Clin Chem Lab Med 2008;46(1):73–79 © 2008 by Walter de Gruyter • Berlin • New York. DOI 10.1515/CCLM.2008.021

Relationship between methionine synthase, methionine synthase reductase genetic polymorphisms and deep vein thrombosis among South Indians

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Abstract

Background: The rationale behind this study was to examine the relationship between polymorphisms in genes that regulate remethylation of homocysteine to methionine, i.e., methionine synthase (*MTR A2756G*) and methionine synthase reductase (*MTRR A66G*), and risk of deep vein thrombosis (DVT) in a South Indian cohort (163 DVT cases and 163 controls), as elevated homocysteine has been documented as an independent risk factor for DVT in the same cohort.
Methods: Plasma homocysteine analysis was carried out by reverse phase HPLC. The *MTR A2756G* and *MTRR A66G* genetic polymorphisms were detected using PCR-restriction fragment length polymorphism method. For statistical analyses, Fisher's exact test was used for categorical variables and Student's t-test and analysis of variance were used for continuous variables.
Results: The *MTRR 66GG* genotype was associated with a 2.74-fold [95% confidence interval (CI): 1.73, 4.34] risk of DVT. The *MTR A2756G* polymorphism was not a risk factor. *MTRR GG/MTR AG* and *MTRR GG/MTR GG* genotypes cumulatively were found to increase the risk of DVT by 2.38-fold (95% CI: 1.43, 3.96). A positive association was observed between plasma homocysteine and the *MTRR G* allele, and the *MTR G* allele was shown to have an additive effect. The risk associated with the *MTRR 66GG* genotype was further increased in subjects compound heterozygous for methylene tetrahydrofolate reductase (*MTHFR*) [odds ratio (OR): 3.46, 95% CI: 1.38, 8.63].
Conclusions: The *MTRR 66GG* genotype is a risk factor for DVT among South Indians. This risk is increased further in the presence of the *MTHFR 677CT/1298AC* genotype.
Clin Chem Lab Med 2008;46:73–9.

Keywords: deep vein thrombosis; methionine synthase; methionine synthase reductase; single nucleotide polymorphisms.

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Received June 29, 2007; accepted August 6, 2007; previously published online November 22, 2007

Introduction

Earlier, we reported that hyperhomocysteinemia and compound heterozygous status for methylene tetrahydrofolate reductase (MTHFR) are independent risk factors for deep vein thrombosis (DVT) among South Indians (1). Homocysteine for its remethylation requires 5-methyl tetrahydrofolate as the co-substrate, methyl cobalamin as the cofactor and methionine synthase (MTR) as the biocatalyst. 5-Methyl tetrahydrofolate is formed by flavin adenine dinucleotide (FAD)-dependent reduction of 5,10-methylene tetrahydrofolate in the presence of MTHFR. Methionine synthase reductase (MTRR) reduces inactive cobalamin II to active cobalamin I and methylates it to methylcobalamin. Methionine combines with adenosine to form S-adenosyl methionine (SAM), the universal methyl donor, which on donating methyl moieties to DNA, proteins and catecholamines forms S-adenosyl homocysteine (SAH). SAH gives back homocysteine by releasing adenosine. Homocysteine can be converted to cystathionine in the presence of vitamin B_6 and cystathionine β-synthase (CBS) and subsequently forms cysteine and glutathione. Any aberration in these metabolic reactions can cause elevated homocysteine. Recently, there have been several attempts to evaluate whether elevated homocysteine itself is the causative agent or factors influencing homocysteine are involved in the pathophysiology of DVT. Cattaneo et al. have shown a positive association between low vitamin B_6 and DVT risk, independent of fasting and post-methionine load homocysteine levels (2). Quere et al. have shown that plasma methionine and red blood cell methyl folate are inversely related to DVT risk (3). Oger et al. have shown that hyperhomocysteinemia, low serum folate and low vitamin B_{12} were associated with a 1.48-fold, 3.14-fold, 1.42-fold risk for DVT, respectively (4).

Leclerc et al. have identified *MTRR A66G* genetic polymorphism resulting in I22M substitution (5). Serum B_{12} deficiency was found to act in conjunction with this polymorphism in decreasing the enzyme activity. This polymorphism was found to be a risk factor for neural tube defects (6) and Down syndrome (7), with hyperhomocysteinemia being the underlying cause for both disorders.

MTR A2756G genetic polymorphism resulting in D-to-G substitution was identified by Leclerc et al. (8) and Chen et al. (9). This polymorphism was found to be a risk factor for maternally inherited neural tube defects (10), for Down syndrome both in case and case mother (11) and maternally inherited orofacial clefts (12).

Song et al. have reported early onset stroke in a 21-year-old male with homozygous mutant status for *MTHFR* (*677TT* genotype) and *MTR* (*2756GG* genotype) (13). The 21-year-old male had elevated homocysteine, normal vitamin B_{12} and slightly low folate levels. The University of Toronto Thrombophilia Study in Women revealed a higher frequency of the *MTRR 66GG* genotype among venous thromboembolism cases than controls (32.55% vs. 27.13%) (14). Although individually *MTR A2756G* polymorphism is not found to be a risk factor for thrombosis in different studies (15, 16), compound *MTR/MTRR* genotype is reported to increase the risk for DVT (17).

Wide variations in allele frequency distribution of these single nucleotide polymorphisms (SNPs) among different ethnic groups and populations, significantly higher plasma homocysteine levels among South Indians and the lack of studies on these SNPs among Indians prompted us to evaluate the role of these two genetic polymorphisms in the pathophysiology of DVT among South Indians.

Materials and methods

Participants

A total of 163 DVT patients (105 men and 58 women) referred to our center between October 1999 and February 2004 participated in this study. The mean age of onset for DVT in men was 37.4 ± 13.7 years and in women 32.4 ± 13.8 years ($p < 0.05$). The diagnosis of DVT was based on impendence plethysmography, Doppler ultrasonography, compression ultrasonography or contrast venography. A total of 163 age-, sex- and race-/ethnicity-matched controls were selected. The controls did not have any history of thrombosis. Informed consent was obtained from all the subjects. All of the study population was from South India. The Bioethical Committee of the Center for DNA Fingerprinting and Diagnostics approved the study protocol. Patients younger than 20 years and over 70 years of age and those with known malignant diseases were excluded from the study.

Methods

Fasting EDTA anticoagulated blood samples were collected on ice, and plasma was separated immediately, frozen until the time of analysis and used for plasma homocysteine estimation. Genomic DNA was isolated from the buffycoat using phenol-chloroform extraction method.

Plasma homocysteine analysis

To 300 μL of plasma, 30 μL of 10% (v/v) of Tri n-butyl phosphine in dimethyl formamide was added and mixed well. The resultant mixture was incubated at 4°C for 30 min. After incubation, deproteinization was carried out by adding 300 μL of 10% trichloro acetic acid in 1 mM EDTA and mixed by vortexing followed by centrifugation at 3000 rpm ($704 \times g$) for 15 min. To 100 μL of clear supernatant, 20 μL of 1.55 N NaOH solution, 250 μL of 125 mM borate buffer (125 mM boric acid, 2 mM EDTA, pH 9.5), and 100 μL of ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (1 mg/mL in 125 mM borate buffer) were added sequentially. The resultant mixture was mixed well and incubated at 60°C for 1 h. The above solution was transferred to auto injector vials. Reverse phase HPLC (Waters Corporation, USA) was performed using a Hichrom C18, 250×4.6 mm column (Hichrom Ltd., Berkshire, UK) as stationary phase and 0.1 M KH_2PO_4 buffer (pH 2.1 adjusted with orthophosphoric acid):acetonitrile mixture in the ratio of 96:4 as mobile phase. Isocratic separation was carried out with a flow rate of 2 mL/min and with a fluorescent detector set at Ex 385 nm/Em 515 nm. The retention time of each sample was calculated by using standards and calibrators and a calibration curve was plotted for different concentrations.

In plasma samples, known concentrations of homocysteine were added. The concentrations in samples with added calibrators were determined in replicates and analytical recoveries were calculated. Mean recoveries of homocysteine were 95%–107%. The inter-assay precision was 5% and intra-assay precision was 4%. Bland-Altman statistical analysis was carried out with the HPLC method and the ELISA based method and was found to be of good correlation (correlation coefficient, $r = 0.99$).

Study of genetic polymorphisms

***MTHFR C677T* polymorphism** For analysis of the *MTHFR C677T* genetic polymorphism, a 173-bp fragment of exon 4 was amplified using specific primers 5′-TTT GAG GCT GAC CTG AAG CAC TTG AAG GAG-3′, and 5′-GAG TGG TAG CCC TGG ATG GGA AAG ATC CCG-3′. Each 20 μL of PCR mixture contained 100 ng DNA, 2 μL PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0)], 1.5 mM $MgCl_2$, 0.2 mM each of deoxynucleoside triphosphate, and 0.5 mM of each primer and 1 unit of Taq DNA polymerase. The PCR conditions were: initial denaturation, 95°C for 5 min; denaturation, 95°C for 1 min; annealing, 60°C for 1 min; extension, 72°C for 1 min; and number of cycles, 30. To test for the polymorphism, 15 μL of PCR product was digested by the addition of 2.9 μL double distilled water, 2 μL New England Biolabs (NEB) buffer 2 and 0.1 μL *Hin*fI. *C677T* mutation creates *Hin*fI restriction site causing cleavage of the 173-bp fragment into 125-bp and 48-bp.

***MTHFR A1298C* polymorphism** PCR amplification of exon 7 of the *MTHFR* gene using primers 5′-CTT TGG GGA GCT GAA GGA CTA CTA C-3′, and 5′-CAC TTT GTG ACC ATT CCG GTT TG-3′ resulted in a 163-bp product. Each 20 μL of PCR mixture contained 100 ng DNA, 2 μL PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0)], 2.5 mM $MgCl_2$, 0.2 mM each of deoxynucleoside triphosphate, 0.5 mM of each primer, and 1 unit of Taq DNA polymerase. The PCR conditions were: initial denaturation, 95°C for 10 min; denaturation, 95°C for 1 min; annealing, 51°C for 1 min; extension, 72°C for 1 min; and number of cycles, 40. To test for the polymorphism, 15 μL of PCR product was digested by the addition of 2.8 μL double distilled water, 2 μL NEB buffer 2, and 0.2 μL *Mbo*II. A1298C mutation abolishes one restriction site of *Mbo*II, resulting in the merger of the 56-bp and 28-bp bands into 84-bp. The common bands in all genotypes were 31-bp, 30-bp and 18-bp.

***MTRR A66G* polymorphism** For the analysis of *MTRR A66G* genetic polymorphism, restriction digestion analysis was performed with an artificially created *Nde*I restriction site using the sense primer: 5′-GCAAAGGCCATCGCAGAAGACAT-3′, and antisense primer: 5′-GTGAAGATCTGCAGAAAATCCATGTA-3′, where the underlined C replaces the A to generate an *Nde*I restriction site in the normal sequence. Each 20 μL of PCR mixture contained 100 ng DNA, 2 μL PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0)], 1.5 mM $MgCl_2$, 0.2 mM each of deoxynucleoside triphos-

phate, 0.5 mM of each primer, and 1 unit of TaqDNA polymerase. The PCR conditions were: initial denaturation, 95°C for 10 min; denaturation, 95°C for 1 min; annealing, 55°C for 30 s; extension, 72°C for 30 s; and number of cycles, 30. To test for the polymorphism, 15 μL of PCR product was digested by the addition of 2.6 μL double distilled water, 2 μL NEB buffer 4 and 0.4 μL *Nde*I. The PCR fragment of 66-bp remains uncut in the presence of the G (methionine) allele but is digested into fragments of 44-bp and 22-bp in the presence of the A (isoleucine) allele.

***MTR A2756G* polymorphism** For the analysis of *MTR A2756G* genetic polymorphism, 211-bp PCR product was amplified using the sense primer: 5′-TGTTCCCAGCTGTTAGATGAAAATC-3′, and antisense primer: 5′-GATCCAAAGCCTTTACACTCCTC-3′. Each 20 μL of PCR mixture contained 100 ng DNA, 2 μL PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0)], 1.5 mM $MgCl_2$, 0.2 mM each of deoxynucleoside triphosphate, 0.5 mM of each primer, and 1 unit of TaqDNA polymerase. The PCR conditions were: initial denaturation, 95°C for 10 min; denaturation, 95°C for 30 s; annealing, 60°C for 30 s; extension, 72°C for 30 s; and number of cycles, 40. To test for the polymorphism, 15 μL of PCR product was digested by the addition of 2.6 μL double distilled water, 2 μL NEB buffer 2 and 0.5 μL *Hae*III. The PCR fragment of 211-bp remains uncut in the presence of the A allele but is digested into fragments of 131-bp and 80-bp in the presence of the G allele.

All primers were obtained from Sigma Genosys (80-51127272; St. Louis, MO, USA), all restriction enzymes from New England Biolabs (Ipswich, MA, USA), all deoxynucleoside triphosphates from MBI Fermantas (Glen Burnie, MD, USA) and Taq polymerase from Bangalore Genie (MME5J; Bangalore, Karnataka, India).

DNA quantity was kept uniform in all samples so that uniform intensities could be obtained under identical conditions. All restriction enzymes were checked for their quality by digesting the plasmids with known restriction sites. Every PCR was accompanied with three controls: a) a negative control without any genomic DNA, b) a negative control with genomic DNA that does not digest with the particular restriction enzyme, and c) a positive control with genomic DNA that digests completely with the particular restriction enzyme. All samples were tested in duplicates to avoid genotyping errors (6, 18).

Statistical analysis

The categorical variables, such as genotype and allele frequencies, were tabulated individually in 2×2 contingency tables based on the presence or absence of the variable in cases and controls. Odds ratio (OR), 95% confidence interval (CI) and p-values were obtained using the statistical software based on Fisher's exact test. Bivariate analysis was performed using Fisher's exact test and logistic regression analysis. A p-value of <0.05 was considered as statistically significant. The plasma homocysteine values were considered as continuous variables. Association of plasma homocysteine with *MTRR* and *MTR* genotypes was assessed using Student's t-test and analysis of variance (ANOVA). The software used for statistical analyses was obtained from the web page: www.Statpages.org.

Results

The baseline characteristics of the study population are presented in Table 1.

The *MTRR 66AA* genotype was present in 0.6% of cases and 9.8% of controls. The *MTRR 66AG* genotype was present in 26.4% of cases and 40.5% of controls. The *MTRR 66GG* genotype was present in 73.0% of cases and 49.7% of controls. Among all the genotypes, the *MTRR 66GG* genotype was associated with a 2.74-fold risk (95% CI: 1.73, 4.34; $p<0.0001$) of thrombosis.

The frequencies of the *MTR 2756AA*, *-AG* and *-GG* genotypes in cases were 53.3%, 39.3% and 7.4%, respectively. The corresponding frequencies in controls were 54.0%, 39.3% and 6.7%, respectively, with no significant association with thrombosis.

Among the combined genotypes, the *MTRR GG/MTR AG and MTRR GG/MTR GG* genotypes cumulatively were found to increase 2.38-fold the risk of DVT (Table 2). A gene-dosage effect was evident by the constant increase in the OR, with an increase in the number of mutated alleles in the *MTRR* and *MTR* loci (Table 3).

The *MTRR 66GG* genotype was found to increase the risk of DVT by 3.46-fold in the presence of the *MTHFR 677CT/AC* genotype and 1.86-fold the risk in the absence of this genotype. No significant risk was observed in the *MTR* genotypes in either the presence or absence of the *MTHFR 677CT/1298AC* genotype (Table 4).

Table 1 General characteristics of the study population.

Parameter	Cases	Controls	p-Value
Male and female ratio	105 men and 58 women	105 men and 58 women	1.00
Age, years	37.4±13.7 (men)	37.3±13.5 (men)	0.96
	32.4±13.8 (women)	32.5±13.6 (women)	0.97
Weight, kg	60.5±10.2 (men)	59.8±9.6 (men)	0.61
	48.2±9.8 (women)	47.8±8.8 (women)	0.82
Education			
Primary level	0	0	–
More than secondary level	163	163	1.00
Smoking	35/163	34/163	1.00
Factor V Leiden	14/163	10/163	0.53
Prothrombin G20210A	0/163	0/163	–
Hyperhomocysteinemia	29/163	5/163	<0.0001
Diabetes	5/163	4/163	1.00
Hypertension	18/163	15/163	0.71

Table 2 Distribution of the *MTRR A66G* and *MS A2756G* genotypes in DVT cases and controls.

Genotype	Cases (%)	Control (%)	OR (95% CI)	p-Value
MTRR A66G				
AA	1 (0.6)	16 (9.8)	0.06 (0.01, 0.34)	0.0002*
AG	43 (26.4)	66 (40.5)	0.53 (0.33, 0.84)	0.01*
GG	119 (73)	81 (49.7)	2.74 (1.73, 4.34)	<0.0001*
A allele	45 (13.8)	98 (30.1)	0.37 (0.25, 0.55)	<0.0001*
G allele	281 (86.2)	228 (69.9)	2.68 (1.81, 3.97)	<0.0001*
MTR A2756G				
AA	87 (53.4)	88 (54)	0.98 (0.63, 1.51)	1.00
AG	64 (39.3)	64 (39.3)	1.00 (0.64, 1.56)	1.00
GG	12 (7.4)	11 (6.7)	1.10 (0.48, 2.52)	1.00
A allele	238 (73)	240 (73.6)	0.97 (0.69, 1.37)	0.93
G allele	88 (27)	86 (26.4)	1.03 (0.73, 1.46)	1.00
MTRR/MTR				
AA/AA	0 (0)	8 (4.9)	0.00 (0.00, 0.46)	0.007*
AA/AG	0 (0)	4 (2.5)	0.00 (0.00, 0.95)	0.12
AA/GG	1 (0.6)	4 (2.5)	0.25 (0.04, 1.65)	0.37
AG/AA	25 (15.3)	29 (17.8)	0.84 (0.47, 1.50)	0.66
AG/AG	16 (9.8)	33 (20.2)	0.43 (0.23, 0.81)	0.01*
AG/GG	2 (1.2)	4 (2.5)	0.49 (0.10, 2.34)	0.69
GG/AA	62 (38)	51 (31.3)	1.35 (0.85, 2.13)	0.24
GG/AG	48 (29.4)	27 (16.6)	2.10 (1.24, 3.57)	0.008*
GG/GG	9 (5.5)	3 (1.8)	3.12 (0.89,10.83)	0.14
GG/AG and *GG/GG*	57 (35)	30 (18.4)	2.38 (1.43, 3.96)	0.001*

*p-Value is significant. OR, odds ratio; 95% CI, 95% confidence interval.

Table 3 Gene-dosage effect.

Number of G alleles at *MTRR* and *MTR* loci	Cases (%)	Controls (%)	OR (95% CI)	p-Value
0	0 (0)	8 (4.9)	0.00 (0.00, 0.46)	0.007*
1	25 (15.3)	33 (20.2)	0.71 (0.41, 1.26)	0.31
2	79 (48.5)	88 (54)	0.80 (0.52, 1.24)	0.38
3	50 (30.7)	31 (19.0)	1.46 (0.87, 2.45)	0.20
4	9 (5.5)	3 (1.8)	3.12 (0.89, 10.83)	0.14

*p-Value is significant. OR, odds ratio; 95% CI, 95% confidence interval. Gene dosage effect, p=0.0002.

A constant increase in mean plasma homocysteine was observed with an increase in the number of *MTRR G* alleles. The *MTR G* allele was found to act additively in increasing homocysteine further. The combined genotypes with the highest mean homocysteine were *MTRR GG/MTR AG* and *MTRR GG/MTR GG* (Table 5).

Discussion

The current study was undertaken to examine the relationship between the *MTR A2756G* and *MTRR A66G* genetic polymorphisms and risk of DVT.

The *MTRR 66GG* genotype was shown to confer a 2.74-fold risk of thrombosis. The *MTR A2756G* genetic

Table 4 Stratifying the subjects based on the presence or absence of the compound heterozygous *MTHFR* genotype.

Genotype	Compound heterozygous for *MTHFR*			
	Yes		No	
	Cases/controls	OR (95% CI)	Cases/controls	OR (95% CI)
MTRR				
AA	0/0	–	1/16	0.06 (0.01, 0.34)*
AG	0/0	–	43/66	0.53 (0.33, 0.84)*
GG	19/6	3.46 (1.38, 8.63)*	100/75	1.86 (1.20, 2.89)*
MTR				
AA	10/4	2.60 (0.84, 7.99)	77/84	0.84 (0.55, 1.30)*
AG	9/2	4.71 (1.12, 19.58)	55/62	0.83 (0.54, 1.30)
GG	0/0	–	88/86	1.05 (0.68, 1.62)

*p-Value is significant. OR, odds ratio; 95% CI, 95% confidence interval.

Table 5 Plasma homocysteine distribution according to genotype in DVT cases and controls.

Genotype	Mean plasma homocysteine ± standard deviation, μmol/L			
	Males		Females	
	Cases	Controls	Cases	Controls
MTRR A66G				
AA	12.0 (n = 1)	4.3 ± 2.4 (n = 10)	– (n = 0)	4.0 ± 1.7 (n = 6)
AG	13.0 ± 3.8 (n = 24)	8.2 ± 2.8 (n = 37)	10.2 ± 1.3 (n = 19)	7.7 ± 2.5 (n = 29)
GG	17.1 ± 7.2 (n = 80)	13.9 ± 4.0 (n = 58)	14.3 ± 7.9 (n = 39)	11.7 ± 1.8 (n = 23)
p-Value	0.001[a]	< 0.0001[b]	< 0.05[a]	0.0001[b]
MTR A2756G				
AA	14.2 ± 4.9 (n = 50)	10.7 ± 4.9 (n = 52)	11.7 ± 2.9 (n = 39)	7.9 ± 3.2 (n = 36)
AG	17.0 ± 7.5 (n = 45)	11.0 ± 4.8 (n = 46)	15.8 ± 8.2 (n = 18)	10.8 ± 2.4 (n = 18)
GG	20.7 ± 8.1 (n = 11)	13.2 ± 6.0 (n = 7)	13.0 (n = 1)	9.5 ± 5.0 (n = 4)
p-Value	< 0.006[b]	0.46[b]	< 0.01[a]	< 0.01[b]
MTRR/MTR				
AA/AA	– (n = 0)	3.3 ± 1.8 (n = 5)	– (n = 0)	2.9 ± 1.7 (n = 3)
AA/AG	– (n = 0)	4.3 ± 2.3 (n = 3)	– (n = 0)	4.6 (n = 1)
AA/GG	12.0 (n = 1)	6.5 ± 1.0 (n = 2)	– (n = 0)	5.0 ± 0.8 (n = 2)
AG/AA	13.3 ± 1.7 (n = 8)	7.3 ± 3.1 (n = 10)	9.9 ± 1.0 (n = 17)	6.2 ± 1.0 (n = 19)
AG/AG	12.4 ± 4.3 (n = 15)	8.0 ± 2.5 (n = 24)	12.0 (n = 1)	10.5 ± 1.6 (n = 9)
AG/GG	20.0 (n = 1)	12.5 ± 0.9 (n = 3)	13.0 (n = 1)	12.1 (n = 1)
GG/AA	14.3 ± 5.3 (n = 41)	12.1 ± 1.0 (n = 37)	13.1 ± 3.1 (n = 22)	11.4 ± 1.4 (n = 14)
GG/AG	19.4 ± 7.8 (n = 30)	12.6 ± 4.0 (n = 19)	16.0 ± 8.2 (n = 17)	11.9 ± 2.1 (n = 8)
GG/GG	21.8 ± 8.4 (n = 9)	15.7 ± 2.6 (n = 2)	– (n = 0)	15.3 (n = 1)

[a]p-Value based on Student's t-test, [b]p-Value based on analysis of variance. n, number of subjects.

polymorphism showed no significant association individually. The combined genotypes *MTRR GG/MTR AG* and *MTRR GG/MTR GG* cumulatively were shown to confer a 2.38-fold risk of DVT. The risk associated with the *MTRR 66GG* genotype was found to be increased further in the presence of the *MTHFR 677CT/1298AC* genotype.

A positive association was observed between plasma homocysteine and *MTRR G* allele. The *MTR G* allele was found to have an additive effect as evidenced by the highest mean homocysteine values in subjects with the *MTRR GG/MTR AG* and *MTRR GG/MTR GG* genotypes.

The Thrombophilia Study in Women from the University of Toronto showed a higher frequency of the *MTRR 66GG* genotype in cases (32.56%) than in controls (27.13%), although this was not significant (14). The lack of direct association of the *MTR A2756G* polymorphism with thrombosis was also observed by Salomon et al. in a cohort of 171 venous thromboembolism cases (15), by Yu et al. in a Chinese cohort of 103 DVT cases and 250 controls (19) and by D'Angelo et al. in an Italian cohort of 170 cases with early onset thrombotic events and 182 controls (20). Although the *MTR A2756G* was individually not reported to be a risk factor, its co-segregation with *MTHFR C677T* (13) or *MTRR A66G* was shown to increase the risk of thrombosis (17).

Silaste et al. have studied the impact of the *MTR A2756G* genetic polymorphism in healthy women and found no significant difference in plasma homocysteine among individual genotypes. However, subjects with the *2756 G* allele responded more rapidly to a high folate diet than subjects with the *2756 A* allele with a subsequent reduction in plasma homocysteine (21).

Yates et al. have studied the impact of the *MTR/MTRR* genotypes on the distribution of homocysteine, total cellular 5-methyl tetrahydrofolate and vitamin B_{12} and have shown that these two polymorphisms influence the distribution of these three metabolites, thereby co-segregation of these variant alleles is associated with the risk of thrombotic event (17). This synergetic effect of these two polymorphisms was also evident in the current study. Using purified recombinant human proteins, Yamada et al. (22) found that *MTRR* maintained *MTR* activity at a 1:1 stoichiometric ratio. In the presence of *MTRR* and NADPH, holoenzyme formation from apo*MTR* and methylcobalamin was significantly enhanced due to stabilization of apo*MTR* in the presence of *MTRR*. *MTRR* was also able to reduce aquacobalamin to cob(II)alamin in the presence of NADPH, which stimulated conversion of apo*MTR* and aquacobalamin to holo*MTR*. They concluded that *MTRR* serves as a chaperone for *MTR* and as an aquacobalamin reductase, rather than acting solely in reductive activation of *MTR* (22).

Laraqui et al. have shown that combined *MTR* and *MTRR* polymorphisms, i.e., *MTR 2756AG+MTRR 66AG*, *MTR 2756AG+MTRR 66GG* genotypes were significant risk factors for hyperhomocysteinemia (23). This is in agreement with our findings. Botto et al. have studied the impact of the *MTHFR C677T* and *MTRR A66G* genetic polymorphisms on plasma homocysteine and DNA damage. They have observed that in subjects with the *677CC* genotype, *66GG* genotype individuals had higher levels of homocysteine

than *66AA* genotype individuals did. In subjects with the *677TT* genotype, individuals with the *66GG* had higher frequency of micronuclei than individuals with the *66AG* or *66AA* genotypes, indicating greater genomic instability in double homozygous mutant genotype (24).

Wang et al., in in vitro experiments, have shown that individuals who are deficient in CBS or methionine synthase not only develop hyperhomocysteinemia, but also have vascular endothelium susceptible to damage by homocysteine compared to individuals having normal enzyme activities (25).

The *MTRR A66G* genetic polymorphism being a risk factor for thrombosis, the *MTR A2756G* having an additive role in increasing the risk, impair remethylation of homocysteine to methionine. This metabolic block results in hyperhomocysteinemia and lowering of methionine levels. Hyperhomocysteinemia and low methionine were both reported to be positively associated with risk of thrombosis.

The *MTRR A66G* and *MTR A2756G* genotypes showed a large difference in their distribution in different ethnic groups. The present data revealed the *MTRR 66GG* genotype as a significant risk factor, at least in South Indians, whereas no such significant association was observed in other ethnic groups. The reason for this discrepancy could be due to increased prevalence of B_{12} deficiency among Indians, which is partly due to a vegetarian diet (26) that can act in conjunction with the *MTRR 66GG* genotype in increasing homocysteine, and thus increasing the risk of DVT.

To conclude, the *MTRR 66GG* genotype is a risk factor for thrombosis among South Indians and its co-segregation with *MTHFR* compound heterozygous state further increases the risk. We recommend including *MTRR* mutation analysis in the thrombophilia panel along with *Factor V*, *Factor II*, *MTHFR* and plasma homocysteine in South Indian subjects. The limitations of this study are its small sample size and lack of data on the nutritional status of these subjects. A large-scale study is needed taking into account both genetic and nutritional factors for more precise risk evaluation.

Acknowledgements

The authors thank the Department of Biotechnology, Government of India for the financial support through the core grant. We thank all the subjects who participated in the study. We also thank Mr. Ram Prakash Singh and Mr. G. Srinivas for technical support.

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