# The Association Between Common C677T Mutation in Methylenetetrahydrofolate Reductase Gene and the Risk of Venous Thrombosis in an Iranian Population

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## Abstract

***Background:*** Venous thrombosis is a multicausal disease involving acquired and genetic factors. The prevalence of methylenetetrahydrofolate reductase (MTHFR) C677T genotypes and its association with venous thrombosis is not established in the Iranian population. In this study we investigated a possible association between fasting hyperhomocysteinemia and C677T mutation in the MTHFR gene with venous thrombosis.

***Materials and Methods:*** We studied 200 venous thrombotic patients and 100 healthy controls, of similar age and sex. Mutation analysis was carried out by PCR-RFLP, and the homocysteine level was measured by EIA.

***Results:*** No significant differences in the frequency of C677T genotypes were observed between patients and controls ($P$=0.2). The frequency of the T allele was 21% and 27.2% in controls and patients, respectively (odds ratio, 1.27; 95% CI, 0.83–1.94, $P$=0.15). Fasting homocysteine level was significantly higher in patients than controls ($P$=0.001).

***Conclusions:*** We concluded that hyperhomocysteinemia, but not MTHFR C677T mutation, is a significant risk factor for venous thrombosis in the Iranian population, and measuring the level of homocysteine is less expensive and more useful than the genetic test for the MTHFR mutation.

Venous thrombosis, including deep-vein thrombosis and pulmonary embolism, is a common cause of morbidity and mortality, particularly in older people.[1] Venous thrombosis (VT) is multifactorial, and its exact pathogenesis has not been fully elucidated. Most cases of venous thrombosis arise due to prolonged immobilization, major surgery, trauma, or cancer, but genetic or acquired hemostatic abnormalities, including elevated plasma homocysteine (Hcy) levels, have also been implicated.[2] Elevated concentrations of total homocysteine have been associated with an increased risk of arterial and venous thrombosis.[3-5] A point mutation, C to T substitution at the nucleotide 677, in the coding sequence of the gene for methylenetetrahydrofolate reductase (MTHFR) is the most common enzyme defect associated with moderately-raised homocysteine concentrations, particularly in the presence of a suboptimal folate intake.[6] Genetic analyses studying the prevalence of the 677 C to T mutation in the MTHFR gene have recently shown divergent results in patients with venous thrombotic disease from different geographic regions. Several studies in patients with venous thrombosis failed to demonstrate an association between MTHFR C677T polymorphism and increased risk of venous thrombotic disease,[7,8,19-22] whereas other studies have reported a positive association.[9,17,18] In view of this controversy, our study was conducted with the purpose of evaluating the potential association of the C677T mutation of the MTHFR gene with venous thrombosis in adult patients.

## Materials and Methods

### Patients and Controls

The present case control study included 200 patients with VT and 100 healthy controls. Cases and controls were recruited simultaneously from the same geographic area. Diagnosis of VT was made by ultrasonography, radioisotope venography, and magnetic resonance imaging angiography.[22] Patients who had an acquired disorder that predisposed them to VT were excluded from the study. These acquired disorders included malignancy, myeloproliferative disorder, nephrotic syndrome, liver disease, antiphospholipid syndrome, or pregnancy. The inclusion criteria for controls were: routine biochemical values within the normal range, nonsmokers, and no history of metabolic, renal, malignant, or vascular pathology. The exclusion criteria also included history of thrombosis and supplementary intake of vitamins. Homocysteine assay was carried out by ELISA method using Axis Homocysteine kit (Axis-Shield, Dundee, Scotland).

### Genetic Analysis

Genomic DNA was extracted from the peripheral blood leukocytes by a previously described method.[10] The C677T mutation in the MTHFR gene was analyzed by PCR-RFLP using forward primer 5'-TGAAGGAGAAGGTGTCTGCGGGA-3' and reverse primer 5'-AGGACGGTGCGGTGAGAGTG-3'. PCR was carried out in a total volume of 40 µL containing 0.5 µmol/L of each primer, 200 µmol/L of all 4 dNTPs, 10 mmol/L Tris-HCl (pH 8.9), 2.2 mmol/L $MgCl_2$, 10% glycerol, 50 mmol/L KCl, 1.5 U of AmpliTaq DNA polymerase (Applied

Biosystems, Foster City, CA), and 200 ng of template DNA. The reaction conditions were as follows: initial denaturation at 94°C for 2 minutes and 35 subsequent cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 30 seconds. PCR product (22 µL) was digested with 0.5 µL (5 u) of Hinf I for 12 hours at 37°C. Digestion of the 198 bp fragment of the 677CT genotype results in 3 fragments of 198 bp, 175 bp, and 23 bp, whereas the 677 TT genotype results in 2 fragments of 175 bp and 23 bp. DNA fragments were separated by electrophoresis on a 3% agarose gel and visualized with ethidium bromide. Due to its small size, the 23 bp fragment was not seen on the gel.

### Statistical Analysis

Allele frequencies were calculated by gene counting in patients and controls. The Hardy-Weinberg equilibrium was analyzed by the chi-square test for all the genotypes, and category variables were analyzed with the chi-square test. Mean quantitative variables between the groups were detected using Student's t-test.

Logistic regression was used to assess the association of MTHFR C677T mutation with VT, and odds ratio (OR) and 95% confidence interval (CI) were calculated after adjustment for age and sex, and a *P* value <0.05 was considered significant.

## Results

In total, 200 patients and 100 controls were included in genetic analyses. Between the patients with VT and control subjects, no significant differences were observed in mean age and gender (**Table 1**). Mean plasma homocysteine levels were significantly higher in the VT group (17.4 ± 7.1 µmol/L) than in the control group (10.9 ± 4.1 µmol/L) (**Table 1**).

The genotype and allele frequencies of the MTHFR gene C677T mutation in VT patients and controls are shown in **Table 2**. The genotype frequency did not differ significantly between the 2 groups. The distributions of genotype in the 2 groups were in agreement with those predicted by Hardy-Weinberg equilibrium. The frequency of the T allele in the VT and control groups after adjusting for age and sex were 27.2% and 21%, respectively (odds ratio=1.27, 95% CI, 0.832–1.940, *P*=0.15).

The association between MTHFR genotypes and homocysteine concentration is significant in both patients (*P*=0.004) and controls (*P*=0.03), as shown respectively in **Figures 1A** and **1B**.

## Discussion

Hyperhomocysteinemia is an independent risk factor for cardiovascular disease that is influenced by factors such as nutritional deficiencies, malignancies, medications, and mutations in the MTHFR gene.[11-13] C677T is the most well-described mutation of this gene that affects homocysteine metabolism.[14] The defect causes a reduction in MTHFR activity and may lead to hyperhomocysteinemia, a condition included among the disorders of venous and arterial occlusive disease.[15-16] Nevertheless, the relationship between the TT genotype of the MTHFR gene

**Table 1_Homocysteine Levels and Other Characteristics in VT Patients and Control Group**

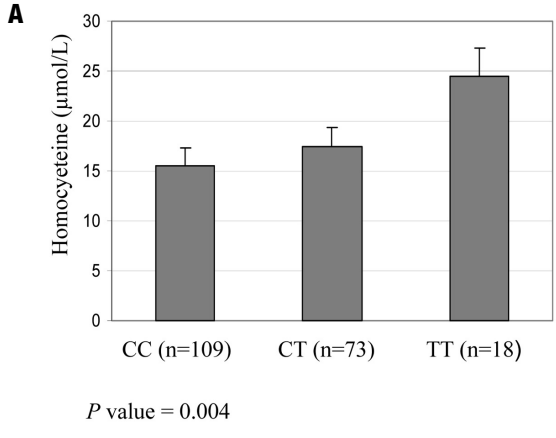
| | VT Group | Control Group | *P* value |
|---|---|---|---|
| Number | 200 | 100 | – |
| Age | 42.03 ± 13.49 | 38.17 ± 8.18 | 0.4 |
| Gender (male/female) | 95/105 | 45/55 | 0.85 |
| Homocysteine (µmol/L) | 17.4 ± 7.1 | 10.9 ± 4.1 | 0.001 |

*Values are mean ± standard deviation. Chi-square test was used to compare gender between the 2 groups and mean values of age and homocysteine were analyzed by Student's t-test. VT, venous thrombosis.*

**Table 2_MTHFR Genotypes and Allelic Frequencies of VT Patients and Controls**

| | Genotype | | | Allele | |
|---|---|---|---|---|---|
| | CC | CT | TT | C | T |
| Control (n=100) | 62 (62.0) | 34 (34.0) | 4 (4.0) | (79.0) | (21.0) |
| VT (n=200) | 109 (54.5) | 73 (36.5) | 18 (9.0) | (72.8) | (27.2) |
| *P* value | | 0.2 | | 0.15 | |

*CC, wild type; CT, heterozygosity; TT, homozygosity; VT, venous thrombosis. Numbers in parentheses represent percentages.*



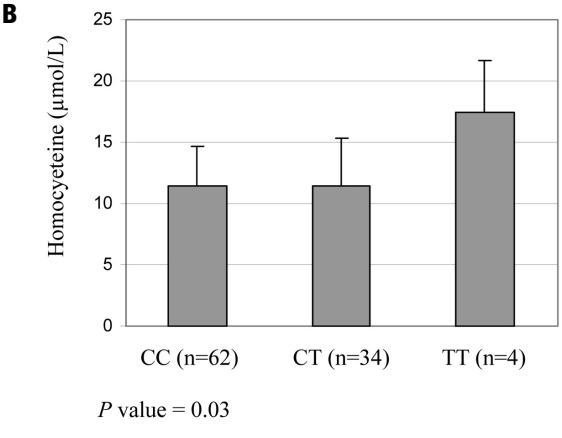




**Figure 1_Effects of MTHFR genotypes on tHcy levels in patients (A) and controls (B). Analysis of covariance was used to compare homocystein levels among the genotypes (*P*=0.004 for A and *P*=0.03 for B).**

and VT remains controversial. Some reports[17-18] identified the TT genotype as a genetic risk for VT; however, subsequent reports[19-22] did not confirm a positive relationship between the TT genotype and VT.

In the present study, homozygous (TT) mutation of MTHFR was not associated with VT, but had higher plasma Hcy levels than normal (CC) or heterozygous (CT) genotypes. This complies with the notion that both congenital and acquired factors affect plasma Hcy levels,[23-24] and thus homozygous MTHFR mutation contributes indirectly to thrombosis via influencing plasma Hcy levels.[25] Our study is consistent with many case-control studies[19-21,26,27] showing no association between MTHFR C677T mutation and VT. Our results also are inconsistent with studies conducted in French,[28] Italian,[29] Chinese,[30] and Spanish[31] populations. The reasons for nonreplication of associated studies are numerous and many factors, such as population heterogeneity, ethnic stratification, and sample size, may contribute to variable association results. Population-specific linkage disequilibrium between markers and causal variants, variation in study design, confounding sampling bias, misclassification of phenotypes, and gene-gene and gene-environment interactions are other factors that influence genetic association results.

The frequency of T allele in our study was 25%, which is lower than the frequency reported in Italian[32] (44%) and Turkish[33] (33.6%) populations and higher than Canadian Inuit[34] (6%) and South African[35] (10.3%) populations. Our result was also comparable with frequency of T allele in Brazilian[36] (24%) and Taiwanese Chinese[37] (24.4%) populations.

A large number of epidemiological studies[9,11] have demonstrated that mild to moderate hyperhomocysteinemia is a prevalent risk factor for cardiovascular disease and venous thromboembolism with some exceptions.[38-39] A meta-analysis by den Heijer and colleagues[40] demonstrated a significant odds ratio of 2.5 (95% CI, 1.8 to 3.5) for VT in patients with fasting hyperhomocysteinemia. In another meta-analysis[41] including 27 studies relating homocysteine to atherosclerotic vascular disease, the odds ratio was estimated to increase 1.6 for every 5 µmol/L of elevated plasma homocysteine. In accordance with these reports, our study implied a positive association between hyperhomocysteinemia and VT.

In conclusion, our results from an Iranian population provide evidence that plasma homocysteine level but not C677T mutation of the MTHFR gene is a significant risk factor in VT and measuring the level of homocysteine is cheaper and more useful than the genetic test for the MTHFR mutation. LM

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