

Influence of Methylenetetrahydrofolate Reductase (*MTHFR*) C677T Polymorphism, B Vitamins and Other Factors on Plasma Homocysteine and Risk of Thromboembolic Disease in Chinese

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Background: Thromboembolic disease is a major cause of morbidity and mortality in many countries. Our previous study found that Chinese subjects carried the same polymorphism of the methylenetetrahydrofolate reductase (*MTHFR*) gene as described in Western studies. The aim of the present study was to determine the influence of *MTHFR* polymorphism, B vitamins and other factors on plasma homocysteine (Hcy) levels and risk of thromboembolic disease in Chinese.

Methods: One hundred and six subjects were enrolled into the study. They were categorized into 4 groups: healthy individuals ($n = 42$); those with diabetes mellitus ($n = 20$); those with deep vein thrombosis (DVT) ($n = 11$); and those with coronary artery disease (CAD) ($n = 33$). Plasma levels of folic acid, vitamins B6 and B12, Hcy, and fasting blood sugar were measured; total cholesterol, triglycerides, complete blood count, and 677 C→T mutation in *MTHFR* were determined.

Results: Plasma Hcy was lowest in the healthy subjects, higher in diabetics, followed by patients with DVT, and highest in patients with CAD ($p < 0.001$, ANOVA). *MTHFR* C677T polymorphism was the common factor affecting plasma logHcy levels in all 4 groups of subjects. Triglycerides affected plasma logHcy in the CAD patients. For the 4 groups as a whole, *MTHFR* polymorphism, triglycerides, and vitamin B12 were the most significant factors influencing plasma Hcy.

Conclusion: We suggest that high plasma Hcy is an important risk factor for CAD. Other factors including *MTHFR* polymorphism, vitamin B12, triglycerides, total cholesterol, and gender might affect Hcy levels in different diseases and conditions. [*J Chin Med Assoc* 2005;68(12):560–565]

Key Words: folic acid level, methylenetetrahydrofolate reductase polymorphism, plasma homocysteine level, thromboembolic disease, vitamin B6 and B12 levels

Introduction

Thromboembolic disease remains a major cause of morbidity and mortality in many countries. Since 1993, factor V Leiden and PT20210 A allele have been found to be the major causes of thromboembolic disease in the West. However, Chinese people are less

prone to thromboembolic disease.^{1–3} While our recent studies in Chinese populations found neither factor V Leiden nor PT20210 A allele,^{4–6} we did find that the Chinese carried the same methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphism as described in Western studies,⁶ with the C→T substitution at nucleotide 677 being another possible cause of

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thrombosis. Whether or not this polymorphism can affect homocysteine (Hcy) levels in Chinese patients deserves our investigation. The present study was, thus, conducted to determine the influence of *MTHFR* gene polymorphism, B vitamins and other possible factors on plasma Hcy levels and risk of thromboembolic disease in Chinese.

Methods

A total of 106 subjects were enrolled into the study and divided into 4 groups. Group A comprised healthy subjects without a history of diabetes mellitus (DM), thromboembolic disease, or of taking any medication in the previous 2 weeks. Group B included patients with type 2 DM with normal renal function. Group C had patients with deep vein thrombosis (DVT) as shown on sonogram. Group D included patients with coronary artery disease (CAD) as shown on angiogram, with at least 1 major vessel having more than 75% stenosis.

After signed informed consent was obtained from each subject, blood samples were taken (between 9 and 10 a.m.) after a 12-hour fast, and not at the acute stage for patients with DVT, in order to test for *MTHFR* gene mutations, and to measure plasma Hcy levels, complete blood count, total cholesterol, triglycerides, fasting blood sugar (FBS), and vitamin B6, vitamin B12 and folate levels.

DNA analysis for the *MTHFR* 677C→T polymorphism was performed using the methods described by Frosst et al.⁷ DNA was extracted from the buffy coat of the collected peripheral blood as previously described⁷ and approximately 0.5 µg was used for amplification by the polymerase chain reaction (PCR). The DNA samples were stored at 4°C for not more than 12 months before they were analyzed.

To detect the 677C→T transition in the *MTHFR* gene, PCR was performed with 5 µM of forward and reverse primers⁷ in 80 µM dNTPs, 10 mM Tris-HCl, pH 8.8 at 25°C, 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100 and 0.4 U DynaZyme™ II DNA Polymerase, Recombinant (Finnzymes Oy, Espoo, Finland) in a total volume of 50 µL. Denaturation was first carried out for 5 minutes at 94°C, followed by another 35 cycles of denaturation for 30 seconds at 94°C, primer annealing for 50 seconds at 57°C, and primer extension for 50 seconds at 72°C. Finally, extension was performed for 5 minutes at 72°C, and then at 4°C for 30 minutes. *Hinf*I restriction enzyme (New England Biolabs Inc, Ipswich, MA, USA) analysis and subsequent electrophoresis in a 2.5% MetaPhor®

agarose gel (Cambrex Corp, East Rutherford, NJ, USA) revealed the mutational status of the subject.

Each time PCR was performed to detect *MTHFR* C677T polymorphism, known positive and negative controls were used in order to obtain accurate results. We used *MTHFR*-TT to represent the homologous polymorphism, *MTHFR*-CT to represent the heterologous polymorphism, and *MTHFR*-all to represent any 1 of the polymorphisms.

Plasma Hcy level was measured using an enzyme immunoassay (Axis® Homocysteine EIA, Axis Biochemicals ASA, Oslo, Norway), according to the manufacturer's instructions. The range for measurement was 2.0–50.0 µmol/L.

Serum vitamin B12 and folate levels were measured with a radioimmunoassay (Radioassay Kit Vitamin B12 [⁵⁷Co]/Folate [¹²⁶I], ICN Pharmaceuticals Inc, Costa Mesa, CA, USA). Vitamin B6 was measured with a radioenzymatic assay (RK-VB6, Buhlmann Laboratories AG, Allschwil, Switzerland). Procedures were performed according to the manufacturers' instructions.

Complete blood count was measured with an automated hematology analyzer (Sysmex SE9000, Toa Medical Electronics Co Ltd, Kobe, Japan). Total cholesterol, triglycerides and FBS were determined with the SMAC-II Analyzer (Technicon Corp, Tarrytown, NY, USA).

ANOVA and the Chi-squared test were used to check the statistical significance of trends among parameters. The Scheffé test was used for multiple comparison if ANOVA results were significant. Multivariate linear regression was used to detect the effect of logHcy levels after adjusting for other variables. As plasma Hcy levels were known to be not normally distributed, we used the Kolmogorov-Smirnov method to test whether logHcy or the square root of Hcy was better at representing Hcy. The results showed that logHcy represented Hcy levels best, although Hcy, logHcy and the square root of Hcy were not normally distributed. Thus, we used logHcy instead of Hcy.

Results

Of the 106 subjects, 42 were in Group A (healthy), 20 in Group B (type 2 DM), 11 in Group C (DVT), and 33 in Group D (CAD). The mean ages in groups A, B, C, and D were 64.9 ± 5.4 years (range, 60–78 years), 64.3 ± 15.0 years (range, 35–86 years), 63.5 ± 13.9 years (range, 40–83 years), and 69.3 ± 11.4 years (range, 33–85 years), respectively.

Table 1. Mean of various parameters and their differences

	Group A (Healthy) n = 42	Group B (DM) n = 20	Group C (DVT) n = 11	Group D (CAD) n = 33	Total (N = 106)	p
Age (yr)	64.9 ± 5.4	64.3 ± 15.0	63.5 ± 13.9	69.3 ± 11.4	66.0 ± 10.7	0.201*
Platelet (× 10 ⁹ /μL)	271 ± 354	188 ± 121	214 ± 53	238 ± 240	239 ± 265	0.699*
Cholesterol (mg/dL)	178 ± 50	173 ± 64	183 ± 39	187 ± 40	180 ± 49	0.778*
Triglycerides (mg/dL)	121 ± 76	132 ± 55	127 ± 44	168 ± 113	139 ± 86	0.107*
FBS (mg/dL)	101.7 ± 26.0	173.1 ± 90.3	117.8 ± 34.8	109.0 ± 46.7	119.1 ± 56.8	< 0.001*
Hcy (μmol/L)	9.0 ± 7.7	9.4 ± 7.0	14.8 ± 9.6	19.8 ± 11.9	13.0 ± 10.3	< 0.001*
Folate (μg/L)	9.1 ± 3.9	9.4 ± 5.9	8.1 ± 3.1	9.4 ± 5.5	9.2 ± 4.7	0.874*
B6 (nmol/L)	59.3 ± 42.2	53.0 ± 55.4	34.2 ± 26.9	70.3 ± 54.1	58.9 ± 48.6	0.175*
B12 (ng/L)	581 ± 354	609 ± 346	581 ± 425	463 ± 482	546 ± 406	0.548*
Gender (M/F)	21/21	12/8	6/5	26/7	65/41	0.016†
<i>MTHFR</i> -TT [†] (%)	7.3	10.0	27.3	9.1	10.5	0.580†
<i>MTHFR</i> -CT [†] (%)	26.8	25.0	18.2	27.3	25.7	0.964†
History of smoking (%)	26.2	35.0	36.4	39.4	33.0	0.232†
History of alcohol consumption (%)	21.4	30.0	18.2	21.2	22.6	0.867†

*ANOVA; †χ² (linear-by-linear association); **MTHFR*-TT = homologous polymorphism and *MTHFR*-CT = heterologous polymorphism. CAD = coronary artery disease; DM = diabetes mellitus; DVT = deep vein thrombosis; FBS = fasting blood sugar; Hcy = homocysteine; *MTHFR* = methylenetetrahydrofolate reductase gene.

Differences in the various parameters

Table 1 shows the various parameters in the 4 groups. FBS, Hcy and gender were significantly different among the 4 groups, based on ANOVA ($p < 0.001$, $p < 0.001$, $p = 0.016$, respectively). Plasma Hcy was lowest in the healthy subjects, higher in diabetics, followed by patients with DVT, and highest in patients with CAD. Differences were significant between healthy subjects and CAD patients, and between DM and CAD patients, based on the Scheffe test ($p < 0.001$, $p = 0.002$, respectively) (Table 2). There was a greater proportion of males in Group D (CAD) than in Group A (healthy) ($p = 0.016$). On the other hand, there was no significant difference among the groups regarding *MTHFR* 677TT and *MTHFR* 677CT polymorphism ($p = 0.580$, $p = 0.964$, respectively).

Influence of the various parameters and *MTHFR* mutations on plasma Hcy

Multivariate analysis showed that *MTHFR*-all could affect plasma logHcy levels in all 4 groups ($p < 0.005$, Table 3), *MTHFR*-TT polymorphism and triglycerides could affect plasma logHcy level in the CAD patients ($p = 0.005$, $p = 0.006$, respectively). Putting all groups together, we found that triglycerides and vitamin B12 could affect logHcy level ($p < 0.005$, $p = 0.026$, respectively).

Table 2. Scheffe test for the 3 significant parameters from Table 1

	p
Fasting blood sugar	
Healthy vs DM	< 0.001
Healthy vs DVT	0.833
Healthy vs CAD	0.945
DM vs DVT	0.044
DM vs CAD	< 0.001
DVT vs CAD	0.969
Homocysteine	
Healthy vs DM	0.999
Healthy vs DVT	0.341
Healthy vs CAD	< 0.001
DM vs DVT	0.492
DM vs CAD	0.002
DVT vs CAD	0.507
Gender	
Healthy vs DM	0.588
Healthy vs DVT	1.0
Healthy vs CAD	0.016
DM vs DVT	1.0
DM vs CAD	0.209
DVT vs CAD	0.139

CAD = coronary artery disease; DM = diabetes mellitus; DVT = deep vein thrombosis.

Multivariate analysis, using the stepwise method, showed that triglycerides had the highest degree of influence on logHcy in the healthy. The most influential factors were cholesterol in the diabetics and triglycerides and *MTHFR*-TT polymorphism in the CAD group. Putting all groups together, the weight of effect was in order of triglycerides, *MTHFR*-TT and vitamin B12 (Table 4).

Discussion

Thromboembolic disease occurs less frequently in Chinese than in Caucasians.¹⁻³ During the past few years, much effort has been made to better understand the causes of idiopathic venous thrombosis, i.e. the discovery of factor V Leiden, PT20210A allele and *MTHFR* gene polymorphism.⁷⁻¹⁴ Although factor V Leiden and PT20210A allele have been found to be the most frequent causes of thromboembolic disease in the West, they are absent in the Chinese.^{4-6,15} However, *MTHFR* C677T polymorphism can be found in Chinese subjects.⁶ *MTHFR* polymorphism itself is not a risk factor of thromboembolic disease,

but high plasma levels of the amino acid Hcy are.¹⁶ One of the main functions of *MTHFR* is remethylation of Hcy into methionine.¹⁷ A common *MTHFR* polymorphism, an alanine-to-valine substitution, renders the enzyme thermolabile, elevating plasma levels of Hcy,¹⁸ which increases the risk of occlusive vascular disease.¹⁹ However, in addition to genetic factors, plasma Hcy levels can also be influenced by environmental factors such as folate, and vitamins B6 and B12.²⁰⁻²² Although the sample size in the present study was not large, this is 1 of the few studies on Chinese subjects. The influence of genetic and environmental factors on Hcy levels was compared in 4 groups representing different degrees of risk for thromboembolic disease in Chinese.

We found that plasma Hcy was lowest in the healthy subjects, higher in diabetics, followed by patients with DVT, and highest in patients with CAD (Table 1). The Scheffe test showed significantly higher plasma Hcy in CAD patients than in DM patients and healthy subjects (Table 2). However, there was no significant difference in the percentage of *MTHFR* C677T polymorphism, either -CT or -TT, among the 4 groups. This demonstrated that *MTHFR* poly-

Table 3. Influence of different variables on logHcy* levels (*p*, multivariate analysis)

	<i>MTHFR</i> -all [†]	<i>MTHFR</i> -TT [†]	<i>MTHFR</i> -CT [†]	TC	TG	PLT	B12	B6	Folate	Age	Gender
Group											
A (Healthy)	< 0.005	0.33	0.046	0.56	0.84	0.36	0.26	0.45	1.00	0.38	0.33
B (DM)	< 0.005	0.89	0.62	0.35	0.36	0.86	0.38	0.86	0.92	0.22	0.72
C (DVT)	< 0.005	-	-	-	-	-	-	-	-	-	-
D (CAD)	< 0.005	0.005	0.48	0.06	0.006	0.05	0.84	0.12	0.11	0.14	0.12
A+B+C+D	< 0.005	0.34	0.03	0.06	< 0.005	0.83	0.026	0.21	0.64	0.06	0.21

*LogHcy = dependent variable; [†]*MTHFR*-TT = homologous polymorphism, *MTHFR*-CT = heterologous polymorphism, and *MTHFR*-all = *MTHFR*-TT + *MTHFR*-CT. CAD = coronary artery disease; DM = diabetes mellitus; DVT = deep vein thrombosis; Hcy = homocysteine; *MTHFR* = methylenetetrahydrofolate reductase gene; PLT = platelets; TC = total cholesterol; TG = triglycerides.

Table 4. Degree of influence of different variables on logHcy levels (stepwise method)

	Model 1	Model 2	Model 3
Group			
A (Healthy)	Triglycerides	Vitamin B6	-
B (DM)	Cholesterol	-	-
C (DVT)	Gender	<i>MTHFR</i> -all*	-
D (CAD)	Triglycerides	<i>MTHFR</i> -TT*	-
A+B+C+D	Triglycerides	<i>MTHFR</i> -TT*	Vitamin B12

The order of influence = Model 1 > Model 2 > Model 3.

**MTHFR*-all = homologous + heterologous polymorphism and *MTHFR*-TT = homologous polymorphism. CAD = coronary artery disease; DM = diabetes mellitus; DVT = deep vein thrombosis; Hcy = homocysteine; *MTHFR* = methylenetetrahydrofolate reductase gene.

morphism itself was not a risk factor for thromboembolic disease, but plasma Hcy level was. The cause of this discrepancy in the influence of *MTHFR* polymorphism and plasma Hcy in our study is not known; perhaps the small sample size or other unknown factors played a role. Further studies with a larger sample size should be performed to obtain clearer conclusions.

Although there was no significant difference in the percentages of *MTHFR* polymorphism among the 4 groups, on multivariate analysis, we found that *MTHFR* polymorphism affected the logHcy level in all 4 groups (Table 3). *MTHFR* 677TT polymorphism affected the logHcy level in CAD patients, and *MTHFR* 677CT polymorphism affected Group A (healthy) and all groups combined (A+B+C+D). Thus, *MTHFR* seemed to be able to affect plasma Hcy levels in the different groups of subjects in our study. Vitamin B12 affected plasma Hcy in all groups of subjects, and triglycerides affected logHcy in CAD patients and all groups combined ($p = 0.006$ and $p < 0.0005$, respectively). This demonstrated that plasma Hcy levels were affected by multiple factors.

Males usually have higher plasma Hcy;²³ male gender is a well-known risk factor for CAD, and this was also shown in our study (Table 2). On the other hand, since the status of male gender did not affect DVT, there must be multiple influences leading to DVT, and plasma Hcy probably plays a less important role in the cause of DVT.

From the present preliminary study, we conclude that plasma Hcy level is a risk factor for CAD. Many factors including *MTHFR* polymorphism, vitamin B12, triglycerides, gender and total cholesterol might affect Hcy levels in different diseases and conditions.

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References

1. Chan CW, Hoaglund FT. Pulmonary thromboembolism and venous thrombosis in the Chinese. *Clin Orthop Relat Res* 1980;150:253–60.
2. Cheng KK, Lai ST, Yu TJ, Duo SM. Postoperative deep vein thrombosis in the Taiwanese Chinese population. *Am J Surg* 1987;153:302–5.
3. Woo KS, Tse LK, Tse CY, Metreweli C, Vallance-Owen J. The prevalence and pattern of pulmonary thromboembolism in the Chinese in Hong Kong. *Int J Cardiol* 1988;20:373–80.
4. Ho CH. Prevalence of activated protein C resistance in the Chinese population. *Thromb Res* 1997;88:409–12.
5. Ho CH, Chau WK, Hsu HC, Gau JP, Chih CM. Prevalence of factor V Leiden in the Chinese population. *J Chin Med Assoc* 1999;62:875–8.
6. Ho CH. Prevalence of prothrombin 20210 A allele and methylenetetrahydrofolate reductase C677T genetic mutations in the Chinese population. *Ann Hematol* 2000;79:239–42.
7. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 1995;10:111–3.
8. Dahlback B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc Natl Acad Sci USA* 1993;90:1004–8.
9. Bertina RM, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, van der Velden PA, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 1994;369:64–7.
10. Hajjar KA. Factor V Leiden—an unselfish gene? *N Engl J Med* 1994;331:1585–7.
11. Dahlback B. Resistance to activate protein C, the Arg506 to Gln mutation in the factor V gene, and venous thrombosis. Functional tests and DNA-based assays, pros and cons. *Thromb Haemost* 1995;73:739–42.
12. Dahlback B. Molecular genetics of venous thromboembolism. *Ann Med* 1995;27:187–92.
13. Rees DC, Cox M, Clegg JB. World distribution of factor V Leiden. *Lancet* 1995;346:1133–4.
14. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood* 1996;88:3698–703.
15. Chan LC, Bourke C, Lam CK, Liu HW, Brookes S, Jenkins V, Pasi J. Lack of activated protein C resistance in healthy Hong Kong Chinese blood donors—correlation with absence of Arg506-Gln mutation of factor V gene. *Thromb Haemost* 1996;75:522–3.
16. Selhub J, D'Angelo A. Hyperhomocysteinemia and thrombosis: acquired conditions. *Thromb Haemost* 1997;78:527–31.
17. Selhub J, Miller JW. The pathogenesis of homocysteinemia: interruption of the coordinate regulation by S-adenosylmethionine of the remethylation and transsulfuration of homocysteine. *Am J Clin Nutr* 1992;55:131–8.
18. Jacques PF, Bostom AG, Williams RR, Ellison RC, Eckfeldt JH, Rosenberg IH, Selhub J, et al. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. *Circulation* 1996;93:7–9.
19. Boushey CJ, Beresford SA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease: probable benefits of increasing folic acid intakes. *JAMA* 1995;274:1049–57.
20. Guttormsen AB, Schneede J, Fiskerstrand T, Ueland PM, Refsum HM. Plasma concentrations of homocysteine and other aminothiols are related to food intake in healthy human subjects. *J Nutr* 1994;124:1934–41.
21. Selhub J, Jacques PF, Wilson PW, Rush D, Rosenberg IH. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. *JAMA* 1993;270:2693–8.

22. Daly L, Robinson K, Tan KS, Graham IM. Hyperhomocysteinaemia: a metabolic risk factor for coronary heart disease determined by both genetic and environmental influences? *QJ Med* 1993;86:685-9.
23. Ho CH. The influence of age, sex, vitamin B12, folate levels and methylenetetrahydrofolate reductase C677T genetic mutations on plasma homocysteine in the Chinese population. *Haematologica* 2000;85:1051-4.