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Original Article

Association between the *MTHFR* C677T polymorphism, blood folate and vitamin B12 deficiency, and elevated serum total homocysteine in healthy individuals in Yunnan Province, China

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Abstract

Background: An increased serum total homocysteine (tHcy) concentration is typically associated with genetic defects involved in Hcy metabolism or related nutritional deficiencies. In this study, the combined effects of methylenetetrahydrofolate reductase (*MTHFR*) C677T polymorphism and folate and vitamin B12 deficiency on serum total Hcy (tHcy) levels were evaluated in a healthy Chinese population in Yunnan Province, China.

Methods: The *MTHFR* C677*T* polymorphism was genotyped in 330 volunteers (164 men and 166 women) using polymerase chain reaction-restriction fragment length polymorphism analysis. Folate, vitamin B12, and tHcy concentrations were determined by corpuscle immune chemiluminescence assays. The tHcy concentration was determined using an enzymatic assay.

Results: Significant negative correlations ($p < 0.001$) were observed between the serum levels of tHcy and folate ($r = -0.252$) and vitamin B12 ($r = -0.243$). Men had significantly higher serum tHcy concentrations than women ($p < 0.001$). Individuals with the *MTHFR* TT genotype had significantly higher serum tHcy concentrations than individuals with the CC and CT genotypes ($p < 0.001$). The folate level of red blood cells was significantly increased in individuals with the TT genotype than in individuals with the CC genotype ($p < 0.05$). Moreover, in the low vitamin group, the serum tHcy level was significantly correlated with the levels of folate ($r = -0.334$, $p = 0.001$) and vitamin B12 ($r = -0.212$, $p = 0.046$).

Conclusion: The *MTHFR* C677T polymorphism, folate deficiency, and B12 deficiency were significantly associated with elevated serum tHcy levels. Among these three factors, folate deficiency had the greatest contribution to the serum tHcy concentration, followed by (in order of decreasing effect) *MTHFR* C677T and vitamin B12 deficiency. Thus, folic acid and vitamin B12 supplementation could help prevent diseases associated with tHcy accumulation, especially in individuals with the *MTHFR* 677TT genotype.

Keywords: folate; homocysteine; *MTHFR* C677T polymorphism; vitamin B12

1. Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid that has a key role in methionine metabolism. Disturbance of this metabolic pathway may result in the accumulation of Hcy and related abnormal outcomes such as cognitive disorders,[1]

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cancer,[2] and birth defects.[3] In particular, elevated plasma total homocysteine (tHcy) is an independent risk factor for cardiovascular disease.[4]

Homocysteine is metabolized by two major pathways: (1) the remethylation pathway and (2) the transsulfuration pathway (Figure 1). In the remethylation pathway, 5-methyltetrahydrofolate (5-MTHF), the predominant folate formed in the blood, acts as a methyl donor for Hcy remethylation mediated by the vitamin B12-dependent enzyme methionine synthase. This process results in the formation of tetrahydrofolate. Homocysteine is then finally converted to methionine. In the transsulfuration reaction, Hcy reacts with serine to form cystathionine, which is catalyzed by cystathionine β-synthase and uses vitamin B6 as a cofactor. Cystathionine is then converted into cysteine, which is finally converted to sulfates and excreted in the urine.[5] Therefore, because of their important roles in these pathways, a deficiency in folic acid and vitamin B12 may affect the normal metabolism of Hcy.

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme that irreversibly catalyzes the conversion of 5,10-MTHF to 5-MTHF, and has a crucial role in controlling the distribution of folic acid through the whole metabolic pathway. Thus, genetic polymorphisms in the *MTHFR* gene may affect enzyme activity. The most common polymorphism of *MTHFR* is C677T, which causes MTHFR to become thermolabile, and consequently have reduced activity.[6] The reduction of MTHFR activity may then decrease the concentration of 5-MTHF and ultimately elevate the Hcy level.

The use of folate and vitamin B12 as dietary supplements to decrease Hcy level has been evaluated previously;[7] however, the overwhelming majority of such studies were conducted in populations such as patients with myocardial infarction[8] and coronary heart disease.[9] These findings have not been clearly substantiated in healthy populations. The literature on the relationship between the *MTHFR* polymorphism and tHcy concentration overall remains controversial. Joachim et al[10] found that tHcy levels did not differ between individuals with the CC genotype who had venous thromboembolism, compared to individuals with the TT or CT genotype. By contrast, several studies have found significant associations between the tHcy concentration and *MTHFR* polymorphisms.[11–13]

The aim of this study was to clarify the association of the serum tHcy level with the combination of the *MTHFR* C677T polymorphism, folate deficiency, and vitamin B12 deficiency within a healthy Chinese population in Yunnan Province. To our knowledge, this is the first study to evaluate the contribution of these three factors to variations in tHcy levels in healthy people. This work will help elucidate the effect of an individual's genetic background and daily dietary environmental determinants on serum tHcy concentrations. This knowledge could then facilitate the monitoring of at-risk individuals for disease prevention.

2. Methods

2.1. Recruitment and sampling

The study was approved by our institutional review board and ethics committee. We randomly recruited 330 volunteers (164 males and 166 females) who had no history of cancer, cardiovascular disease, or neurodegenerative disease; who had

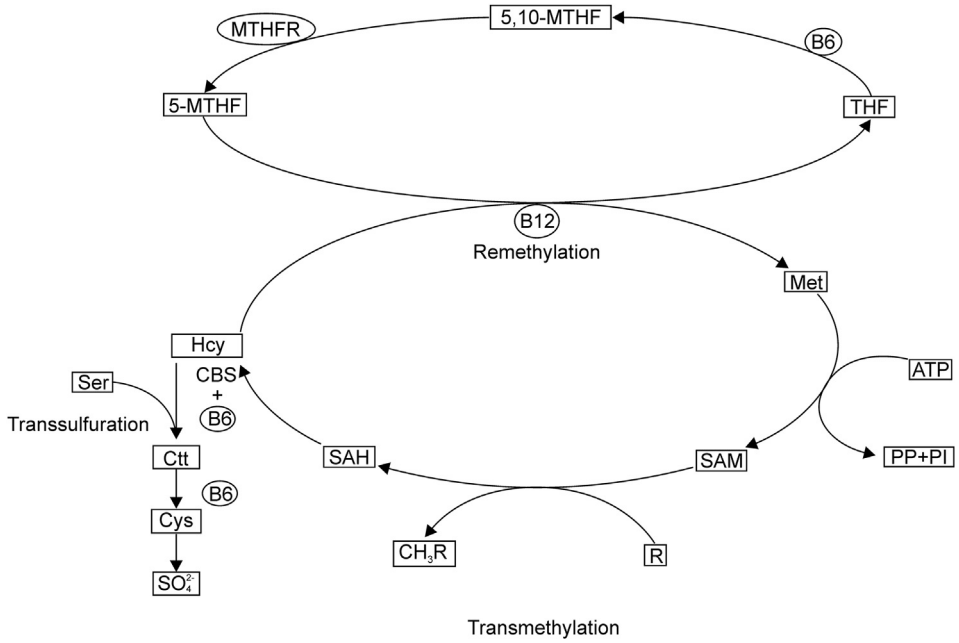

Fig. 1. Schematic representation of homocysteine metabolism.[58,59] See the text for details. 5-MTHF = 5-methylenetetrahydrofolate; 5,10-MTHF = 5,10-methylenetetrahydrofolate; B6 = vitamin B6; B12 = vitamin B12; CBS = cystathionine β-synthase; Ctt = cystathionine; Cys = cysteine; Hcy = homocysteine; Met = methionine; MTHFR = N5,N10-methylenetetrahydrofolate reductase; SAH = S-adenosyl homocysteine; SAM = S-adenosyl methionine; Ser = serine; THF = tetrahydrofolate.

no exposure to chemical carcinogens and radiation; who practiced a healthy lifestyle and dietary habits; and who were 18−81 years old (Table 1). Basic information about the volunteers such as weight, height, age, sex, and smoking and drinking habits was also collected using a questionnaire.

Blood samples from volunteers were collected after they provided informed consent and filled out the questionnaire. Blood (3 mL) was collected by venipuncture and placed into tubes without an anticoagulant. Another blood sample (1 mL) was collected and placed into tubes containing ethylenediaminetetraacetic acid (EDTA) from overnight-fasted study participants who had responded to a food-frequency questionnaire. Blood samples were collected for the measurement of serum folate, red blood cell (RBC) folate, serum vitamin B12, and serum tHcy concentrations, and for the determination of the *MTHFR* C677T genotype.

2.2. Genomic DNA extraction

The EDTA-preserved whole-blood samples were used for the extraction of genomic DNA using the TIANamp Blood DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China). The DNA samples were stored at −20°C until use.

2.3. Genotyping of the MTHFR C677T polymorphism

The *MTHFR* C677T polymorphism was detected via polymerase chain reaction amplification of genomic DNA, followed by restriction fragment length polymorphism (RFLP) analysis.[14] The genotype assay was performed with a forward primer (i.e., 5′-TGAAGGAGAAGGTGTCTGCGGGA-3′) and a reverse primer (i.e., 5′-AGGACGGTGCGGTGAGAGTG-3′).[15] For the RFLP analysis, the samples were incubated with the restriction enzyme HinFI at 37°C for 1 hour. The bands were then resolved by 3% agarose gel electrophoresis and visualized using an ultraviolet light. Genotypes were identified, based on the expected fragment lengths: two bands containing 175 bp and 23 bp for TT; three bands containing 198 bp, 175 bp, and 23 bp for CT; and one band containing 198 bp for CC. All determinations were repeated twice in two separate runs.

2.4. Biochemical assays for serum folate, RBC folate, serum vitamin B12, and serum tHcy concentrations

Within 1 hour of collection, the blood samples were collected into tubes without an anticoagulant and centrifuged at 2000 *g* for 5 minutes to obtain the serum sample. All samples were stored at −20°C until analysis.

Table 1
Demographics of the study population.

	Men	Women	*p*
Age (y)	37.26 ± 14.99	36.19 ± 1.12	0.433
Age range	18−77	18−81	
No. of individuals	164	166	

The data are presented as the mean ± standard deviation or as the number.

The RBCs were separated from the EDTA-preserved whole-blood sample, washed, and broken down. They were then diluted 1:21 by mixing 50 μL of the RBCs with 1 mL of 0.2% vitamin C solution. Samples were stored in dark at −20°C until analysis.

Serum levels of tHcy, folate, and vitamin B12 were estimated using commercial kits (Access Folate Kit [A98032]/ Access Vitamin B12 kit [33000]; Beckman Coulter, Fullerton, CA, USA; and Homocysteine Assay Kit [AB200]; Ausa Pharmed Co., Ltd., Shenzhen, China). Folate and vitamin B12 levels were also determined by a corpuscle immune chemiluminescence assay (ACCESS2 Immunoassay System; Beckman Coulter, Fullerton, CA, USA). The tHcy concentration was determined using an enzymatic assay (OLYMPUS AU5400 Automatic Biochemistry Analyzer; Olympus-Beckman, Tokyo, Japan). The assays were performed in accordance with the manufacturers' protocols.

2.5. Statistical analysis

Statistical software SPSS 15.0 was used for data analysis. Allele frequencies were calculated by allele counting. Concordance of genotype frequencies with the Hardy−Weinberg equilibrium was tested by the Chi-square test. The data were summarized as the mean and standard deviation. Bivariate analyses were used to evaluate the correlation between the serum tHcy levels and the folate, vitamin B12, and RBC folate levels. One-way analysis of variance (ANOVA) was performed to compare the serum levels of tHcy, vitamin B12, folate, and RBC folate between the different *MTHFR* C677T genotypes. The influence of sex on the concentrations of serum tHcy, vitamin B12, folate, and RBC folate was evaluated using the Student *t* test. Two-way ANOVA was used to compare the relative effects of folate, vitamin B12, and *MTHFR* C677T on the serum tHcy concentrations. Statistical significance was accepted at $p < 0.05$.

3. Results

The demographics of the study population are shown in Table 1. Serum levels of tHcy were significantly negatively correlated with folate levels ($r = -0.252$, $p < 0.001$) and vitamin B12 levels ($r = -0.243$, $p < 0.001$). However, there was no significant correlation between the levels of tHcy and RBC folate ($r = -0.032$, $p = 0.564$).

Table 2 shows the influence of sex on the concentrations of serum tHcy, vitamin B12, folate, and RBC folate. Men had a significantly higher mean tHcy concentration than women ($p < 0.001$), and a significantly a lower mean serum folate level than women ($p < 0.05$). There was no difference in RBC folate and vitamin B12 levels between men and women.

The *MTHFR* C677T genotype distribution deviated from the expected Hardy−Weinberg distribution. The overall T allele frequency was 35.2% (Table 3). Individuals with the TT genotype had a significantly higher tHcy concentration than individuals with the CC and CT genotypes ($p < 0.001$). Moreover, the RBC folate level was significantly increased in

Table 2
The influence of sex on the concentrations of total homocysteine, vitamin B12, folate, and red blood cell folate.

Sex	tHcy (μM)	B12 (pM)	folate (nM)	RBC folate (nM)
Men ($n = 164$)	15.77 ± 8.80	416.18 ± 189.69	19.46 ± 14.12	674.91 ± 365.92
Women ($n = 166$)	11.77 ± 6.32*	446.74 ± 216.70	22.99 ± 13.84**	737.01 ± 427.62
Total ($n = 330$)	13.76 ± 7.90	431.55 ± 203.99	21.24 ± 14.07	706.15 ± 398.76

The data are presented as the mean ± the standard deviation.
* $p < 0.01$ and ** $p < 0.05$ for men versus women (based on the Student t test).
B6 = vitamin B6; B12 = vitamin B12; RBC = red blood cell; tHcy = total homocysteine.

Table 3
The distribution of the *MTHFR* C677T genotype and allele frequencies in the study population.

	Genotype frequency			Allele frequency		χ^2 [a]	p
C677T	CC	CT	TT	C	T		
	146 (44.2%)	136 (41.2%)	48 (14.5%)	64.8%	35.2%	3.013	0.222

The data are presented as the number of individuals (percentages).
MTHFR = methylenetetrahydrofolate reductase gene.
[a] The Chi-square test was used to assess the Hardy–Weinberg equilibrium.

individuals with the TT genotype, compared to individuals with the CC genotype ($p < 0.05$; Table 4).

There was no significant correlation between the tHcy level and RBC folate level; therefore, we used two-way ANOVA to analyze the relative contributions of serum folate, the C677T genotype, and vitamin B12 to variations in tHcy concentrations. Intervention studies in humans taking folate and/or vitamin B12 supplements have shown that a plasma concentration of vitamin B12 greater than 300pM and a plasma folate concentration greater than 34nM could be the reference values for maintaining genome stability.[16–22] Therefore, these levels served as the thresholds for defining low vitamin levels. We accordingly found that the relative contribution of the three factors to tHcy level (in decreasing order) was folate, the C677T genotype, and vitamin B12 (Table 5). Moreover, there was a significant correlation between the tHcy concentration and low serum folate levels ($r = -0.334$, $p = 0.001$) and low serum vitamin B12 levels ($r = -0.212$, $p = 0.046$). However, there was no significant correlation between the tHcy concentration and high serum folate levels ($r = 0.051$, $p = 0.763$) and high vitamin B12 levels ($r = -0.124$, $p = 0.054$).

4. Discussion

Increased plasma tHcy levels are associated with several diseases such as cardiovascular disease,[23,24] osteoporosis,[25,26] dementia, Alzheimer's disease,[27,28] pregnancy complications,[29] and psychiatric disorders.[30] Plasma tHcy is a sensitive marker of folate and vitamin B12 status, and defects in the metabolism of either factor may lead to increased plasma Hcy levels.[31,32] Our study demonstrated a significant negative correlation between the mean serum levels of tHcy and the levels of folate and vitamin B12, thus supporting previous findings.[33,34] Several human studies have shown that folate and vitamin B12 intake and biochemical status are important determinants of plasma tHcy concentrations.[8,9,35] It has therefore been proposed that supplementation with vitamin B12 could help normalize blood tHcy levels.[7] Thus, dietary folate deficiency and drugs that interfere with folate metabolism may lead to Hcy accumulation and the consequent cellular efflux of Hcy.[36]

In our study, the mean serum tHcy concentration was notably higher than previously reported concentrations.[37–41] We speculate that this difference may have resulted from the unique cooking and dietary habits among people in Yunnan Province. People in Yunnan traditionally eat pickled and fried foods, which may not be conducive to the intake of folate and other vitamins.

Table 5
The association and relative contribution of serum folate and vitamin B12 levels and *MTHFR* C677T genotype to the serum total homocysteine concentration.

Vitamin status	C677T CC (mean ± SD)	C677T CT (mean ± SD)	C677T TT (mean ± SD)	Association and contribution
High folate	10.08 ± 2.77	10.74 ± 3.79	9.50 ± 0.28	Folate > C677T
Low folate[a]	11.89 ± 3.94	13.73 ± 5.64	21.79 ± 15.35*	
High B12	11.25 ± 3.26	12.41 ± 5.12	17.43 ± 13.97*	C677T > B12
Low B12[b]	13.32 ± 5.34	15.64 ± 5.85	26.67 ± 15.61*	

* $p < 0.01$, based on pairwise comparisons between genotypes.
B6 = vitamin B6; B12 = vitamin B12; *MTHFR* = methylenetetrahydrofolate reductase gene; SD = standard deviation.
[a] A folate level ≤ 34nM is the criterion for a low folate level.
[b] A vitamin B12 level ≤ 300pM is the criterion for a low B12 level.

Table 4
The serum levels of total homocysteine, vitamin B12, folate, and red blood cell folate, based on the *MTHFR* C677T genotype.

Genotype	tHcy (μM)	B12 (pM)	Folate (nM)	RBC folate (nM)
CC ($n = 146$)	11.69 ± 3.87	439.81 ± 202.81	21.86 ± 13.97	648.14 ± 385.20
CT ($n = 136$)	13.32 ± 5.50	426.40 ± 201.35	21.81 ± 14.85	731.22 ± 388.00
TT ($n = 48$)	21.28 ± 15.22 *	421.03 ± 218.01	17.74 ± 11.65	811.54 ± 446.41**

* $p < 0.01$ between TT and CC/CT.
** $p < 0.05$, between TT and CC.
B6 = vitamin B6; B12 = vitamin B12; *MTHFR* = methylenetetrahydrofolate reductase gene; RBC = red blood cell; tHcy = total homocysteine.

We confirmed that men had significantly higher serum tHcy concentrations than women, which is in line with the findings of previous reports.[42,43] Nienaber-Rousseau et al[44] believe that this finding may result from gene–sex interactions or may result from an inherent difference in creatinine levels, and that it may be influenced in men by increased alcohol consumption. Based on literature reports,[42,45] the tHcy value in men is, on average, 1μM higher than in women. This difference could be caused by the larger muscle mass and thus greater creatine phosphate synthesis in men,[46] a reduced effect of estrogens in women,[47] and/or different Hcy metabolism processes between the sexes.[46] By contrast, our results showed that the level of serum folate was significantly decreased in men than in women. A folate-B12 intervention trial revealed that the micronuclear frequency of peripheral blood lymphocytes was reduced by 37.1% in women and by 30% in men after supplementation, but the difference in men did not achieve statistical significance.[48] It is possible that this differential response stems from an initial difference in vitamin status because vitamin intake is usually lower in men than in women; moreover, men consume more alcohol than women, which may further affect the absorption of the B vitamins.

In addition, our results showed that individuals homozygous for the C677T variant allele (T) displayed elevated serum tHcy concentrations and that TT-homozygous individuals had the highest serum tHcy concentrations, whereas wild-type CC-homozygous individuals had the lowest serum tHcy concentrations. This result is consistent with the finding of Zidan et al,[11] who found that the tHcy level was significantly increased in Egyptian children with coronary heart disease harboring the *MTHFR* 677TT and *MTHFR* 1298CC genotypes. Furthermore, de Bree et al[12] and Ozarda et al[13] found that healthy individuals with the TT genotype had a significantly higher tHcy concentration than those with the CC and CT genotypes. The increased tHcy concentrations could be attributed to thermolability induced in MTHFR, which results in dissociation of the active dimer into inactive monomers with a subsequent loss of flavin adenine dinucleotide-binding capacity.[49] As a consequence, MTHFR would be unable to efficiently reduce 5,10-MTHF to 5-MTHF, which is necessary for the conversion of Hcy to methionine. Furthermore, the T allele frequency in our study was 35.2%. Schneider et al[50] reported that the distribution of the *MTHFR* C677T mutation ranged 4.5%–44.9% in populations from Europe, Africa, the Middle East, Asia, Asia Minor, Australasia, and the Americas. The *MTHFR* C677T polymorphism is associated with increased tHcy levels and has been implicated in the increased risk of a wide range of adverse health conditions throughout life from birth defects[51] to cardiovascular disease and osteoporosis[26] in the elderly.

We also found that the RBC folate level was significantly increased in individuals with the TT genotype compared to individuals with the CC genotype. The plasma folate concentration fluctuates in relation to diet, and is thus a useful dynamic measure that reflects recent nutritional uptake. Therefore, serum folate is commonly used as a marker for the short-term folate status, whereas RBC folate is used as a marker for long-term folate status because it reflects the folate status during erythropoiesis.[52] The reduced stability and activity of the MTHFR enzyme associated with the *MTHFR* C677T polymorphism may diminish the utilization of folate, thereby leading to its accumulation. Fohr et al[53] showed that individuals with the TT genotype had a greater increase in RBC folate after supplementation with a folate derivative, compared to individuals with the CT or CC genotype.

Previous intervention studies[16–22] in humans taking folate and/or vitamin B12 supplements showed that DNA hypomethylation, chromosome breaks, uracil misincorporation, and micronucleus formation were minimized when the plasma concentration of vitamin B12 was >300pM, the plasma folate concentration was >34nM, the RBC folate concentration was >700 M, and the plasma Hcy concentration was <7.5μM. Therefore, these levels served as our criteria for defining low vitamin levels, and a cutoff level below 7.5μM was considered a reasonable fasting tHcy concentration.

To our knowledge, this is the first study to report the relative contribution of the *MTHFR* C677T gene polymorphism and folate and vitamin B12 deficiency on serum tHcy levels. Our data suggest no significant correlation between the mean serum levels of tHcy and the C677T polymorphism when the folate level is high (>34nM); however, at a low folate level (≤34 nM), individuals with the TT genotype had significantly higher tHcy levels. Thus, the C677T polymorphism is associated with increased serum Hcy levels when combined with a low folate status. This finding is consistent with previous work showing that most T-homozygous individuals with low serum folate levels had increased tHcy levels, whereas T-homozygous individuals with high serum folate levels had normal tHcy levels,[54] and that dietary folate is a key risk modifier that can negate the risk associated with the *MTHFR* C677T polymorphism by directly controlling tHcy levels.[55] Riboflavin and folate levels are significant predictors of Hcy levels in individuals homozygous for the *MTHFR* T677 allele, which suggests that T-homozygous individuals require a higher riboflavin and folate intake to maintain low Hcy levels.[56,57] Therefore, the impact of folate on tHcy levels is greater than the impact of the *MTHFR* C677T gene polymorphism.

Moreover, we found a significant correlation between mean serum levels of tHcy and B12, but only for individuals with low serum vitamin B12 levels. This finding may have resulted from other factors that interfered with detecting the specific contribution of vitamin B12 to the tHcy level. The tHcy level was significantly correlated with the C677T polymorphism, independent of the vitamin B12 status, which suggests that the impact of the C677T polymorphism on tHcy levels is greater than the impact of vitamin B12. The tHcy level overall appears to significantly increase in the presence of a low serum vitamin status.

In conclusion, the *MTHFR* C677T polymorphism and folate and vitamin B12 deficiency were all associated with elevated serum tHcy levels in healthy individuals in Yunnan Province, China. Among these three factors, folate deficiency appears to be much more important than the *MTHFR* C677T

polymorphism for elevating the tHcy concentration, whereas B12 deficiency was the weakest factor. These results suggest that appropriate doses of folic acid and vitamin B12 supplementation could help to normalize the blood tHcy level, especially in individuals with the *MTHFR* 677TT genotype. These results may provide new strategies for preventing diseases related to Hcy accumulation, particularly cardiovascular disease.

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