

METHYLENETETRAHYDROFOLATE REDUCTASE (MTHFR) POLYMORPHISM (C677T) IN RELATION TO HOMOCYSTEINE CONCENTRATION IN OVERWEIGHT AND OBESE THAIS

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Abstract. We analyzed the association between MTHFR (C677T) gene polymorphism with serum concentrations of homocysteine, folate, and vitamin B₁₂ in 37 male and 112 female overweight/obese Thai volunteers (BMI \geq 25.00 kg/m²), and compared them with 23 male and 90 female control subjects (BMI=18.5-24.99 kg/m²). Statistically significant higher levels of serum homocysteine were found in the overweight/obese subjects than the control subjects ($p < 0.05$). Serum folic acid levels in the overweight/obese subjects were significantly lower than the control subjects ($p < 0.05$). When the data were grouped according to homocysteine concentration and MTHFR gene polymorphism, there were significantly higher homocysteine concentrations in the overweight/obese subjects than the control subjects in wild type gene polymorphism (CC) in the hyperhomocysteine group (homocysteine >10.0 mmol/l) ($p < 0.05$), but in genotype polymorphism (CC, CT, TT) there were lower folic acid and vitamin B₁₂ concentrations in the overweight/obese subjects than in the control subjects. In the hyperhomocysteine groups, there was no significant difference in the frequencies of MTHFR (C677T) gene polymorphism between the overweight/obese subjects and the control subjects. Folic acid and gene polymorphism were found to be significantly related to the overweight/obese and control groups in logistic regression analysis ($p < 0.05$). The results support the supposition that folic acid is more important than vitamin B₁₂.

INTRODUCTION

In Thailand, transition from the rural to the urban environment affects health in various aspects, such as changes in eating habits, drinking alcohol and smoking (Osuntokun, 1985; Hamburg, 1987; Epstein, 1989). The rapid approach towards the status of a newly industrialized country is well reflected in some demographic and economic indicators (Pongpaew and Schelp, 1997). A total of 23.6% of Thai female construction-site workers were reported to be obese (Pongpaew *et al*, 1994). Obesity is found

in 11% of the Thai elderly (Pongpaew *et al*, 1991). Moderate to severe obesity in Thailand is increasingly found. Obesity is associated with clear health risks, including hypertension, diabetes and dyslipidemia; this leads to a higher risk of cardiovascular disease (Kushner, 1993).

Methylenetetrahydrofolate reductase (MTHFR) catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which serves as a methyl donor in the reaction converting homocysteine (Hcy) to methionine (Ueland *et al*, 2000, 2001). An increased plasma Hcy level has consistently been shown to be an independent risk factor for atherosclerotic disorders in several meta-analyses (Boushey *et al*, 1995; Ueland *et al*, 2000, 2001). Thermolability of MTHFR is caused by a missense mutation in exon 4 of the MTHFR gene, a cytosine (C) to thymine (T) substitution at nucle-

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otide (C677T) occurs, which converts an alanine to a valine (Frosst *et al*, 1995). The C677T mutation is common in different populations, with reported homozygote frequencies of 5-28% (Schneider *et al*, 1998). Moreover, the TT genotype is associated with mildly higher elevations of plasma homocysteine than the wild type (CC) and heterozygous genotypes, indicating that activity of the thermolabile MTHFR is also impaired *in vivo* (Rozen, 1997). A significant elevation of plasma homocysteine in TT genotype individuals occurs only under low folate conditions (Jacques *et al*, 1996). A high proportion of people with the C677T homozygous genotype show a satisfactory homocysteine-lowering response to modest daily folate supplements in the range of 100-200 µg/day (Guttormsen *et al*, 1996). The polymorphism is also associated with a lower plasma folate concentration (DeLoughery *et al*, 1996; Harmon *et al*, 1996; Ma *et al*, 1996; Schwartz *et al*, 1997; Nelen *et al*, 1998; Zittoun *et al*, 1998; McQuillan *et al*, 1999; Hustad *et al*, 2000). These metabolic changes are postulated to modify the risk of chronic diseases, including cardiovascular disease (Gallagher *et al*, 1996; Brattstrom and Wilcken, 2000; Ueland *et al*, 2000), cancer (Chen *et al*, 1999; Schiavon *et al*, 2004), dementia (Nishiyama *et al*, 2000; Yoo *et al*, 2000), and the risk of neural tube defects (Ma *et al*, 1996; van der Put *et al*, 1997; Christensen *et al*, 1999). Knowledge of the homocysteine and vitamin levels of overweight and obese Thais is needed to reduce their risk of chronic diseases, especially cardiovascular diseases, and increase their life-span. However, information on the association between the C677T MTHFR genotype, serum homocysteine level and the vitamin of overweight and obese individuals is limited, especially in developing countries, such as Thailand. We evaluated the association of both plasma homocysteine concentrations and the MTHFR genotype, including folic acid and vitamin B₁₂ levels in overweight and obese people compared with healthy subjects in Bangkok.

MATERIALS AND METHODS

Study population

Thirty-seven male and 112 female overweight and obese Thai volunteers, and 23 male

and 90 female normal subjects, comprised the study population. Thai volunteers who attended the Out-patient Department, General Practice Section, Rajvithi Hospital, Bangkok, for a physical check-up, were investigated for this study. All of them visited the clinic on their own. Except for minor ailments and typical diseases of obese people, such as hypertension, mild to moderate degrees of cardiovascular disease and non-insulin-dependent diabetes mellitus, these persons were still fairly healthy. They were evaluated by physical and biochemical laboratory examinations for inclusion criteria. The age, marital status, place of origin, drinking and smoking habits were assessed by standardized questionnaire. The same medical doctor conducted the physical examinations throughout the study.

Analytical methods

The body weight of each individual dressed in light clothing was measured using a carefully calibrated beam balance (Detecto®, Detecto Scale Manufacturing, USA). Height measurements were taken using a vertical measuring rod. The BMI or Quetelet's index was conventionally calculated as weight in kg/(height in meters)². The classifications of BMI employed were those used by the WHO Expert Committee, 2000 (WHO, 2000): normal BMI = 18.5-24.99 kg/m²; overweight BMI = 25.00-29.99 kg/m²; grade I (obesity) BMI = 30.00-34.99; grade II (obesity) BMI = 35.00-39.99 kg/m²; grade III (obesity) BMI ≥ 40 kg/m².

From the subjects under study, about 10 ml of venous blood was taken in the morning after an overnight fast.

Serum vitamin B₁₂ and folic acid levels were assessed using a commercially available radioimmunoassay Dual-count solid-phase no-boil assay for vitamin B₁₂/folic acid [Diagnostic Products Corporation (DPC), Los Angeles]. Values indicating deficiencies were serum vitamin B₁₂ < 200 pg/ml, serum folic acid < 3.0 ng/ml (Tungtrongchitr *et al*, 1997).

Serum homocysteine was measured by a fluorescence polarization immunoassay on an automated analyzer (IMx system; Abbott). Optimal procedures in blood sample collection and handling were followed to prevent the passage of homocysteine from the red cells to the se-

rum, to ensure reliable measurements. The cut-off separating normal from abnormally increased serum concentrations was 10.0 $\mu\text{mol/l}$, which is in agreement with the literature (Nygard *et al*, 1999).

DNA was extracted from the peripheral blood leukocyte-rich buffy coat fraction of centrifuged, EDTA-treated whole blood by using the Flexi Gene DNA kit (Qiagen, Hilden, Germany). The polymerase chain reaction was done on a Gene Amp PCR system 9700 (Applied Biosystem, USA). Primers (5'-TGA AGG AGA AGG TGT CTG CGG GA-3' and 5'-AGG ACG GTG CGG TGA GAG TG-3') were used to amplify a portion of the MTHFR sequence from 100 ng of genomic DNA in a 50 μl reaction containing PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 15 mM MgCl_2], 0.2 mM deoxynucleotide triphosphates, 0.2 μM each primer, and 1 unit Taq DNA polymerase. Upon amplification for 30 cycles, the 198-bp MTHFR fragment was digested with *HinfI* in a 20 μl reaction containing 10 μl of PCR fragment, 2 μl of 10xbuffer (Bio Basic Inc, Canada; supplied by the manufacturer), and 4 units of *HinfI* at 37°C for 1 hour. The digestion products were separated on a 3% Seakem[®]LE agarose. (BioWhittaker Molecular Application, Rockland, ME, USA), and the

ethidium bromide-stained bands were photographed on a UV transilluminator. Wild-type (CC) individuals were characterized by a 198-bp fragment, heterozygotes (CT) by fragments of 198, 175, and 23 bp (less visible), and homozygote variants (TT) by fragments of 175 and 23 bp (Frosst *et al*, 1995). Repeat genotyping was undertaken for 20 DNA samples. Reproducibility of the genotype was 100%.

Statistical analysis

The results were expressed as median, range, and 95% confidence interval (CI). The data were coded and analyzed using a standard statistical method provided by the Minitab computer program (Ryan *et al*, 1985).

RESULTS

The median, range, and 95% confidence intervals (CI) for age, folic acid, vitamin B₁₂, and homocysteine levels in the overweight/obese and control subjects are shown in Table 1. There were significantly higher levels of serum homocysteine in the overweight/obese subjects than in the control subjects. Serum folic acid levels in the overweight and obese subjects were found to be significantly lower than in the control subjects. Homocysteine, folic acid, and vitamin B₁₂ concen-

Table 1
Medians, ranges, and 95% confidence interval (CI) of age, body mass index, folic acid, vitamin B₁₂, and homocysteine in blood of overweight/obese and control subjects.

	Overweight/obese (N=149) Median (range)	95%CI	Control (N=113) Median (range)	95%CI	p-value ^a
Age (yrs)	40 (18.0-61.0)	38.0-41.0	38 (18.0-58.0)	36.0-40.0	0.195
BMI (kg/m ²)	31.5 (25.1-56.2)	30.6-32.3	21.8 (18.52-24.97)	21.19-22.2	0.000
Folic acid(ng/ml)	5 (1.7-19.0)	4.4-5.3	10.5 (1.9-24.5)	9.93-11.5	0.000
Vitamin B ₁₂ (pg/ml)	500 (120.0-1,600.0)	430.0-560.0	580 (100.0-1,850.0)	500.0-643.7	0.104
Homocysteine ($\mu\text{mol/l}$)	9.98 (5.71-22.86)	9.53-10.53	9.91 (2.65-16.61)	8.73-9.66	0.005

^aMann-Whitney *U*-Wilcoxon rank sum *W* test (two-tailed). Significant difference between overweight/obese and control subjects $p < 0.05$.

BMI = Body mass Index

Table 2
Homocysteine, folic acid, and vitamin B₁₂ concentrations according to MTHFR gene polymorphism and homocysteine levels in overweight/obese and control subjects.

	Genotype					
	CC			CT+TT		
	Overweight/ obese Median (range)	Control Median (range)	p-value ^a	Overweight/ Obese Median (range)	Control Median (range)	p-value*
Group I: Homocysteine levels <10.0 µmol/l						
	N=39	N=25		N=10	N=8	
Homocysteine µmol/l	8.64 (5.71-9.98)	8.26 (6.17-9.9)	0.436	7.54 (5.76-9.63)	7.54 (6.14-9.66)	1.000
Folic acid ng/ml	5.6 (2.0-15.0)	12.5 (9.0-24.0)	0.000	5.75 (4.4-8.0)	11.5 (3.1-17.5)	0.006
Vitamin B ₁₂ pg/ml	535 (120.0-1,450.0)	750 (210.0-1,850.0)	0.001	505 (285.0-900.0)	770 (420.0-1,750.0)	0.043
Group II: Homocysteine levels >10.0 µmol/l						
	N=28	N=9		N=13	N=8	
Homocysteine µmol/l	12.67 (8.58-22.86)	11.06 (10.05-12.74)	0.04	11.53 (10.05-17.42)	12.12 (10.32-13.78)	0.916
Folic acid ng/ml	4.8 (2.0-8.6)	14.5 (8.2-21.0)	0.000	3.9 (1.7-5.6)	11.5 (6.2-15.5)	0.000
Vitamin B ₁₂ pg/ml	560 (220.0-1,550.0)	630 (480.0-1,100.0)	0.188	400 (140.0-590.0)	640 (330.0-875.0)	0.025

^aMann-Whitney *U*-Wilcoxon rank sum *W* test (two-tailed). Significant difference between overweight/obese and control subjects *p*<0.05

CC=wide type, C=heterozygous, TT=homozygous

trations were compared according to the MTHFR gene polymorphisms (C677T) of the overweight/obese subjects and controls using a cut-off point in the 2 groups (normal=a homocysteine level <10.0 µmol/l and hyperhomocysteinemia=a homocysteine level >10.0 µmol/l) (Table 2). There were significantly higher homocysteine concentrations in the overweight/obese subjects than in the controls in the wild type MTHFR gene polymorphism (CC) in the hyperhomocysteine group. In all the genotype polymorphisms (CC, CT, TT) there were lower folic acid and vitamin B₁₂ concentrations in the overweight/obese subjects than in the controls.

Using the homocysteine concentration cut-off point higher than 10 µmol/l to differentiate hyperhomocysteinemia in overweight/obese subjects and controls, there were no statistically

significant differences in the MTHFR (C677T) gene polymorphisms between the overweight/obese subjects and the controls. However, hyperhomocysteine may have a tendency to have MTHFR gene polymorphism (C677T), both heterozygous and homozygous, in both the overweight/obese subjects and the controls (Odd's ratio=1.915, Table 3).

To determine whether the gene polymorphism is related to the increasingly obese status of the population (logistic variable, overweight/obese and not obese) or whether it was a confounder, logistic regression analysis was carried out, where folic acid, vitamin B₁₂, homocysteine and gene polymorphisms were taken as independent variables, and overweight/obese and normal subjects status was a dependent logistic variable (Table 4). Folic acid and gene

Table 3

Frequencies of the methylenetetrahydrofolate reductase gene normal and mutant (C677T) in overweight/obese and control groups.

MTHFR genotype	Overweight/obese n (%)	Control n (%)	Odd's ratio (95% CI)	p-value ^a
Group I: Homocysteine level <10.0 µmol/l				
CC	39 (79.59)	25 (75.76)	1.248	0.441
CT+TT	10 (20.41)	8 (24.24)	(0.38-4.05)	
Group II : Homocysteine level > 10.0 µmol/l				
CC	28 (68.29)	9 (52.94)	1.915	0.209
CT+TT	13 (31.71)	8 (47.06)	(0.52-7.12)	

^aPearson chi-square

MTHFR = Methylenetetrahydrofolate reductase

CC=wide type, CT=heterozygous, TT=homozygous

Table 4

Covariance analysis of folic acid and gene polymorphism as independent variables, and overweight/obese and control subjects as dependent variables in the model.

Model	Unstandardized coefficients		Standardized coefficients	t	Significance
	B	Std Error			
1 (Constant)	1.803	0.068		26.584	0.000
Folic acid	-0.051	0.007	-0.517	-7.100	0.000
2 (Constant)	2.029	0.125		16.230	0.000
Folic acid	-0.052	0.007	-0.531	-0.735	0.000
Gene polymorphism	-1.680	0.079	-1.550	-2.141	0.034

polymorphisms were found to be significantly related to overweight/obese and normal status.

DISCUSSION

A C677T single nucleotide polymorphism was localized in the MTHFR gene which codes for the substitution of the amino acid valine for alanine at position 226 of the protein (Frosst *et al*, 1995). The C677T variant is less resistant to heat inactivation and is associated with reduced enzyme activity (Engbersen *et al*, 1995). It is also associated with increased homocysteine levels (Jacques *et al*, 1996; Rozen, 1997; Schneider *et al*, 1998). A preponderance of the recent literature supports the concept that serum homocysteine concentration is more strongly and consistently associated with clinical end-points

of atherosclerosis, such as myocardial infarction and stroke (Engbersen *et al*, 1995; Guttormsen *et al*, 1996; Kluijtmans *et al*, 1996; Verhoef *et al*, 1997; Brattstrom *et al*, 1998; Gemmati *et al*, 2004; Gutierrez Revilla *et al*, 2004; Schwahn *et al*, 2004).

Although the level of serum homocysteine in the overweight/obese subjects was found to be higher than in the control subjects, there were no significant differences in homocysteine concentrations in the heterozygous and homozygous mutations between the overweight/obese subjects and the control groups (Table 2). This may have been caused by the small numbers of heterozygous (CT) and homozygous (TT) mutations in each group, which are difficult to analyze.

When the data were arranged according to homocysteine concentration groups, the frequencies of methylenetetrahydrofolate reductase (C677T) were proposed (Table 3). Although a significant difference was not shown, hyperhomocysteine in the control group tended to be more prevalent among the heterozygous and homozygous mutations, than in the overweight/obese group (Odds ratio=1.915, $p=0.209$). The hyperhomocysteine levels in the overweight/obese group may have been caused by a lower folate status than in the control group, while there were more abnormal genetic defects of the MTHFR (C677T) in the control group than in the overweight/obese group. These results suggest that serum hyperhomocysteine may be derived from 2 causes: genetic defects and a low folate status. In overweight/obese people, hyperhomocysteine, caused by a low folate level, may be related more to an insufficiency in dietary intake than due to a genetic defect. It has been reported that hyperhomocysteine, but not the MTHFR genotype, is significantly associated with carotid atherosclerosis (Spence *et al*, 1999). Elevated homocysteine may be caused by mutation in the cystathionine β -synthase (CBS) gene 844ins68bp, the key enzyme in the trans-sulfuration of homocysteine to cystathionine (Janosikova *et al*, 2003). However, this mutation was not determined in our study, which may have limited our interpretations. Further studies of genetic defects may be required.

Logistic regression analysis was carried out, where folic acid, vitamin B₁₂, homocysteine and gene polymorphisms were taken as independent variables, and overweight/obese and normal status as dependent logistic variables. Folic acid and gene polymorphisms were found to have a significant relation to overweight/obese and normal status. The results support the supposition that folic acid is more important than vitamin B₁₂. It can be concluded that gene polymorphism C677T, which is also an important factor in elevated homocysteine, may be associated with overweight/obese subjects and controls.

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