

MTHFR C677T Polymorphism, Homocysteine and B-Vitamins Status in a Sample of Chinese and Malay Subjects in Universiti Putra Malaysia

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ABSTRACT

Introduction: Methylene tetrahydrofolate reductase (MTHFR) C677T is involved in folate and homocysteine metabolism. Disruption in the activity of this enzyme will alter their levels in the body. **Methodology:** This study assessed MTHFR C677T polymorphism and its relationship with serum homocysteine and B-vitamins levels in a sample of Chinese and Malays subjects in UPM, Serdang. One hundred subjects were randomly selected from among the university population. Folate, vitamin B₁₂, B₆, and homocysteine levels were determined using MBA, ECLIA, and HPLC, respectively. PCR coupled with *HinfI* digestion was used for detection of MTHFR C677T polymorphism. **Results:** The frequency of T allele was higher in the Chinese subjects (0.40) compared to the Malay (0.14). Folate, vitamin B₁₂ and B₆ levels were highest in the wild genotype in both ethnic groups. Subjects with heterozygous and homozygous genotype showed the highest homocysteine levels. The serum folate and homocysteine were mainly affected by homozygous genotype. **Conclusion:** MTHFR C677T polymorphism plays an important role in influencing the folate and homocysteine metabolism.

Keywords: Chinese, folate, homocysteine, Malay, MTHFR C677T

INTRODUCTION

Folate is essential for nucleic acid synthesis and repair and is required for regulation of homocysteine metabolism through remethylation reactions. Genetic defects in the enzyme 5,10-methylene tetrahydrofolate reductase (MTHFR) or dietary deficiency of B-vitamin cofactors and substrates involved

in this metabolism result in elevated homocysteine levels. Hyperhomocysteinemia is an independent risk factor for cardiovascular disease (Hao *et al.*, 2003), depression (Tiemeier *et al.*, 2002), and impairment in neuronal plasticity and degeneration which subsequently cause dementia and Alzheimer's disease (Mattson & Shea, 2003).

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MTHFR catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is the main circulating form of folate and the methyl donor for the vitamin B₁₂-dependent remethylation of homocysteine to methionine. A common C677T transition in the MTHFR gene results in a thermolabile variant with specifically decreased enzyme activity. Under conditions of impaired folate status, the genetic variant predisposes to high homocysteine levels probably because mutation impedes the formation of 5-methyltetrahydrofolate (Guttormsen *et al.*, 1996). The presence of MTHFR genotype has been linked to lower serum folate (Jacques *et al.*, 1996), elevated homocysteine (Russo *et al.*, 2003), and altered distribution of different folates in cells (Bagley & Selhub, 2000).

Individuals who are homozygous (TT) for MTHFR polymorphism may have enzyme activities up to 50% lower than those without the polymorphism (Bailey & Gregory, 1999). In Malaysia, data on MTHFR polymorphism is limited. Thus this study was undertaken with Chinese and Malay adults as subjects. Both ethnic groups are known to be at high risk of displaying the effect on MTHFR polymorphism, particularly the TT genotype. In Singapore, Lu *et al.* (2007) and Kasiman *et al.* (2009) reported that Chinese population had 12.7% and 7.5% of homozygous for the C677T MTHFR polymorphism, respectively. Up to the time of this study, no research had been done on Malaysian Chinese. As for Malay subjects, in the study of Lu *et al.* (2007), none was reported with TT genotype but in the study of Kasiman *et al.* (2009), Malay subjects were found to show 4.9% of homozygosity for this polymorphism. In another study by Ling *et al.* (2003) on Malaysian Malay subjects, 8% of subjects were found to have TT genotype. However, Hayati *et al.* (2008) found this homozygosity absent in Malay subjects. This uncertainty leads to conflict in interpreting the exact gene polymorphism in the Malay population.

There have been no data generated on relating this polymorphism with blood parameters such as folate, homocysteine, vitamin B₁₂ and B₆. Therefore, the aim of this study was to investigate the frequency of the MTHFR C677T polymorphism in a small population in Malaysia, to determine the levels of homocysteine, folate, B₁₂ and B₆ according to the specific genotype, and to investigate the relationships among MTHFR C677T with homocysteine and B-vitamin status.

METHODOLOGY

Recruitment of subjects

This is a cross-sectional study involving a total of 100 subjects (54 Chinese and 46 Malays) consisting of staff and post-graduate students of Universiti Putra Malaysia by using a simple random sampling procedure. Approval from the Medical Research Ethics Committee of the Faculty of Medicine and Health Sciences (FMHS) was obtained for the study. Subjects were given an information sheet explaining the purpose and methodology of the study. Those who agreed to participate were asked to sign an informed consent form prior to their participation. The inclusion criteria were as follows: 20-45 years old male and female, Chinese or Malay, not pregnant or breastfeeding, not consuming alcohol habitually, and not a smoker. The exclusion criteria were: use of any type of B-vitamin supplement in the past three months as well as oral contraceptives. All data (demographic background, medical history, and dietary intake) were collected during face-to-face interviews with the subjects.

Anthropometric assessment

Body weight was taken to the nearest 0.1 kg using a digital weighing scale (TANITA THD Model 306, Germany). Height was measured using a portable body meter (SECA wall stadiometer Model 206, Germany) to the nearest 0.1 cm.

Dietary assessment

The twenty-four hour dietary recall method was used to record dietary folate intake by an interviewer. Detailed descriptions of all foods and beverages consumed during the 24-hour period before the interview including the quantity, cooking method and the brand name were recorded. Food quantities were assessed by the use of household measurements. Dietary folate intakes were then tabulated based on the USDA Nutrient Database for Standard Reference using the Nutritionist Pro, version 2.5 food databases (First Databank, California, USA). In addition, an additional Food Frequency Questionnaire (FFQ), which consisted of a list of folate-rich foods in several food categories (vegetables, fruits, cereals, legumes, and dairy products), common to the Chinese and Malay communities, was developed.

Biochemical assessment

Subjects were required to fast (10-12 hr) and from each subject, 10 ml of blood was drawn in order to test for MTHFR polymorphism, complete blood count, folate, homocysteine, vitamin B₁₂ and vitamin B₆. The obtained serum was stored at -80°C until analysis of folate, vitamin B₁₂, vitamin B₆, and homocysteine. Determination of serum folate and red blood cell folate was based on microbiological assay (MBA) with *Lactobacillus casei* (*L. casei*) (Riken, Japan) as test microorganism (O'Broin & Kelleher, 1992). This is the gold standard method used for folate determination. *L. casei* was cryopreserved by the methods according to Wilson & Horne (1982). Serum vitamin B₁₂ was determined using electrochemiluminescence immunoassay (ECLIA) with Elecsys vitamin B₁₂ reagent kit on Elecsys 2010 (Roche Diagnostics GmbH, Germany). The kit was used according to the manufacturer's instruction. This assay employs a competitive test principle by using the intrinsic factor, specific to vitamin

B₁₂. Reagent kit for high-performance liquid chromatography (HPLC) (Agilent 1100, Hewlett-Packard, Germany) analysis of vitamin B₆ (Chromsystems, GmbH, Germany) which uses a simple isocratic HPLC system with an attached fluorescence detector (λ Ex 320; λ Em 415) was used for vitamin B₆ determination. As for serum homocysteine, it was determined by HPLC, using a Chromsystems Reagent Kit for HPLC analysis of homocysteine in serum (Chromsystems, GmbH, Germany), which uses a simple isocratic HPLC system with an attached fluorescence detector (λ Ex 385; λ Em 515).

Determination of MTHFR C677T polymorphism

Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) from whole blood drawn from the subjects. The purity and concentration of the polymerase chain reaction (PCR) products was determined using Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) at absorbance of 260 and 280. The sample was considered free of protein impurity when the ratio of optical density of A₂₆₀ to A₂₈₀ was between 1.7-1.9. The MTHFR 677C->T mutation site was amplified using a set of primers (Eurogentec Ait, Aitbiotech, Singapore) previously described by Frosst *et al.* (1995). The primers used were 5'-TGA AGG AGA AGG TGT CTG GGG GA-3' (forward) and 5'-AGG ACG GTG CGG TGA GAG TG-3' (reverse). The PCR conditions were as follows: an initial denaturation step of 4 min at 94°C, followed by 30 cycles of denaturation (94°C, 60 s), annealing (55°C, 60 s), extension (72°C, 60 s) and final extension (72°C, 5 min) before cooling at 15°C (Kou *et al.*, 2001). The amplification was carried out using Mastercycler Gradient Thermalcycler (Eppendorf, Germany). The amplified PCR products were then electrophoresed in a 2.0% agarose gel

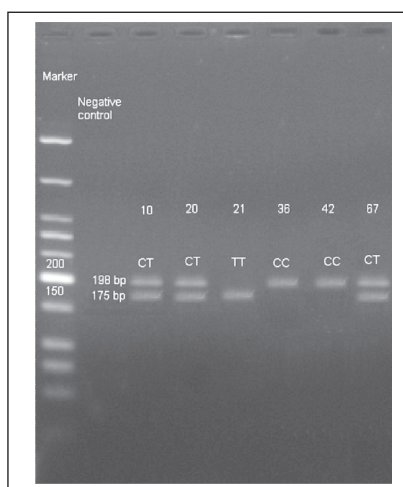


Figure 1: Different MTHFR genotype. DNA samples are amplified using PCR and digested with *HinfI* restriction enzyme. The presence of C to T mutation creates a *HinfI* restriction site. CT (heterozygous) contains two bands (198 and 175 bp), TT (homozygous) contains one band (175 bp), and CC (wild) contains one band (198 bp).

(Seakem, Rockland, ME, USA), which was stained with ethidium bromide (Sigma Chemical, St Louis, MO, USA) and viewed under ultraviolet light (Alpha Innotech Corp, San Leandro, CA, USA). The PCR products were subsequently purified using a PCR DNA Fragment Extraction Kit (Yeastern Biotech Co, Ltd, Taiwan) in order to remove excess primers. Upon purification, the samples were digested with *HinfI* restriction enzyme (New England Biolabs, Beverly, MA, USA). The restriction digested PCR products were then analysed in 2.0% agarose gel stained with ethidium bromide. Each time PCR was performed to detect MTHFR C677T polymorphism, known positive and negative controls were used in order to obtain accurate results. The purified PCR products were sent to First Base Laboratories Sdn. Bhd., Kuala Lumpur, Malaysia for sequencing.

Statistical analysis

Data was analysed using Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) version 17 and expressed as mean \pm SD. Independent sample T-tests and

ANOVA were carried out to examine the mean differences for categorical independent variables with continuous variables, while Pearson's correlation was used to examine the relationship between two normally distributed continuous variables. Chi-square analysis was used to test Hardy-Weinberg equilibrium (HWE). The differences in slopes of the relation between serum folate, RBC folate, serum homocysteine, vitamin B₁₂ and B₆ by MTHFR genotype were examined by linear regression with an interaction term. ANCOVA analysis was conducted to investigate the effect of MTHFR genotype on serum and RBC folate, serum homocysteine, vitamin B₁₂ and B₆ controlling for ethnicity, gender, and age. Linear regression analysis with genotype as the independent variable was used to find the variables that influence the folate and homocysteine concentration in this sample according to genotypes.

RESULTS

MTHFR C677T polymorphism

Figure 1 shows an agarose gel illustrating the different genotypes of the 677C>T

Table 1. Distribution of MTHFR genotype according to ethnicity and genders (n = 100)

Genotype	n	Chinese		Malay	
		Male	Female	Male	Female
CC	57	13	11	12	21
CT	30	4	13	5	8
TT	13	8	5	0	0

mutation on 2.0% agarose gel electrophoresis after the digestion of PCR products with *HinfI*. This enzyme digests the fragments into two parts: a shorter fragment (23bp) and a longer fragment (175bp).

Table 1 shows the results for parameters studied which are classified according to three genotypes of MTHFR C677T polymorphism. Of the 100 subjects studied, more than half of the subjects (57%) were normal (CC). Both Malay men and women showed no tendency of having the TT genotype whilst among the Chinese subjects, men had a higher occurrence of this genotype. The C677T polymorphism was present in 13% of the Malay subjects and 30% of the Chinese subjects in this study (Table 1). The expected genotype frequencies were calculated from the allele frequencies and compared to observed frequencies under the assumption of Hardy-Weinberg equilibrium. The T allele frequency for the Chinese and Malay subjects was 0.40 and 0.14, respectively. As for the C allele, a higher occurrence was detected among the Malay subjects (0.86) than the Chinese subjects (0.60).

MTHFR C677T polymorphism and homocysteine, folate, vitamin B₁₂ and B₆ levels

Apart from dietary intake, folate metabolism was strongly influenced by genetic changes of MTHFR. Based on Table 2, there was a

significant difference in the serum and RBC folate among three different genotypes between the Chinese and Malay subjects. The highest serum and RBC folate was found in subjects with normal genotype (CC), followed by heterozygous (CT) and homozygous (TT) genotype. Among these three genotypes, CT and TT types subjects (14.53 $\mu\text{mol/L}$ in Chinese and 19.79 $\mu\text{mol/L}$ in Malay) had a significantly higher level of homocysteine than normal subjects (13.69 $\mu\text{mol/L}$ in Chinese and 14.68 $\mu\text{mol/L}$ in Malay) (Table 2). From Table 2, it is apparent that there is no significant difference between the C677T polymorphism with serum vitamin B₁₂ and B₆ levels.

For homogeneity-of-regression assumption, all the parameters were tested for the covariate on ethnicity, gender, and age. From the results obtained, only one parameter (RBC folate) was significant when tested for ethnicity. This suggests that the differences on the RBC folate among genotypes vary with ethnicity. Based on the one-way analysis of covariance (ANCOVA), a significant result was obtained for RBC folate (controlling for gender and age), serum folate and serum homocysteine (controlling for ethnicity, gender, and age). Hence, this shows that by controlling for gender and age, serum folate, RBC folate and serum homocysteine differ significantly with MTHFR genotypes. Based on the linear regression analysis, only 3 parameters were shown to be significant; serum folate, RBC

Table 2. Serum folate, RBC folate, serum homocysteine, serum vitamin B₁₂ and serum vitamin B₆ against MTHFR genotype according to ethnicity

	Subjects						
	All (n = 100)	CC (n = 57)		p value	CT + TT (n = 43)		p value
		Chinese (n = 24)	Malay (n = 33)		Chinese (n = 30)	Malay (n = 13)	
Serum folate (nmol/L)	9.42 ± 3.98	11.45	8.90 ± 3.85	0.035	10.51 ± 3.35	7.22 ± 3.68	0.010
RBC folate (nmol/L)	209.73 ± 11.85	254.28 ± 77.23	237.49 ± 28.51	0.028	183.73 ± 25.20	135.33 ± 16.33	0.010
Serum homocysteine (µmol/L)	14.54 ± 2.35	13.69 ± 2.35	14.68 ± 3.22	0.027	14.53 ± 2.21	19.79 ± 4.57	0.040
Serum vitamin B ₁₂ (pmol/L)	460.37 ± 35.93	445.03 ± 27.37	513.51 ± 35.71	0.128	417.88 ± 23.62	439.31 ± 78.98	0.950
Serum vitamin B ₆ (nmol/L)	22.14 ± 2.14	23.45 ± 1.03	25.77 ± 6.08	0.334	18.22 ± 6.46	23.45 ± 1.00	0.420

Differences between ethnicities were assessed by using an Independent-Sample *T*-Test. *P*-value < 0.05 was considered significant.

folate and homocysteine. For these three genotypes, RBC folate was the most affected. Serum folate and homocysteine were mainly influenced by the TT genotype (Table 3).

DISCUSSION

The agarose gel is unable to retain the shorter fragment due to its low size and is suspected to have migrated out of the gel as claimed by Frosst *et al.* (1995). Hence, a single band at the 198 base pair (Bp) indicates homozygous wild type (CC), a single band at the 175 base pair indicates homozygous mutation (TT), whilst two bands (198 and 175 base pairs) are identified as heterozygous mutation (CT). The same findings are reported in most of the studies (Ling *et al.*, 2003; Frosst *et al.*, 1995). In addition, the bands obtained showed a high quality of extracted DNA as

they appear solid and smeared with no traces of RNA at the bottom of the gel. Overall, the effect of purification is good and there is less tailing while running for the gel electrophoresis. This might be due to the fewer shears formed from pipetting (Kou *et al.*, 2001). Known positive and negative controls were run to test the accuracy of the results. No DNA was added in the negative control.

The proportion of Malay subjects is lower than the one observed in a previous study done at our institution (19%) for 53 subjects as reported by Ling *et al.* (2003) due to the differences in sample size as well as changes in dietary and environmental factors over the years. However, it is comparable with a national study done by Hayati *et al.* (2008) where none of the Malay subjects were found to have the

Table 3. Coefficient of linear regression for the concentrations of serum folate, RBC folate and serum homocysteine stratified by MTHFR genotypes

	CC		CT		TT	
	Unstandardised Coefficients	p	Unstandardised Coefficients	p	Unstandardised Coefficients	p
Serum folate ¹	0.155	0.049	-0.277	0.036	-0.479	0.048
RBC folate ²	71.273	0.002	-23.578	0.036	-110.678	0.001
Serum homocysteine ¹	-0.068	0.049	0.605	0.024	0.976	0.016

The regression coefficient is the average that the dependent variable increases when the independent variable increases by one unit and other variables are held constant. Significantly different from genotypes ($p < 0.05$). CC = wild type, CT = heterozygous, TT = homozygous, UC = unstandardised coefficients

¹ Controlled for ethnicity, gender and age

² Controlled for gender and age

Table 4: The prevalence of MTHFR mutation in Asian populations

Asian Countries	No of subjects (n)	T/T	T/C	C/C	Year	References
China	121	0.14	0.44	0.42	2004	Li <i>et al.</i>
Indonesia	68	0.00	0.16	0.84	2002	Sadewa <i>et al.</i>
Japan	244	0.13	0.48	0.39	2002	Sadewa <i>et al.</i>
Malaysia, Malay	53	0.04	0.17	0.79	2003	Ling <i>et al.</i>
Singapore, Chinese	477	0.08	0.35	0.57	2001	Saw <i>et al.</i>

homozygous genotype. In the present study, the observed versus expected 677 genotype frequencies were not in Hardy Weinberg equilibrium. This may be due to the consequence of genetic selection found in the population in the absence of other causes such as gene flow (Jennings *et al.*, 2010). Besides, they further found that the undigested PCR product in the analysis of the MTHFR 677 polymorphism may be another cause of deviation from HWE. In this study, the T allele frequency for both genders is comparable with published data worldwide, 0.052-0.487 (Rady *et al.*, 1999). When compared to other studies in the Asian population (Table 4), the CT and TT genotype were almost similar.

Results in this study are in agreement with other studies (Molloy *et al.*, 1997; Jacques *et al.*, 1996) in the sense that mutant genotypes tend to have lower folate levels than normal genotype. These findings imply that, in order to compensate for the presence of T allele, heterozygous and homozygous subjects tend to require more folate compared to normal subjects. This is because with an adequate consumption of folate, the effect portrayed by mutant genotypes can be reduced. Hence, it is clear that lower serum folate levels in homozygous or heterozygous subjects are not solely due to dietary insufficiencies but may reflect an effect of the presence of MTHFR C677T polymorphism. It can be concluded that low dietary folate coupled with mutation in the MTHFR may contribute to a reduced 5-

methyltetrahydrofolate pool, subsequently leading to hyperhomocysteinemia as well as NTDs.

In this study, the highest homocysteine levels were noted in mutants' genotype and this is supported by the study of Huh *et al.* (2006) on the Korean population. Our findings also showed that MTHFR significantly affected serum homocysteine levels in both the ethnic groups. Besides gene polymorphism, serum homocysteine levels can also be influenced by environmental factors such as the intake of folate, vitamin B₁₂ and vitamin B₆. Based on the results of this study, MTHFR C677T polymorphism is shown not to be related to serum vitamin B₁₂ and B₆. This is in agreement with the present and other studies (Huh *et al.*, 2006; Ozarda *et al.*, 2009). In other words, genotype differences do not play a role in determining the status of both vitamins.

Based on the linear regression analysis, the results from this study suggest that the susceptibility to hyperhomocysteinemia and neural tube defects may be more prominent in homozygous subjects. However, the effect of the homozygous genotype could be compensated by increasing folate intake (Malinow *et al.*, 1997).

CONCLUSION

In conclusion, this study describes an important gene-environment interaction in the general healthy Malaysian population. Our findings indicate that subjects with the C677T mutation, especially those with the mutation in homozygous form, need more dietary folate as their folate status were lower than in normal genotype. Therefore, it is suggested that the thermolabile MTHFR genotype should be taken into account in the design of studies aiming to identify the optimum dose of folic acid required to lower homocysteine levels, as the effectiveness of folate supplementation is likely to vary with genotypes.

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