR (95%CI)	*p*†
rs3733890						
Genotype						
GG	171 (52.6)	136 (43.5)	Ref.		Ref.	
GA	131 (40.3)	141 (45.0)	1.35 (0.98,1.88)	0.070	1.43 (1.02,2.06)	0.034*
AA	23 (7.1)	36 (11.5)	1.97 (1.11,3.48)	0.018*	2.03 (1.21,3.43)	0.008*
GA+AA	154 (47.4)	177 (56.5)	1.45 (1.06,1.97)	0.021*	1.65 (1.20,2.29)	0.009*
Allele						
G	473 (72.8)	413 (66.0)	Ref.			
A	177 (27.2)	213 (34.0)	1.38 (1.09,1.75)	0.008*		
rs585800						
Genotype						
TT	2 (0.6)	1 (0.3)	Ref.		Ref.	
TA	56 (17.2)	55 (17.6)	1.96 (0.17,22.29)	0.596	5.10 (0.34,76.61)	0.239
AA	267 (82.2)	257 (82.1)	1.91 (0.17,21.20)	0.598	4.54 (0.32,64.15)	0.263
TA+AA	323（99.4）	312 (99.7)	0.50 (0.17,21.28)	0.571	4.61 (0.33,64.82)	0.257
Allele						
T	60 (0.09)	57 (0.09)	Ref.			
A	590 (0.91)	569 (0.91)	1.01 (0.69,1.48)	0.967		

Ref.: referent values.
Data are presented as n (%). OR, 95CI% and p were calculated using unconditional logistic regression.
†Adjusted age, sex, smoking, alcohol consumption, history of diabetes, stroke, hypertension, CHD, nephropathy and biochemical indicators.
*p value<0.05 was considered statistically significant.

Table 3. The correlation between BHMT gene polymorphisms and folate therapy efficacy

Gene	Methylation level†	Success group VS. failure group‡			
		Crude OR(95%CI)	*p*	Adjusted OR(95%CI)	*p*§
BHMT	<0.1898	Ref.		Ref.	
	≥0.1898	0.626 (0.396, 0.990)	0.045*	0.485 (0.268, 0.879)	0.017*
BHMT_1	<0.1948	Ref.		Ref.	
	≥0.1948	0.576 (0.364, 0.913)	0.019*	0.515 (0.285, 0.930)	0.028*
BHMT_2	<0.1849	Ref.		Ref.	
	≥0.1849	0.843 (0.535, 1.327)	0.460	0.592 (0.328, 1.068)	0.082

Ref.: referent values.
OR, 95CI% and *p* were calculated using logistic regression.
†Mean of total or fragment gene methylation.
‡OR, *p* values were from logistic regression analysis.
§Adjusted age, sex, smoking, alcohol consumption, history of diabetes, stroke, hypertension, CHD, nephropathy and biochemical indicators.
*p value<0.05 was considered statistically significant.

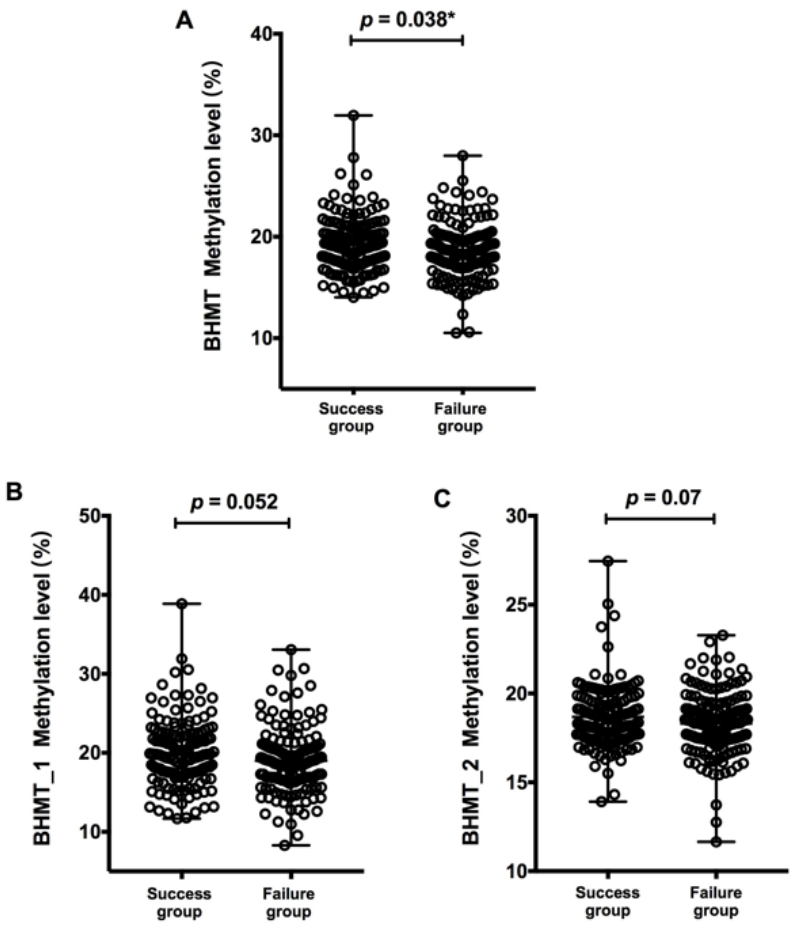

Figure 1. Methylation level of promoter in BHMT, BHMT_1 and BHMT_2 genes in success and failure groups. (A) Methylation level in BHMT gene; (B) Methylation level in BHMT_1 gene; (C) Methylation level in BHMT_2 gene. Each dot represents an individual. *p* were calculated using t-test, the t-test $^{*}p<0.05$ was considered statistically significant.

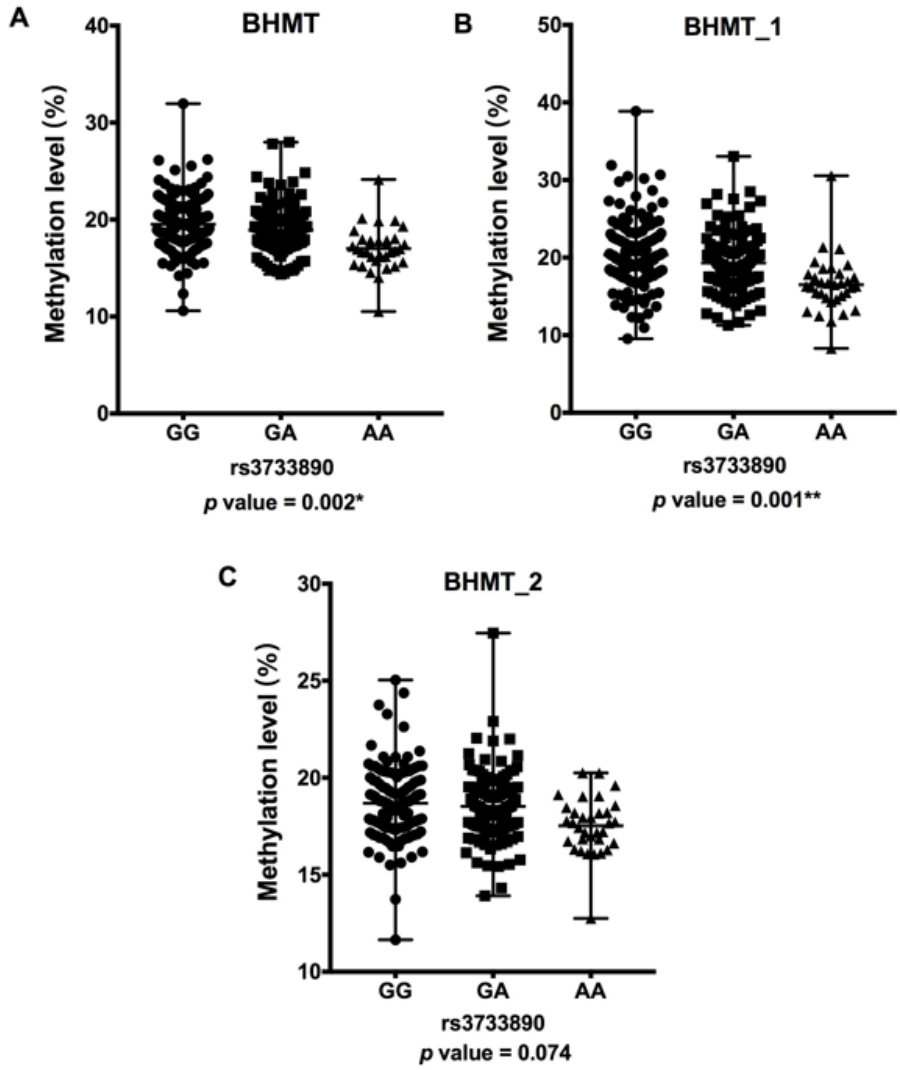

Figure 2. The association between promoter methylation in BHMT, BHMT_1 and BHMT_2 genes and rs3733890 SNPs. (A) Methylation level in BHMT gene; (B) Methylation level in BHMT_1 gene; (C) Methylation level in BHMT_2 gene. Each dot represents an individual. The statistical significance (*p* value) of association between genotype and DNA methylation, measured by linear regression model, is indicated at the bottom of the plot. The linear regression $^{*}p<0.05$, $^{**}p<0.001$ was considered statistically significant.

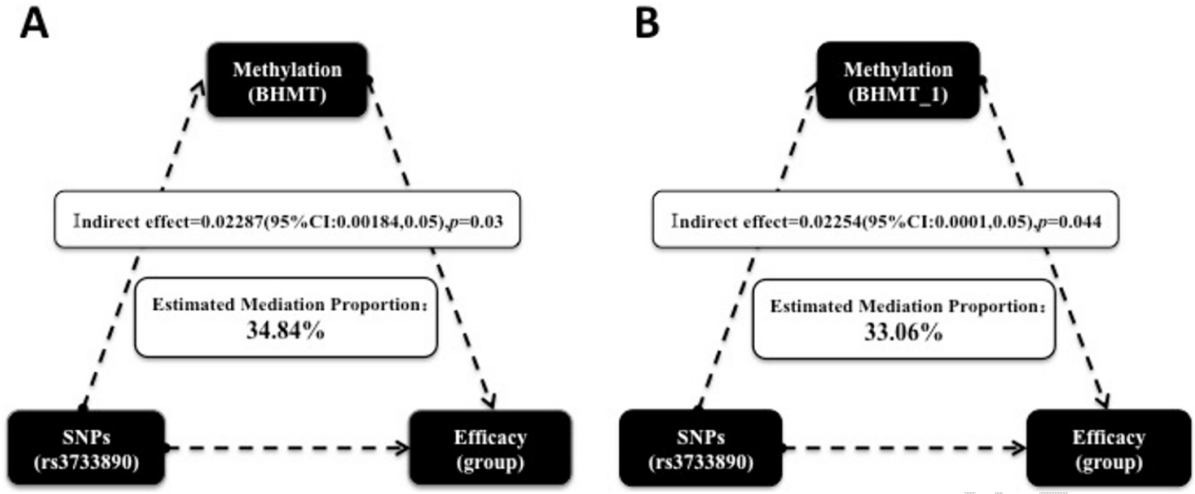

Figure 3. Mediation analysis of DNA methylation levels. Mediation analysis of (A) the rs3733890 SNPs (predictor), methylation levels of BHMT (mediator), and folate therapy efficacy (outcome); (B) the rs3733890 SNPs (predictor), methylation levels of BHMT_1 (mediator), and folate therapy efficacy (outcome). The figure shows, for the two potential mediators (BHMT or BHMT_1 methylation), the estimates of indirect effect (IEs), and proportion of mediation (%).