Association study of methylenetetrahydrofolate reductase A1298C mutation with cerebral venous thrombosis risk in an Iranian population

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Abstract

Background: Cerebral venous thrombosis (CVT) is an uncommon condition characterized by severe clinical manifestations and high mortality rate. There is limited data on the role of methylenetetrahydrofolate reductase (MTHFR) A1298C mutation as a risk factor for CVT development in Iranians. Aim: The aim was to investigate a possible association between fasting plasma homocysteine (Hcy) levels, MTHFR A1298C mutation, and CVT in Iranian population. Materials and Methods: The study population consisted of 50 patients with a diagnosis of CVT (20–63 years old) and 75 healthy subjects (18–65 years old) as control. Genotyping of the MTHFR A1298C mutation and Hcy measurement was carried out by polymerase chain reaction-restriction fragment length polymorphism technique and enzyme immunoassay method, respectively. Results: Fasting plasma total Hcy levels were significantly higher in CVT patients than controls (P = 0.015). No significant differences were observed in the MTHFR A1298C genotypes frequency between CVT patients and controls (P > 0.05). The frequency of the 1298C allele was 36% and 37.5% in CVT patients and controls, respectively and did not differ significantly between the two groups (P = 0.16). Conclusions: Our study demonstrated that MTHFR A1298C mutation is not a significant risk factor for CVT.

Key words: Homocysteine, methylenetetrahydrofolate reductase, mutation, polymerase chain reaction-restriction fragment length polymorphism, thrombosis

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Introduction

Cerebral venous thrombosis (CVT) is a rare type of cerebrovascular disease that can occur at any age even in neonate. It accounts for 0.5% of all strokes with an annual incidence of 3–4 cases per million populations.[1] CVT is a multifactorial disease, and at least one predisposing factors can be identified in 80% of patients.[2] Thrombophilia, either genetic or acquired, is among the most frequent risk factors identified in more than 20% of the CVT patients.[3] Hyperhomocysteinemia, an established independent risk factor for venous and arterial thrombosis, may result from genetic or environmental factors or a combination of both.[4] One of the main causes of mild to moderate hyperhomocysteinemia involves genetic defects in the enzyme methylenetetrahydrofolate reductase (MTHFR), which plays a central role in homocysteine (Hcy) and folate metabolism.[5] Deficiencies in MTHFR enzyme leads to hyperhomocysteinemia which may result in arterial and venous thrombotic disorders.[6]

Two common mutations in MTHFR gene, the C677T and A1298C, have been associated with decreased MTHFR activity and elevated plasma Hcy levels.[6] The association between MTHFR C677T mutation with CVT has been studied extensively with conflicting results.[7,8]
Unlike MTHFR C677T mutation, studies investigating the role of MTHFR A1298C mutation in CVT are limited. Until date, only a few studies investigated the role of MTHFR A1298C in CVT. More recently, a case–control study by Fekih-Mrissa et al. demonstrated a significant association of MTHFR A1298C mutation with CVT.

Since, there is little information on the prevalence of MTHFR A1298C mutation in Iranian CVT patients, we aimed to perform a case–control study to investigate the relationship between the MTHFR A1298C mutation, total Hcy levels, and CVT development in an Iranian population.

Materials and Methods

Patients and control subjects
Fifty-five unrelated patients with CVT were referred to the coagulation center of Iranian blood transfusion organization for thrombophilia screening. The clinical records and objective documentation of CVT were reviewed by a neurologist to confirm the diagnosis. Five patients were excluded from the analysis because the diagnosis was uncertain. Altogether, 50 patients (17 men and 33 women; mean age 38 years ranging 20–63 years) with objectively confirmed diagnosis of CVT were included in the study.

The controls consisted of 75 healthy individuals (25 men and 50 women; mean age 36 years; ranging 18–65 years) matched for age and sex with the patients, and without any history of thrombosis and/or use of vitamin B supplements and folate. Informed consent was obtained from all participants. The study was approved by Iranian Blood Transfusion Organization Ethic Committee.

Total homocysteine assay of fasting plasma
Blood was drawn in ethylenediaminetetraacetic acid containing vacutainer tubes from fasting subjects for analysis of total plasma Hcy. Samples were centrifuged at 4°C and plasma fraction was aspirated and transferred to plastic tube and stored at –20°C until analysis for total Hcy. Total plasma Hcy levels were determined by microplate enzyme immunoassay using a commercial kit (Axis® Shield Diagnostic Ltd., UK) according to manufacturer’s instruction. The assay’s detection range was 2.5–50.0 μM.

Methylenetetrahydrofolate reductase A1298C genotype analysis
DNA was extracted from blood leukocyte according to the method described by Lahiri and Nurnberger. MTHFR A1298C mutation was analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method as previously described by van der van der Put et al. with a slight modification.

The sequences of the primers were: 5’-CTT TGG GGA GCT GAA GGA CTA CTA C-3’ and 5’-CAC TTT GTG ACC ATT CCG GTT TG-3’ which amplified a 163 bp fragment of DNA.

Polymerase chain reaction conditions were optimized for the Hybaid thermal cycler and included an initial denaturation at 92°C for 120 s and 40 subsequent cycles of denaturation at 92°C for 60 s, annealing at 62°C for 60 s and extension at 72°C for 30 s. This was followed by a final extension period at 72°C for 4 min. The amplified products were digested with 2.5 U of MboII (Fermentas, Vilnius, Lithuania) for 3 h at 37°C. The A1298C mutation abolishes an MboII restriction site and digestion of the 163 bp amplicon results in 84, 31, 30, and 18 bp fragments in the presence of the 1298C allele, and 56, 31, 30, 28, and 18 bp fragments in the presence of the 1298A allele [Figure 1]. DNA fragments were separated by electrophoresis on a 4% agarose gel and visualized with ethidium bromide.

Statistical analysis
Allele frequencies were calculated by gene counting in CVT patients and control subjects. Mean ages and plasma total Hcy levels differences between CVT patients and control subjects were assessed by Student’s t-test. Furthermore, a t-test was used to assess the association of MTHFR A1298C mutation with CVT and plasma total Hcy levels. Moreover, analysis of different genetic models including dominant, recessive, and co-dominant was done using Chi-square test. Statistical analysis was performed by Statistical Package for the Social Sciences version 15 (SPSS Inc., Chicago, IL) and the statistical significance was set at P < 0.05.

Results

Our study population consisted of 50 patients with CVT and 75 apparently healthy subjects matched for age and sex as control. The mean age of CVT patients and control subjects were 38 ± 28.2 and 36 ± 30.6, respectively which did not differ significantly (P = 0.5). Of 50 CVT patients, 17 were male and 33 were female while out of 75 control subjects, 25 were male and 50 were female. There was no significant difference in sex distribution between CVT patients and control subjects, 25 were male and 50 were female. There was no significant difference in sex distribution between CVT patients and control subjects.
and control subjects (P = 0.85). Mean plasma total Hcy levels were significantly higher in CVT patients than control subjects (13.7 ± 4.5 vs. 9.9 ± 3.6 µmol/L, P = 0.015).

The prevalence of MTHFR A1298C genotypes was similar between CVT patients and control subjects and did not differ significantly [Table 1]. Neither 1298AC heterozygote genotype (P = 0.33) nor 1298CC homozygote genotype (P = 0.32) was significantly associated with CVT. Moreover, no significant differences were observed in the frequency of mutant 1298C allele between CVT patients and control subjects (P = 0.16).

We also evaluated the risk of CVT development in codominant, dominant, and recessive models of MTHFR A1298C polymorphism. As shown in Table 2, no evidence of significant association was found between CVT patients and control subjects in any genetic model (codominant models: CC vs. AA, odds ratio [OR] = 0.93, 95% confidence interval [CI]: 0.66–1.27, P = 0.33; AC vs. AA, OR = 0.82, 95% CI: 0.62–1.12, P = 0.29; dominant model: AC + CC vs. AA, OR = 0.85, 95% CI: 0.58–1.16, P = 0.31; recessive model: CC vs. AC + AA, OR = 1.06, 95%, CI: 0.69–1.39, P = 0.43).

As shown in Table 3, the association between MTHFR A1298C genotypes and plasma total Hcy levels were not significant in both CVT patients (P = 0.92) and control subjects (P = 0.78), indicating lack of impact of various genotypes of this polymorphism in determining levels of plasma total Hcy.

In addition, statistical analysis using t-test showed that the wild-type, heterozygote, and homozygote genotypes in patients had significantly higher plasma total Hcy levels than corresponding genotypes in control subjects (P < 0.01).

The relative genotype and allele frequency in our population compared to some other populations was shown in Table 4. As shown in Table 4, the MTHFR A1298C mutation is relatively prevalent in our population.

**DISCUSSION**

Cerebral vein thrombosis is a relatively rare but severe thrombotic disorder characterized by severes clinical manifestation and a high mortality rate. Elevated plasma total Hcy have been proposed as a significant risk marker for CVT in several studies. The study by Martinelli et al. demonstrated that hyperhomocysteinemia increased the risk of CVT by four-fold. In another study by Cantu et al. the estimated risk of association between hyperhomocysteinemia and CVT was reported 4.6. In agreements with these reports, our study also demonstrated a positive association between hyperhomocysteinemia and CVT (P = 0.001).

Plasma Hcy is influenced by factors such as nutritional deficiencies, malignancies, medications, and mutations in the MTHFR gene. The A1298C mutation of the MTHFR gene has been reported to result in a less active enzymatic

| Table 1: Prevalence of MTHFR A1298C allele and genotypes in CVT patients and controls |
|------------------------------------------|-------------|-------------|---|
| MTHFR A1298C gene polymorphism | CVT patients (n=50) (%) | Controls (n=75) (%) | P* |
| I298C allele | 36 (36) | 56 (75) | 0.16 |
| I298AC genotype | 21 (42) | 30 (40) | 0.32 |
| I298AC genotype | 22 (44) | 34 (45.33) | 0.33 |
| I298CC genotype | 7 (14) | 11 (14.67) | 0.32 |

*P<0.05; I298AA: Wild type; I298AC: Heterozygote; I298CC: Homozygote; CVT: Cerebral venous thrombosis; MTHFR: Methylene tetrahydrofolate reductase |

| Table 2: Analysis of MTHFR A1298C in CVT patients and control subjects using codominant, dominant, and recessive models |
|------------------------------------------|-------------|-------------|---|
| Model | Genotype | CVT patients (n=50) (%) | Controls (n=75) (%) | OR (95%CI) | P |
| Codominant | AA | 22 (44) | 34 (45.33) | 0.82 (0.62-1.12) | 0.29 |
| | AC | 7 (14) | 11 (14.67) | 0.93 (0.66-1.27) | 0.33 |
| Dominant | AA | 30 (40) | | 1 |
| | AC+CC | 30 (40) | | 0.85 (0.58-1.16) | 0.31 |
| Recessive | AA+AC | 43 (86) | 64 (85.33) | 1 |
| | CC | 7 (14) | 11 (14.67) | 1.06 (0.69-1.39) | 0.43 |

Cerebral venous thrombosis; MTHFR: Methylene tetrahydrofolate reductase; OR: Odds ratio; CI: Confidence interval |

| Table 3: Effects of the MTHFR A1298C genotypes on total Hcy levels in CVT patients and controls |
|------------------------------------------|-------------|-------------|---|
| Genotypes | Mean Hcy levels (µmol/L)±SD | CVT patients | Controls |
| I298AA | 14.1±5.2 | 9.8±3.3 |
| I298AC | 13.6±4.1 | 10.5±4.1 |
| I298CC | 12.5±2.7 | 8.3±3.1 |
| P | 0.92 | 0.78 |

I298AA: Wild type; I298AC: Heterozygote; I298CC: Homozygote; CVT: Cerebral venous thrombosis; MTHFR: Methylene tetrahydrofolate reductase; Hcy: Homocysteine |

| Table 4: Genotype and allele frequency of MTHFR A1298C in different populations |
|------------------------------------------|-------------|-------------|---|
| Population | I298C allele frequency (%) | I298bCC genotype frequency (%) | References |
| Lebanese | 49 | 23.9 | [14] |
| Pakistan (Punjab) | 59 | 23.0 | [15] |
| Iran | 37.34 | 14.67 | Present study |
| USA | 29 | 7.9 | [16] |
| Portugal | 28.2 | 5.98 | [6] |
| Brazil | 20 | 5 | [17] |
| Poland | 20 | 4 | [18] |
| Tunis | 3.5 | 0 | [11] |

MTHFR: Methylene tetrahydrofolate reductase
variant that may lead to an increased total Hcy and thrombosis.\textsuperscript{[6,23]} However, conflicting data have been reported about the association of the MTHFR A1298C mutation with hyperhomocysteinemia and CVT.\textsuperscript{[6,24-26]}

Plasma Hcy levels are affected with both genetic and environmental factors and it appears that the A1298C polymorphism alone does not significantly affect plasma Hcy levels.\textsuperscript{[1,23]} Environmental factors such as folate and vitamin B12 status in different populations could significantly modify the effect of this polymorphism on plasma Hcy levels.\textsuperscript{[27]} Therefore, the variable results of association studies may be explained by the presence of other genetic or environmental risk factors affecting plasma Hcy levels and subsequently CVT development.

Our results showed that MTHFR A1298C mutation in either heterozygote or homozygote state did not affect plasma Hcy levels and thus were in agreement with the results of Zetterberg et al. and Guéant-Rodriguez et al. studies.\textsuperscript{[24,25]}

Numerous studies have reported MTHFR A1298C mutation as a significant risk factor for CVT.\textsuperscript{[9,10]} In the most recent case-control study,\textsuperscript{[11]} the frequency of MTHFR 1298CC genotype in CVT patients (37.14%) was significantly higher compared with control subjects (0%), (OR = 10.25, 95%, CI: 5.6–18.7, \(P = 0.001\)). That study implied MTHFR A1298C mutation as a potentially risk factor for CVT.

Results of the present study showed that heterozygosity or homozygosity for MTHFRA1298C mutation did not confer an increased risk for CVT. Therefore, our study was inconsistent with those studies that showed significant association between MTHFR A1298C mutation and CVT.\textsuperscript{[9-11]} The reasons for non-consistency of association studies are numerous and many factors such as population heterogeneity, sample size, variation in study design and gene-gene, and gene-environment interactions may contribute to variable association results.\textsuperscript{[28]}

The A1298C mutation appears relatively prevalent in the Iranian population and the frequency of the 1298CC genotype (14.67%) in Iranian population is much higher than the frequency reported for Portuguese (5.98%), American (7.9%), Brazilian (5%), and Polish (4%) populations.\textsuperscript{[6,16-18]} However, the frequency of the 1298CC genotype in the Iranian population is lower than the frequency reported for Pakistan (23%) and Lebanese (23.9%) populations.\textsuperscript{[14,15]}

Taken together, the high frequency of the MTHFRA1298C mutation in our population, its similar distribution between CVT patients and control subjects and its lack of impact in determining levels of total Hcy, is opposite with a plausible role for this common mutation as a risk marker for CVT.

In the current study, we used PCR-RFLP technique for A1298C genotype analysis. In comparison to some of the newer techniques for single nucleotide polymorphism (SNP) analysis such as DNA sequencing, PCR-RFLP is a laborious and time consuming technique that requires specific endonucleases and long time from start to completion of the analysis. It also requires that a genetic variation creates or abolishes a recognition site for a specific restriction enzyme.\textsuperscript{[29]} Moreover, star activity may result in alteration of the specificity of restriction enzyme and in non-specific digestion of DNA in non-optimized reaction condition.\textsuperscript{[30]} In addition, PCR-RFLP analysis is not suitable for high-throughput analysis.

However, in the current study we used PCR-RFLP analysis for A1298C SNP analysis, as it is an inexpensive and easy-to-design method that requires no expensive equipment. We also followed exactly the instructions of kit in order to minimize the possible star activity of restriction enzyme. The limitation of this study is the small size of the study group, so the results can be considered as preliminary. Further information is needed from more studies over the world to analyze the contribution of MTHFR A1298C genotype to the risk of CVT.

**Conclusion**

Based on this study, we suggest that MTHFR A1298C mutation is not a significant risk marker for CVT development.

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