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Original Article

Association of Methylenetetrahydrofolate Reductase C677T Polymorphism with Hyperhomocysteinemia and Deep Vein Thrombosis in the Iranian Population

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Purpose: Deep venous thrombosis (DVT) is a common but elusive condition characterized by a high morbidity and mortality rate. The aim of the present study was to investigate the correlation between methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism with plasma total homocysteine (tHcy) levels and DVT risk in an Iranian population.

Materials and Methods: Our study population consisted of 67 patients with a diagnosis of DVT and 67 healthy subjects as controls. Genotyping of MTHFR C677T polymorphism was performed by the polymerase chain reaction technique combined with restriction enzyme fragment length polymorphism (PCR-RFLP) and measurement of tHcy levels was done by enzyme immunoassay method.

Results: Plasma tHcy levels were significantly higher in DVT patients than controls (18.09±7.6 vs. 10.5±4.3, P=0.001). Also, plasma tHcy levels were significantly higher in MTHFR 677TT genotypes compared to 677CC genotypes in both DVT patients (P=0.016) and controls (P=0.03). Neither heterozygote nor homozygote genotypes of MTHFR C677T polymorphism was significantly correlated with DVT (P>0.05). The distribution of MTHFR C677T genotypes was similar between men and women in both DVT patients and controls (P>0.05). Moreover, the frequency of mutant 677T allele did not differ significantly between the two groups (28.3% vs. 21.6%, P=0.15).

Conclusion: Based on this study, we propose that hyperhomocysteinemia but not homozygosity for MTHFR C677T polymorphism is a significant risk factor for DVT in the Iranian population. Also, MTHFR 677TT genotype is a determinant of elevated plasma tHcy levels.

Key Words: Homocysteine, Deep venous thrombosis, Methylenetetrahydrofolate reductase, Genetic polymorphism

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INTRODUCTION

Deep venous thrombosis (DVT) is an elusive and a common preventable cause of morbidity and mortality worldwide. DVT with an annual incidence of 48 per 100,000 populations is a common disorder in the elderly and its incidence rises markedly with age. According to some studies, genetic factors account for about 60% of the risk for DVT [1]. A large number of epidemiological studies have shown that elevated plasma total homocysteine (tHcy) level is a significant risk factor for venous thrombotic disorders [2,3] with some exceptions [4,5]. Mild hyperhomocysteinemia may result from nutritional deficiency or mutation in methylenetetrahydrofolate reductase (MTHFR) gene [3,6,7]. MTHFR is a key regulatory enzyme of Hcy metabolism, catalyzing the remethylation of Hcy to methionine [8]. The most common polymorphism in MTHFR gene, namely the C677T, causes a reduction in MTHFR activity and may lead to hyperhomocysteinemia, a condition linked with venous and arterial thrombotic disease [2]. Studies investigating the role of MTHFR C677T polymorphism in DVT have yielded conflicting results [3,9-13]. Some studies identified MTHFR C677T polymorphism as important risk factors for DVT [13], while other studies did not confirm such an association [9-11]. So, in the present study we investigated a potential association of MTHFR C677T polymorphism with DVT in Iranian adult patients.

MATERIALS AND METHODS

1) Study population

Sixty-seven patients including 30 men and 37 women (mean age, 39.12 years; ranging 25-73 years) with a diagnosis of DVT were referred to the coagulation center of the Iranian blood transfusion organization for thrombophilia screening. DVT was diagnosed in all patients based upon clinical presentation, D-dimer test results and duplex ultrasound imaging technique. All of the DVT patients investigated in this study had idiopathic venous thrombosis described by the DVT occurrence in the absence of predisposing risk factors including prolonged immobility, oral contraception use/hormonal replacement treatment, obesity, congestive heart failure, surgery under general anesthesia, active cancer and its treatment, stroke or paralysis, acute infection, previous DVT occurrence, and pregnancy/post-partum.

These patients received unfractionated heparin, low-molecular-weight heparin and warfarin for the treatment of DVT. For the prophylaxis of DVT warfarin was mainly used in men and non-pregnant women. All of the patients investigated in the current study were outpatients referred to coagulation laboratory of the Iranian blood transfusion organization. So, we were not able to follow up all of the patients. However, a few number of patients were again referred to the coagulation laboratory to test for plasma tHcy levels in order to evaluate the efficacy of plasma tHcy lowering treatment. Moreover, no recurrent cases were referred to the coagulation laboratory of the Iranian blood transfusion organization. The controls included 67 healthy individuals (31 men and 36 women; mean age, 38.17 years; ranging 23-70 years) matched for age and sex with the patients and without any history of thrombosis and/or use of vitamin B supplements. Informed consent was obtained from all participants. This work was approved by the Iranian blood transfusion organization ethics committee with the assigned authentication number "IBTO- P82-1000".

2) Total plasma homocysteine assay

Blood samples were collected from fasting subjects in ethylenediaminetetraacetic acid (EDTA)-containing vacutainer tubes (Pole Ideal Pars Ltd., Tehran, Iran) for analysis of tHcy in plasma. Immediately, samples were centrifuged at 4°C and the plasma fraction was aspirated and stored at –20°C until analysis for tHcy. Additionally, the cellular fraction was used for DNA extraction. Measurement of plasma tHcy levels were performed by an enzyme linked immunosorbent assay using a commercially available kit (Axis Shield Diagnostics Ltd., Dundee, United Kingdom) and according to manufacturer's instructions. The assay's detection range was 2.5-50.0 µM.

3) MTHFR polymorphism analysis

Genomic DNA was extracted from blood leukocytes according to the method described by Lahiri and Nurnberger [14]. The C677T polymorphism on the MTHFR gene was analyzed by the polymerase chain reaction technique combined with restriction enzyme fragment length polymorphism (PCR-RFLP) as described in previous studies with slight modifications [15]. The sequences of primers for MTHFR C677T polymorphism were as follows: 5'-TGA AGG AGG TGT CTG CGG GA-3' and 5'-AGG ACG GTG CGG TGA GAG TG-3'. These primers amplified a 198 bp fragment of DNA. The PCR conditions included an initial denaturation at 94°C for 120 seconds, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 7 minutes. The amplified products were digested with 5 units of Hinf I (Promega,

Madison, WI, USA) at 37°C for 12 hours. Electrophoresis of digested PCR products on a 3% agarose gel resulted in a 198 bp band in wild type; 198 bp,175 bp and 23 bp bands in heterozygote; 175 bp and 23 bp bands in homozygotes. Due to small size, the 23 bp band was not seen.

4) Statistical analysis

Age and plasma tHcy levels were expressed as mean± standard deviation. Student t-test was used for analysis of differences in mean age and tHcy levels between DVT patients and controls. Chi-square test was used to assess the association of MTHFR C677T polymorphism with venous thrombosis and odds ratio (OR) along with 95% confidence interval (CI) were calculated. Allele frequencies were calculated by gene counting in DVT patients and control subjects. Statistical analysis was performed by SPSS software ver. 16.0 (SPSS Inc., Chicago, IL, USA) with a statistical significance level of $P<0.05$.

RESULTS

The study enrolled 67 DVT patients and 67 healthy controls. The sex distribution (P=0.73) and mean age (P=0.80) were not significantly different between DVT patients and control subjects. Mean plasma tHcy levels in the DVT patients were significantly higher than in the control subjects (18.09±7.6 vs. 10.5±4.3, P=0.001) (Table 1). The distribution of different MTHFR C677T genotypes was comparable among DVT patients and controls. Neither MTHFR 677CT heterozygotes (P=0.37) nor MTHFR 677TT homozygotes (P=0.17) was significantly associated with DVT (Table 2). Moreover, the frequency of the minor T allele was 28.3% and 21.6% in DVT patients and controls, respectively and did not differ significantly (P=0.15; Table 2).

Additionally, to assess the associations of the MTHFR C677T polymorphism with DVT risk, dominant and recessive genetic models were applied. Results showed that the association between MTHFR C677T polymorphism and DVT risk was not significant in either genetic model that was analyzed (dominant model: CT+TT vs. CC, OR=1.63, 95% CI 0.56-2.76, P=0.32; recessive model: TT vs. CT+CC, OR=1.44, 95% CI 0.87-2.42, P=0.15) (Table 3). The distribution of different MTHFR C677T genotypes was similar between men and women in both DVT patients (0.79) and controls (0.86) and didn't differ statistically (Table 4).

Moreover, the associations of different genotypes of MTHFR C677T polymorphism with plasma levels of tHcy were determined in DVT patients and controls. Results indicated that the correlation between MTHFR 677TT genotype with plasma tHcy levels was statistically significant in both DVT patients (P=0.016) and controls (P=0.03) (Fig. 1).

Table 1. Mean plasma homocysteine levels and other characteristics in DVT patients and controls

Study population	DVT patient (n=67)	Control (n=67)	P-value
Age (y)	39.12±28.3	38.17±30.6	0.80
Gender (male/female)	30/37	31/36	0.73
Plasma total homocysteine (μmol/L)	18.09±7.6	10.5±4.3	0.001

Values are presented as mean±standard deviation or number only.
DVT, deep vein thrombosis.
Chi square test was used to compare gender between the two groups, and mean values of age and homocysteine levels were analyzed by Student's t-test.

Table 2. Prevalence of MTHFR C677T allele and genotypes in DVT patients and controls

MTHFR C677T gene polymorphism	DVT patient (n=67)	Control (n=67)	P-value
677 T allele	38 (28.3)	29 (21.6)	0.15
677 CC genotype	34 (50.8)	41 (61.2)	0.54
677 CT genotype	28 (41.8)	23 (34.3)	0.37
677 TT genotype	5 (7.5)	3 (4.5)	0.17

Values are presented as number (%).
MTHFR, methylenetetrahydrofolate reductase; DVT, deep vein thrombosis; 677 CC, wild type; 677 CT, heterozygote; 677 TT, homozygote.

Table 3. Analysis of MTHFR C677T polymorphism in DVT patients and control subjects using dominant and recessive models

Genetic model	Genotype	DVT patient (n=67)	Control (n=67)	OR (95% CI)	P-value
Dominant	CC	34 (50.8)	41 (61.2)	1	
	CT+TT	33 (49.3)	26 (38.8)	1.63 (0.56- 2.76)	0.32
Recessive	CC+CT	62 (92.5)	64 (95.5)	1	
	TT	5 (7.5)	3 (4.5)	1.44 (0.87-2.42)	0.15

Values are presented as number (%).
MTHFR, methylenetetrahydrofolate reductase; DVT, deep vein thrombosis; OR, odds ratio; CI, confidence interval; CC, wild type; CT, heterozygote; TT, homozygote.

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Table 4. The genotype distribution of MTHFR C677T polymorphism according to gender in DVT patients and control subjects

MTHFR C677T gene polymorphism	Male	Female	Total
DVT patient	30 (100)	37 (100)	67 (100)
677 CC genotype	16 (53.3)	18 (48.7)	34 (50.8)
677 CT genotype	12 (40.0)	16 (43.2)	28 (41.8)
677 TT genotype	2 (6.7)	3 (8.1)	5 (7.5)
P-value		0.79	
Control subjects	31 (100)	36 (100)	67 (100)
677 CC genotype	19 (61.3)	22 (61.1)	41 (61.2)
677 CT genotype	11 (35.5)	12 (33.3)	23 (34.3)
677TT genotype	1 (3.2)	2 (5.6)	3 (4.5)
P-value		0.86	

MTHFR, methylenetetrahydrofolate reductase; DVT, deep vein thrombosis; CC, wild type; CT, heterozygote; TT, homozygote.

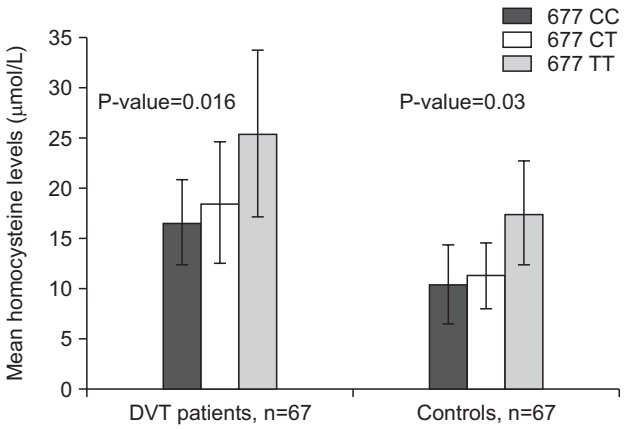

Fig. 1. Association between methylenetetrahydrofolate reductase (MTHFR) C677T genotypes and plasma concentration of total homocysteine (µmol/L) in deep vein thrombosis (DVT) patients and controls.

DISCUSSION

The main findings of this study were that (i) elevated plasma tHcy levels correlated significantly with DVT risk; (ii) the MTHFR 677TT genotype caused significantly higher plasma tHcy levels in DVT patients and controls in the Iranian population; and (iii) the MTHFR C677T polymorphism did not increase the risk of DVT in either heterozygote or homozygote state.

DVT is a potentially dangerous clinical condition that can lead to preventable morbidity and mortality [1]. The association between elevated plasma tHcy levels and venous thrombotic disorders have been studied extensively with conflicting results [2-5]. Our results indicated hyperhomocysteinemia as a frequent and significant risk factor for DVT development. Our results are consistent with some previous studies that identified elevated plasma tHcy levels as a risk marker in DVT patients [2,3]. However, in contrast to our result, some other studies did not find hyperhomocysteinemia as a risk factor for DVT [4,5]. The reasons for these conflicting results may be related to the concomitant presence of other environmental and genetic factors affecting plasma tHcy levels in different populations [16,17]. According to some studies, there is an inverse relationship between plasma tHcy levels and plasma vitamin B12 and folate concentrations. So, decreased plasma levels of vitamin B12 and folate can significantly induce elevated plasma tHcy levels in DVT patients [16].

The C677T polymorphism in the MTHFR gene associated with decreased MTHFR activity and elevated plasma tHcy levels has been proposed as an important genetic risk factor for DVT, although the results are controversial [3,9-11,13]. In the present study the MTHFR 677TT homozygote genotype did not correlate with DVT risk but had a higher plasma tHcy level than wild type (677CC) and heterozygote (677CT) genotypes. This finding may be explained with the notion that both genetic and acquired factors affect plasma tHcy levels and thus homozygote MTHFR 677TT genotype contributes indirectly to thrombosis via influencing plasma tHcy levels [3,6]. Differences in environmental factors such as vitamin B12 and folate levels in different populations can significantly alter the association of this polymorphism with DVT risk [6,16,17].

Our study was inconsistent with those studies that indicated MTHFR 677TT homozygote genotype as a risk factor for DVT [3,13]. The reasons for the apparent discrepancies between different studies are various and multifactorial and many factors such as diet, racial diversity, sample size, sample selection criteria, study design and gene-gene interactions may influence the results of association studies [18].

The prevalence of MTHFR 677T allele in our study (24%) was comparable with the frequency of T allele in Brazilian (24%) and Taiwanese Chinese (24.4%) populations [19,20] However, the frequency of MTHFR 677T allele in our study was higher than that reported in Bahraini Arab (11%), West African (9%) and South Indian (7.5%) populations [21-23] and lower than that reported in Chinese (55.2%) and Turkish (33.3%) populations [24,25].

Some studies have shown that vitamin supplementation can successfully treat hyperhomocysteinemia [9]. So, plasma tHcy levels should be routinely measured in DVT patients and all cases with even slightly elevated plasma tHcy levels should be considered for vitamin supplementation in hopes

of reducing the risks of DVT. Lack of data on serum folate and vitamin B12 levels and the retrospective design of our study are some limitations in this study.

CONCLUSION

Based on this study, we suggest that hyperhomocysteinemia but not MTHFR C677T polymorphism is significant risk factor for DVT. Moreover, MTHFR C677T polymorphism is a determinant of elevated plasma tHcy levels.

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